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Promoting Thymopoiesis with Age: Potential Role of the Transcription Factor Foxn1

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PROMOTING THYMOPOIESIS WITH AGE:
POTENTIAL ROLE OF THE TRANSCRIPTION FACTOR FOXN1

A DISERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN CELL BIOLOGY, NEUROBIOLOGY, AND ANATOMY

BY
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.......................................................................................... iii

LIST OF FIGURES................................................................................................ vii

LIST OF TABLES.................................................................................................... xii

LIST OF ABBREVIATIONS....................................................................................... xiii

CHAPTER I: STATEMENT OF PROBLEM......................................................... 1

CHAPTER II: REVIEW OF THE LITERATURE.................................................. 4
  Introduction to the aged immune system....................................................... 4
  The thymus......................................................................................................... 5
    The discovery of the thymus as an organ that produces T cells............. 5
    Cellular and structural composition of the thymus............................... 7
    Thymopoiesis................................................................................................. 13
    Thymic organogenesis.................................................................................. 22
  Age associated thymic involution............................................................... 25
    Thymic structure and aging....................................................................... 26
    Aging and thymopoiesis.......................................................................... 28
    Peripheral effects of thymic involution.................................................... 29
  HSC and hematopoiesis.............................................................................. 31
    Embryonic origins of HSC...................................................................... 32
    BM HSC niche.......................................................................................... 35
    Development of lymphoid and myeloid progenitors from HSC........... 43
    Development of T cell progenitors in the BM...................................... 45
    Cytokine and Transcriptional Regulation of the Development of
    Myeloid and Lymphoid Progenitors from HSC...................................... 50
  Hematopoiesis and aging.......................................................................... 55
    HSC and aging.......................................................................................... 55
    Aging and the HSC niche........................................................................ 60
  Foxn1 and nude mice.................................................................................. 60

CHAPTER III: OVER EXPRESSION OF FOXN1 ATTUNATES THYMIC
  INVOLUTION.................................................................................................... 64
  Introduction.................................................................................................. 64
  Results.......................................................................................................... 65
<table>
<thead>
<tr>
<th>Summary</th>
<th>99</th>
</tr>
</thead>
</table>

**CHAPTER IV: OVER EXPRESSION OF FOXN1 PROMOTES THE GENERATION OF T CELL PROGENITORS IN THE BM.**

- Introduction: 102
- Results: 103
- Summary: 146

**CHAPTER V: B CELL AND MYELOID PROGENITORS IN FOXN1 TG BM.**

- Introduction: 147
- Results: 148
- Summary: 161

**CHAPTER VI: FOXN1 EXPRESSION IN THE BM.**

- Introduction: 166
- Results: 167
- Summary: 183

**CHAPTER VII: DISCUSSION.**

- Foxn1 and the thymus: 188
  - Regulation of Foxn1 in the thymus: 188
  - A role for Foxn1 in thymopoiesis with age: 190
  - A role for Foxn1 in maintaining thymic architecture with age: 194
- Foxn1 and the bone marrow: 197
  - The effect of Foxn1 on HSC: 197
  - A role for Foxn1 in hematopoiesis: 207
  - A role for Foxn1 in the development of CTP and CIP: 207
  - Expression and regulation of Foxn1 in the bone marrow: 210
- Concluding remarks: 215
  - Putting it all together: 215
  - Significance: 216

**CHAPTER VIII: MATERIALS AND METHODS.**

- Wt mice, H2-VEX mice, Foxn1cre-LacZ mice: 220
- Foxn1 transgenic mice (Foxn1Tg): 220
- Isolation of thymocytes from thymic stroma: 221
- Isolation of BM cells: 221
- Digestion of thymus and isolation of thymic epithelial cells: 224
- Separation of BM cells using percoll: 225
- Quantification of Foxn1 mRNA levels in thymic stroma and BM: 225
- Quantitative RT-PCR analysis of Foxn1 in different BM populations: 226
- Quantification of HSC, MPP, CLP, ETP, CTP and CIP frequency and total cell number: 228
- Electronically sorting of ETP: 228
Electronic Isolation of HSC, MPP, CTP and CIP………………………………………230
Cell cycle analysis………………………………………………………………………………230
Flow cytometers and antibodies…………………………………………………………….232
OP9 cell cultures…………………………………………………………………………………232
Colony forming assay methylcellulose cultures………………………………………..232
Non-irradiated adaptive transfers………………………………………………………….233
Irradiated adaptive transfers…………………………………………………………………233
Immunofluorescence and hematoxylin and eosin (H&E) staining of thymi……………234
Immunohistochemistry of sternums for Foxn1……………………………………….234
Identification of Foxn1\textsuperscript{pos} Cells within the Sorted Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} BM cells ……………………………………………………………………………………………………………………………235
Statistical analysis………………………………………………………………………………236

REFERENCES…………………………………………………………………………………237

VITA……………………………………………………………………………………………271
LIST OF FIGURES

Figure 1. Thymus structure and localization of thymocyte populations........... 10
Figure 2. Development of TEC and TEC subsets................................. 12
Figure 3. Cell surface phenotype of developing thymocytes...................... 14
Figure 4. TCR Vα chain rearrangement and generation of sjTRECs............. 18
Figure 5. Check points of important stages of T cell development............. 19
Figure 6. Endosteal and vascular HSC niches..................................... 36
Figure 7. Hematopoiesis in mouse BM............................................. 44
Figure 8. Simplified diagram of hematopoiesis with CTP and CIP............. 47
Figure 9. Cytokine and transcriptional regulation of hematopoiesis.... ........ 56
Figure 10. Changes in the BM and thymic populations with age.............. 58
Figure 11. Design and specificity of primer to the Foxn1 transgene........... 67
Figure 12. Endogenous and transgenic Foxn1 expression in thymic stroma.... 69
Figure 13. Foxn1 expression in Wt and Foxn1Tg thymi.......................... 70
Figure 14. Distribution of thymocyte populations with age in Wt and Foxn1Tg................................................................. 72
Figure 15. Thymocyte number in Wt and Foxn1Tg with age....................... 74
Figure 16. Gross morphology of aged Wt and Foxn1Tg thymi................... 76
Figure 17. Flow cytometry identification of ETP.................................. 77
Figure 18. ETP frequency and number in young and aged Wt and Foxn1Tg.... 78
Figure 19. Flow cytometry analysis of ETP cultures on OP9-DL1(GFP)...... 81
Figure 20. ETP commitment to T lineage and differentiation .......... 82
Figure 21. Recombination of the VEX construct in H2-SVEX cells .... 84
Figure 22. Identification of donor CD45.1\textsuperscript{pos} VEX\textsuperscript{pos} ETP in aged Wt and \textit{Foxn1Tg} hosts.......................... 86
Figure 23. Frequency of donor ETP and percent of donor ETP that express VEX in aged Wt and \textit{Foxn1Tg} hosts......................... 87
Figure 24. Gross thymic morphology and histology of old Wt and \textit{Foxn1Tg} hosts.................................................. 88
Figure 25. Keratin 8 and keratin 5 staining of Wt and \textit{Foxn1Tg} thymi ...... 91
Figure 26. Identification of TEC subsets by flow cytometry................. 93
Figure 27. Number of cTEC in young and aged Wt and \textit{Foxn1Tg}........ 94
Figure 28. Number of MHCII\textsuperscript{pos} mTEC in young and aged Wt and \textit{Foxn1Tg}... 95
Figure 29. Number of MHCII\textsuperscript{hi} mTEC in young and aged Wt and \textit{Foxn1Tg}..... 96
Figure 30. Frequency of Ki67\textsuperscript{pos} MHCII\textsuperscript{hi} mTEC in young and aged Wt and \textit{Foxn1Tg} ................................................................. 97
Figure 31. Identification of HSC and MPP..................................... 105
Figure 32. Frequency and number of MPP in young and aged Wt and \textit{Foxn1Tg}................................................................. 106
Figure 33. Frequency and number of HSC in young and aged Wt and \textit{Foxn1Tg}................................................................. 108
Figure 34. Frequency of LSK cells within the Lin\textsuperscript{neg} population of Wt and \textit{Foxn1Tg} mice with age........................................ 111
Figure 35. Frequency and number of LSK in young and aged Wt and \textit{Foxn1Tg} ................................................................. 112
Figure 36. Total number of nucleated cells in the BM of Wt and \textit{Foxn1Tg} with age ............................................................ 115
Figure 37. Flow cytometry gating for cell cycle analysis of HSC and MPP .... 117
Figure 38. Cell cycle analysis of HSC and MPP in young and aged Wt and Foxn1Tg mice ................................................................. 118

Figure 39. Mean fluorescent intensity of CD117 on HSC and MPP from young and aged Wt and Foxn1Tg mice ................................. 121

Figure 40. Morphology of colonies generated in methylcellulose.............. 124

Figure 41. Total number of functional HSC in young and aged Wt and Foxn1Tg................................................................. 125

Figure 42. Identification of CD45.1pos donor HSC and MPP ............... 127

Figure 43. Generation of donor MPP in aged Foxn1Tg and Wt hosts........ 128

Figure 44. Flow cytometry analysis of HSC and MPP cultures on OP9-DL1(GFP)................................................................. 131

Figure 45. Commitment of MPP to T lineage and differentiation to DN2/DN3................................................................. 132

Figure 46. Commitment of HSC to T lineage and differentiation to DN2/DN3................................................................. 133

Figure 47. Expression of Notch 1 mRNA in aged Wt and Foxn1Tg BM....... 135

Figure 48. Expression of Notch 3 mRNA in the BM of Wt and Foxn1Tg with age ................................................................. 136

Figure 49. Flow cytometry identification of CTP and CIP ......................... 138

Figure 50. Number of CTP and CIP and the generation of CIP in Wt and Foxn1Tg with age ................................................................. 139

Figure 51. Cell cycle activity of CTP and CIP in Wt and Foxn1Tg mice with age ................................................................. 141

Figure 52. Flow cytometer gating on CD45.1pos donor CTP and CIP ........ 143

Figure 53. Generation of donor CIP in aged Foxn1Tg and Wt hosts.......... 144

Figure 54. Analysis of hematopoietic lineages with age in Wt and Foxn1Tg mice ................................................................. 149
Figure 55. Number of B lineage cells in young and aged Wt and Foxn1Tg BM ................................................................. 153

Figure 56. Identification of CLP .................................................... 154

Figure 57. Frequency and number of CLP in young and aged Wt and Foxn1Tg ................................................................. 155

Figure 58. Mean fluorescent intensity of CD135 on MPP from young and aged Wt and Foxn1Tg mice .............................................. 158

Figure 59. Number of myeloid lineage cells in young and aged Wt and Foxn1Tg BM ................................................................. 160

Figure 60. Number of myeloid CFU in the BM of young and aged Wt and Foxn1Tg BM ................................................................. 162

Figure 61. Number of CFU-M, CFU-GM, CFU-G, and CFU-E colonies generated from young and aged Wt and Foxn1Tg mice ................. 163

Figure 62. Expression of Foxn1 mRNA in the bone marrow of Wt and Foxn1Tg mice ................................................................. 168

Figure 63. In situ staining of young and aged Wt and Foxn1Tg sternums with Foxn1 ................................................................. 171

Figure 64. Foxn1 is not expressed in HSC and MPP from Wt and Foxn1Tg BM ................................................................. 173

Figure 65. Analysis of Foxn1 mRNA expression in CD45pos and CD45neg Foxn1Tg BM cells ................................................................. 175

Figure 66. Foxn1 mRNA expression in Wt BM cells separated by percoll gradients ................................................................. 176

Figure 67. Identification of EpCAMpos cells in the BM of young and aged Wt and Foxn1Tg ................................................................. 178

Figure 68. Analysis of Foxn1 expression in sorted Linneg/low EpCAMpos cells from aged Foxn1Tg ................................................................. 179

Figure 69. Analysis of Foxn1 expression in sorted Linneg/low EpCAMpos Syndecan-1neg cells from aged Wt and Foxn1Tg BM ................. 180
Figure 70. Frequency of Foxn1 expressing cells in the BM of aged Wt and Foxn1 Tg…………………………………………………………….. 181

Figure 71. Analysis of β-galactosidase expression in sorted Lin<sup>neg/low</sup> EpCAM<sup>pos</sup> Syndecan-1<sup>neg</sup> cells from Foxn1cer-lacZ BM………………………… 182

Figure 72. MPP differentiation into ETP and CLP in aged Foxn1 Tg mice …… 203

Figure 73. Skewing of MPP in Foxn1 Tg mice toward T lineage……………. 206

Figure 74. Age-associated alteration in T lineage progenitors within the BM of Wt mice………………………………….………..……... 209

Figure 75. Working model of how over expression of Foxn1 promotes thymopoiesis…………………………………………………………. 219

Figure 76. Design of primers for genotyping Foxn1 Tg mice…………… 223
# LIST OF TABLES

Table I. Two way ANOVA analysis summary of data that provides information for a function of *Foxn1* in the thymus or the BM........................................ 185

Table II. Two way ANOVA analysis summary of data that does not provided information for a function of *Foxn1* in the thymus or the BM........................................................................... 186

Table III. TEC subsets in young and aged Wt and *Foxn1*Tg ......................... 98

Table IV. Changes in the BM lineage compartments of Wt and *Foxn1*Tg mice with age..................................................................................... 151

Table V. Primer used for quantification of RT-PCR........................................... 227

Table VI. Antibodies used for flow cytometry analysis................................. 229

Table VII. Summary of cell surface markers of thymic and BM populations.... 231
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIRE</td>
<td>autoimmune regulator</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>AGM</td>
<td>aorta gonad-mesonephros</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CIP</td>
<td>committed intermediate progenitors</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
</tr>
<tr>
<td>cTEC</td>
<td>cortical thymic epithelial cells</td>
</tr>
<tr>
<td>CTP</td>
<td>committed T cell progenitors</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>DL1</td>
<td>delta like 1</td>
</tr>
<tr>
<td>DL4</td>
<td>delta like 4</td>
</tr>
<tr>
<td>DN</td>
<td>double negative</td>
</tr>
<tr>
<td>DP</td>
<td>double positive</td>
</tr>
<tr>
<td>EBF</td>
<td>early B cell factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial mesenchymal transition</td>
</tr>
<tr>
<td>ETP</td>
<td>early T cell progenitors</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Foxn1</td>
<td>forkhead box N1</td>
</tr>
<tr>
<td>FSP-1</td>
<td>fibroblast specific protein -1</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GMP</td>
<td>granulocyte-macrophage progenitor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and Eosin</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cells</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>Lin</td>
<td>lineage (in reference to specific T, B, NK, and myeloid cell surface markers)</td>
</tr>
<tr>
<td>LMPP</td>
<td>lymphoid primed multipotent progenitors</td>
</tr>
<tr>
<td>LT-HSC</td>
<td>long term hematopoietic stem cells</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>mTEC</td>
<td>medullary thymic epithelial cells</td>
</tr>
<tr>
<td>MPP</td>
<td>multipotent progenitors</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodine</td>
</tr>
<tr>
<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>pTα</td>
<td>pre- T cell receptor alpha</td>
</tr>
<tr>
<td>RSS</td>
<td>recombination signal sequence</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>SJ-TREC</td>
<td>signal joint T cell receptor excision circle</td>
</tr>
<tr>
<td>SP</td>
<td>single positive</td>
</tr>
<tr>
<td>ST-HSC</td>
<td>short term hematopoietic stem cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEC</td>
<td>thymic epithelial cells</td>
</tr>
<tr>
<td>TLR</td>
<td>toll like receptors</td>
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<td>Vs.</td>
<td>versus</td>
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CHAPTER I

STATEMENT OF PROBLEM

The elderly population is more susceptible to illnesses and infections (1). The increased mortality and morbidity due to infections in the elderly population is the result of a decline in functions of the immune system (1). The mammalian immune system functions to protect against various pathogens and can be divided into two branches: the innate immune system and the adaptive immune system. The innate immune system is the body’s first line of defense against invading pathogens and is non specific to the pathogens. In contrast, the adaptive branch is pathogen specific and results in immunological memory that mounts a faster and more robust response to the pathogens upon re-infection. With age, the functions of both the innate and adaptive immune branches decline. The adaptive branch of the immune system is primarily composed of T cells and B cells. In response to an invading pathogen and upon activation, B cells produce antibodies to fight against the infections. T cells function to activate B cells as well as to kill the infected cells. Aging results in both a decrease in T cell functions, and more importantly, a reduction in the continued generation of T cells from the thymus to replenish and maintain the peripheral T cell pool.

In post natal life, the thymus is the primary organ for thymopoiesis, a developmental program that orchestrates the generation of T cells from T cell progenitors derived from the bone marrow (BM) (2, 3). The thymus is composed of an intricate 3-
dimensional network of thymic epithelial cells (TEC) that are essential for the
development of T cells (4-9). T cell progenitors in the thymus are non-self renewing and
the thymus relies on the BM for a continuous supply of T cell progenitors to maintain
thymopoiesis (10, 11). Despite its critical role in the generation and production of T
cells, the thymus decreases in size and functions, as well as undergoes alterations in the
3-dimensional epithelial network; this age-associated deterioration of thymic function is
 termed thymic involution. Within the involuting thymus, contraction of the epithelial
compartments and a decline in the numbers of T cell progenitors coming from the BM
contribute to decline in T cell production (4-9, 12, 13). Currently, the exact cellular and
molecular mechanisms that govern thymic involution are not completely known.

Previously, it was shown that the initial decline in thymocyte number correlates
with a decline in the expression of the epithelial specific Forkhead box N1 (Foxn1) in
thymic stroma (14). Foxn1 is a transcription factor that is required for thymic
organogenesis and TEC differentiation during embryonic development and mutations in
Foxn1 result in hairless and athymic conditions observed in nude mice (15, 16). Induced
deletion of the Foxn1 gene causes reduced Foxn1 expression and premature thymic
involution in the thymus of young mice, suggesting that expression of Foxn1 is important
for maintaining thymic function in the post natal thymus (17, 18). Besides not having a
functional thymus, nude mice also have alterations in their BM. Nude BM has decreased
number of nucleated cells and is ineffective in preventing against lethal irradiation when
adoptive transferred (19); together, the above observations lead to the notion that Foxn1
has a novel function in the BM. A direct demonstration of Foxn1 in the BM and its effect on hematopoiesis in the BM has not been investigated.

I hypothesized that maintaining Foxn1 expression with age prevents alterations in TEC compartments and is important for the production and survival of T cell progenitors in the thymus and in the BM.

A Foxn1 transgenic mouse model (Foxn1Tg) in which Foxn1 is over expressed under the control of the human keratin 14 promoter was utilized to test this hypothesis. **Aim 1** was to test if over expression of Foxn1 prevents age-associated changes in TEC architecture and T cell progenitors in the thymus, thus preventing a decline in thymopoiesis. **Aim 2** was to determine if over expression of Foxn1 promotes the generation of T cell progenitors in the BM and prevents their decline with age. In **Aim 3**, I examined whether over expression of Foxn1 affects other BM lineages including the B cell lineage and the myeloid lineage. **Aim 4** was to identify Foxn1 expressing cells in the BM.

Improving and maintaining T cell development with age require preventing age-associated alterations in the thymus and in the BM. The results from this dissertation firmly establish a function of Foxn1 in thymic function and thymopoiesis, thus providing a functional framework for further development of novel approaches to manipulate Foxn1 expression and providing a potential therapeutic intervention to boost immune responses in the elderly.
CHAPTER II

REVIEW OF THE LITERATURE

Introduction to the Aged Immune System

Several clinical reports have documented that the elderly are more susceptible to bacterial and viral infections (20, 21). Furthermore, vaccines designed to protect against various infections have been shown less effective in the elderly population (22). Changes with age in both the innate and adaptive branches of the immune systems contribute to the reduced immune response reported in the elderly. Toll like receptors (TLR) recognize molecular patterns on pathogens; however, expression of TLR on innate macrophages is reduced in the elderly (23). Furthermore, aged macrophages display a reduced response to TRL ligands compared to young macrophages (23). In the adaptive branch of the immune system, the number of naïve B cells is reduced with age, as well as the production of high affinity antibodies from activated B cells (24-26). Perhaps the best characterized adaptive immune cell type affected by aging is the T cell. Both CD4^{pos} T helper cells and CD8^{pos} cytotoxic T cells have altered functions with age. Helper T cells provide stimulatory signals to B cells that are required for the activation of B cells to secrete antibodies. The activation of B cells by aged CD4^{pos} T helper cells is reduced, contributing to decreased antibody protection (27, 28) and reduced vaccine efficacy (29, 30). One function of cytotoxic T cells is to lyse virally infected cells; the number and diversity of cytotoxic CD8^{pos} T cells that respond to influenza decline with age resulting
in an increased risk of infection (31). Several factors contribute to decline in immune function with age. However, perturbations in the number and function of peripheral T cells with age are the result of thymic involution, which is the age-related decrease in thymus size, organization, and thymopoiesis.

The Thymus

The discovery of the thymus as an organ that produces T cells

The thymus is a lymphoid-epithelial organ that is located in the mediastinal cavity deep to the sternum and superior to the heart. For many years, it was believed that the thymus did not have any biological significance. A protective role of the thymus in viral infection was observed when neonatal thymectomized mice died shortly after inoculation with a virus (32, 33). It was later shown that neonatal thymectomized mice did not reject allogeneic skin grafts leading to a hypothesis that the thymus produces immunocompetent cells (34). At this point in time, it was known that immune cells produce antibody or contribute to an immune response in a cell-mediated manner; however, it was thought that the immune cells were a homogenous population (35). Together these findings led to a false assumption that the thymus produced antibody secreting cells. Histological examination of lymph nodes and spleens from neonatal thymectomized mice revealed that the areas where antibody producing cells are found were histologically normal; however, the areas that contained the cell mediated immunity were disrupted (36). This suggested that the thymus may not be the organ responsible for the production of antibody cells after all. Experiments performed by Claman et al. in
irradiated mice demonstrated that antibody production required cells from both the bone marrow (BM) and the thymus (37). A series of experiments later demonstrated that antibody producing cells were derived from the BM and that thymus derived cells were essential in assisting the production of antibody from BM-derived cells (38-40). The cells produced in the BM were called “B cells” and the cells produced in the thymus were called “T cells”.

Since the 1960s, a great deal of information about the function of the thymus has been revealed. In the mid 70’s, it was discovered that the thymus produces two types of lymphocytes, which could be distinguished based on the expression of different cell surface molecules (41-43). These two subsets are known as CD4pos T helper cells and CD8pos cytotoxic T cells. The thymus does not contain any stem cells; to maintain the production of naïve T cells the thymus relies on a continuous seeding of progenitors from the BM (10, 11). Upon arrival in the thymus, T cell progenitors interact with thymic epithelial cells (TEC) that provide signals for T cell development (4, 5, 7-9, 44, 45). Committed T cell progenitors proceed through a series of differentiation stages, resulting in the production of naïve T cells. Naïve T cells exit the thymus and function in the periphery to protect against invading pathogens. The thymus of a young mouse produces approximately 10^8 T cells a day but only exports 10^6 naïve T cells per day (46, 47).

The T cell receptor (TCR) is expressed on T cells and is responsible for antigen specific recognition of pathogens (48). CD4pos T cells recognize antigen presented in context of major histocompatibility complex (MHC) class II and CD8pos T cells recognize antigen presented in MHC class I (49, 50). As thymopoiesis progresses, T cells that
express a TCR that recognizes antigen in the context of MHC molecules are signaled to survive in a process termed positive selection (51-54). The thymus must produce naïve T cells with a broad repertoire of T cell receptors (TCR) that are capable of recognizing a plethora of different pathogens. To generate a broad spectrum of TCR specificity, genes encoding TCR undergo gene rearrangement resulting in a diversification of TCRs that have the potential of responding to a multitude of different pathogenic antigens. This rearrangement of TCR genes results in the production of TCRs that may also react to host antigens. To protect against a possible attack on the body by T cells, the thymus must also function to ensure that potentially auto-reactive T cells are deleted in a process termed negative selection (52, 55).

The thymus also functions as an endocrine organ to promote intra thymic and extra thymic T cell maturation. TEC produce cytokines and growth factors that influence the development and proliferation of thymocytes, and in turn thymocytes secrete cytokines that affect TEC proliferation. The hormone thymulin is secreted from TEC (56-58). When bound to zinc, thymulin assists in the proliferation of IL2 receptor expressing mature T cells in the thymus as well as in the periphery (59). Thymulin secretion by TEC is regulated by IL-1 (60). Thus, the thymus not only produces T cells but regulates their proliferation in the periphery through thymulin secretion.

**Cellular and Structural Compositions of the Thymus**

The thymus can be divided into two parts: the cortex and the medulla. The cortex is composed primarily of cortical TEC (cTEC) and immature or developing thymocytes
while the medulla is primarily composed of medullary TEC (mTEC) with predominantly mature thymocytes (61-63). The cortical-medullary junction (CMJ) is the boundary between the cortex and the medulla. The CMJ is the location at which T cell progenitors enter the thymus from the BM though high endothelial venules (64-70). Surrounding the thymus is the capsule, which is composed of primarily fibroblasts. The capsule gives rise to trabeculae that penetrates the thymus forming and dividing the two thymic lobes into smaller incomplete lobules. Also present in the thymus are dendritic cells (DC) and macrophages which play an important role in presentation of antigens to the developing T cells as well as in the removal of apoptotic cells (7, 71).

In H&E histological sections, the cortex of the thymus appears dark while the medulla has a lighter appearance due to the different density of thymocytes within each region (Fig. 1). The dense appearance of the cortex is the result of the numerous immature T cells that occupy that region. The most immature T cells are negative for the cluster of differentiation (CD) markers 4 and 8, thus are referred to as double negative (DN) cells (72). The DN subset can further be subdivided into 4 distinct cell populations based on the expression of CD44 and CD25: the DN1 are CD44\textsuperscript{pos} CD25\textsuperscript{neg}, DN2 are CD44\textsuperscript{pos} CD25\textsuperscript{pos}, DN3 are CD44\textsuperscript{neg} CD25\textsuperscript{pos}, and DN4 are CD44\textsuperscript{neg} CD25\textsuperscript{neg} (73). DN cells in the cortex migrate in an arch pattern from the cortical-medullary junction toward the subcapsular space and then back towards the cortical-medullary junction as they differentiate from the DN1 stage to the DN4 stage (Fig. 1). The migration of the DN subsets through the cortex requires CXCR4 expression on DN cells and CXCL12 production by cTEC (74). After completion of the DN stages, the developing T cells
**Figure 1**: Thymus structure and localization of thymocyte populations. The thymus can be divided into the cortex (C) and the medulla (M) which is demarcated by the corticomedullary junction. Mature single positive (SP) CD4 and CD8 T cell are located in the medulla. Developing immature thymocytes are located in the cortex and can be divided into DN1, DN2, DN3 and DN4 subsets. As the DN subsets differentiate towards the double positive stage (DP), the cells migrate from the corticomedullary junction toward the subcapsular space and back towards the corticomedullary junction and medullary region.
T Cell progenitors

Cortical-medullary junction (CMJ)

Capsule

CMJ
express both CD4 and CD8 on their cell surface and are referred to as double positive (DP) thymocytes. Developing T cells that have successfully passed through the DP stage will then mature into either single positive (SP) CD4 or CD8 T cells and will migrate to the medulla (75). The number of single positive T cells in the medulla is not as numerous as the immature T cells in the cortex, and thus, the medulla is less dense in appearance (Fig. 1).

TEC form a three dimensional network in which the developing thymocytes physically interact with TEC. The three dimensional structure of TEC is critical for their ability to support T cell development (76). Cortical TEC and medullary TEC can be distinguished from each other based on the anatomical regions within the thymus, the patterns of keratin expression, and the expression of specific cell surface molecules. Functionally mature cortical TEC express predominantly keratin 8 and lack keratin 5 while medullary TEC express keratin 5 and lack keratin 8 (77). In contrast, TEC progenitors reside within the corticomedullary junction and express both keratin 5 and keratin 8; these cells have the potential to differentiate into either cTEC or mTEC (77-79) (Fig. 2). Recently, various cell surface markers have been used to distinguish cTEC and mTEC. Both cTEC and mTEC lack the expression of CD45 and express the major histocompatibility complex (MHC) II. Cortical TEC express Ly51 and EpCAM (80). Medullary TEC are EpCAMpos but do not express Ly51 (81). A population of mTEC that express high levels of MHCII are transient amplifying cells; these cells are actively dividing and are responsible for maintain the mTEC pool (81) (Fig. 2). Taken together,
Figure 2: Development of thymic epithelial cells (TEC) and TEC subsets. TEC progenitors reside in the corticomedullary junction and express both keratin (K) 5 and K8. These TEC progenitors can give rise to cTEC that are K5^neg K8^pos or mTEC that are K5^pos K8^neg. Within the mTEC population, the MHCII^{hi} mTEC are transient amplifying cells that are responsible for maintaining the mTEC pool and are the precursor for the more mature MHCII^{pos} mTEC.
mTEC can be identified as CD45\textsuperscript{neg} MHCII\textsuperscript{pos/hi} LY51\textsuperscript{neg} and EpCAM\textsuperscript{pos}, while cTEC are CD45\textsuperscript{neg} MHCII\textsuperscript{pos} LY51\textsuperscript{pos} and EpCAM\textsuperscript{pos}.

**Thymopoiesis**

Thymopoiesis is the development of naïve T cells from T cell progenitors in the thymus (Fig. 3). The majority of T cells produced in the thymus have TCRs composed of an alpha chain and a beta chain (αβ T cells). Within the DN1 subset, the earliest T cell progenitor (ETP) has been identified as negative for lineage-specific T, B, NK, and myeloid markers (Lin\textsuperscript{neg}) as well as negative for CD25 and CD127, but express CD117 and CD44 (82, 83). ETP have not yet committed to T lineage as they possess myeloid and B lineage potential (82, 84, 85). Upon initial commitment to the T lineage, the first cell surface molecules that are up regulated is CD90 (Thy 1) and CD25; expression of these molecules marks the differentiation to DN2 or DN3 stages of T cell development (86, 87) (Fig. 3). DN2 cells lost B lineage potential, however retain myeloid potential (88). Irreversible and full commitment to the T lineage occurs at the DN3 upon successful rearrangement of the Vβ genes (89, 90). Signaling of the completion of β chain gene rearrangement in the DN4 stage results in differentiation to the DP subset, which begins to rearrange the α chain genes of the TCR (91, 92). Upon rearrangement of α TCR and expressing TCRα and β, the DP cells undergo positive and negative selection and mature into the SP stage.

Thymopoiesis requires Notch signaling for the commitment and differentiation of ETP toward T lineage (88, 93). The Notch 1 receptor expressed on ETP binds to its
Figure 3. Cell surface phenotype of developing thymocytes. Cell surface molecules are used to identify different stages of thymopoiesis. The DN1 subset is defined as Lin\textsuperscript{neg} CD44\textsuperscript{pos} and CD25\textsuperscript{neg}. Within the DN1 subset, the earliest T cell progenitor (ETP) is defined as Lin\textsuperscript{neg} CD117\textsuperscript{pos} CD44\textsuperscript{hi} CD127\textsuperscript{neg} and CD25\textsuperscript{neg}. The DN2 subset is defined as Lin\textsuperscript{neg} CD44\textsuperscript{pos} CD25\textsuperscript{pos}. The DN3 subset is defined as Lin\textsuperscript{neg} CD44\textsuperscript{neg} CD25\textsuperscript{pos}. The DN4 subset is defined as Lin\textsuperscript{neg} CD44\textsuperscript{neg} CD25\textsuperscript{neg}.
Lin^{neg} CD44^{pos} CD25^{neg}

Lin^{neg} CD44^{pos} CD25^{pos} CD90^{pos}

Lin^{neg} CD44^{neg} CD25^{pos} CD90^{pos}

Lin^{neg} CD44^{neg} CD25^{pos} CD90^{pos}

CD4^{pos} CD8^{pos} CD90^{pos}

CD4^{+} CD3^{pos} CD8^{+} CD90^{pos}
ligand, delta like 1 or 4 (Dl1 and Dl4) expressed on TEC (44, 45). While both DL1 and DL4 signal through Notch to induce T lineage commitment and differentiation, DL4 is believed to be the physiological ligand (94). The importance of Notch signaling in T cell development has been demonstrated both in vivo and in vitro. When Notch is deleted from T cell progenitors in vivo, B cells develop in the thymus instead of T cells (85). Similarly, when intracellular Notch is over expressed in the BM, T cell development occurs instead of B cell development (95). Prior to the discovery that Notch signaling is required for T lineage development thymocytes could not be generated in vitro using a two dimensional monolayer of stromal cells. Instead generation of thymocytes from T cell progenitors and could only be achieved in the fetal thymus organ culture system. The establishment of the OP9 bone marrow murine cell line that over expresses DL1(OP9-DL1) made it possible to generate T cells in vitro from T progenitors; however, these developing thymocytes only develop to the DP stage as this system does not support positive selection (45, 96). T cell progenitors cultured on OP9 cells that lack DL1 results in the development of B cells (97). Taken together these data demonstrate that Notch signaling is critical for T lineage commitment and development toward T lineage and that without Notch signaling T cell progenitors default to B lineage.

In the cortex, rearrangement of the TCR genes is initiated in the DN2 stage and completed in the DP stage. The TCR functions to identify foreign pathogens. To combat the vast number of pathogens that the body is exposed to, developing T cells randomly rearrange a combination of variable (V), diversity (D), and joining (J) genes to increase the diversity of the β chain of the TCR. T cells also rearrange a combination of V genes
and J genes for the α chain of the TCR. Rearrangement of the DNA requires expression of the recombination activation gene (RAG) 1 and 2; mice deficient in RAG expression do not have mature T cells or B cells (98, 99). The β chain rearrangement is initiated at the DN2 stage and is completed in the DN3 stage (100-102). The TCR β chain rearrangement consists of one of two D genes rearranging to one of twelve J genes. The rearranged DJ segment then rearranges with one of 28 V β genes. Completion of the β chain gene rearrangement occurs when the VDJ segment joins with a constant region.

The TCR β chain gene rearrangement is completed in the DN3 stage, at which point the rearranged β chain pairs with a surrogate pre-T alpha chain (ptα) forming a pre-TCR complex (103-108). Signaling through the pre-TCR is essential for progression to the double positive (DP) stage, leading to TCRα gene rearrangement; the TCR α chain rearrangement is completed in the DP stage (91, 92). During α chain rearrangement one of 70-100 V α genes combines to one of 50 J α genes. The rearrangement of the α TCR genes results in the production of signal joint TCR excision circles (sj-TREC), which are the circular DNA segment that contain the deleted δ genes during rearrangement of the TCR α chain (Fig. 4). Successful rearrangement of both the β chain and the α chain genes result in a complete TCR complex that is required for positive and negative selections. Figure 5 summarizes the molecular events that occur at each stage of thymopoiesis.

T cells that express TCRαβ on the cell surface undergo both positive selection and negative selection. As previously mentioned, the random rearrangement of TCR genes may result in a TCR that has the potential to recognize host antigenic peptides.
Figure 4: TCR Vα chain rearrangement and generation of a sjTRECs. During Vα chain rearrangement, the δRec (a Vα pseudogene) and the ψJa rearrange resulting in the deletion of δ locus. This process requires expression of Rag and generates a circular sjTREC DNA. The presence of sjTREC marks recently produced naïve T cells. Recombination signal sequences (RSS) are represented by yellow triangles.
Figure 5: Check points of important stages in T cell development. ETP are a subset of the DN1 population and in a young mouse comprise approximately 0.01% of total thymocytes and 0.001% of thymocytes in an aged mouse. Notch signaling-induced ETP progresses through a series of different stages and develops into naïve T cells. Notch signaling is required for all stages of T cell development. ETP progress to DN2, however up to this point myeloid potential is maintained. TCR β chain rearrangement is initiated at the end of the DN2 stage. In the DN3 stage, β chain rearrangement is completed resulting in T lineage commitment. TCRα gene rearrangement is initiated and completed in the DP stage. Single positive cells that express a functional TCRαβ that recognize self MHC/peptide undergo positive selection and negative selection.
Myeloid, B, and T lineage potential

ETP

DN1

DN2
TCRβ chain rearrangement

DN3
TCRβ chain rearrangement completion
T lineage commitment

DN4
Pre-TCR signaling

DP
TCRα chain rearrangement

CD4+

CD8+

Positive and Negative Selection
Peptides are presented to T cells in the context of MHC molecules. In order to fully mature and be exported from the thymus, T cells must express a TCR that recognizes and binds to MHC/peptide but does not bind too strongly to MHC or self peptide presented in MHC. T cells that have rearranged a TCR that will bind and recognize MHC will receive a signal to survive in a process termed positive selection (51-54). T cells that have not successfully received TCR stimulation at the DP stage though interaction with MHC and antigens will undergo cell death. In the thymus, self peptides are presented to DP T cells on MHC expressed by TEC (109). Expression of autoimmune regulator (AIRE) by mTEC ensures that certain organ specific peripheral antigens are expressed in the thymus and function in the deletion of auto-reactive T cells (110). T cells that express a TCR that recognize self peptides in MHC with high affinity are signaled to undergo cell death through a process termed negative selection (52, 55). Thus, the strength of a TCR signal determines survival or deletion of T cells (109); TCRs that bind with low affinity survive, while TCRs that bind with high affinity to a peptide presented in MHC will be deleted (109).

Selection in the thymus is dependent on how endogenous peptides are loaded onto MHC I and exogenous peptides onto MHC II. CD8^{pos} T cells recognize MHC class I molecules and CD4^{pos} T cells recognize MHC class II (49, 50). Intracellular peptides are loaded onto MHC class I and MHC class II are known to present exogenous peptides acquired externally from the endocytic pathway (111-114). Intracellular proteins can be presented in MHC class II (115); however, the mechanism by which self peptides are presented on MHC class II for the deletion of auto-reactive CD4^{pos} T cells remains to be
fully elucidated. One mechanism in which it is believed that self peptides are presented on MHC class II is through the regular turn over of TEC. When TEC undergo apoptosis they are phagocytosed by surrounding dendritic cells and macrophages. Dendritic cells and macrophages in the thymus can then present self peptide on MHC class II through the exogenous pathway (116). Recently, it was identified that autophagy in TEC contributes to the presentation of self antigens onto MHC class II molecules and assists in shaping the T cell repertoire (117). Interruption of autophagy in TEC resulted in the escape of auto-reactive T cells that are responsible for colitis and inflammation, indicating that autophagy in TEC is required for the generation of self-tolerant T cells (118). Furthermore, mTEC with high levels of MHC class II demonstrated high autophagy while MHCII\text{low} mTEC had little autophagy (118); thus, it is likely that the MHCII\text{hi} mTEC play a critical role in negative selection.

**Thymic Organogenesis**

Organogenesis of the thymus is a complex process as different cellular components of the thymus arise from different embryonic germ layers. Developing T lymphocytes are hematopoietic in origin and are derived from mesoderm. The capsule of the thymus is derived from neural crest cells of the ectoderm (119). While it was once proposed that cTEC are ectodermaly derived and mTEC are endodermal in origin, current data suggest that both cTEC and mTEC are derived from a common precursor that is endodermal in origin (120, 121).
TEC are the predominant stromal cell type of the thymus and are derived from endodermal cells of the third pharyngeal pouch, which also gives rise to the parathyroid (122). The earliest TEC progenitors can be identified at embryonic day (E) 11.5 (123). At E11.5 to 11.75 the thymic/parathyroid primordium detaches from the pharynx and begins its decent toward the thoracic cavity. During its migration, toward the chest cavity the parathyroid detaches from the thymic primordium and remains in the neck region while the thymic primordium continues its decent to its final anatomical location. Due to its course of migration, functional secondary thymic tissue has been identified in the neck (124, 125). Detailed signaling event that result in endodermal cell commitment of the third pharyngeal pouch to the thymus or to the parathyroid remain to be elucidated. It is known that the differential pattern expression of two proteins dictates differentiation of cells toward either the parathyroid or thymus. At E 9.5, the differentiating endodermal cells destined to become the parathyroid express glial cell missing homolog 2 gene (Gcm2), while cells differentiating into thymic tissue begin to express forkhead box n1 (Foxn1) at embryonic day 11.5 (123). It is not known what controls the patterning of Gcm2 and Foxn1 expression. Signals generated from interaction between the endodermal cells of the third pharyngeal pouch and neural crest cells that surround the primordium play a role. When the number of neural crest cells that surround the thymic and parathyroid primordium are reduced, the patterning of Gcm2 and Foxn1 are altered, resulting in an increased number of progenitor cells expressing Foxn1 and fewer cells expressing Gcm2. This ultimately results in a larger thymus and a smaller parathyroid (126). Sonic hedgehog (Shh) also contributes to the patterning of the primordium. Shh
null embryos have a larger portion of the third pharyngeal pouch expressing Foxn1, suggesting that expression of Shh may negatively regulate Foxn1 expression limiting the number of cells that develop into TEC (127).

Development of mature TEC requires interaction of TEC progenitors with hematopoietic stem cells. At E11.5, hematopoietic cells migrate to the thymic primordium prior to vascularization (E15.5) due to secretion of the chemokines CCL25 and CCL21 by the thymic primordium (128). Epithelial cells of the thymic primordium express MTS24 and these cells also express both keratin 8 and keratin 5, suggesting that these cells are the early thymic epithelial cell progenitors which subsequently differentiate into keratin 5pos mTEC or keratin 8pos cTEC (129-131). Hematopoietic stem cells interactions with thymic epithelial progenitor cells are also required for further functional differentiation to cortical and medullary thymic epithelial subsets (132-134).

The single most important gene for thymic organogenesis is Foxn1. The Foxn1 gene was first identified in nude mice, which lack a functional thymus (16). Identification of the gene that results in the athymic condition has allowed for the examination of a functional role of Foxn1 in thymic organogenesis. Mutation in Foxn1 results in normal formation of the thymic primordium; however, further development is arrested at E11.5 (15). Despite its arrested development, the thymic anlage migrates to its final location in the thoracic cavity indicating that expression of Foxn1 is not required for migration (135). Deletion of the N terminal region of Foxn1 demonstrated that Foxn1 is not required for the development of thymic epithelial progenitors but is needed for their functional maturation as seen by the block in differentiation of the keratin 5 and keratin 8
double positive epithelial cells (136). In nude mice, thymic epithelial cells are not colonized by hematopoietic cells despite the presence of the hematopoietic cells in the surrounding mesenchyme, indicating that Foxn1 is required for functional TEC to recruit hematopoietic cells (137). The nude thymic anlage does not express the Notch ligands Dl1 and Dl4 required for T cell development, suggesting that Foxn1 may be required for their expression (138, 139). Although direct evidence has not been proved, Foxn1 levels are directly proportional to the expression of DL4 and CCL25 in TEC at E 12.5, suggesting that Foxn1 regulates these genes during thymic organogenesis (140). Together, an abundance of data shows that Foxn1 expression is critical for thymic organogenesis, yet regulation of Foxn1 during thymic organogenesis has not been clearly delineated.

**Age-Associated Thymic Involution**

Despite being the primary site for the development of naïve T cells in post natal life, with age the thymus involutes. In humans, thymic involution begins within the first year of life, but is accelerated with puberty (4). Using sj-TREC as a molecular maker for naïve T cells, it was showed that with age the number of T cells in the periphery that contain sj-TREC is reduced, indicative of reduced T cell development and export (116, 141, 142). To maintain the peripheral T cells numbers, memory T cells undergo homeostatic proliferation, resulting in the expansion of the memory T cells and in contraction of the T cell repertoire (143-146). In addition to skewing of the peripheral T cell pool to a memory phenotype, peripheral T cell functions are also reduced, resulting
in a poor immune response to various viral and bacterial pathogens (147, 148).
Reduction in the number of naïve T cells exported from the aged thymus is the result of both changes in the number and function of T cell progenitors, as well as alteration in the thymic environment.

**Thymic Structure and Aging**

T cell progenitors require interaction with TEC for the development of naïve T cells. With age, the organization of TEC and the architecture of thymus are disrupted (4, 5, 149). The once well defined cortical-medullary junction becomes unorganized and distinction of the cortex from medulla compartment is lost (149). Histological analysis on the aged thymus demonstrates a reduction in the cortical and medullary TEC networks (149). Mesenchymal tissue that does not support thymopoiesis expands with age, while the cortex and medulla, which are required for thymopoiesis contracts (4, 5). In the post natal thymus, the number of CD45neg MHCIIpos TEC and their proliferation decline with age (81). On the other hand, the number of fibroblasts and adiopcytes present in the aged thymus increases (150, 151).

It has been suggested that epithelial to mesenchymal transition (EMT) is one mechanism responsible for the increase in adiopcytes and fibroblasts, with a simultaneous reduction in the number of TEC in the aged thymus (152). TEC that once expressed Foxn1 were shown to differentiate to fibroblasts via EMT (152). Fibroblasts generated from epithelial cells express fibroblast specific protein -1 (FSP-1), a strong indicator of the EMT pathway (153). Thymic involution is associated with an increase in FSP-1
protein expression in mTEC (152, 154), suggesting that the EMT pathway contributes to age associated increase in fibroblasts during thymic involution. EMT cells in the aged thymus express the peroxisome proliferator-activated receptor gamma (PPARγ), a pro-adipogenesis transcription factor (150, 154), suggesting that TEC may transition to adiopcytes through the EMT pathway. Thus the decline in TEC number and the increase in the number of fibroblast and adiopcytes in the aged thymus may be the result of TEC transitioning to fibroblast and adipocytes.

The decline in the number of TEC with age contributes to the decline in the deletion of auto-reactive T cells as well as a reduction in the secretion of thymulin from the thymus. Autophagy in TEC assists in the negative selection of auto-reactive T cells (118). With age the number of autophagic vesicles declines in cTEC and mTEC (155), suggesting that cross presentation of self peptides required for deletion of auto-reactive CD4<sup>pos</sup> T cell may be compromised, consequently leading to autoimmune disease (118). Examination of autophagy in aged TEC and its contribution to shaping the T cell repertoire is only beginning to be examined. Thymulin affects T cell proliferation and is produced by cTEC and mTEC; however, expression of thymulin is more prominent in mTEC (156, 157). With age, production of thymulin decreases as the number of TEC that produce thymulin decline (158). These data suggest that preventing the decline in TEC would prevent a decline in autophagy and thymulin secretion.

*Foxn1* expression is required for thymic organogenesis (15); however, a role for *Foxn1* in age-associated thymic involution remains to be fully elucidated. Expression of *Foxn1* in thymic stroma, which is comprised primarily of TEC, declines with age and
correlated to the decline in thymocyte number (14). In the post natal thymus, mTEC express the highest level of Foxn1 (17). When Foxn1 expression levels are reduced in the postnatal thymus, the numbers of mTEC are decreased and mTEC proliferation declines, suggesting that expression of Foxn1 is required for the maintenance of the postnatal mTEC pool (17). The number of mTEC is greater than cTEC; however, in 12 month old mice the ratio of mTEC to cTEC declined compared to mice 4 weeks of age (81), indicating that mTEC are more sensitive to the effects of aging. Taken together, these data suggest that preventing the decline in Foxn1 expression may result in maintained TEC number and architecture required for the development of thymocytes.

**Aging and Thymopoiesis**

The number of DN1 cells in the thymus increases with age (159). At first, this led to the hypothesis that the age-associated alterations in T cell development were not the result of a decrease in the number of T cell progenitors. Later, it was revealed that only a small subset of the DN1 population contains the ETP (82, 83). With age, there is a decline in the frequency and number of ETP (12); ETP from aged thymi also have decreased cell cycle and increased cell death (12). When cultured, aged ETP gave rise to fewer thymocytes compared to young ETP indicating a functional decline in aged ETP (12). Taken together these data indicate that with age there is not only a decline in the number of ETP but also their function, both contributes to reduced thymopoiesis.

Despite the decline in number and function of ETP, naïve T cells are still produced in the aged thymus but at a lower number (142, 160). The distribution of
thymocyte populations are not altered with age (161, 162), suggesting that thymopoiesis is functionally active. Although thymopoiesis is active in the aged thymus, functions of the thymocytes produced from the aged thymus are reduced. Aged thymocytes express lower levels of CD3 and CD3 signaling is required for T cell activation (163). The lower level of CD3 expression may explain the diminished activation and function of naïve T cells produced from the aged thymus (147, 164). Of the aged T cells that are activated, these T cells demonstrate reduced proliferation \textit{in vitro} (162). Thus, while the aged thymus is capable of producing naïve T cells, the activation and function of the naïve T cells as well as their number are diminished.

**Peripheral Effects of Thymic Involution**

Functional defects in aged T cells in the periphery contribute to the reduced immune response in the elderly. The use of TCR transgenic mice that recognized pigeon cytochrome c allowed for functional examination of aged naïve T cells. The function of both CD8\textsuperscript{pos} and CD4\textsuperscript{pos} T cells are altered with age. Naïve CD4\textsuperscript{pos} T cells from aged TCR transgenic mice showed reduced recruitment of signaling molecules to the immunological synapse required for the activation of naïve T cells by antigen presenting cells (165, 166). Reduced proliferation and reduced production of IL2 were observed in aged naïve CD4\textsuperscript{pos} T cells compared to young, suggesting a reduction in effector functions (167-171). Aged naïve CD4\textsuperscript{pos} T cells were ineffective in their ability to activate B cells. When the same number of young and aged CD4\textsuperscript{pos} TCR transgenic cells were adoptively transferred into young host and immunologically challenged, host mice
that received aged cells showed a reduction in antibody production and B cell expansion (27). While the production of naïve T cells decreases with age, the thymus is still functional and naïve T cell are produced at low numbers (161, 172). During thymic involution, it is believed that the longevity of naïve T cells in the periphery is increased as a result of decreased thymic output (167-171, 173). It has also been documented that recent emigrates from an aged thymus also have reduced IL2 production and proliferation (147, 164). With age, both recently produced naïve T cells as well as aged naïve T cells in the periphery demonstrate defects in their activation and effector functions.

Age associated reductions in naïve T cell function are not specific to CD4<sup>pos</sup> T cells as aged CD8<sup>pos</sup> T cells also have reduced function. Aged CD8<sup>pos</sup> T cells demonstrate reduced clearance of viruses after infection and aged CD8<sup>pos</sup> T cells produced less INF-γ in response to viral infection (174, 175). Naïve CD8<sup>pos</sup> T cells show decreased TCR diversity when subjected to DNA spectratyping (176), which results in the inability to respond and clear viral infections rapidly. Again, a decrease in TCR diversity is the result of decreased thymic output due to thymic involution. Specifically, a decrease in diversity of the CD8<sup>pos</sup> T cell repertoire was directly shown to affect the ability of CD8<sup>pos</sup> T cells to protect against influenza (31). Aged CD8<sup>pos</sup> T cells also show diminished ability to generate memory T cells after infection (175). The number of memory CD8<sup>pos</sup> T cells generated after infection with influenza is reduced and results in decreased clearance upon secondary infection (31). Thus, thymic involution results in the expansion of memory peripheral T cells leading to decreased TCR diversity as well as an
increase in the life span of peripheral T cells whose functions are decreased. Preventing or reversing thymic involution may restore proper function of peripheral T cells.

**HSC and Hematopoiesis**

In the 1800s, Franz Ernst Christian Neumann first identified the BM as the location for the development of blood cells, also known as hematopoiesis (177). The BM is located in the central cavity of bones and contains two types of marrow. Yellow marrow that is made up of fat cells, and red marrow which contains the hematopoietic cells. Hematopoietic cells consist of T cell progenitors that will seed the thymus for further maturation as well as developing and mature red blood cells, B cells, and myeloid cells. The stroma of the BM is the non-hematopoietic tissue, which consists of fibroblasts, macrophages, osteoclasts, osteoblasts, endothelial cells, adipocytes, and adventitial reticular cells, and provides the supportive environment required for the development new blood cells from HSC (178, 179). Franz Ernst Christian Neumann was also the first to propose that all blood cells develop from stem cells (177). However, it wasn’t until the 1960s when the stem cell theory was supported though experiments in which BM cells were adoptively transferred into irradiated mice and the number of myeloid colonies generated in the host spleen (CFU-S) was proportional to the number of cells injected (180, 181). It is now well accepted that all hematopoietic cells developed from hematopoietic stem cells (HSC).
Embryonic Origins of HSC

HSC maintain the development of all mature blood cells, but the embryonic origin of HSC has been challenging to identify. The difficulty in identifying the origin of HSC was due to multiple reasons: 1) hematopoiesis occurs in two waves, 2) HSC can be identified in multiple locations in the developing embryo, and 3) cell surface markers used to identify adult HSC are not exclusively expressed on embryonic HSC (182-184).

At E7.5-8, the first wave of HSC develops from the yolk sac and generates erythrocytes and a few myeloid cells thought to be required primarily for oxygen exchange of the developing embryo (185, 186). The second wave generates the postnatal HSC from the aorta gonad-mesonephros (AGM) region that will eventually seed the BM and generate all mature blood cells in the adult (187). While it is now generally accepted that there are two waves of HSC development, the fact that HSC could be identified in the yolk sac as well as the AGM in the developing embryo led to conflicting results and extensive effort were examined to clarify the origin of HSC.

To determine the origin of embryonic HSC, colony forming assays were used to detect stem cell activity within a group of isolated cells since cell surface molecules could not be used to identify embryonic HSC. Cells believed to contain HSC can be injected into lethally irradiated hosts and after several weeks the spleen can be isolated and examined for colony forming unit spleen (CFU-S). HSC give rise to colonies in the spleen when transferred into irradiated host. Each colony counted in the irradiated host’s spleen developed from one individual HSC. Stem cells and progenitor cell activity can also be measured in vitro in semi-solid methylcellulose cultures. Each individual stem
cell or progenitor cell capable of proliferating and differentiating will generate a cluster of cells or colony in the methylcellulose media. Each CFU can be examined to determine if the cells in the colony are homozygous and developed from a lineage specific progenitor, or heterogeneous and developed from a progenitor that contains multiple types of lineage potential. HSC give rise to the most heterogeneous CFU as these cells have the potential to give rise to all blood cells.

At first, it was believed that the extra embryonic yolk sac was the origin of HSC as cells from the yolk sac were able to generate different CFU in vitro and in vivo (185). However, experiments in which quail embryos were grafted to a chick yolk sac demonstrated that mature cells in the spleen and thymus were quail in origin and not chick, indicating that adult HSC do not originate from the yolk sac but from an intra embryonic source (119). A model of HSC embryogenesis suggests that the first wave of hematopoiesis originates in the yolk sac from blood islands forming primitive HSC but that these cells do not contribute to the adult HSC pool. HSC in the adult BM develop independently from the yolk sac and are established in the second wave of hematopoiesis. HSC that contribute to the adult HSC pool can be identified in the yolk sac but only after E11 and after generation of HSC from the AGM region (188, 189).

The identification that cells from the wall of the aorta of the chick can form CFU of monocytes (CFU-M), granulocytes (CFU-G), granulocyte/macrophage (CFU-GM), and erythroid (CFU-E), first implicated the aorta as a possible origin for HSC that subsequently establish the pool of HSC in the adult (190). Later it was identified that cells from the AGM region contained long term repopulation capacity when transplanted
into primary host as well as when serially transplanted into several hosts (189). The confirmation that AGM-derived cells give rise to HSC was demonstrated when cells isolated from AGM autonomously generated HSC (191). At E11.5, HSC are formed in the dorsal aorta (192, 193). HSC develop from the dorsal aortic endothelium (194); transition of endothelial cells of the dorsal aorta to HSC is regulated by Runx1 (195, 196). Notch signaling regulates Gata2 expression which in turn regulates the expression of Runx1 in the development of HSC (197-199). Thus, proper Notch signaling regulates the development of HSC during embryonic development from the dorsal aorta.

At E11.5, HSC from the AGM seed the fetal liver and this is the major organ for hematopoiesis during embryogenesis. In the fetal liver, HSC rapidly proliferate and expand in number (200). It remains to be determined if HSC from the AGM are the only cells that seed the fetal liver or if HSC found in the yolk sac, placenta, and umbilical and vitelline arteries contribute to the fetal liver pool as all of these anatomical sites also demonstrate HSC activity (192, 201-203). At E17.5, HSC from the fetal liver seed the BM which will be the permanent site of hematopoiesis in the adult mice. Seeding of the BM by fetal HSC requires signaling mediated by the CXCR4 receptor on HSC and CXCL12 (SDF-1) expressed in the BM (204). During embryogenesis, the size of the adult HSC pool in the BM is regulated by the sry-related high mobility group box 17 (sox17) (205). At four weeks post birth, Sox17 expression is down regulated as highly proliferative fetal HSC transition to the slowly proliferating adult phenotype, establishing the adult HSC pool within the BM (205). After the establishment of the adult HSC pool, HSC acquire the quiescent phenotype and display limited proliferative activity (206).
The balance between the quiescent state and proliferation of adult HSC is regulated by the HSC niche.

**BM HSC Niche**

The concept of a HSC niche was first demonstrated in 1978 by Schofield who showed that HSC self renewal ability is lost when HSC are removed from the BM (207). This suggested that regulation of HSC self renewal potential requires interaction with other BM cells. Since then, two distinct HSC niches have been proposed: a vascular niche and an endosteal niche (Fig. 6). The endosteal niche is thought to predominantly contain the dormant long term HSC (LT-HSC) that have high self renewal capacity but proliferate infrequently (208). On the other hand, the vascular niche supports the more proliferative short term HSC (ST-HSC) with limited self-renewal that are differentiating into multipotent progenitors (MPP) (208). HSC niches function to maintain HSC numbers, regulate HSC self renewal, and induce HSC differentiation.

The endosteal niche is located adjacent to the endosteum, or the lining of the inner bone surface within the marrow cavity. Evidence of an endosteal niche was demonstrated *in vivo* as CFSE labeled HSC injected into host mice could be identified next to the BM endosteum within 15 hours (209). A higher number of HSC are found in the BM when the number of osteoblasts is increased, suggesting that the osteoblasts are a key cell type of the endosteal HSC niche (210, 211). The interaction between osteoblasts and HSC was also demonstrated *ex vivo* as the number of HSC increased
Figure 6: Bone marrow endosteal and vascular niches. Two niches that support HSC localization, proliferation, self renewal, and differentiation were identified in the BM; the endosteal niche and the vascular niche. The endosteal niche is located adjacent to the endosteum. Osteoblasts, osteoclasts, mesenchymal stem cells (MSC), osteomac, and reticular cells that express CXCL12 (CAR cells) maintain HSC in the endosteal niche. In the vascular niche HSC reside adjacent to endothelial cells and their self renewal, proliferation, and differentiation are regulated by CAR cells, MSC, macrophages, and megakaryocytes.
when cultured with osteoblasts, indicating that osteoblasts promote HSC self-renewal (212).

Regulation of HSC activity by osteoblasts occurs through secretion of cytokines as well as direct interaction between cell surface molecules. Osteoblasts regulate HSC number, proliferation, and survival though secretion of thrombopoietin and angiopoietin. Osteoblasts secrete thrombopoietin and angiopoietin that regulate the quiescent state of HSC by binding to their respective receptors MPL and Tie 1 and 2 expressed on HSC, respectively (213-215). Mutant mice that do not express thrombopoietin or its receptor MPL have a reduced number of HSC that fail to provide long term reconstitution in Wt host (213, 215). In thrombopoietin deficient mice, HSC display increased cell cycling due to reduced expression of p57 and p19$^{INK4D}$ leading to HSC exhaustion (213). Mice deficient in Tie1 and 2 also have a reduced number of HSC; these HSC and their down stream progenitors display a decline in their survival (214).

Cell-to-cell interactions between osteoblasts and HSC also regulate HSC renewal. Osteoblasts express the Notch ligand Jagged; however, a role for Notch signaling in the maintenance of HSC remains controversial. Deletion of Jagged 1 and Notch 1 signaling in vivo suggests that Notch is not important for HSC self renewal and hematopoiesis (216, 217). However, in vitro cultures demonstrated that Notch 1 signaling in HSC promotes self renewal and prevents cell cycling-mediated exhaustion (218, 219). In vivo, Notch 2 is required for the self renewal of HSC and generation of MPP following BM damage as Notch 2 deficient mice show reduced ability to recover from 5-fluorouracil treatment (220). Thus, the controversial role of Notch signaling in HSC hematopoiesis
may be the result of redundant function of multiple Notch receptors and ligands during homeostasis, which may only become important in times of stress.

Changes in the expression of N-cadherin can alter HSC localization in the BM and thus affect HSC proliferation and differentiation. N-Cadherin is an adhesion molecule that is expressed on HSC and osteoblasts and is responsible for sequestering HSC in the endosteal niche. N-Cadherin molecules mediate homotypic cell-cell interaction by binding to other N-Cadherin molecules; both the short term and long term HSC express N-Cadherin (221, 222). Reduced N-Cadherin by osteoblasts or HSC promotes HSC migration from the niche. In experiments in which HSC over expressed N-Cadherin, a larger number of HSC were found at the endosteal surface; these HSC displayed a slower cell cycling (221). HSC that express higher levels of N-Cadherin proliferate less and are important in long term maintenance of hematopoiesis (222). In contrast, lower levels of N-Cadherin are associated with HSC that have higher proliferation and differentiation potential (222).

Beside osteoblasts, osteoclasts also contribute to the HSC endosteal niche by inducing a release of growth factors trapped in the bone matrix. The number of long term HSC are reduced in mice with decreased bone matrix reapportion by osteoclasts; these HSC demonstrate increased proliferation and differentiation (223). Osteoclasts degrade bone matrix, releasing growth factors such as TGFβ1 and bone morophogenic proteins (BMP) into the BM environment. In vitro, HSC cultured with physiological levels of TGFβ1 maintained their quiescent state and showed lower proliferation and reduced cell death (224). BMP-4 also prevents the proliferation and differentiation of HSC (225).
During embryogenesis, prior to endochondral ossification, HSC in the fetal liver undergo expansion, suggesting that HSC can be supported by a second niche separate from the endosteal niche (200). The development of HSC from endothelial cells of the dorsal aorta led to the belief that a vascular niche may also support HSC. HSC expansion occurred when HSC were cultured with endothelial cells isolated from E9.5 yolk sac; these HSC were capable of repopulating lethally irradiated hosts (226). Adult endothelial cells isolated from various organs and cultured with adult HSC were also able to expand HSC that are capable of generating CFU when transferred in to lethally irradiated hosts (227). Together these experiments provided evidence that endothelial cells and the vascular niche support the expansion of HSC. There is also *in vivo* evidence to support a functional vascular niche in the BM. Endothelial cells in the BM of mice deficient in the expression of gp130 fail to support hematopoiesis from Wt HSC (228). Imaging analysis revealed that a large number of HSC localize to sinusoids in the BM (208), illustrating the localization of HSC in the vicinity endothelial cells and further providing support for a vascular niche.

Reticular cells that express CXCL12 (CAR cells) are important for maintaining HSC number and cell cycling. CXCL12-GFP reporter mice demonstrate that Sca-1 and c-kit expressing cells co-localized with CXCL12-GFP cells (CAR cells), suggesting that HSC which express Sac-1 and c-kit are associated with CAR cells (229). Deletion of CAR cells results in a decrease in the number of HSC and decreased HSC cell cycling as measured by Ki-67 staining, indicating that CAR cells regulate HSC proliferation (230).
In addition, CXCL12 CAR cells also secrete large amounts of stem cell factor (SCF) known to induce HSC proliferation (230).

Megakaryocytes indirectly regulate HSC in the vascular niche. Megakaryocytes are located within the vascular niche and are in contact with endothelial cells of sinusoids (231). A potential mechanism by which megakaryocytes regulate HSC has been suggested. Thrombopoietin was shown to maintain HSC in a quiescent state (213). The serum level of thrombopoietin is inversely correlated with the number of megakaryocytes (232, 233). It was shown that mice with an increased number of megakaryocytes have a depletion of thrombopoietin from the circulation by megakaryocytes and starvation of HSC (234). This results in HSC proliferation and differentiation, ultimately resulting in HSC depletion (234). Thus, megakaryocytes can affect HSC by sequestering thrombopoietin required to maintain HSC quiescence.

Recently, Nestin\textsuperscript{pos} mesenchymal stem cells (MSC) have been identified as another cellular component of the HSC vascular niche. In the BM, 88% of HSC are localized to Nestin\textsuperscript{pos} MSC and the majority of the Nestin\textsuperscript{pos} MSC are located outside the endosteal niche in the central marrow (235). When the number of Nestin\textsuperscript{pos} MSC are selectively deleted, the number of HSC in the BM as well as the number of HSC that are able to home to the BM from the circulation are reduced (235). Nestin\textsuperscript{pos} MSC also express CXCL12, SCF, and angiopoietin-1 which have all been shown to be important for maintaining HSC (235).

Surprisingly, the expression of CXCL12, SCF, and angiopoietin-1 by Nestin\textsuperscript{pos} MSC was reduced when CD169\textsuperscript{pos} macrophages were depleted, suggesting that
macrophages also regulate the HSC niche; depletion of CD169\textsuperscript{pos} macrophages also led to increased mobilization of HSC out of the BM (236). However, depletion of CD169\textsuperscript{pos} macrophages did not result in a decrease in the number of Nestin\textsuperscript{pos} MSC (236). The molecular mechanism by which macrophages regulate Nestin\textsuperscript{pos} MSC expression of CXCL12, SCF, and angiopoietin remains to be determined. A similar observation was also made in the endosteal niche. When macrophage located near the endosteal surface (osteomacs) were depleted, the expression of CXCL12, SCF, and angiopoietin-1 was reduced in the endosteal stoma and the number HSC in BM was reduced (237). Thus macrophages are an important cellular component of both the endosteal and vascular HSC niche.

Evidence that an endosteal niche and a vascular niche are capable of supporting HSC and hematopoiesis has been obtained, however it can not be ruled out if these two niches are functionally distinct or that the two niches function together to regulate HSC. While a higher percent of CAR cells and Nestin\textsuperscript{pos} MSC are found in contact with HSC in the vascular niche, both CAR cells and Nestin\textsuperscript{pos} MSC have been identified in the endosteal niche (229, 235). Further, CAR cells and Nestin\textsuperscript{pos} MSC can also differentiate into osteoblasts (230, 235), which are the key cellular component of the endosteal niche. Megakaryocytes are located in the vascular niche but increased number of megakaryocytes results in an increase in the number of osteoblasts and bone mass (238). Megakaryocytes can increase osteoblasts proliferation in a cell to cell contact manner (238-240). Taken together, these data demonstrate a possible interaction between the endosteal niche and the vascular niche.
Development of Lymphoid and Myeloid Progenitors from HSC

The most immature BM progenitors are the Lin<sup>neg</sup> CD117<sup>pos</sup> (c-kit) and Sca1<sup>pos</sup> cells and thus are termed LSK cells. The LSK population contains HSC and their immediate progeny the multipotent progenitors (MPP). Expression of Flt3 distinguishes HSC from MPP within the LSK population; while HSC lack the expression of Flt3, MPP express Flt3 (241). As previously mentioned, HSC are self-renewing and can be divided into LT-HCS and ST-HSC. LT-HSC lack the expression of CD34 while ST-HSC express CD34 (242). LT-HSC proliferate infrequently and maintain self-renewal and multipotency throughout life. ST-HSC are derived from LT-HSC, have limited self-renewal ability (243), and give rise to MPP (244). MPP have the ability to generate erythroid, myeloid, and lymphoid progenitors (Fig. 7). The progression to MPP is the first step in lineage commitment since MPP no longer have self renewal ability (241).

The development of myeloid and lymphoid progenitors from MPP is still being untangled as the direct progenitor/progeny relationships have proven to be complicated. Evidence to support a common myeloid progenitor (CMP) and a common lymphoid progenitor (CLP) led to the hypothesis that these two lineages diverge from the MPP (245). However, it was later identified that erythroid and megakaryocytic lineage is the first lineage divergence from MPP (246-248) (Fig. 7). Current evidence demonstrates that several lymphoid progenitors still contain myeloid potential (249, 250). Thus, hematopoiesis is more complicated than the original CMP and CMP models predicted.

In an attempt to identify populations that contain predominantly myeloid or predominantly lymphoid potential, MPP population was separated into several different
Figure 7: Hematopoiesis in the mouse bone marrow. All blood cells develop from HSC.

For clarification the HSC population contains LT-HSC and ST-HSC. (Adapted from Zlotoff et al 2011).
subsets based on different cell surface molecules as well as expression of the recombination activating gene (Rag). MPP that express low levels of Flt3 can give rise to CMP (247). In contrast, MPP with high expression of Flt3 expression display high lymphoid potential and low myeloid potential (246, 247). MPP that do not express VCAM-1 gave rise to T and B cells \textit{in vivo}; whereas, MPP that expressed VCAM-1 were able to generate myeloid cells (248). A subpopulation of MPP has been shown to express Rag and is termed early lymphoid progenitors (ELP) or lymphoid primed multipotent progenitors (LMPP); from here on, the ELP/LMPP population will be identified as ELP (246, 251) ELP have both lymphoid and myeloid potentials (251). ELP gave rise to CLP, which have high B lineage potential but retain some myeloid potential and T lineage potential (82, 252). ELP also express CCR9 and are thought to contain the thymic seeding progenitor as these cells efficiently seed the thymus (253-255). A current paradigm of hematopoiesis is presented in Figure 7.

**Development of T cell Progenitors in the Bone Marrow**

The thymus does not contain lymphoid stem cells to maintain the development of naïve T cells. The BM is the source of stems cells that maintain thymopoiesis throughout life (10, 11). While several populations of cells in the BM have T lineage potential, not all are capable of settling in the thymus. The exact phenotypes of cells from the BM that circulate through the blood to enter the thymus and become ETP are not known due to the fast transition to ETP phenotype upon arrival in the thymus (82). HSC, MPP, and CLP all have T lineage potential; however, HSC do not express the chemokine receptors
required for homing to the thymus (82, 254). Both CLP and MPP are able to home to the thymus when intravenously transferred. MPP gave rise to a higher number of DP T cells than CLP, suggesting that the MPP contains the predominant thymic seeding progenitor (254). Because a subpopulation of ETP expresses both Flt3 and CCR9, and thus is phenotypically similar to MPP, it is suggested that the MPP population contains the BM precursor to thymic ETP (256, 257) (Fig. 8).

Several chemokine receptors including CXCR4, CCR7, and CCR9 have been implicated in the homing of T cell progenitors to the thymus as their respective chemokines CXCL12, CCL21, and CCL25 are expressed in the thymus (258). It is likely that all three contribute to homing of T cell progenitor to the adult thymus as all three are important and play redundant roles in homing to of T cell progenitors to the embryonic thymus (258). Deficient CXCR4 signaling does not result in altered homing to the thymus; however, CXCR4−/− mice have a block in thymopoiesis as CXCR4 is required for migration of DN subsets through the cortex (74, 259). CCR7−/− mice, CCR9−/− mice, as well as CCR7−/− CCR9−/− double knock out mice have reduced number of ETP, but display normal thymic cellularity (254, 260). These data suggest that while recruitment of T cell progenitors is reduced, T lineage potential and expansion is intact and the thymus can compensate for the low number of thymic seeding progenitors (254, 260). CCR7 and CCR9 are expressed on MPP and CLP (254, 257, 260). Flt3 signaling is required for the generation of CCR9+ MPP as FLT3−/− or FLt3L−/− MPP do not express CCR9 (254). While several progenitors in the BM have T lineage potential, the ability to home to the thymus for further maturation is not equivalent. Current data support the notion that the
**Figure 8**: Simplified diagram of hematopoiesis containing the CTP and CIP populations. Dotted lines diagram cells that can potentially travel to the thymus for further development into T cells.
MPP population is the predominate BM population that seeds the thymus as they express the chemokine receptor required for thymic homing and efficiently give rise to a large number of thymocytes when intravenously transferred (252).

HSC give rise to T cell progenitors (likely MPP) that travel to the thymus for further maturation and development. In addition, the presence of both γ/δ and α/β T cells in athymic mice indicate that extrathymic sites for T cell development also exist (261-263). The presence of T cell in the BM of athymic and normal mice has identified the BM as a possible organ for extra thymic T cell development (264-267). Identification of sj-TREC in athymic bone marrow cells further implicates the BM as a possible site that supports T lineage commitment and T cell development (268). A novel population of T cell progenitors has been identified independently by two groups (269-271). These two groups that identified this population of T cell progenitors have different views on where these T cell progenitors develop and whether expression of Foxn1 is required for the generation of this population of T cell progenitors.

Strober and colleagues identified a committed T cell progenitor (CTP) in the BM that they believe gives rise to T cells, independently of the thymus (269, 270, 272). CTP are defined as Linneg CD90pos and CD2neg and develop into an intermediate CIP stage that requires up regulation of CD2 (269, 273). When CTP are cultured, single positive T cells are generated; however, the presence of mature TCRα/βpos T cells inhibit this thymic independent pathway at the CIP stage (273). It could be argued that the presence of T cells in the cultures are due to small amounts of contaminating mature T cells that expand in vitro and that the CTP and CIP populations require the thymic environment for
development. When CTP were injected into lethally irradiated congenic mice, donor TCRα/β^pos^ T cells were found in the thymus; however, the number was low compared to that seen in the BM or spleen, indicating to Strober and colleagues that the CTP preferentially develop by an extra thymic process (270). The presence of donor TCRα/β^pos^ T cells in the thymus may be an artifact of irradiation that grants access of CTP to the thymus and does not reflect the in vivo ability of CTP to home the thymus. Alternatively, CTP may in fact home to the thymus as proposed by Krueger et al. (271).

Independently, Krueger et al. identified a circulating T cell progenitor in the blood which was also termed “CTP” (271). Phenotypically, Krueger’s CTP overlap with Strober’s CTP (271). Krueger et al. also showed that CTP express preTα and have high T lineage potential with very limited B and myeloid potential (271). Krueger demonstrated that the CTP in the blood express thymic homing molecules such as CCR9 and P-selectin and home to the thymus (271). Thus, whether CTP are thymic independent or thymic dependent is still debatable. Figure 8 is a simplified diagram of hematopoiesis that includes the CTP and CIP populations that may home to the thymus and generate T cells.

It also remains controversial whether the functional development of CTP requires Foxn1 expression. When isolated donor CTP from nude Foxn1^-/-^ BM were injected into nude congenic hosts, donor T cells failed to develop suggesting to Strober’s group that Foxn1 is required for the generation of functional CTP from HSC (274). Furthermore, Strober’s group demonstrated that the number of CTP in nude BM was equivalent to Wt; however, CTP from nude BM showed reduced proliferation and increased cell death,
resulting in a reduced total number of CIP (275). Kruger et al. also reported that the number of CTP in nude mice was equivalent to Wt (271), just as reported for CTP in the BM of nude mice by Strober (275). Contrary to Strober’s findings, Krueger demonstrated that CTP from nude mice develop into DP cells at levels equivalently to that found in Wt CTP when cultured on the OP9-DL1 (271). These data demonstrate that either the functional development of CTP does not require Foxn1, or that any dysfunction of CTP from nude mice can be overcome by a high level of Notch signaling.

Cytokine and Transcriptional Regulation of the Development of Myeloid and Lymphoid Progenitors from HSC

Cytokines and transcription factors can influence myeloid and lymphoid lineage decision in hematopoietic progenitors. In few instances, expression of a specific transcription factor or signaling though a cytokine receptor is required for lineage commitment of a progenitor population. In other instances, the presence or absence of transcription factors or cytokine does not directly indicate specification toward or away from a specific lineage. Differentiation to myeloid or lymphoid lineage is a complicated balance between multiple signals that have the potential to influence lineage choice fate. Interest in identifying factors that influence the development of myeloid and lymphoid progenitors was made possible with the bone marrow colony assay. In this assay methylcellulose media allows for the formation of identifiable granulocyte and macrophage colonies (276). This assay set into motion investigations to identify colony stimulating factors, also known as hematopoietic growth factors and cytokines that
promote the development of different hematopoietic lineages (277). Currently, several different cytokines that influence lineage development from HSC as well as cytokines responsible for maintaining HSC function have been identified (summarized in Fig. 9). HSC express c-kit (278) and signaling by its ligand SCF is important for HSC survival (279, 280). SCF assists in maintaining HSC in their quiescent state and prevents HSC differentiation (281). HSC also express the G-CSF receptor (282), and G-CSF signaling mobilizes HSC from their vascular niche (283).

Cytokines and signaling that promote the development of MPP from HSC remains to be resolved; however, several cytokines have been identified to play a role in lymphoid lineage commitment and development. Mice deficient in Flt3 signaling have a reduced number of CLP, indicating that Flt3L is important for the generation of CLP and their B lineage progeny (284-286). IL7 was also demonstrated to be critical for B cell development. Addition of IL7 to methylcellulose cultures allowed for the development of pre-B cell colonies whereas these colonies do not develop without IL7 (287). The ability of IL7 to support the development of lymphoid lineage has also been demonstrated in vivo. B cell development is arrested when there is a deficiency in IL7 or IL7 receptor as signaling through IL7 is required for the up regulation of transcription factors required for B lineage development (288, 289).

Cytokines that promote the development of myeloid and erythroid cells have also been identified. Perhaps the most well known hematopoietic cytokine is erythropoietin (EPO), which promotes the development of red blood cells. GM-CSF promotes the development of granulocytes and monocytes; however, mice deficient in GM-CSF have
normal hematopoiesis (290), suggesting redundant functions among the cytokines that can promote the generation of granulocytes and monocytes. In fact, GM-CSF, G-CSF, and M-CSF all support the development of granulocytes and monocytes. The number of neutrophils and monocytes are reduced in mice deficient in either M-CSF or GM-CSF (291). The number of monocytes and neutrophils are present but reduced in tripled knock out mice deficient in M-CSF, G-CSF, and GM-CSF suggesting that these cytokines are not essential for lineage commitment, but are required for the survival and expansion of progenitors (292).

Two models of how cytokines regulate hematopoiesis have been proposed, the stochastic model and the instructional model (293). The stochastic model suggests that cytokines are not required for the commitment of multipotent cells to a specific lineage, but that cytokines promote survival and expansion of lineage specific progenitors. In the instructive model, cytokines transmit signals that result in lineage commitment. The finding that triple knock out mice deficient in M-CSF, G-CSF, and GM-CSF have monocytes and granulocytes but at a reduced number supports the stochastic cytokine model (292). M-CSF, G-CSF, and GM-CSF is not required for lineage commitment as granulocytes and monocytes are present; however, they are required for the expansion and survival as the number of granulocytes and monocytes are reduced. The stochastic model was also supported by studies in which the cytoplasmic tail of the G-CSF receptor was replaced with erythropoietin, which did not result in the development of erythrocytes at the expense of granulocytes (294, 295). Support for the instructional model of cytokines was observed when the IL-2 receptor is ectopically expressed on CLP and
signaling through IL2 receptor by its ligand IL2 resulted in the differentiation of CLP to myeloid cells (296, 297). When CLP ectopically expressed the GM-CSF receptor and not the IL7 receptor, CLP differentiated to myeloid cells, indicating that GM-CSF cannot replace the critical role of IL7 in the development of B cells (297). Thus, the current data in the literature support both the stochastic model and the instructional model of how cytokines influence hematopoiesis.

Several transcription factors have been identified to play a role in lineage commitment during hematopoiesis. However, both myeloid and lymphoid lineage progenitors expresses the same transcription factors, but at different levels. The transcription factor PU.1 is expressed in common myeloid progenitors (CMP) as well as lymphoid lineage progenitors (298-300). It is thought that the timing and level of expression of transcription factors during hematopoiesis dictates lineage development. PU.1 is expressed at high levels in CMP and at lower levels in B lineage progenitors (299). The expression level of PU.1 is crucial for lineage specification as high levels of PU.1 in progenitors of the fetal liver was shown to induce macrophage generation whereas lower levels resulted in B lineage formation (301). PU.1-/- B cell progenitors are able to give rise to B cells at reduced rates indicating that PU.1 assists in B lineage development, but is not required (302). Thus, the expression level of PU.1 can influence lineage decisions.

The transcription factor E2A regulates B lineage commitment and is required for the development of mature B cells (303). E2A-/- HSC give rise to T, NK, erythroid and myeloid cells but fail to develop into mature B cells when adaptively transferred (304).
A binding site for E2A has been identified in the regulatory region of early B cell factor (EBF) and forced expression of E2A was shown to up regulate EBF expression (305, 306). Forced expression of EBF in the absence of E2A or PU.1 can promote B lineage differentiation, suggesting that E2A and PU.1 function to up regulate EBF in B lineage commitment and development (307, 308).

Ikaros is required for both B and T lineage development; Ikaros expression in hematopoietic progenitors represses expression of transcription factors and signaling molecules required for erythroid and myeloid lineage, and induces those required for lymphoid (313,309). Ikaros is required for the development of CLP as Ikaros<sup>−/−</sup> BM does not contain CLP (310). Ikaros null mice contain the ELP population suggesting that Ikaros is not required for the development of ELP (311). Ikaros<sup>−/−</sup> mice also retain normal levels of ETP indicating that ETP develop independently of CLP and that Ikaros is not required for the development of T cell progenitors (82); however, Ikaros is required for development of T cells in the thymus and T cell progenitors in Ikaros null mice have a slow progression to the DN2 and DN3 stages (312).

As mentioned previously, T cell development required Notch signaling and that T cell commitment does not occur until the DN3 stage of thymopoiesis (89, 90, 314). Down regulation of the transcription factors PU.1 and C/EBPα at the DN3 stage is required for T lineage commitment (315-317). PU.1 and C/EBPα expression in early thymocytes can result in the development of dendritic cells and macrophages (317). Notch activation results in the inhibition of PU.1 and C/EBPα thus promoting T lineage
commitment in the thymus (317). A summary of transcription factors that influence lineage commitment is summarized in Figure 9.

**Hematopoiesis and Aging**

**HSC and Aging**

Aging results in intrinsic changes in HSC and thus hematopoiesis. Intrinsic alterations in aged HSC was demonstrated by transferring young and aged HSC in to irradiated young hosts. Aged HSC had reduced homing and engrafting potential compared to young HSC (318, 319). Intrinsic changes in HSC contribute to differences in lineage development and commitment. The occurrence of leukemia increases with age (320). Interestingly, there is a difference in the types of leukemia found in the elderly compared to young individuals. The predominant form of leukemia in adults is myeloid lineage, while the predominant form of leukemia in young is lymphoid lineage, suggesting alterations in HSC lymphoid and myeloid potential with age (321). Young BM cells transformed with the BCR-ABL (P210) oncogene gave rise to both myeloid and lymphoid malignancies; however, transformed aged BM cells predominantly gave rise to myeloid with rare instance of lymphoid malignancies (321). This is in line with previous reports showing that the lymphoid lineage potential is reduced with age while myeloid potential of HSC is maintained with age (318, 322-324). Experiments by Kim et al. demonstrated that changes in the HSC potential with age are intrinsic as aged HSC adaptively transferred to a young environment still resulted in a reduced B lineage engraftment and higher myeloid engraftment compared to young HSC transferred into a
Figure 9: Cytokines and transcription factors that influence lineage commitment decisions and the stages in which they are important.
young environment (322). Furthermore, lymphoid specific genes are expressed at lower levels in aged LT-HSC while myeloid specific genes are expressed at higher levels compared to young, suggesting intrinsic skewing toward myeloid lineage in aged HSC (288, 289, 323).

Despite the decline in lymphoid potential in aged HSC, the number of HSC increase with age (318, 323, 324). Changes in the number of HSC with age are mouse strain dependent (325). In C57BL/6 mice (318, 323, 324), as well as in human (326, 327), the number of HSC increase with age. It was hypothesized that the increase in the number of HSC results from a feedback mechanism compensating for functional decline in the lymphoid potential of HSC (324, 328). It was also suggested that the increase in the number of HSC is the result of a differential block in the development of HSC to MPP as supported by a reduced number of MPP with age (13). The exact mechanism for the increase in the number of HSC with age remains to be determined.

The MPP give rise to CLP, ETP, and myeloid progenitors. With age, the number of MPP decline, which is thought to contribute to the reduced number of ETP in the aged thymus (13). This finding suggests that thymic involution is initiated in the BM with a reduction in the number of thymic seeding progenitors. Besides giving rise to ETP, the MPP population gives rise to CLP which also decline in numbers with age, contributing to reduced B lymphopoiesis in aged BM (329, 330). Despite the decline in the number of MPP, the numbers of myeloid progenitors do not decline with age (330). A summary of the age associated changes in the number of BM progenitors is shown in Figure 10.
Figure 10: Changes in BM and thymic populations with age. The number of ETP decreases with age and the decline in the BM precursor to ETP, MPP also declines in aged mice. The number of CLP and B cells decreased in aged BM. No changes in the number of myeloid progenitors occurred with age. It is not known how CTP and CIP are affected in aged mice.
Aging and the HSC Niche

A limited number of studies have examined effects of the aged microenvironment on HSC and the HSC niche. Early studies in which young and aged bones were placed subcutaneously in young mice demonstrated that young HSC reconstituted the aged BM environment at lower levels then the young environment (331). This experiment suggested that there are age-associated differences between the young and aged HSC niches. This notion is supported by the fact that long term BM stroma cultures from aged mice fail to completely support the development of hematopoietic progenitors (332). Furthermore, aged HSC are located a greater distance away from the endosteal niche than their young counterparts (333), suggesting that the aged endosteal HSC niche does not retain HSC as well as the young. This was also shown when young and aged mice were treated with GM-CSF. GM-CSF is known to mobilize HSC from their BM niches into circulation; however, a larger number HSC are mobilized in aged mice treated with GM-CSF than young mice suggesting reduced adhesion of aged HSC to their niche (283). Taken together these experiments demonstrate age-associated alterations in the HSC niche that can affect HSC function.

Foxn1 and Nude Mice

Nude mice were first described in 1966 (135). These mice are born without hair or vibrissae, have reduced body weight by 3 weeks of age, reduced fertility, and increased mortality (135). Survival of nude mice past 2 weeks of age is 45% and past 12 weeks of age is approximately 10% (135). It was later identified that nude mice also
lacked a thymus (334). It took another 30 years before the gene responsible for the nude phenotype was identified as Foxn1 (15, 16). Foxn1 is located on chromosome 11 in mice (chromosome 17 in humans) and contains 9 exons with two alternative first exons and two promoters (335, 336).

In the fifteen years since the discovery that the Foxn1 gene is responsible for the athymic condition in nude mice, little information into the regulation of Foxn1 has been published. The existing known information about genes that regulate Foxn1 as well as genes regulated by Foxn1 was obtained from studying skin. Nude mice produce hair fibers, but the fiber coils in the hair canal and fails to penetrate the epidermis due to defective keratinization (337, 338-340), suggesting that Foxn1 regulates keratin expression.

Beside alterations in the skin and thymus, Zipori et al. showed that nude mice also have alterations in their BM. The average number of nucleated cells in the BM of a nude mouse is 30 million compared to 40 million found in Wt BM (19). The frequency of nude BM to form CFU in Wt spleen when adoptively transferred were equivalent to Wt mice however the total number of CFU are reduced based on the reduced number of total nucleated cells (19). Nude BM also failed to protect against lethal irradiation when adaptively transferred into lethal irradiated Wt hosts (19), suggesting a role for Foxn1 in hematopoiesis. Of the few irradiated mice that survived to 100 days after receiving BM from nude, the antibody titer to hemagglutinin and hemolysin after injection with sheep red blood cells was lower than host mice that received Wt BM (19). Taken together, these data suggest a decreased in HSC homeostasis and hematopoiesis in nude BM.
Despite these observations the numbers of HSC and MPP in nude mice have not been determined. However, the number and function of CTP and CIP from nude mice have been examined.

It was shown that the cell cycle activity of CTP and the number of CIP are reduced in nude mice (275), suggesting a role for Foxn1 in the development of T cell progenitors. As mentioned previously, a role for Foxn1 in the generation of functional CTP and whether CTP develop extrathymically remains controversial. With age, nude mice experience an increase in the number of CD90$^{pos}$ T cells found in the periphery (341). Thus it is possible that the increase in the number of T cells in the periphery of aged nude mice is the result of an age-associated increase in extrathymic T cell development pathway.

The nude mouse model served a valuable system in identifying Foxn1 as the critical gene responsible for thymic organogenesis and TEC differentiation (15, 16). A role for Foxn1 in the postnatal thymus is just starting to be elucidated. It has been shown that the decline in Foxn1 expression correlates with the decline in thymocyte number with age and that postnatal reduction of Foxn1 results in premature thymic involution as seen by a decrease in the number and proliferation of mTEC (14, 17, 18). However, it is not known if overexpression of Foxn1 could prevent thymic involution. As mentioned above, the BM of nude mice have alterations in hematopoiesis and possibly the development of T cell progenitors (19, 275). It has not been shown whether Foxn1 affects the development of progenitors in the BM and if Foxn1 is expressed by BM cells.

To better understand the role of Foxn1 in postnatal life, it is important to know how
expression of Foxn1 affects the postnatal thymus and if Foxn1 has a role in BM which produces T cell progenitors.
CHAPTER III
OVER EXPRESSION OF FOXN1 ATTENUATES AGE-ASSOCIATED THYMIC INVOLUTION

Introduction

The thymus is the primary site for the development of naïve T cells in post natal life (2, 3). Despite this important role, the thymus involutes with age and the number of naïve T cells produced from the thymus is reduced (4, 5). To compensate for reduced naïve T cell output, memory T cells in the periphery undergo homeostatic proliferation to maintain the size of the peripheral T cell pool (143-146). The switch from a naïve T cell phenotype in the periphery to a memory phenotype, combined with alterations in functions of the peripheral T cells, culminates in a reduced immune response in the elderly (147, 164, 167-171, 174, 175). Thus, thymic involution has a profound effect on the number and functions of naïve T cells and memory T cells. Approaches to prevent or reverse thymic involution and to promote the development of naïve T cells with age could ameliorate the immune system of the elderly.

A reduction in thymopoiesis with age is reflected by changes in both the number and function of T cell progenitors as well as alterations in the thymic architecture. The number of ETP as well as their T lineage potential is reduced in the aged mice (12). ETP require interaction with TEC for proper development. TEC are the predominate stromal cell of the thymus responsible for thymopoiesis (4-9). With age, the number of TEC
declines (81) and the architecture of cTEC and mTEC is disrupted (4, 5, 149). Expression of Foxn1 in thymic stroma decreases with age and correlates with a decrease in thymocyte number, suggesting that Foxn1 has a role in thymic involution (14). Foxn1 is expressed predominantly in mTEC but scattered cTEC also are positive for Foxn1 (124). Studies in which Foxn1 was deleted postnataley have shown that thymic involution occurs prematurely with reduced number and cycling of mTEC, suggesting that Foxn1 is required to maintain mTEC in the postnatal thymus (17, 18, 342). Using a Foxn1 transgenic (Foxn1Tg) mouse model that over expressed Foxn1 under the human keratin 14 (K14) promoter, experiments in this chapter were designed to test the hypothesis that over expression of Foxn1 prevents thymic involution. We generated two Foxn1Tg lines (line 5 and line 60) on the C57BL/6 background. Data from these lines were pooled as we observed no functional differences among the transgenic lines.

Results

Expression of Foxn1 in thymic stroma of young and aged Wt and Foxn1Tg mice.

The levels of Foxn1 expression in the thymic stroma of Foxn1Tg were measured using quantitative RT-PCR. Primers that do not distinguish between endogenous Foxn1 (Foxn1endo) transcripts and transgene transcripts (Foxn1Tg) were used to determine the total levels of Foxn1 (Foxn1Total). To determine the contribution of the transgene to total levels a second set of primers was used that only recognize the transgene transcripts (Foxn1Tg). The specificity and locations of the primers in endogenous and transgene loci are shown in Figure 11. The endogenous Foxn1 (Foxn1Endo) RNA levels were calculated
by subtracting transgene levels from total Foxn1 (Foxn1\textsuperscript{endo} = Foxn1\textsuperscript{Total} – Foxn1\textsuperscript{Tg}). In two month old Foxn1\textsuperscript{Tg} mice, expression of the transgene in the thymic stroma was 4-fold higher than endogenous Foxn1 (Fig. 12A). In both lines of Foxn1\textsuperscript{Tg} mice, line 5 and line 60, the expression of total Foxn1 was equivalent and did not decline with age even in thymi from very old transgenic mice 35 months of age (Fig. 12B). Similar to BALB/c mice (14), expression of Foxn1 declined in aged Wt C57BL/6 thymic stroma (Fig. 12B). The endogenous Foxn1 expression in 2 months old (line 60) thymic stroma was 7.2-fold higher than endogenous levels in 2 month Wt (Fig. 12 A compared to 12B), suggesting that expression of the transgene induced expression of the endogenous Foxn1.

**High levels of Foxn1 expression do not affect thymic architecture in young mice.**

Immunofluorescence staining was performed on young Wt and Foxn1\textsuperscript{Tg} thymi to determine if high levels of Foxn1 alter the thymic architecture. A rabbit polyclonal antibody raised against the mouse Foxn1 peptide revealed that Foxn1 was expressed predominantly in the medulla of a Wt thymus (2-3 mo) with scattered Foxn1\textsuperscript{pos} cells in the cortex (Fig. 13, panels A, B inset b). Expression of Foxn1 was similar in Foxn1\textsuperscript{Tg} thymus with Foxn1 detected primarily in the thymic medulla and scattered Foxn1\textsuperscript{pos} cells in the thymic cortex; however, the staining appeared more prominent in Foxn1\textsuperscript{Tg} compared to the Wt thymus (Fig. 13, panels A, B, D and E, insets b, e). The enhanced intensity staining of Foxn1 reflected the increase in the expression at the mRNA levels of both the transgene and endogenous Foxn1. Thus, the staining pattern of Foxn1 in young Wt and Foxn1\textsuperscript{Tg} thymi were similar with an apparent higher intensity in Foxn1\textsuperscript{Tg}. 
Figure 11. A-B: Locations of Foxn1 primers and specificity by RT-PCR. A. RT-PCR shows specificity of primers that detect both the endogenous and transgene Foxn1 and primers that only detect transgene Foxn1 transcripts. Each lane represents results from a Wt or Foxn1Tg mouse (line 60). B. Diagram showing the relative positions of the forward and reverse primers that detect either both endogenous and transgene (black arrows) or only transgenic (red arrows) Foxn1 transcripts.
A

Transgene Foxn1

Exon

5' 6' 7' 8' 9'

Endogenous Foxn1

5' 6' 7' 8' 9'

Gapdh

Endogenous and transgene Foxn1 (200 bp)

Transgene Foxn1 (338 bp)

WT Foxn1

Tg Foxn1

B

Endogenous Foxn1

5' 3'

Transgene Foxn1

5' 3'

Human K14 Poly A

Foxn1 Poly A
**Figure 12**: Quantification of endogenous and transgenic *Foxn1* expression by quantitative RT-PCR. 

A. Expression of the endogenous and transgene *Foxn1* in 2 mo (n=3) and 23-28 mo (n=3) old transgenic line (line 60). The numbers of transcripts/µg total RNA were calculated from a standard curve after normalization with the mouse house keeping gene *Gapdh*; each sample was performed in triplicates. The endogenous *Foxn1* levels were determined by subtracting the total *Foxn1* (endogenous plus transgene) from that of the transgene. White bars denote the total *Foxn1* levels; gray bars are transgenic *Foxn1*; black bars are endogenous *Foxn1*. Errors bars are standard deviations; 

B. Expression of total *Foxn1* in Wt (n= 2 at 2 mo; n= 3 at 24 mo) and in the two *Foxn1* transgenic lines (line 60: n=3 at 2 mo and n= 3 at 24 mo; line 5: n= 4 at 25-35 mo). Wt: white bar is 2 mo of age and gray bar is 24 mo of age. *Foxn1*Tg: white bar is 2 mo, and gray bar is 24 mo (line 60); crossed bars are 25-35 mo of age (line 5). Y axis is in logarithmic scale; errors bars are standard deviations.
Figure 13. Detection of Foxn1 expressing stroma cells in the thymus of Wt and Foxn1Tg mice (2-3 mo) by immunofluorescent staining. Frozen sections (4-5 µm) were fixed in acetone and stained with a rabbit anti-Foxn1 antibody (IgG fraction) or purified rabbit IgG control antibody, followed by incubation with FITC-conjugated goat anti-rabbit IgG F(ab)2’. Panels A, B, D and E were stained with anti-Foxn1 antibody; C and F were stained with purified rabbit IgG antibody. Slides were studied using Zeiss confocal microscopy at 25X (A, D, C and F) and 50X (B, E). Insets b and e represent an enlargement of the medulla regions in B and E respectively. Bar scales are 50 µm. M=medulla. C=cortex.
High levels of *Foxn1* expression do not affect the distribution of thymocyte subsets and thymopoiesis.

To determine if high levels of *Foxn1* affect the distribution of thymocytes populations, and thus disrupt thymopoiesis, the frequencies of DN, DP, SP CD4, and SP CD8 cells were measured using flow cytometry. No differences were observed in the relative proportions of these populations between young Wt and young *Foxn1*Tg (Fig. 14A). The distribution of DN, DP, and SP CD4 and SP CD8 was not affected by age in Wt or in *Foxn1*Tg mice (Fig. 14B). The only noticeable difference between young Wt and young *Foxn1*Tg was the percent of CD8\textsuperscript{pos} T cells that expressed CD3. There was a slightly higher percent of CD8\textsuperscript{pos} CD3\textsuperscript{pos} cells in the thymi of young *Foxn1*Tg compared to young Wt (Fig. 14). Taken together over expression of *Foxn1* does not affect thymopoiesis and the distribution of thymocyte population in young or aged mice.

**Over expression of *Foxn1* attenuates the age-associated decline in thymocyte number.**

Because thymopoiesis did not appear to be affected by high expression levels of *Foxn1*, total numbers of thymocytes were enumerated to determine if over expression of *Foxn1* prevented the decline in their number with age. Thymocyte numbers in young *Foxn1*Tg were equivalent to young Wt (Fig. 15). With age, the total number of thymocytes declined; however, the decline was less severe in *Foxn1*Tg compared to Wt. When compared to 3 months of age, Wt mice 16-10 months had a 3.7- fold decline while *Foxn1*Tg experienced a 1.4-fold decrease. By 20-26 months of age Wt experienced a
Figure 14: Distributions of CD4⁰⁰, CD8⁰⁰ thymocyte populations and expression of CD3 within the CD4⁰⁰, CD8⁰⁰, DP and DN thymocyte subsets in Foxn1Tg and Wt mice with age. Young Wt and Foxn1Tg mice were 2-3 months of age. Aged Wt and Foxn1Tg were 16-32 months of age. Representative flow cytometric profiles of one young and aged Wt and one young and aged Foxn1Tg mouse. Number in each quadrant represents the average percentage ± SD of each subset from 5 young Wt, 5 young Foxn1Tg, 4 aged Wt, and 6 aged Foxn1Tg mice.
A  

Young

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<th>CD4pos CD8pos</th>
<th>CD4neg CD8neg</th>
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<td>88±1.9</td>
<td>87±2.3</td>
<td>59±6.2</td>
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<td>5±.5</td>
<td>6±.8</td>
<td>41±6.2</td>
<td>6±.7</td>
</tr>
<tr>
<td>Foxn1Tg</td>
<td>3±1</td>
<td>86±2</td>
<td>89±2.5</td>
<td>73±5.5</td>
</tr>
<tr>
<td></td>
<td>6±.8</td>
<td>27±1.6</td>
<td>5±.5</td>
<td>26±1.6</td>
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B  

Aged

<table>
<thead>
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<th></th>
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<th>CD8pos</th>
<th>CD4pos CD8pos</th>
<th>CD4neg CD8neg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>.5±.1</td>
<td>93±1.5</td>
<td>75±9</td>
<td>5±1</td>
</tr>
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<td>2±1</td>
<td>12±7.2</td>
<td>77±8.2</td>
<td>56±4.5</td>
</tr>
<tr>
<td>Foxn1Tg</td>
<td>9±.2</td>
<td>87±4</td>
<td>33±16</td>
<td>48±28</td>
</tr>
<tr>
<td></td>
<td>5±3</td>
<td>71±12</td>
<td>66±17</td>
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<td></td>
<td>6±2</td>
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Figure 15: Thymocyte numbers in 3 mo, 16-19 mo and 20-26 mo old Wt (white bars) and Foxn1Tg (line 60) (black bars). p values from comparison between Wt and Foxn1Tg as determined by t-test or Mann-Whitney are: *p = 0.043 ** p = 0.012, respectively.

Numbers in parentheses denote the number of mice.
5.3-fold reduction while Foxn1Tg had a 3-fold reduction compared to mice 3 months of age. In aged thymi, not only did Foxn1Tg have a higher number of thymocytes compared to aged matched Wt, but the thymi were larger with few foci of adipose tissue (Fig. 16, fat denoted with *). Thus, over expression of Foxn1 attenuated the decline in thymopoiesis with age as seen by a higher total thymocyte number with a normal thymocyte population distribution. In agreement with this interpretation, two way ANOVA analysis shows that while age negatively affects the number of thymocytes in Wt mice, over expression of Foxn1 in aged mice results in a higher number of thymocytes and minimizes the affect of age. Table I and Table II summaries of all two-way ANOVA and can be found on pages 185-186.

**Over expression of Foxn1 prevents the decline in ETP frequency and attenuates the decline in ETP number in aged mice.**

Naïve T cells develop from ETP in the thymus (82, 83). In aged Wt thymi, the frequency and total number of ETP decline, contributing to the decline in thymopoiesis (12). To begin elucidating a mechanism by which over expression of Foxn1 attenuates the decline in thymocyte number and thymopoiesis, frequency and total number of ETP in young and aged Wt and Foxn1Tg mice were determined using flow cytometry. ETP were identified as thymocytes that are Linneg CD117pos CD44hi CD25neg and CD127neg (82, 83) (Fig. 17). In agreement with others, the frequency of ETP/10^5 cells declined in aged Wt (#p=0.009) (12); however, aged Foxn1Tg mice had an ETP frequency equivalent to young and 2.6-fold higher than aged Wt (Fig. 18A *p=0.038). Two way ANOVA
**Figure 16.** Gross morphology of thymi from 2 Wt (Top) and 2 Foxn1 transgenic (line 60) mice 13 months of age (bottom). The numbers denote total number of thymocytes obtained from each thymus. *Denotes translucent/fatty areas of the thymus.
Figure 17: Identification of ETP using flow cytometry. ETP were identified as thymocytes that are Lin$^{\text{neg}}$ CD117$^{\text{pos}}$ CD44$^{\text{hi}}$ CD25$^{\text{neg}}$ and CD127$^{\text{neg}}$. 
**Figure 18:** Frequency and total number of ETP in young and aged Wt and Foxn1Tg mice. A. Frequencies of ETP in Wt (white bars) and Foxn1Tg (black bars). ETP are defined as Linneg CD117pos CD44hi CD25neg CD127neg. Wt 3 mo vs. Wt 18-24 mo #p=0.009; Foxn1Tg 25-36 mo vs. Wt 18-24 mo *p=0.038. B. Total number of ETP in thymi from Wt (white bars) and Foxn1Tg (black bars) mice. Wt 3 mo vs. 21-24 mo #p=0.003; Foxn1Tg 3 mo vs. 21-30 mo ##p<0.001; Foxn1Tg 21-30 mo vs. Wt 21-24 mo *p=0.047. Error bars are SD. Numbers in parentheses represent the number of mice in each group.
analysis of ETP frequency revealed that over expression of Foxn1 has an affect on ETP frequency and that over expression of Foxn1 prevents the decline with age. Age also affected the total number of ETP. The total number of ETP in aged Wt and Foxn1Tg thymi was reduced compared to young (*p=0.003 Wt; **p<0.001 Foxn1Tg), but aged Foxn1Tg have a 2.7-fold higher ETP number per thymus compared to aged Wt (Fig. 18B *p=0.047). Two way ANOVA analysis of ETP number demonstrates that age results in a decrease in the number of ETP in Wt mice; however, over expression of Foxn1 results in a higher number of ETP in aged mice and minimizes the effect of age. Taken together, over expression of Foxn1 prevented the decline in ETP frequency and attenuated the decline in ETP number potentially contributing to higher number of thymocytes observed in the aged thymus.

**ETP from Foxn1Tg and Wt mice display similar T lineage potential.**

ETP in the thymus have high T lineage potential, however they also have myeloid and B lineage potential (82, 84, 85). To determine if over expression of Foxn1 in TEC increases T lineage potential of ETP, ETP were electronically sorted and cultured on a BM stroma cell line that over expresses the Notch ligand DL1 (OP9-DL1). After two weeks in culture, cells were harvested and flow cytometry was used to determine T lineage commitment and differentiation as measured by the expression of CD90 and CD25. OP9-DL1 cells that express GFP were excluded from the analysis by gating on GFP negative cells and GFP negative cells were analyzed for the expression of CD90 and CD25 (Fig. 19). The cell surface molecule CD90 is the first molecule that is up regulated
upon commitment to T lineage, and CD25 is expressed in the developing thymocytes at the DN2 and DN3 stages (86, 87) (Fig. 19). Approximately 95% of cells in culture from Wt ETP 1-4 months of age expressed CD90 (Fig. 20A). The commitment potential of ETP to T lineage did not change in aged Wt ETP cultures (Fig. 20A). The percent of cells that expressed CD90 in cultures from Foxn1Tg ETP 1-4 mo of age was equivalent to aged matched Wt (Fig. 20A). Similar to that found in aged Wt mice, T lineage potential of ETP did not change with age in Foxn1Tg mice, suggesting that over expression of Foxn1 does not affect ETP T lineage potential with age.

Differentiation of ETP to the DN2/DN3 stages of T cell development is marked by the up regulation of CD25 (86, 87). To determine if over expression of Foxn1 affected ETP differentiation, expression of CD25 was measured amongst the cells that expressed CD90. No differences were seen between young and aged Wt ETP cultures (Fig. 20B). There was also no age difference in the expression of CD25 amongst CD90<sup>pos</sup> cells from young and aged Foxn1Tg ETP cultures. Furthermore, there was no difference in T lineage differentiation between Foxn1Tg and aged matched Wt. Together, these data indicate that over expression of Foxn1 does not affect T lineage differentiation potential of ETP to the DN2/DN3 stages (Fig. 20B).

**Aged Foxn1Tg thymic microenvironment does not increase the generation of ETP but may increase Rag activity.**

To determine if the higher number of ETP found in aged Foxn1Tg thymi resulted from an increase in the generation of ETP, adaptive transfer experiments were performed
Figure 19: Flow cytometry analysis of ETP cultured on OP9-DL1 (GFP). Cells that were GFP\textsuperscript{neg} were analyzed for the expression of CD90 to determine T lineage commitment. Within the GFP\textsuperscript{neg} CD90\textsuperscript{pos} population cells that expressed CD25 were measured to determine T lineage differentiation.
**Figure 20:** ETP commitment to T lineage and differentiation to DN2/DN3 stage. ETP were electronically sorted from 1-4 and 21-32 months of age Wt and Foxn1Tg mice. Cells were cultured for 2 weeks on OP9-DL1 in the presence of SCF, Flt3L, and IL7. A. Percent of cells in culture that have committed to T lineage as indicated by expression of CD90. B. Differentiation of T lineage cells to DN2/DN3 as measured by expression of CD25. Error bars are SD. Numbers in parentheses denote the number of mice.
using BM LSK cells from the H2-SVEX transgenic mice (CD45.1 background, a gift from Dr. Rachel Gerstein, University of Massachusetts). If the Foxn1Tg thymic environment promotes the generation of ETP, then we would expect a higher number of donor derived ETP in the aged Foxn1Tg thymus compared to Wt thymus. H2-SVEX LSK cells also allowed us to measure Rag activity in donor ETP generated in the Foxn1Tg hosts versus Wt hosts. H2-SVEX mice contain a construct in which the H2-K promoter drives expression of a violet activated GFP (VEX) (343). The VEX gene is orientated in the antisense direction and is flanked by V(D)J recombination signal sequences (RSS) (343)(Fig. 21). When a cell from the H2-SVEX mice expresses Rag, Rag binds to the RSS and rearranges the VEX gene such that it reverses to the sense orientation allowing VEX expression (343) (Fig. 21). The expression of VEX reflects Rag activity in developing lymphocytes (343). Once the VEX construct is inverted the cell will continue to express VEX even after a cell no longer expresses Rag (343). If the Foxn1Tg thymic environment promotes Rag activity in ETP, then we would expect to see more VEXpos donor ETP in the aged Foxn1Tg thymis compared to aged Wt.

Donor LSK cells (8,000-16,000 cells) from young CD45.1pos H2-VEX mice were transferred into non-irradiated Wt and Foxn1Tg hosts 17-21 months of age. Ten weeks post transfer, flow cytometry was used to determine the generation of ETP by measuring the frequency of ETP among donor cells in aged Wt and Foxn1Tg thymi. The expression of VEX amongst donor ETP was used to determine Rag activity in ETP which is required for TCR gene rearrangement. The gating of donor ETP frequency and VEX expression among donor ETP is shown in Figure 22.
Figure 21: Recombination of the VEX construct. The VEX construct is driven by the H2-K promoter and is flanked by RSS (yellow triangles). In cells that do not express Rag the VEX construct is in the antisense orientation. When Rag is expressed in a cell the RSS direct inversion of the VEX construct allowing for its expression.
Aged Wt and Foxn1Tg hosts generated the same frequency of donor ETP per 100,000 donor thymocytes, suggesting that the aged Wt and Foxn1Tg thymic microenvironments are equivalent in the generation of ETP from young donor LSK cells (Fig. 23A). Previously it was shown that in H2-SVEX mice 1-2 months of age, approximately 50% of ETP express VEX, reporting Rag activity (84). We found that approximately 36% of the CD45.1 donor ETP in aged Wt thymi expressed VEX (Fig. 23B). The frequency of VEX^pos donor ETP in aged Foxn1Tg host was approximately 60% (Fig 23B). While not statistically different this trend may suggest that over expression of Foxn1 in the thymic microenvironment increase the efficiency of Rag activity.

**Over expression of Foxn1 minimizes age-associated changes in thymic architecture.**

Aged mice experience disruptions in the thymic architecture including a contraction in the thymic epithelial compartment that supports thymopoiesis and an expansion of the perivascular tissue filled with adipose and fibroblasts that do not support thymopoiesis (4, 5). Figure 24A and C shows thymi in situ from a Wt mouse 24 months of age (A) and a Foxn1Tg mouse 31 months of age (C). The yellow arrows in Fig. 24C point to numerous translucent areas representing adipose in the aged Wt thymus; in contrast, a thymus from the older Foxn1Tg appears opaque with no obvious appearance of adipose. Hematoxylin and Eosin (H&E) staining on these thymi revealed that the Wt thymus (Fig. 24B) had histological changes associated with aging such as the loss of the cortical-medullary boundary and adipose tissue deposition (marked with an asterisk). In
Figure 22: Identification of CD45.1<sup>pos</sup> VEX<sup>pos</sup> ETP. Donor cells were gated based on expression of CD45.1. Donor cells that were Lin<sup>neg</sup> CD117<sup>pos</sup> CD44<sup>hi</sup> CD25<sup>neg</sup> and CD127<sup>neg</sup> (ETP) were examined for VEX expression.
Figure 23: Frequency of donor ETP and percent of ETP that express VEX in aged Wt and Foxn1Tg hosts. A. The frequency of CD45.1^pos donor ETP in the thymus of aged Wt and Foxn1Tg hosts. B. Percent of donor ETP that have Rag activity as measured by the expression of VEX in aged Wt and Foxn1Tg thymi. Error bars are SD.
**Figure 24:** Changes in gross thymic morphology and histology of old Wt and Foxn1Tg mice (line 60). A) Gross thymic morphology of a 26 months of age Wt mouse with adipose tissue present (arrows); B) H&E staining of the same thymus shows the loss of cortical-medulla demarcation and the abundance of adipose tissue, denoted with *. C) Gross thymic morphology of a 31 mo Foxn1Tg mouse with intact parenchymal tissue with little adipose tissue deposition; D-F) H&E staining of the same thymus shows a clear cortical-medulla demarcation and little adipose tissue deposition. Bar scales are 50 µm. C=cortex and M=medulla.
comparison, the *Foxn1Tg* thymi showed little adipose tissue deposition and displayed a well defined cortical-medullary boundary (Fig. 24 D-F).

Differences in the thymic architecture of aged Wt and *Foxn1Tg* thymi were further observed when stained for keratin 8 and keratin 5, which is expressed in cortical and medullary TEC, respectively. In aged Wt thymus (26 mo), the intensity of keratin 8 (red) staining was less and areas that were devoid of staining were prominent compared to that seen in young Wt (2 mo) (Fig. 25 B, C versus A). Additionally, keratin 5 staining (green) in aged Wt demonstrated a collapse of the medullary region from several small foci into a large fused filamentous area (Fig. 25 B, C). Contrary to aged Wt, aged *Foxn1Tg* thymus (31 mo) displayed intense keratin 8 staining with few areas that were devoid of keratin 8 expression (Fig. 25 E, F). Aged *Foxn1Tg* thymi also retained numerous, well defined keratin 5$^{pos}$ foci similar to that seen in the young thymi (Fig. 25 D-F). Thus, over expression of *Foxn1* dampened the age-associated alterations in thymic architecture.

*Foxn1Tg* thymus has a larger mTEC pool

With age, the number of TEC declines (81). Preventing or minimizing the decline in number of TEC with age could be one mechanism in which *Foxn1Tg* mice minimize alterations in the thymic architecture. The total numbers of cTEC and mTEC were calculated from the thymi of young and aged *Foxn1Tg* and Wt mice. Flow cytometry was used to determine the total number of cTEC and mTEC amongst the stroma cells. Thymi were digested and stroma cells were isolated using a percoll gradient. Cortical
Figure 25: Double staining of cortical keratin 8 (red) and medullary keratin 5 (green) in the thymus of Wt and Foxn1Tg mice (line 60). Acetone-fixed frozen sections of thymi from 2 mo (A) and 26 mo (B, C) Wt mice and 2 mo (D) and 31 mo old (E, F) transgenic mice were stained with a rabbit-anti mouse keratin 5 (green) and rat-anti mouse keratin 8 (red). Sections were analyzed with a Zeiss confocal microscope. Bar scale is 200 µm.
TEC were identified as cells that were CD45\(^{\text{neg}}\) MHCII\(^{\text{pos}}\) EpCAM\(^{\text{pos}}\) and LY51\(^{\text{pos}}\) (80) (Fig. 26). The number of cTEC per thymus in young Foxn1Tg was equivalent to young Wt (Fig. 27). With age, the number of cTEC per thymus declined in both Wt and Foxn1Tg thymi and aged Foxn1Tg had cTEC numbers equivalent to aged Wt, suggesting that over expression of Foxn1 does not affect the size of the cortical epithelial compartment and does prevent the decline in the number cTEC with age. This is supported by two-way ANOVA in which age results in a decrease in the number of cTEC and over expression of Foxn1 has no affect on the number of cTEC.

In contrast, over expression of Foxn1 significantly affects the mTEC population. Medullary TEC were identified as CD45\(^{\text{neg}}\) EpCAM\(^{\text{pos}}\) Ly51\(^{\text{neg}}\) and were further divided into either MHCII\(^{\text{pos}}\) or MHCII\(^{\text{hi}}\) (81) (Fig. 26). Two–way ANOVA revealed that age negatively affects the number of MHCII\(^{\text{pos}}\) and MHCII\(^{\text{hi}}\) mTEC and over expression of Foxn1 increases the number of MHCII\(^{\text{pos}}\) and MHCII\(^{\text{hi}}\) TEC. However, over expression of Foxn1 can also attenuate the affect of age on both MHCII\(^{\text{pos}}\) and MHCII\(^{\text{hi}}\) mTEC number. Young Foxn1Tg had a 2.5-fold increase in the number of MHCII\(^{\text{pos}}\) mTEC and a 5-fold higher number of MHCII\(^{\text{hi}}\) mTEC compared to young Wt (Fig. 27 and 28). While the number of MHCII\(^{\text{pos}}\) and MHCII\(^{\text{hi}}\) mTEC declined with age in both Wt and Foxn1Tg, aged Foxn1Tg had a 2-fold higher number of MHCII\(^{\text{pos}}\) and MHCII\(^{\text{hi}}\) compared to aged Wt (Fig. 27 and 28). The MHCII\(^{\text{hi}}\) mTEC population has been identified as the transient amplifying population that is responsible for maintaining the mTEC pool (81). To determine if the larger mTEC pool in Foxn1Tg is due to an increase in proliferation of MHCII\(^{\text{hi}}\) mTEC, expression of Ki-67 was examined using flow cytometry (Fig. 26). No
**Figure 26:** Identification of TEC subsets by flow cytometry. Enriched thymic stromal cells were stained for CD45, MHCII, EpCAM, and Ly51. CD45neg cells were gated for MHCII^{hi}, MHCII^{pos}, and MHCII^{neg} and then analyzed for expression of EpCAM and Ly51. Within each MHC subsets medullary TEC were identified as EpCAM^{pos} Ly51^{neg} and cortical TEC were identified as EpCAM^{pos} Ly51^{pos}. The percent of MHCII^{hi} mTEC in cell cycle was determined using Ki67. Numbers represent percent of cells in each quadrant. Figure represents data from a 3 mo Wt mouse.
Figure 27: Number of cTEC in thymi from 2-3 and 18-23 mo Wt and Foxn1Tg mice. Cortical TEC were defined as CD45<sup>neg</sup> MHCII<sup>pos</sup> EpCAM<sup>pos</sup> and Ly51<sup>pos</sup>. Numbers in parentheses denote the number of mice. Error bars are SD.
Figure 28: Number of MHCI$^{\text{pos}}$ mTEC in thymi from 3 mo and 18-20 mo Wt and Foxn1Tg mice. MHCI$^{\text{pos}}$ mTEC were identified as CD45neg MHCI$^{\text{pos}}$ EpCAM$^{\text{pos}}$ and Ly51$^{\text{neg}}$. The number of MHCI$^{\text{pos}}$ mTEC declined in aged Wt (#p=0.012) and Foxn1Tg (*p=0.016). Foxn1Tg had a higher number of MHCI$^{\text{pos}}$ mTEC compared to aged matched Wt (p=0.012 young Foxn1Tg vs. young Wt; p=0.023 aged Foxn1Tg vs. aged Wt). Numbers in parentheses denote the number of mice in each group. Error bars are SD.
Figure 29: Number of MHCII$^\text{hi}$ mTEC in thymi from young and aged Wt and Foxn1Tg mice. MHCII$^\text{hi}$ mTEC were identified as CD45neg MHCII$^\text{hi}$ EpCAM$^\text{pos}$ and Ly51$^\text{neg}$.

The number of MHCII$^\text{hi}$ mTEC declined in aged Wt (p<0.001) and Foxn1Tg (p=0.016). Foxn1Tg had a higher number of MHCII$^\text{pos}$ mTEC compared to aged matched Wt (p<0.001 young Foxn1Tg vs. young Wt; p=0.04 aged Foxn1Tg vs. aged Wt). Numbers in parentheses denote the number of mice in each group. Error bars are SD.
Figure 30: MHCII$^{hi}$ mTEC cell cycle activity in 3 mo and 18-20 mo Wt and Foxn1Tg. The percent of MHCII$^{hi}$ mTEC in cell cycle was determined using Ki-67. The percent of MHCII$^{hi}$ mTEC in cell cycle decreased with age (#p<0.001 aged Wt vs. young Wt; *p=0.002 aged Foxn1Tg vs. young Foxn1Tg). Aged Foxn1Tg had a larger percent of MHCII$^{hi}$ mTEC expressing Ki-67 compared to aged Wt (p=0.017). Numbers in parentheses denote the number of mice. Error bars are SD.
Table III

<table>
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<th>Mouse Strains</th>
<th>EpCAM(^{\text{pos}}) \text{Ly5}^{1\text{pos}} \text{MHCII}^{\text{pos}} \text{cTEC}</th>
<th>EpCAM(^{\text{pos}}) \text{Ly5}^{1\text{pos}} \text{MHCII}^{\text{pos}} \text{mTEC}</th>
<th>EpCAM(^{\text{pos}}) \text{Ly5}^{1\text{neg}} \text{MHCII}^{\text{hi}} \text{mTEC}</th>
<th>Percent \text{Ki6}^{7\text{pos}}</th>
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<td>Number</td>
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<td>13,100 (7,490)</td>
<td>27,481 (11,622)</td>
<td>4,996 (902)</td>
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<td>n=7</td>
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<td>27,481 (11,622)</td>
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The numbers of EpCAM\(^{\text{pos}}\) \text{Ly5}^{1\text{pos}} \text{MHCII}^{\text{pos}} \text{cTEC}, EpCAM\(^{\text{pos}}\) \text{Ly5}^{1\text{neg}} \text{MHCII}^{\text{hi}} \text{mTEC} and MHCII\(^{\text{hi}}\) \text{Ly5}^{1\text{neg}} \text{mTEC} were calculated based on the total cell number in the CD45\(^{\text{neg}}\) compartment and the percentage cells within the MHCII\(^{\text{pos}}\) and MHCII\(^{\text{hi}}\) subsets as determined by flow cytometry. Statistical significance was determined by t-test (SigmaStat, Version 2). \(^\# p = 0.012, \text{vs. young Wt; } \ast p = 0.016, \text{vs. young Foxn1Tg; } \dagger p = 0.003, \text{vs. young Wt; } \ddagger p = 0.023, \text{vs. age Wt; } \#\# p <0.001, \text{vs. young Wt; } \ast\ast p = 0.016, \text{vs. young Foxn1Tg; } \ddagger\ddagger p <0.001, \text{vs. young Wt; } \% p = 0.040, \text{vs. age Wt; } \%\% p = 0.002, \text{vs. young Foxn1Tg; } \#\#\# p = 0.017, \text{vs. age Wt. Numbers in parentheses are SDs.}
difference was seen in the percent of Ki-67 positive in MHCII$^{\text{hi}}$ mTEC in young Foxn1Tg versus young Wt (Fig. 29). With age the percent of Ki-67$^{\text{pos}}$ MHCII$^{\text{hi}}$ mTEC decreased in both Wt and Foxn1Tg (Fig. 29). However, aged Foxn1Tg had a larger percent of MHCII$^{\text{hi}}$ mTEC in cell cycle compared to aged Wt, suggesting that the higher mTEC pool in aged Foxn1Tg was due to increased cell cycle of the MHCII$^{\text{hi}}$ mTEC (Fig. 29). Data on all TEC subsets are summarized in Table III.

**Summary**

Results from experiments in this chapter showed that Foxn1 is over expressed in the thymic stroma of Foxn1Tg mice. Contrary to Wt mice (14), expression of Foxn1 did not decline with age in Foxn1Tg thymic stroma. Foxn1 was expressed primarily in the medulla with scattered positive cells in the cortex in both Wt and Foxn1Tg thymi. Furthermore, high levels of Foxn1 did not alter thymopoiesis in Foxn1Tg, establishing Foxn1Tg mice as a model to determine if high levels of Foxn1 could can prevent age-associated thymic involution. The Foxn1Tg mouse model also revealed the novel finding that Foxn1 is auto-regulated. While the target genes of Foxn1 are not known, expression of the Foxn1 transgene resulted in an increase in the level of endogenous Foxn1. Thus, the Foxn1Tg mouse model also proves to be a potential model to determine other targets of Foxn1.

With age, the decline in thymocyte number was attenuated in Foxn1Tg mice. In Wt mice, the frequency and number of ETP declined with age contributing to reduced thymopoiesis (12). We also demonstrated that the frequency and number of ETP
declined with age in Wt mice; however, in Foxn1Tg the frequency of ETP was not changed and the decline in ETP was attenuated. Interestingly, when young CD45.1\textsuperscript{pos} donor LSK cells were adaptively transferred into aged Foxn1Tg and aged Wt host, no difference was observed in the frequency of donor ETP. This may be the result of young donor cells in an aged host environment. It is possible that the young donor LSK cells can compensate for the alteration in the aged Wt thymic microenvironment resulting in a donor ETP frequency equivalent to aged Foxn1Tg.

While not significantly different, a higher percent of donor ETP expressed VEX in aged Foxn1Tg hosts than aged Wt hosts. This may suggest that the aged Foxn1Tg thymic microenvironment promotes Rag activity in ETP with a higher efficiency than aged Wt thymic microenvironment. However, ETP from aged Foxn1Tg did not show an increased T lineage potential or differentiation when cultured on the OP9-DL1 stroma cell line. It was previously shown that in wild type mice, aged ETP have reduced T lineage potential as measured by the number of DP cells generated from fetal thymic organ cultures (12). However, I was not able to confirm these conclusions using the OP9-DL1 stromal cell culture system. This may result form an efficient culture system as evident by the fact that approximately over than 90% of cells in all cultures committed to T lineage by two weeks. Since this system does not allow us to distinguish differences in Wt ETP T lineage potential with age, this system may not be able to discriminate difference in ETP T lineage potential from Foxn1Tg compared to Wt.

With age in Wt mice age-associated alterations in the thymic environment contributes to reduced thymopoiesis (4, 5). Aged Foxn1Tg had decrease adipose
accumulation and maintained distinction of the corticomedullary junction. While the number of cTEC and mTEC decrease with age, aged Foxn1Tg had a higher number of mTEC compared to aged Wt. This is likely the result of increased MHCII$^{hi}$ mTEC cell cycle activity seen in aged Foxn1Tg. Taken together, data from this chapter support my conclusion that over expression of Foxn1 attenuates thymic involution.
CHAPTER IV
OVER EXPRESSION OF FOXN1 PROMOTES THE GENERATION OF T CELL PROGENITORS IN THE BM

Introduction

Thymopoiesis requires a functional thymus that is capable of recruiting BM-derived T cell progenitors to the thymus and supports their development toward T lineage (252). Because the thymus does not contain self renewing cells, it relies on the BM for a continuous supply of T cell progenitors to maintain thymopoiesis (10, 11). The most immature BM derived T cell progenitors are the early T cell progenitors or ETP (82, 83). While the exact BM precursor to ETP has not been clearly identified, a subpopulation of ETP expresses both Flt3 and CCR9 and is phenotypically similar to the BM multipotent progenitors (MPP); thus, suggesting that at least a subset of MPP population is the precursor of ETP (256, 257). With age both ETP in the thymus and MPP in the BM decline in number and function (12, 13). These findings support the hypothesis that a decline in ETP contributing to age-associated thymic involution is initiated in the BM with a decline in MPP (13). In this chapter, I tested if the maintenance of ETP frequencies and higher numbers of ETP in aged Foxn1Tg results from amelioration of BM function toward the generation of MPP.

Beside the MPP, another population of T cell progenitors in the BM has been identified. Work by Strober and colleagues identified a T cell progenitor population in
the BM whose development is altered in *Foxn1* mutated nude mice (269). Phenotypically these cells are lineage marker negative (Lin\textsuperscript{neg}) CD90\textsuperscript{pos} CD2\textsuperscript{neg} and are referred to as committed T cell progenitors or CTP. Subsequent development of CTP toward the T lineage progresses through an intermediate stage, the CIP, which depends on the proliferation of CTP (269, 270, 274, 275). In nude mice, Chatterjea-Matthes et al. reported that CTP are present but are not functional, and the development of CTP toward CIP is reduced (274, 275). However, Krueger et al. reported that CTP from nude mice are functional when cultured on OP9-DL1 (271). Thus, while a role for *Foxn1* in the development of CTP has been suggested, it remains a controversial issue. In this chapter I also determined whether *Foxn1* affects CTP proliferation and development of CIP.

**Results**

**Over expression of *Foxn1* prevents the decline in MPP with age.**

MPP are the BM precursor to ETP (256, 257). With age, the number of MPP declines, contributing to the decline in ETP with age, and suggesting that thymic involution is initiated in the BM (13). Because the ETP frequency did not decline with age in *Foxn1* Tg mice, we were compelled to ask whether the higher level of ETP in aged *Foxn1* Tg mice was the result of preventing the age-associated decline in MPP. A representative flow cytometry gating of MPP is shown in Figure 31. In Wt mice, the frequency and total number of MPP decreased by 20 months, and further decreased by 24-25 months of age (Fig. 32A ##p<0.001 Wt 1-4 vs. Wt 24-25 mo; Fig. 32B. #p=0.006 Wt 1-4 mo vs. Wt 24-25 mo). Over expression of *Foxn1* resulted in the maintenance of
the frequency and number of MPP as the frequency and number of MPP were not
different among the three age groups, even in Foxn1Tg mice that were 24-35 months of
age (Fig. 32). Moreover, the frequency and total MPP number in the Foxn1Tg 24-35
months of age were higher than aged Wt 24-25 months of age (Fig. 32A ††p=0.001; Fig.
32 B †p<0.001). Two-way ANOVA analysis of MPP frequency and number
demonstrates that with age the MPP pool decreases. However, over expression of Foxn1
prevented the age associated changes in the frequency and number of MPP.

*Foxn1Tg mice have a larger HSC pool.*

One potential contributing factor to the maintenance of MPP population with age,
could be due to alterations in the immediate precursor to the MPP, the HSC. A
representative gating of HSC is shown in Figure 31. In agreement with previous reports,
the frequency and total number of HSC in Wt mice increased from 1-4 to 20-21 months
of age (Fig. 33A **p=0.007; Fig. 33B *p=0.004) (318, 318, 324). However, Wt mice that
were 24-25 months of age showed a significant reduction of in HSC frequency and
number compared to 20-21 months and 1-4 months of age (Fig. 33A **p<0.001 Wt 1-4
mo vs. Wt 24-25 mo; 33B #p=0.006 Wt 1-4 mo vs. Wt 24-25 mo). Compared to Wt
mice, I first observed that the number of HSC in 1-4 months old Foxn1Tg was
significantly higher (1.3-fold) (Fig. 33B, †p=0.036). As in Wt mice, there was an
increase in HSC frequency and number in the 20-21 months old Foxn1Tg and the number
of HSC was higher than age matched Wt (Fig. 33A ††p=0.03 Foxn1Tg 1-4 mo vs.
Foxn1Tg 20-21mo; Fig. 33B †p<0.001 Foxn1Tg 1-4mo vs. 20-21 mo; ‡p=0.01). The
Figure 31: Identification of HSC and MPP. Nucleated cells that were Lin<sup>neg</sup> CD117<sup>pos</sup> and Sca1<sup>pos</sup> (LSK) cells were gated on CD135 to identify HSC from MPP. HSC were defined as Lin<sup>neg</sup> CD117<sup>pos</sup> Sca1<sup>pos</sup> and CD135<sup>neg</sup>. MPP were defined as Lin<sup>neg</sup> CD117<sup>pos</sup> Sca1<sup>pos</sup> and CD135<sup>pos</sup>.
Figure 32: Frequency and number of MPP in Wt and Foxn1Tg 1-4, 20-21, and 24-35 mo.
A) Frequency of MPP per $1 \times 10^6$ nucleated cells. $^{##}p<0.001$ Wt 24-25 mo vs. Wt 1-4 mo; $^{\dagger\dagger}p=0.001$ Foxn1Tg 24-25 mo vs. Wt 24-25 mo. B) Total number of MPP in two tibia and femur. $^{#}p=0.006$ Wt 24-25 mo vs. Wt 1-4 mo; $^{\dagger}p<0.001$ Foxn1Tg 24-35 mo vs. Wt 24-25 mo. Each symbol represents one animal. Half filled triangles represent Foxn1Tg mice older than 25 months of age. Error bars are SD.
**Figure 33:** Frequency and total number of HSC in Wt and Foxn1Tg 1-4, 20-21, and 24-25 months of age. A) Frequency of HSC per 1x10^6 nucleated cells. **p=0.007 Wt 1-4 mo vs. Wt 20-21 mo; ##p<0.001 Wt 1-4 mo vs. Wt 24-25 mo; ††p=0.03 Foxn1Tg 20-21 vs. Foxn1Tg 1-4 mo; §§p=0.004 Foxn1Tg 24-25 mo vs. Wt 45-25 mo. B) Total HSC in two tibia and two femurs. *p=0.004 Wt 20-21 mo vs. Wt 1-4 mo; †p=0.006 Wt 24-25 mo vs. Wt 1-4; ‡p=0.036 Foxn1Tg 1-4 mo vs. Wt 1-4 mo; ††p<0.001 Foxn1Tg 20-21 mo vs. Foxn1Tg 1-4 mo; ‡‡p=0.01 Foxn1Tg 20-21 mo vs. Wt 20-21 mo; ¶p<0.001 Foxn1Tg 24-35 mo vs. Wt 24-25 mo. Each symbol represents one animal. Half filled triangles represent Foxn1Tg mice older than 25 months of age. Error bars are SD.
frequency and number of HSC in the 24-35 months old Foxn1Tg group were only reduced to the level found in 1-4 months of age (Fig. 33A-B) whereas, Wt HSC at 24-25 months of age were about one third the level found in 1-4 months old mice. The frequency and number of HSC in the 24-35 months old Foxn1Tg were higher than aged Wt 24-25 months of age even though half of the mice in this age group were older than the advanced aged Wt (Fig. 33A §§p=0.004; Fig. 39B §p<0.001). Analysis of HSC frequency and total number using two-way ANOVA showed that aging results in an initial increase in both frequency and number of HSC, followed by a decrease in frequency and number. While over expression of Foxn1 resulted in a higher HSC frequency and number, it did not affect changes associated with aging.

LSK frequency decreases with age in Wt mice, but not Foxn1Tg.

Previously, it was reported that the LSK population which includes HSC and MPP does not change with age (13). It was shown that as the size of the MPP population decreases, the HSC population increased, thus the LSK population remains unchanged with age (13). Since, we observed a decline in both HSC and MPP in Wt mice at the 24-25 months of age group we analyzed if these differences were reflected in a decline in the LSK population. Figure 34 contains a representative gating on LSK cells with age in Wt and Foxn1Tg mice. In Wt mice, from 1-4 to 20-21 months of age the LSK frequency remained unchanged (Fig. 34 and 35A). This is because in 20-21 months of age Wt mice as the MPP decreased the HSC increases. In Wt mice 24-25 months of age, the LSK population is drastically reduced as a result of a decline in both HSC and MPP (Fig. 34
Figure 34: Frequency of LSK cells within the Lin<sup>neg</sup> population of Wt and Foxn1Tg mice 1-4, 20-21, and 24+ months of age. Each graph represents the gating of one mouse from the following groups: Wt 1-4 mo (n=10), Wt 20-21 mo (n=5), Wt 24-25 mo (n=8), Foxn1Tg 1-4 mo (n=14), Foxn1Tg 20-21 mo (n=6), and Foxn1Tg 24-35 mo (n=12). Numbers in graph are the percent of LSK cells within the lineage marker negative population ±SD. *p<0.001 Wt 20-21 vs. 24-45 mo.
Figure 35: Frequency and total number of LSK in Wt and Foxn1Tg 1-4, 20-21, and 24-35 months of age. A) LSK frequency per 1x10^6 nucleated cells. *p<0.001 Wt 20-21 vs. 24-45 mo; †p=0.002 Foxn1Tg 24-35 vs. Wt 24-25 mo. B) Total LSK in two tibias and two femurs. ‡p=0.01 Wt 20-21 mo. vs. Wt 24-25 mo; ‡p=0.006 Foxn1Tg 20-21 mo vs. 1-4 mo; ‡p=0.02 Foxn1Tg 20-21 vs. Wt 20-21 mo. ‡p=0.003 Foxn1Tg 20-21 mo vs. 24-35 mo. Foxn1Tg 24-35 mo vs. Wt 24-25 mo. Each symbol represents one animal. Half filled triangles represent Foxn1Tg mice older than 25 months of age. Error bars are SD.
A

LSK / 1 x 10^6 Nucleated Cells (10^3)

0 1 2 3 4 5

1-4 mo 20-21 mo 24-25 mo 1-4 mo 20-21 mo 24-35mo

Wt Foxn1Tg

B

Total LSK (10^5)

0 1 2 3 4 5

1-4 mo 20-21 mo 24-25 mo 1-4 mo 20-21 mo 24-35mo

Wt Foxn1Tg
and 35A \(^{6}p=0.002\). With age, the LSK frequency remained unchanged in Foxn1Tg mice (Fig. 34 and 35A).

In agreement with previous reports, when the LSK frequency was used to calculate the total number of LSK cells, we found no difference in the total number of LSK between 1-4 and 20-21 months of age in Wt mice (Fig. 34 B) (13). However, we found that the total number of LSK decreased in Wt BM from 20-21 to 24-25 months of age (Fig. 35B \(^{†}p=0.01\)). The total number of LSK in Foxn1Tg increased from 1-4 to 20-21 months of age, and then decreased from 20-21 to 24-35 months of age (Figs. 35B; \(^{5}p=0.006\) Foxn1Tg 1-3 vs. Foxn1Tg 20-21 mo; \(^{‡}p=0.003\) Foxn1Tg 20-21 vs. Foxn1Tg 24-35 mo). The number of LSK cells in Foxn1Tg BM of 20-21 and 24-35 months of age was higher than Wt 20-21 and 24-25 mo respectively (Fig. 35B, \(^{6}p=0.02\) Foxn1Tg 20-21 vs. Wt 20-21; \(^{**}p=0.006\) Foxn1Tg 24-35 mo vs. Wt 24-25 mo). When the frequency and number of LSK cells in young and aged Wt and Foxn1Tg were analyzed using two way ANOVA, it was shown that both age and over expression of Foxn1 affect LSK frequency and number (Fig. 35A and B). It also demonstrated that over expression of Foxn1 can interact with the affect of age in regulating LSK frequency and number. Thus, over expression of Foxn1 results in a higher number of LSK in aged mice.

Previous work has demonstrated that the number of nucleated cells is reduced in Foxn1 mutated nude BM, suggesting that Foxn1 plays a role in BM cellularity (19). The total number of nucleated cells in Wt and Foxn1Tg BM was calculated to determine if over expression of Foxn1 affects BM cellularity. Wt and Foxn1Tg mice of 1-4 months of age had equivalent numbers of BM nucleated cells (Fig. 36). As previously reported, I
**Figure 36**: Total number of nucleated cells in the BM of Wt and Foxn1Tg mice 1-4, 20-21, and 24-35 months of age. Tibias and femurs were flushed and the total numbers of nucleated cells from Wt and Foxn1Tg mice were counted using a hemocytometer. Each symbol represents one mouse. Half filled triangles in Foxn1Tg 24-35 months of age represent mice 26 months of age or older. Error bars are SD.
found that the number of BM nucleated cells increased from 1-4 to 20-21 months of age in Wt; a similar change was also observed in Foxn1Tg mice (Fig. 36) (344). While the number of nucleated cells remained unchanged in Wt mice from 20-21 to 24-25 months of age, the number of nucleated cells decreased from 20-21 to 24-35 months of age in Foxn1Tg mice (Fig. 36). Foxn1Tg 20-21 months of age have a higher number of cells compared to Wt 20-21 months of age. Two way ANOVA analysis demonstrates that both age and over expression of Foxn1 affects the number of nucleated cells in the BM, and that over expression of Foxn1 and aging interact to regulate the number of BM nucleated cells.

**HSC and MPP in Foxn1Tg mice are resistant to age-associated cell death.**

To begin elucidating a cellular mechanism by which the numbers of HSC and MPP in advanced age were higher than aged matched Wt, cell cycle activities of HSC and MPP in young (2-5 mo) and aged (20-29 mo) Wt and Foxn1Tg mice were analyzed. Flow cytometry gating for DNA content in single cells is presented in Figure 37. We confirmed that there was no increase in the percentage of HSC in S,G2/M with age in Wt mice as previously reported (345) (Fig. 38A). However, aged Foxn1Tg mice had a 1.7-fold increase in the percent of HSC in S,G2/M compared with young Foxn1Tg ($p=0.005$) and this increase was significantly higher than in aged Wt ($^*p=0.001$). We observed no changes in the frequency of MPP in S,G2/M with age in either Wt or Foxn1Tg (Fig. 38C). In aged Wt mice, we detected a significant increase the percent of cells in the subG₀ fraction of HSC (Fig. 38B $^#p=0.04$). While there was a similar trend in
**Figure 37:** Flow cytometry gating for cell cycle analysis of HSC and MPP. Sorted HSC or MPP were fixed and stained with propidium iodine (PI). Singlet cells were gated based on the PI width. PI area was used to determine the DNA content.
Figure 38: Cell cycle analysis of HSC and MPP in young and aged Wt and Foxn1Tg mice. HSC (A-B) and MPP (C-D) from Wt 2 mo, Foxn1Tg 2-5 mo, Wt 20 mo and Foxn1Tg 20-29 mo were analyzed for DNA content using propidium iodine. A) Percent of HSC in S,G2/M. $p=0.005$ Foxn1Tg 20-29 mo vs. Foxn1Tg 2-5 mo; $^*p=0.001$ Foxn1Tg 20-29 mo vs. Wt 20 mo. B) Percent of HSC in sub G0. $^#p=0.04$ Wt 2 mo vs. Wt 20 mo; $^{**}p=0.03$ Foxn1Tg 20-29 mo vs. Wt 20 mo. C) Percent of MPP in S,G2/M. D) Percent of MPP in sub G0. $^{###}p=0.005$ Foxn1Tg 20-29 mo vs. Wt 20 mo. Numbers in parentheses denote the number of mice in each group. Error bars are SD.
A S,G2/M

B Sub G0

C S,G2/M

D Sub G0

Percent HSC in S,G2/M (%)

Percent MPP in S,G2/M (%)

Percent HSC in Sub Go (%)

Percent MPP in Sub Go (%)

2-5 mo

20-29 mo

2-5 mo

20-29 mo

2-5 mo

20-29 mo

2-5 mo

20-29 mo
MPP, the increase in the subG₀ fraction was not significant (Fig. 38D). In contrast, the percentages of HSC and MPP in subG₀ were not significantly different between young and aged Foxn1Tg. Strikingly, the subG₀ fractions of HSC and MPP in Foxn1Tg 20-29 months of age were 3.5-fold (Fig. 38B **p=0.03) and 3.7-fold less compared to Wt mice, respectively (Fig. 38D ##p=0.005). Thus, in aged Foxn1Tg mice, there was an increase cell cycling of HSC and reduced cell death of HSC and MPP.

Signaling through the CD117 (c-kit) by SCF is critical for HSC survival (279, 280). Preliminary studies in our laboratory demonstrated that the mRNA levels of SCF were not different between Wt and Foxn1Tg BM (data not shown). Expression of c-kit receptors on HSC and MPP from 1-2 and 20-35 months of age Wt and Foxn1Tg was measured to provide a possible explanation for increased survival of HSC in aged Foxn1Tg. Although the mean fluorescent intensity of CD117 was higher in aged Foxn1Tg HSC and MPP, this difference was not statistically different (Fig. 39).

**HSC from aged Foxn1Tg are able to generate multipotent colonies.**

To determine if HSC from aged Foxn1Tg are functional, we determined their ability to generate CFU-GEMM using methylcellulose bone marrow cultures. The methylcellulose BM cultures contained cytokines required for the developmental growth of myeloid progenitors but not T or B lineage progenitors. Five different types of colony forming units (CFU) can be produced in methylcellulose cultures and can be distinguished based on their morphology in Figure 40. Each myeloid progenitor gives rise to a different colony based on the lineage potentials in that progenitor. HSC have
Figure 39: Mean fluorescent intensity of CD117 on HSC and MPP from young and aged Wt and Foxn1Tg mice. Flow cytometry was used to determine the relative MFI of CD117 on HSC and MPP from 1-2 and 20-35 months of age Wt and Foxn1Tg. Each sample was normalized to the MFI of positively controlled beads coated with anti-CD117. Each symbol represents one animal. Error bars are SD.
lineage potential for granulocyte, erythroid, monocyte, and megakaryocyte. HSC give
rise to the most heterogeneous colony named a GEMM, which has all four lineage
potentials (Fig. 40E). Compared to young Wt, aged Wt cultures produced fewer GEMM
colonies (Fig. 41 *p=0.03). No significant difference was observed in the ability of aged
*Foxn1* Tg HSC to give rise to GEMM colonies compared to young *Foxn1* Tg (Fig 41).
Thus, HSC from *Foxn1* Tg were able to give rise to multipotent progenitors and the
overall number of functional HSC did not decline with age.

**Foxn1* Tg BM microenvironment promotes the development of MPP.**

In order to examine if the *Foxn1* Tg BM microenvironment promotes the
development of MPP from HSC, LSK cells from CD45.1 H2-SVEX BM were adaptively
transferred intravenously into non-irradiated 17-21 months of age CD45.2 Wt and
*Foxn1* Tg hosts. After 10 weeks, the frequencies of donor HSC and MPP were
determined using flow cytometry (Fig. 42). The frequency of donor derived HSC was
lower but not statistically different in aged *Foxn1* Tg BM compared to aged Wt; however,
despite the lower frequency of donor HSC, the frequency of donor MPP in the aged
*Foxn1* Tg BM was equivalent to that found in aged Wt (Fig. 43A-B). When the ratio of
MPP to HSC was examined to determine the efficiency of generating MPP from HSC,
the MPP/HSC ratio was higher in aged *Foxn1* Tg BM compared to Wt, indicating that the
aged *Foxn1* Tg microenvironment was more effective in promoting the development of
MPP from HSC (Fig. 43C *p=0.03). When the CD45.1*pos* LSK cells were intravenously
transferred into sub-lethally irradiated host, the subtle difference in donor HSC frequency
Figure 40: Morphology of colonies generated in methylcellulose from BM nucleated cells. Representative examples of myeloid colonies CFU-M (A), CFU-GM (B), CFU-G (C), CFU-E (D), and CFU- GEMM.
Figure 41: Total number of functional HSC in 2 tibias and 2 femurs of Wt and Foxn1Tg mice 1-4 and 19-25 months of age. The frequency of GEMM colonies generated per 10,000 nucleated cells was multiplied by the total number of nucleated cells isolated from two tibias and femurs to determine the total number of functionally active HSC.

*p=0.031 Wt 19-25 mo vs. Wt 1-4 mo. Numbers in parentheses denotes the number of mice. Error bars are SD.
was abolished and the frequency of donor MPP was again equivalent (Fig. 43D-E). When the efficiency of the generation of MPP was examined by the ratio of MPP to HSC, the difference in irradiated aged Foxn1Tg and Wt hosts was lost (Fig. 43F). Thus, the Foxn1Tg BM microenvironment promotes the generation of MPP from HSC; however, the effect is either neutralized by irradiation or the effector cells are sensitive to the irradiation treatment.

**HSC and MPP from Foxn1Tg display no differences in their intrinsic potential to commit to T lineage.**

The increased generation of MPP from HSC can contribute to a higher number of ETP in aged Foxn1Tg compared to Wt, however it is also possible that over expression of Foxn1 increased T lineage potential of HSC and MPP, thus further contributing to an increase in ETP. T lineage potential of HSC and MPP from 1-4 and 21-32 months of age Wt and Foxn1Tg was determined *in vitro*. HSC and MPP were cultured on the OP9-DL1 stromal cell line and their commitment and differentiation to T lineage was determined by expression of CD90 and CD25. MPP were electronically sorted and cultured on OP9-DL1 (GFP) cells for 2 weeks in the presence of IL7, SCF, and Flt3L. After two weeks cells were harvested and flow cytometry was used to determine T lineage commitment based on expression of CD90 and T lineage differentiation by expression of CD25 (Fig. 44). No difference was seen in the ability of MPP to commit to T lineage between Wt and Foxn1Tg 1-4 or 21-32 months of age (Fig. 45). There was also no difference in the differentiation to the CD25 positive DN2/DN3 stages, indicating that MPP developed in
Figure 42: Identification of CD45.1$^{\text{pos}}$ donor HSC and MPP. CD45.1$^{\text{pos}}$ LSK (8,000-16,000) were intravenously transferred into aged CD45.2$^{\text{pos}}$ Wt and Foxn1/Tg (17-21 months) hosts. Ten weeks post transfer host BM cells were analyzed for donor HSC and MPP. CD45.1$^{\text{pos}}$ donor cells that were Lin$^{\text{neg}}$ Sca1$^{\text{pos}}$ CD117$^{\text{pos}}$ were separated into HSC and MPP based on the expression of CD135. HSC are CD135$^{\text{neg}}$ and MPP are CD135$^{\text{pos}}$. 
Figure 43: Generation of donor MPP in aged Foxn1Tg and Wt non-irradiated (A-C) and irradiated (D-E) hosts. The frequency of CD45.1pos donor HSC per 1x10^6 donor cells in non-irradiated (A) and irradiated (D) Wt and Foxn1Tg hosts. The frequency of CD45pos donor MPP per 1x10^6 donor cells shown in non-irradiated (B) and irradiated (E) Wt and Foxn1Tg hosts. The generation of donor MPP per donor HSC was examined by the ratio of donor MPP to donor HSC in non-irradiated (C) and irradiated (F) hosts. †p=0.06. Each symbol represents one host mouse. Error bars are SD.
the BM of Foxn1Tg do not have an increase in T potential or differentiation toward T lineage (Fig. 45).

The commitment and differentiation of HSC in Wt and Foxn1Tg was also examined. HSC were cultured on OP9-DL1 with SCF, Flt3L, and IL7 for three week prior to flow cytometry analysis. There was a decrease in the percent of harvested cells that expressed CD90 with age in Wt HSC cultures (Fig. 46A *p=0.02). Cells harvested from Foxn1Tg HSC 1-4 months of age had reduced frequency of CD90 expression compared to aged matched Wt (Fig. 46A #p=0.006). Interestingly, with age there was no decline in the percent of CD90 positive cells harvested from Foxn1Tg HSC cultures. Of the committed T lineage cells, there was no difference in the ability to differentiate to DN2/DN3 stages with age or between Wt and Foxn1Tg (Fig. 46B). Taken together, over expression of Foxn1 does not increase T lineage potential or differentiation ability of HSC; in fact, the percent of committed T lineage cells in culture from young Foxn1Tg was less compared to young Wt.

**Notch 3 but not Notch 1 expression is reduced in the BM of Foxn1Tg.**

Notch1 signaling has been shown to increase HSC self-renewal (218, 219). The mRNA levels of Notch 1 were measured in the BM of aged Wt and Foxn1Tg to determine if the reduced level of CD90 expression in Foxn1Tg HSC cultures could possibly be the result of increased Notch 1 signaling in the total BM (Fig.47). No difference in the mRNA expression of Notch 1 was observed in the BM of Wt and Foxn1Tg 17-20 month of age. However, expression of Notch 1 in total BM may not
Figure 44: HSC and MPP commitment to T lineage and differentiation to DN2/DN3. MPP were cultured on OP9-DL1 (GFP\textsuperscript{pos}) for 2 weeks and HSC were cultured on OP9-DL1 for 3 weeks in the presence of SCF, Flt3L, and IL7 (10ng/mL each). Cells in culture that were GFP negative were analyzed for T lineage commitment based on expression of CD90. Differentiation of CD90\textsuperscript{pos} cell to the TN2/TN3 stages were identified by expression of CD25 and CD44.
Figure 45: Commitment and differentiation of MPP from Wt and Foxn1Tg 1-4 and 21-32 months of age. MPP were cultured on OP9-DL1 for 2 weeks and the percent of cells in culture that committed to T lineage was determined by CD90 expression (A). Differentiation of HSC to DN2/DN3 was determined based on CD25 expression within the CD90<sup>pos</sup> population (B). Error bars are SD. Numbers in parentheses denote the number of mice.
Figure 46: Commitment of HSC to T lineage and differentiation to DN2/DN3. HSC were cultured on OP9-DL1 for 3 weeks and the percent of cells in culture that committed to T lineage was determined by CD90 expression (A). Differentiation of HSC to DN2/DN3 was determined based on CD25 expression within the CD90^pos population. (B). *p=0.02 Wt 1-4 mo vs. Wt 21-32 mo; #p=0.006 Foxn1Tg 1-4 mo vs. Wt 1-4 mo. Error bars are SD. Numbers in parentheses denote the number of mice.
accurately measure its expression within HSC or in the HSC niche. It was thought that there is redundancy in the expression of Notch receptors in hematopoiesis; however, recently it was demonstrated that Notch 3 has a different role compared to Notch 1 in thymic lymphopoiesis (346). It remains to be determined if Notch 1 and Notch 3 serve distinct roles in the BM. Interestingly, the expression of Notch 3 mRNA was 58-fold less in young Foxn1Tg and 60-fold less in aged Foxn1Tg compared to aged matched Wt (Fig. 48). Whether Notch 3 signaling has an affect in HSC homeostasis or differentiation remains to be determined.

**CTP from Foxn1Tg BM displayed higher proliferative signature and efficiently generated more CIP.**

In Foxn1 mutated nude mice, proliferation of CTP is reduced and coupled to a reduction in the generation of CIP (275). Thus, we determined if first, over expression of Foxn1 affects CTP proliferation, and promotes their development into CIP and second if over expression of Foxn1 lead to an increase in the numbers of CTP and CIP with age. CTP were identified as Lin\(^{-}\) CD90\(^{+}\) and CD2\(^{-}\), and CIP were identified as Lin\(^{-}\) CD90\(^{+}\) CD2\(^{+}\) (Fig. 49). In mice 2-4 months of age, the total number of CTP was not significantly different between Wt and Foxn1Tg; however, Foxn1Tg had a 2.2-fold higher number of CIP (Fig. 50A *p<0.001). In aged mice, the numbers of CTP and CIP increased in both Wt and Foxn1Tg mice. Aged Wt mice displayed a greater increase in the number of CTP compared to Foxn1Tg (Wt 2-4 mo 101,764 and Wt 21-25 mo 543,883; Foxn1Tg 2-4 mo 130,628 and Foxn1Tg 21-25 mo 295,846) and the number of
**Figure 47:** Expression of Notch 1 mRNA in aged Wt and Foxn1Tg BM. RT-PCR was used to measure the expression of Notch 1 in the BM of Wt and Foxn1Tg 17-20 months of age. Error bars are SD. Numbers in parentheses denote the number of mice.
Figure 48: Expression of Notch 3 mRNA in the BM. Total BM cells from 2 mo and 20-24 mo Wt and Foxn1Tg BM was used to determine expression of Notch 3 expression with RT-PCR. Error bars are SD. Numbers in parentheses denote the number of mice.
CTP in 21-25 month old Foxn1Tg was 1.8-fold lower than Wt (Fig. 50B, \#p=0.002). In contrast, the number of CIP was not significantly different between aged Wt and Foxn1Tg. Thus, while aged Foxn1Tg displayed a lower number of CTP compared to Wt, the number of CIP generated from this pool were similar between Foxn1Tg and Wt mice. Aging results in an increase in both CTP and CIP. Over expression of Foxn1 minimizes the age associated-increase in CTP, resulting in a lower number of CTP in aged Foxn1Tg compared to Wt. In addition, over expression of Foxn1 increases the efficacy of generating CIP from CTP.

When the ratios of CIP over CTP were analyzed using two way ANOVA, over expression of Foxn1 resulted in an increase in the CIP/CTP ratio. The Foxn1Tg mice displayed higher ratios in both the 2-4 and 21-25 month groups (Fig. 50C, \*p=0.047 young; \$p<0.001 aged), suggesting that Foxn1Tg BM is more efficient in generating CIP from CTP. Previously, it was determined that the generation of CIP requires proliferation of CTP \{90 Chatterjea-Matthes,D. 2003\}. To determine a mechanism which promotes more CIP generation from CTP, we examined the cell cycle activity of CTP and CIP in Wt and Foxn1Tg using propidium iodine. CTP from both 2-4 and 21-25 months of age Foxn1Tg mice showed a higher percentage of cells in S,G2/M compared to Wt (Fig. 51 \*p=0.002, 2-4 months; \#p=0.054 21-25 months). There were no differences observed in cell cycle activities of CIP in Wt and Foxn1Tg mice. With age, we observed no changes in the cell cycle activity of CTP and CIP. These findings provide direct evidence that the BM of Foxn1Tg promotes proliferation of CTP and support the previous notion that
Figure 49: Flow cytometry identification of CTP and CIP. CTP were defined as cells that are Lin$^{neg}$ CD90$^{pos}$ and CD2$^{neg}$. CIP cells were defined as Lin$^{neg}$ CD90$^{pos}$ and CD2$^{pos}$. 
**Figure 50:** Number of CTP and CIP and the generation of CIP in Wt and *Foxn1*Tg with age.  A) Total CTP and CIP in two tibias and two femurs young (2-4 mo) *Foxn1*Tg and Wt mice; *p*<0.001 CIP in Wt vs. *Foxn1*Tg.  B) Total CTP and CIP in aged (21-25 mo) *Foxn1*Tg and Wt BM. The total numbers of CTP and CIP increased with age in both Wt and *Foxn1*Tg (p<0.001 young Wt CTP vs. aged Wt CTP; p=0.006 young *Foxn1*Tg CTP vs. aged *Foxn1*Tg CTP; p<0.001 young Wt CIP vs. aged Wt CIP; 0.002 young *Foxn1*Tg CIP vs. aged *Foxn1*Tg CIP). However, aged *Foxn1*Tg had fewer CTP than aged Wt #p=0.002 CTP in Wt vs. *Foxn1*Tg. C) Ratio of CIP over CTP in Wt and *Foxn1*Tg; ‡p=0.047 and §p<0.001 were comparisons of Wt vs. *Foxn1*Tg. Numbers in parentheses denote number of mice in each group. Error bars are SD.
**Figure 51:** Cell cycle activity of CTP and CIP in Wt and Foxn1Tg mice with age.

Electronically sorted CTP and CIP from 2-4 and 21-25 mo Wt and Foxn1Tg were analyzed for DNA content using propidium iodine and flow cytometry. Data represent the percent of cells in S,G2/M phase. *p=0.002 CTP from 2-4 mo Foxn1Tg vs. Wt; #p=0.054 CTP from 21-25 mo Foxn1Tg vs. Wt. Numbers in parentheses denote the number of mice in each group. Error bars are SD.
*Foxn1* plays a role in the proliferation of CTP which is required for the generation of CIP (275).

**Foxn1Tg microenvironment promotes the development of CIP.**

To confirm that the BM environment of *Foxn1Tg* is more efficient in the generation of CIP from CTP, donor CD45.1\textsuperscript{pos} LSK cells (8,000-16,000) were transferred intravenously into non-irradiated CD45.2\textsuperscript{pos} Wt and *Foxn1Tg* 17-21 months of age. After 10 week, the BM was harvested from host mice and the frequency of donor CTP and CIP was determined. Identification of donor CTP and CIP is showed in Figure 52. Although not statistically different, the frequencies of donor CTP was lower in aged *Foxn1Tg* compared to aged Wt host while the frequencies of donor CIP in *Foxn1Tg* and Wt were equivalent (Fig. 53 A-B). The ratios of donor CIP to donor CTP were used to determine the efficacy of generating CIP from CTP. Aged *Foxn1Tg* had a higher ratio of CIP to CTP compared to aged Wt hosts (Fig. 53C). To increase engraftment of donor cells, we repeated the experiment in which aged Wt and *Foxn1Tg* hosts were sub-lethally irradiated 24 hours prior to adaptive transfer of donor CD45.1\textsuperscript{pos} LSK cells. Sub-lethally irradiated Wt and *Foxn1Tg* hosts had equivalent donor frequencies of CTP and CIP (Fig. 53 D and E) and the generation of CIP from CTP in Wt and *Foxn1Tg* was not significantly different as determined by CIP to CTP ratios (Fig. 53F). Thus, as seen with HSC, the *Foxn1Tg* BM microenvironment promotes the generation of CIP from CTP; however, the effect is either neutralized by irradiation or the effector cells are sensitive to the irradiation treatment.
Figure 52: Flow cytometer gating on donor CTP and CIP. Donor CD45<sup>pos</sup> cells were gated for cells that lack lineage specific markers but were CD90<sup>pos</sup> CD2<sup>neg</sup> (CTP) and CD90<sup>pos</sup> CD2<sup>pos</sup> (CIP).
Figure 53: Generation of CIP in aged Foxn1Tg and Wt non-irradiated (A-C) and irradiated (D-E) hosts. The frequency of CD45.1^{pos} donor CTP (A and C) and CIP (B and D) per 1x10^6 donor cells in non-irradiated and irradiated Wt and Foxn1Tg hosts was determined 10 weeks after injection using flow cytometry. The generation of donor CIP donor per CTP was examined by the ratio of CIP to CTP in non-irradiated (C) and irradiated (F) host. †p=0.06. Each symbol represents one host mouse. Error bars are SD.
Summary

Although the number of MPP decline with age in Wt mice, this population remained unchanged in Foxn1Tg. Aged Foxn1Tg had fewer MPP undergoing cell death compared to aged Wt. One progeny of MPP is the ETP, thus the maintained number of MPP in aged Foxn1Tg may contribute to the higher number of ETP found in the aged Foxn1Tg thymus. Over expression of Foxn1 does not prevent changes in the HSC population with age; however, Foxn1Tg mice have a larger HSC pool compared to Wt mice. Aged Foxn1Tg mice 24-35 months of age have higher percent of HSC in cell cycle with reduced cell death. While the number of HSC that generate multipotent progenitors decreased with age in Wt, this did not occur in aged Foxn1Tg. Furthermore, the aged Foxn1Tg BM microenvironment promoted the generation of MPP from HSC. Cultures of HSC and MPP on OP9-DL1 demonstrated that HSC and MPP from Foxn1Tg did not have increased T lineage potential compared to Wt. While a role for Foxn1 in the development and function CTP remains controversial, this chapter demonstrates that over expression of Foxn1 increased CTP cell cycle and promoted the development of CIP from CTP. Taken together, data from this chapter demonstrated a novel function of Foxn1 in promoting the development of T cell progenitors within the BM.
CHAPTER V

B CELL AND MYELOID PROGENITORS IN FOXN1Tg BM

Introduction

MPP are multipotent progenitors that have lost the ability to self renew and are the immediate progeny of HSC (241, 244). MPP are precursors for ETP that are found in the thymus, as well as CLP, and myeloid progenitors in the BM (246, 253, 255). I showed in Chapter III that the number of MPP declined with age in Wt BM but not Foxn1Tg BM. I also determined that the frequency of ETP, precursor for T lineage cells within the thymus declined with age in Wt mice but not Foxn1Tg (347). CLP in the BM display potent B lineage potential and are precursors for B cells (82). With age, the number CLP declines, resulting in reduced B lymphopoiesis (329, 330). I asked whether maintaining the MPP pool with age in Foxn1Tg also maintains CLP and B lymphopoiesis. MPP also give rise to myeloid progenitors; however, the numbers of myeloid progenitors are not affected by age (330). To obtain a comprehensive view of hematopoiesis in Foxn1Tg, I also determined whether high levels of Foxn1 altered the numbers of myeloid progenitors in young and aged Foxn1Tg mice. This chapter will address whether the effect of over expressing of Foxn1 is specific to T lineage progenitors or whether high levels of Foxn1 expression also affect the development of B lineage and myeloid lineage progenitors from MPP.
Results

Distributions of hematopoietic lineages in the BM of Wt and Foxn1Tg mice.

Analysis of BM cells was performed using flow cytometry to determine if high levels of Foxn1 altered the distribution of myeloid-, erythroid-, and B- lineage cells with age. Compared to young (2 mo), aged Wt BM (18 mo) had a 5-fold expansion of Ter 119 positive erythroid lineage cells (p<0.001) and a 2-fold contraction in the B220/CD19 positive B lineage compartment (Fig. 54). There was no change in the percent of GR1/CD11b myeloid lineage population in aged Wt BM compared to young Wt (Fig. 54). Compared to young Wt (2 mo) months of age, young Foxn1Tg (2-5 mo) had a 1.7-fold increase in the percent GR1/CD11b myeloid lineage cells and a 1.8-fold decrease in the percent of the B220/CD19 B lineage cells (Fig. 54 ** p<0.001 myeloid lineage; * p<0.001 B lineage). In aged Foxn1Tg (17-29 mo), there was no significant expansion of the Ter 119 positive erythroid lineage but there was a 1.4 fold expansion in the percent of myeloid lineage cells (p<0.001) compared to young Foxn1Tg (2-5 mo). Similar to Wt mice, with age there was a 3.2-fold contraction of the B lineage population in aged Foxn1Tg compared to young Foxn1Tg (p<0.001). When compared to aged Wt, the BM of aged Foxn1Tg mice had 3 fold less B lineage cells and erythroid cells, and 2.2 fold more myeloid lineage cells (Fig. 54 # p<0.001 B lineage; ### p<0.001 erythroid lineage; ## p<0.001 myeloid lineage). Overall, the percentage of B lineage cells was lower while the percentage of cells in the myeloid compartment was higher in Foxn1Tg compared to aged matched Wt. A summary of all significant comparisons can be found in Table IV.
Figure 54: Analysis of hematopoietic lineages with age in Wt and Foxn1Tg mice. One to two million nucleated cells from the bone marrow of 2-5 and 17-29 months of age Wt and Foxn1Tg were analyzed for cell surface expression of B lineage markers CD19 and B220, myeloid lineage marker Gr1 and CD11b, T lineage marker CD3, NK lineage marker DX5, and erythroid lineage marker Ter119 using flow cytometry. Cells that did not express any of these markers were grouped into an “other” category that includes the lineage marker negative population and stromal cells. *p<0.001 B220/CD19 percent Wt 2-5 mo vs. Foxn1Tg 2-5 mo. **p<0.001 Gr1 / CD11b percent Wt 2-5 mo vs. Foxn1Tg 2-5 mo. #p<0.001 B220/CD19 percent Wt 17-29 mo vs. Foxn1Tg 17-29 mo. ##p<0.001 Gr1/CD11b percent Wt 17-29 mo vs. Foxn1Tg 17-29 mo. ###p<0.001 Ter119 percent Wt 17-29 mo vs. Foxn1Tg 17-29 mo.
2-5 mo

Wt

25 ± 9
9 ± 3

26 ± 3%

1 ± .3

3 ± 1

n = 6

Tg

18 ± 4
13 ± 1

20 ± 7

4 ± 1

44 ± 6

.7 ± .5

n = 11

17-29 mo

Wt

37 ± 3

44 ± 4

n = 5

Tg

19 ± 5

6 ± 1

n = 7

### B220 / CD19

#### CD3

#### Ter 119

#### Gr1 / CD11b

#### DX5

#### Other
Changes in the BM lineage compartments of Wt and Foxn1Tg mice with age. B lineage cells were defined as B220 and/or CD19 positive. Myeloid lineage was identified as Gr1 and/or CD11b positive. Erythroid lineage was defined as Ter119 positive. Cells that did not fall into these categories were grouped into an “other” category which would include the lineage negative cells as well as stromal cells of the BM.

<table>
<thead>
<tr>
<th>Lineage Population</th>
<th>Comparisons</th>
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<tbody>
<tr>
<td>B220 / CD19 lineage</td>
<td>Wt 2-5 mo vs. Wt 17-29 mo</td>
<td>&lt;0.001</td>
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<tr>
<td>B220 / CD19 lineage</td>
<td>Foxn1Tg 2-5 mo vs. Foxn1Tg 17-29 mo</td>
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<td>B220 / CD19 lineage</td>
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<td>Gr1 / CD11b lineage</td>
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Number of B lineage cells and CLP are reduced in *Foxn1Tg*.

Compensatory changes in the frequency of one BM lineage population may result in the expansion or contraction in other hematopoietic lineages. Thus, the absolute numbers of B-lineage cells were calculated to determine if *Foxn1Tg* mice have fewer B-lineage cells compared to aged matched Wt. The total number of B-lineage cells was reduced 1.7-fold in young *Foxn1Tg* mice compared to young Wt (Fig. 55 #_p=0.01). With age, the total number of B-lineage cells decline 1.6-fold in Wt mice and 3-fold in *Foxn1Tg* (Fig. 55 *_p=0.007 Wt; †_p=0.002 Foxn1Tg). Further, aged *Foxn1Tg* mice had a 3-fold lower number of B-lineage cells compared to aged Wt (Fig. 55 §_p=0.004). Two way ANOVA analyses revealed that age results in a reduction in the number of B-lineage cells. Furthermore, over expression of *Foxn1* also results in a lower number of B-lineage cells and does not prevent the decline with age as seen with MPP. In correlation with the reduced frequency of B-lineage cells, the absolute number of B-lineage cells was also significantly reduced in *Foxn1Tg* compared to aged matched Wt.

CLP are B cell progenitors and the number of CLP decline with age in Wt BM, contributing to the reduced number of B-lineage cells in aged Wt mice (82, 329, 330). Based on this knowledge, we hypothesized that the reduced frequency and number of B-lineage cells in *Foxn1Tg* could result from a decrease in the number of CLP. CLP were identified using flow cytometry as Lin$^{neg}$ CD127$^{pos}$ CD117$^{low}$ Sca1$^{pos}$ and CD135$^{pos}$ (Fig. 56). *Foxn1Tg* mice 1-2 months of age had a decreased frequency and number of CLP compared to Wt 1-2 months of age (Fig. 57 A-B). In agreement with previous reports, (329, 330), the frequency and number of CLP declined with age in Wt and *Foxn1Tg* BM.
Figure 55: The number of B lineage cells in 2-3 and 17-18 months of age Wt and Foxn1Tg BM. B lineage cells were identified as lymphocytes that expressed B220 and/or CD19. *p=0.007 Wt 21-22 mo vs. Wt 2-3 mo; #p=0.01 Foxn1Tg 2-3 mo vs. Wt 2-3 mo; †p=0.002 Foxn1Tg 17-18 mo vs. Foxn1Tg 2-3 mo; §§=0.004 Foxn1Tg 17-28 mo vs. Wt 17-18 mo. Numbers in parentheses denotes the number of mice in each group. Error bars are SD. Bone marrow cells from male and female mice were pooled as no sex difference was observed.
Figure 56: Identification of CLP. CLP were identified as nucleated cells that were Lin^{neg} CD127^{pos} CD117^{low} Sca1^{low} and CD135^{pos}.
Figure 57: A: Frequency and number of CLP in Wt and Foxn1Tg 1-2 and 21-23 months of age. A) CLP frequency. *p<0.001 Wt 21-23 vs. Wt 1-2 mo; #p<0.001 Wt 1-2 vs. Foxn1Tg 1-2 mo; **p<0.001 Foxn1Tg 21-23 mo vs. Foxn1Tg 1-2 mo; ##p<0.001 Wt 21-23 mo vs. Foxn1Tg 21-23 mo. B) Number of CLP in two tibias and two femurs. *p=0.004 Wt 1-2 vs. Wt 21-23 mo; #p=0.005 Wt 1-2 mo vs. Foxn1Tg 1-2 mo; **p<0.001 Foxn1Tg 1-2 mo vs. Foxn1Tg; ###p<0.001 Wt 21-23 mo vs. Foxn1Tg 21-23 mo. Each symbol represents one mouse. Error bars are SD.
(Fig. 57 A-B). The frequency and number of CLP in *Foxn1* Tg 21-23 months of age was less than in Wt 21-23 months of age (Fig. 57 A-B). This is supported by the two way ANOVA in which CLP frequency and number are reduced with age. Over expression of *Foxn1* also results in a decline in CLP frequency and number and over expression of *Foxn1* does not prevent the decline with age. Together these data suggest that the reduced number of B-lineage cells in the BM of *Foxn1* Tg results from a reduction in the generation of CLP from MPP or survival of CLP in *Foxn1* Tg BM in both young and aged mice.

The generation of CLP from MPP requires Flt3 signaling (284-286). Preliminarily data from our laboratory suggest that there is no difference in the mRNA expression of Flt3L in total BM of *Foxn1* Tg compared to Wt (data not shown). The expression level of the CD135 or Flt3, the receptor for Flt3L, on MPP was measured via flow cytometry to determine if changes in the expression of CD135 is a possible mechanism for the reduced number of CLP in *Foxn1* Tg BM. Two way ANOVA analysis of the mean fluorescent intensity of CD135 on MPP revealed that over expression of *Foxn1* affects the expression level of CD135 (Fig. 58). Both young and aged *Foxn1* Tg mice have a higher expression level of CD135 on MPP compared to Wt (Fig. 58). From these data we conclude that the reduced number of CLP in *Foxn1* Tg mice is not the result of reduced CD135 expression on MPP. Whether signaling through CD135 on *Foxn1* Tg MPP is effective remains to be determined.
**Figure 58**: Mean fluorescent intensity of CD135 on MPP from young and aged Wt and Foxn1Tg mice. Flow cytometry was used to determine the relative MFI of CD135 on MPP. Each sample was normalized to the MFI of positively controlled beads coated with anti-CD135 and ran in the same experiment. The expression of CD135 (Flt3) is not affected by age, however Foxn1Tg MPP had a higher expression level of CD135 compared to Wt (p=0.002). Each symbol represents one animal. Error bars are SD.
Myeloid progenitors in *Foxn1*Tg BM

As mentioned above, the frequency of myeloid lineage cells was higher in *Foxn1*Tg mice compared to Wt. Thus, the total numbers of myeloid lineage cells were determined in Wt and *Foxn1*Tg with age. *Foxn1*Tg mice 2-5 months of age had a higher number of myeloid lineage cells compared to aged matched Wt (Fig. 59 *p<0.001). With age there was no change in the number of myeloid lineage cells in the BM of Wt; however, there was an increase in aged *Foxn1*Tg (Fig. 59 †p<0.001). Furthermore, *Foxn1*Tg 17-29 months of age had more myeloid lineage cells in the BM compared to aged matched Wt (Fig.59 ‡p<0.001). Two way ANOVA analyses indicated that with age the number of myeloid lineage cells increase in *Foxn1*Tg but not Wt, and over expression of *Foxn1* resulted in a higher number of myeloid lineage cells compared to aged matched Wt. Additionally, over expression of *Foxn1* alters the effect of age on the number of myeloid lineage cells as there is no increase with age in Wt, but a significant increase with age in *Foxn1*Tg.

Methylcellulose cultures were performed to determine if a higher number of myeloid lineage cells in the BM of *Foxn1*Tg were a result of a higher number of myeloid progenitors. Methylcellulose cultures were performed in duplicate using 10,000 nucleated BM cells from Wt and *Foxn1*Tg 1-4 and 19-28 months of age. The number of myeloid colonies and the type of each colony generated per 10,000 cells was used to calculate the total number of myeloid progenitors as well as the total number of each CFU-M, CFU-GM, CFU-G, CFU-E progenitors. The total numbers of myeloid progenitors in *Foxn1*Tg 1-4 months of age was equivalent to aged matched Wt (Fig. 60).
**Figure 59:** Number of Gr1/CD11b myeloid lineage cells in Wt and Foxn1Tg 1-5 and 17-29 months of age. Foxn1Tg have a significantly higher number of myeloid lineage cells compared to aged matched Wt. In aged mice the number of myeloid lineage cells increased in Foxn1Tg but not in Wt. *p<0.001 Foxn1Tg 1-5 mo vs. Wt 1-5 mo; #p<0.001 Foxn1Tg 17-29 mo vs. Wt 17-29; ‡p<0.001 Foxn1Tg 17-29 mo vs. Foxn1Tg 1-5 mo.

Numbers in parentheses denotes the number of mice. Error bars are SD.
With age, the total number of myeloid progenitors did not change in either Wt or Foxn1Tg, and aged Foxn1Tg have the same number as aged Wt (Fig. 60). The total number of CFU-M, CFU-GM, CFU-G, and CFU-E was calculated to determine if there were any differences in the type of myeloid or erythroid progenitors between Wt and Foxn1Tg mice (Fig. 61A-B). No differences between Wt and Foxn1Tg mice were seen in the total number of each type of myeloid progenitor or erythroid progenitor; there were also no changes in the total number of these colonies with age. Thus the increase in the number of myeloid lineage cells in Foxn1Tg compared to Wt is not the result of an increase in the number of myeloid progenitors but is likely to be the result of an increase in the number of mature myeloid cells due to proliferation.

**Summary**

This chapter determined if over expression of Foxn1 affected the B and myeloid lineages of hematopoiesis. Young and aged Foxn1Tg mice had a higher number of myeloid lineage cells compared to aged matched Wt. The increase in the number of myeloid lineage cells was not the result of an increase in the number of myeloid progenitors, and over expression of Foxn1 did not appear to affect the development of myeloid progenitors from MPP. The increased number of myeloid lineage cell is likely the result of increased proliferation and expansion of mature myeloid cells. The number of B-lineage cells was reduced in young and aged Foxn1Tg BM compared to aged matched Wt, thus the number of available niches for myeloid expansion may be higher in
**Figure 60**: Number of myeloid CFU in the BM of Wt and *Foxn1Tg* 1-4 and 18-28 mo.

Numbers in parentheses denotes the number of mice. Error bars are SD.
Figure 61: Number of CFU-M, CFU-GM, CFU-G, and CFU-E colonies in Wt and Foxn1Tg 1-4 and 19-28 months of age. A) Number of CFU-M and CFU-GM in Wt and Foxn1Tg 1-4 and 19-28 months of age. B) Number of CFU-G and CFU-E in Wt and Foxn1Tg 1-4 and 19-28 months of age. Numbers in parentheses denotes the number of mice. Error bars are SD.
Foxn1Tg BM. The reduced number of B-lineage cells in young and aged Foxn1Tg mice compared to Wt was the result of a reduced numbers of CLP, an immediate progeny of MPP (246). In aged Foxn1Tg, while the number of MPP was not reduced with age, the number of CLP declined. Previously we demonstrated that the frequency of ETP did not decline with age in Foxn1Tg. Since ETP are also a progeny of MPP this suggests that over expression of Foxn1 affects lymphoid development at the level of the MPP, resulting in reduced number of CLP but maintained development of ETP. Development of MPP towards ETP instead of CLP may result from differences in the expression of Flt3. Flt3 signaling is required for the generation of CLP and its expression is higher on MPP in Foxn1Tg mice compared to Wt mice. (284-286). However, Flt3 signaling is also required for the generation of CCR9^{pos} MPP (ELP) (254). MPP with the highest level of Flt3 have greater T lineage potential than B lineage, thus the higher level of Flt3 expression on Foxn1Tg MPP may reflect the commitment of this population to T lineage over B lineage.
CHAPTER VI
FOXN1 EXPRESSION IN THE BM

Introduction

It is well known that Foxn1 is expressed in the thymus and skin (16); however, expression of Foxn1 in other organs has not been extensively investigated. Potential functions for Foxn1 in the BM have been suggested from previous study in nude mice in which the Foxn1 gene is mutated. BM from nude mice display reduced HSC function and generation of committed intermediate progenitors (CIP) (19, 275). Interestingly, over expression of Foxn1 in aged Foxn1Tg mice resulted in a higher number of HSC and MPP compared to aged Wt (Refer to Chapter III). Furthermore, the aged Foxn1Tg microenvironment supports the generation of MPP and CIP (refer to Chapter III). Surprisingly, the numbers of CLP and B-lineage cells were reduced in Foxn1Tg BM compared to aged matched Wt (refer to Chapter IV). Taken together, these data indicate that over expression of Foxn1 affects the development of lymphoid progenitors in the BM. The effect of over expression of Foxn1 on progenitors in the BM with age can be the result of endocrine functions of the thymus acting on the BM, or alternatively Foxn1 is expressed in the BM. The focus of this chapter was first to determine if Foxn1 is expressed in the BM, and second to identify Foxn1 expressing BM cells in Wt as well as in Foxn1Tg mice.
Results

*Foxn1* is expressed in the BM of Wt and *Foxn1Tg* mice.

*Foxn1* is expressed in epithelial cells of the thymus and skin and plays a critical role in thymic organogenesis and hair follicle development, respectively (16). Alterations in hematopoietic lineages in the BM of *Foxn1Tg* provides a functional basis to investigate if *Foxn1* is expressed in the BM of Wt and *Foxn1Tg*. Figure 62A reveals that *Foxn1* is expressed in the BM of Wt (1-3 months of age) mice (414 copies / μg of total RNA). Contrary to the thymus (14), expression of *Foxn1* in the BM of Wt mice was not changed with age (Fig. 62A). In *Foxn1Tg*, the total level of *Foxn1* expression (transgene and endogenous) was 98-fold higher than Wt mice (Fig. 62B). In agreement with Wt BM, the mRNA expression level of *Foxn1* did not change with age in *Foxn1Tg* mice (Fig. 62B). As previously shown in the thymic stroma of *Foxn1Tg* mice, expression of the *Foxn1* transgene increased expression of the endogenous *Foxn1* (Fig. 62A-B). The endogenous *Foxn1* levels were 18-24 fold higher in young and aged *Foxn1Tg* mice compared to age matched Wt (Fig. 62B compared to Fig. 62A, young p=0.036; aged p=0.024). Thus *Foxn1* is expressed in the BM of Wt mice, and over expressed in the BM of *Foxn1Tg*.

**Foxn1^pos** cells are present in the BM of Wt and *Foxn1Tg* mice.

*Foxn1*-expressing cells can be detected in the BM of Wt and *Foxn1Tg* mice using immunohistochemistry. On the average, 1-3 *Foxn1^pos* cells were identified per 5 sternum sections (5μm) in young Wt. The *Foxn1^pos* cells appeared as single cell in the central
Figure 62: Expression of Foxn1 mRNA in the bone marrow of Wt and Foxn1Tg mice. Quantitative RT-PCR was used to determine the number of Foxn1 copies per μg of RNA. A) Expression of Foxn1 in Wt BM 2 mo and 20-24 mo. B) Expression on Foxn1 in Foxn1Tg 2 mo and 23-28 mo. Primers that do not distinguish endogenous Foxn1 from transgene Foxn1 were used to determine the total level of Foxn1. Primers specific for just the Foxn1 transgene were used to calculate the contribution of the Foxn1 transgene to the total Foxn1 level. The endogenous Foxn1 level was determined by subtracting the transgene level from the total Foxn1 level. Samples were run in triplicate. Numbers in parentheses denotes the number of mice in each group. Error bars are SD.
A

Copies of Foxn1 / μg RNA

Wt

(5) (6)

2 mo
20-24 mo

B

Copies of Foxn1 / μg RNA

Total Foxn1
Endogenous Foxn1
Transgene Foxn1

(3) (3)

2 mo
23-28 mo

(3)

(3)
marrow cavity in close proximity to sinusoids (Fig. 63). In the BM of young Foxn1Tg mice, Foxn1<sup>pos</sup> cells were readily detectable within the vicinity of sinusoids at approximately 3-6 cells per 5μm-thick section (Fig. 63). Morphologically, the Foxn1 expressing cells were round with abundant cytoplasm and a central nucleus (Fig. 63). Foxn1 protein appeared homogeneous in the cytoplasm or in patches. In aged Wt and Foxn1Tg BM, the number of readily detectable Foxn1<sup>pos</sup> cells per BM section increased; however, it appeared that there were more Foxn1 expressing cells in aged Foxn1Tg BM compared to aged Wt.

**Foxn1 is not expressed in HSC or MPP.**

As shown in the chapter III, HSC and MPP declined in aged Wt mice; however, their numbers were higher in aged Foxn1Tg. The higher number of HSC and MPP in aged Foxn1Tg may be in part the result of increase HSC cell cycle and reduced cell death of HSC and MPP. Based on the morphology of Foxn1<sup>pos</sup> cells identified in the BM it was unlikely that HSC and MPP expressed Foxn1. To definitely rule out that HSC and MPP cell cycle activity in aged Foxn1Tg is the result of intrinsic Foxn1 expression, HSC and MPP from aged Wt and Foxn1Tg were electronically sorted and RT-PCR was used to determine Foxn1 expression. Figure 64 reveals that neither HSC nor MPP from Wt or Foxn1Tg mice express Foxn1.
Figure 63: Foxn1 staining in Wt and FoxnITg BM. Sternums from Wt and FoxnITg mice were fixed, decalcified, embedded in paraffin blocks, and sectioned at 5 microns. Antigen retrieval was performed using Dako’s antigen retrieval solution and sections were stained with either rabbit anti-mouse Foxn1 or rabbit IgG at 2μg/ml. Primary antibodies were incubated overnight at 4°C. Dako Universal LSAB kit or donkey anti-rabbit biotin (6μg/ml) followed by streptavidin-HRP was used for detection of primary antibody. Sections were developed with AEC for 1.5 minutes and counterstained with hematoxylin. Pictures were taken using a Leitz Diaplan microscope with Retiga 2000R camera. Pictures represent staining from approximately 4-5 mice per group. Yellow arrows point to Foxn1 positive cells. Sinusoids are outlined in blue and denoted with an S.
Figure 64: *Foxn1* is not expressed in HSC and MPP. HSC and MPP were electronically sorted from the BM of Wt and *Foxn1*Tg mice and RT-PCR was used to determine if HSC and MPP express *Foxn1*. 
**Foxn1 is expressed in CD45\textsuperscript{pos} cells.**

In effort to begin to determine a phenotype of the Foxn1\textsuperscript{pos} cells, BM cells from an aged Foxn1\textsuperscript{Tg} mouse were electronically sorted based on the expression of CD45, which is a hematopoietic cell marker. RT-PCR was used to determine if Foxn1 was expressed within the CD45\textsuperscript{pos} or CD45\textsuperscript{neg} population. In the thymus, Foxn1 is expressed within CD45\textsuperscript{neg} TEC (17, 18). To our surprise, in Foxn1\textsuperscript{Tg} mice, Foxn1 was expressed within the CD45\textsuperscript{pos} population in the BM (Fig. 65). A percoll gradient was used to separate fractions of BM cells from Wt mice based on cell density. This gradient allowed for isolation of an adipose layer, a middle layer which we would expect to find epithelial cells and stromal cells, and a dense bottom layer that contains predominantly hematopoietic CD45\textsuperscript{pos} cells. Since Foxn1\textsuperscript{Tg} BM cells expressed Foxn1 in CD45\textsuperscript{pos} cells, we expected that the dense layer of the percoll gradient in Wt mice would express Foxn1. Indeed, Foxn1 was expressed in the third percoll layer from young and aged Wt BM cells (Fig. 66).

**A subset of Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} Syndecan-1\textsuperscript{neg} cells express Foxn1.**

In the thymus, medullary thymic epithelial cells (mTEC) are the predominant TEC expressing Foxn1 (17). Medullary TEC also express EpCAM (348). Thus, I set out to determine if a population of cells in the BM of Wt and Foxn1\textsuperscript{Tg} mice expresses EpCAM. EpCAM\textsuperscript{pos} cells were identified in both Wt and Foxn1\textsuperscript{Tg} BM amongst the Lin\textsuperscript{neg/low} population (Fig. 67). With age the frequency of the Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} cells
Figure 65: Foxn1 is expressed in CD45\textsuperscript{pos} cells. Tibias and femurs from a Foxn1Tg mouse were flushed and BM cells were sorted for CD45\textsuperscript{pos} and CD45\textsuperscript{neg} subsets. RT-PCR was used to measure if Foxn1 expressed in CD45\textsuperscript{neg} and CD45\textsuperscript{pos} cells. Vertical lines represent repositioning of gel.
Figure 66: *Foxn1* expression in the third fraction of a percoll gradient in Wt BM.

Young and aged Wt BM cells were separated into three fractions based on density using a percoll gradient. The densest fraction in young and aged Wt BM expressed *Foxn1*. 
increased in both Wt and Foxn1Tg mice; however, the frequency was higher in aged Foxn1Tg compared to aged Wt (Fig. 67).

Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} cells were electronically sorted from aged Foxn1Tg BM and assessed for Foxn1 expression by immunohistochemistry staining. We determined that 12.8% of the Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} cells were positive for Foxn1 in aged Foxn1Tg mice (Fig. 68). Plasma cells also express EpCAM (349) and have a morphology similar to the Foxn1\textsuperscript{pos} cells found in the BM. The Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} population was sorted for cells that were negative for Syndecan-1, a common marker of plasma cells (350) (Fig. 69). We determined that 23% and 40% of the Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} were negative for Syndecan-1 in Wt and Foxn1Tg, respectively. Among the Syndecan-1\textsuperscript{neg} population on average 45% and 68% of these cells were positive for Foxn1 in Wt and Foxn1Tg, respectively (Fig. 69). The frequencies of Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} Syndecan-1\textsuperscript{neg} Foxn1\textsuperscript{pos} cells per 100,000 BM nucleated cells were 3±1 in old Wt mice and 95±49 in old Foxn1Tg mice (Fig. 70). This was the first finding showing that Foxn1 is expressed in the BM by a subset of cells within the Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} Syndecan-1\textsuperscript{neg} population.

To further support that Foxn1 is expressed in the BM, I assessed the endogenous Foxn1 promoter activity in BM isolated from the Foxn1cre-Lac Z reporter mice in which the Foxn1 promoter drives the expression of the E.Coli LacZ gene encoding β-galactosidase. Electronically sorted Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} Syndecan-1\textsuperscript{neg} BM cells from Foxn1cre-LacZ mice were stained with an anti-β-galactosidase antibody. Figure 71 shows that β-galactosidase expressing cells were morphologically identical to the
Figure 67: Identification of Lin$^{\text{neg/low}}$ EpCAM$^{\text{pos}}$ BM cells. Flow cytometry was used to determine the frequency of Lin$^{\text{neg/low}}$ EpCAM$^{\text{pos}}$ cells in the BM of Wt and Foxn1Tg 3-4 and 18-23 months.
Figure 68: Lin$^{\text{neg/low}}$ EpCAM$^{\text{pos}}$ cells from Foxn1Tg mice 18-23 months of age were electronically sorted and stained with rabbit anti-mouse Foxn1 2μg/mL (A) or rabbit IgG (B). Approximately 12.8% of Lin$^{\text{neg/low}}$ EpCAM$^{\text{pos}}$ cells in from aged Foxn1Tg express Foxn1. Panel A represents only positive cells with in the Lin$^{\text{neg/low}}$ EpCAM$^{\text{pos}}$ subset from 3 aged Foxn1Tg mice.
Figure 69: Foxn1 expression in Lin^{neg/low} EpCAM^{pos} Syndecan-1^{neg} cells from aged Foxn1Tg and Wt mice. Lin^{neg/low} EpCAM^{pos} Syndecan-1^{neg} cells from Foxn1Tg (23 mo) and Wt (24 mo) were electronically sorted and stained for Foxn1 (A and C) or rabbit IgG (B and D). Approximately 23% and 40% of Lin^{neg/low} EpCAM^{pos} Syndecan-1^{neg} cells from Wt and Foxn1Tg express Foxn1 respectively. Panels A and C depict only positive cells.
Figure 70: Frequency of Foxn1<sup>pos</sup> cells in the BM of aged Wt and Foxn1<sup>Tg</sup> mice 18-23 mo. The frequency of Foxn1<sup>pos</sup> cells was determined by multiplying the percent of Foxn1<sup>pos</sup> cells within the sorted Lin<sup>neg/low</sup> EpCAM<sup>pos</sup> Syndecan-1<sup>neg</sup> population by the frequency of Lin<sup>neg/low</sup> EpCAM<sup>pos</sup> Syndecan-1<sup>neg</sup> cells in total BM. Numbers in parenthesis denoted the number of mice in each group. Error bars are SD.
Figure 71: Endogenous Foxn1 promoter is active in the aged BM. Lin^{neg/low} EpCAM^{pos} Syndecan-1^{neg} cells from the BM of Foxn1-lacZ mice 24 months of age were stained with rabbit anti-E.coli β-galactosidase or rabbit IgG.
Foxn1\textsuperscript{pos} within the Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} Syndecan-1\textsuperscript{neg} population of Foxn1\textsuperscript{Tg} and Wt mice. Thus, the endogenous Foxn1 promoter is active in the BM cells.

**Summary**

This chapter demonstrated that Foxn1 is expressed in the BM of Wt mice and over expressed in the BM of Foxn1\textsuperscript{Tg} mice. Contrary to the thymus, the mRNA expression level of Foxn1 in the BM did not decline with age. However, with age the number of Foxn1\textsuperscript{pos} cells increased in both Wt and Foxn1\textsuperscript{Tg} BM. The transcriptional and translational regulation of Foxn1 is not known. Since the mRNA levels did not change with age, but the number of Foxn1\textsuperscript{pos} cells detected *in situ* increased, it is possible that expression of Foxn1 is differentially regulated at the protein levels in aged BM compared to young BM. Foxn1\textsuperscript{pos} cells were identified within a population of BM cells that are Lin\textsuperscript{neg/low} and EpCAM\textsuperscript{pos}. In agreement with the immunohistochemistry staining showing that the number of Foxn1\textsuperscript{pos} cells increase with age, the frequency of Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} cells increased in aged Wt and Foxn1\textsuperscript{Tg} BM. Thus, it is possible that the number of Foxn1\textsuperscript{pos} cells found within the Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} population increases with age. However, this scenario does not correlate with the mRNA expression level of Foxn1.

Quantitative RT-PCR analysis on the sorted Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} cells would clarify whether the mRNA expression level of Foxn1 increases with age. However, since the frequency of Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} cells in the BM of young Wt and Foxn1\textsuperscript{Tg} mice is very low, the approach is technically challenging.
Foxn1^pos cells were detected in the BM of Wt and Foxn1Tg mice near sinusoids. It is possible that the Foxn1^pos cells function in the vascular HSC niche where HSC are proliferating and differentiating into MPP. Morphologically, the Lin^{neg/low} EpCAM^{pos} cells appeared similar to plasma cells which a subset express EpCAM (349). However, a majority of plasma cells express Syndecan-1 (350). My finding that the Lin^{neg/low} EpCAM^{pos} Syndecan-1^{neg} BM population expressed Foxn1 indicate that they are unlikely to be plasma cells. Further, sorted Lin^{neg/low} EpCAM^{pos} Syndecan-1^{neg} cells from Foxn1cre-lacZ reporter mice were positive for β-galactosidase, confirming that the endogenous Foxn1 promoter is active in these cells. Taken together, data from this chapter demonstrates that Foxn1 is expressed in the BM within a population of Lin^{neg/low} EpCAM^{pos} Syndecan-1^{neg} cells.
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Two way ANOVA analysis summary (more important findings). Table summarized whether age has an affect on a parameter, whether *Foxn1* expression affects the parameter, and whether over expression of *Foxn1* interacts with the age effect.
Table II.

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Two way ANOVA analysis summary (less important findings). Table summarized whether age has an affect on a parameter, whether Foxn1 expression affects the parameter, and whether over expression of Foxn1 interacts with the age effect.
CHAPTER VII
DISCUSSION

The initial decline in thymocytes number with age correlates to the decline in the transcription factor Foxn1 expressed in thymic stroma (14). Induced deletion of the Foxn1 gene leads to reduced Foxn1 expression and results in premature thymic involution as seen by a decline in the number of thymocytes and mTEC (17, 18); these above findings led me to predict that Foxn1 is required to maintain thymopoiesis in the postnatal thymus. Foxn1 was identified in nude mice that do not have a functional thymus as Foxn1 is required for thymic organogenesis (15). Interestingly, the thymus is not the only primary lymphoid organ disrupted in nude mice. The BM of nude mice display reduced cellularity and hematopoiesis (19, 275). Based on these observations, I predicted that Foxn1 also function in the BM to maintain HSC.

I predicted that manipulating Foxn1 expression affects the decline in thymopoiesis with age and hypothesized that over expression of Foxn1 prevents the decline in thymopoiesis with age. Using the Foxn1Tg mouse model, I demonstrated that age-associated thymic involution is attenuated in Foxn1Tg as seen by a higher number of thymocytes, ETP, and maintained thymic architecture. I also demonstrated that over expression of Foxn1 prevents the decline in MPP. Aged Foxn1Tg had a higher number of HSC with increased proliferation. Additionally, the aged Foxn1Tg BM microenvironment promoted the generation of MPP from HSC. Over expression of
Foxn1 also increased CTP proliferation and CIP generation, demonstrating that over expression of Foxn1 promotes the survival and production of T cell progenitors. The effect of over expression of Foxn1 was specific to the development of T cell progenitors as the number of CLP were reduced in Foxn1Tg and myeloid progenitors were not affected. I identified the Foxn1 expressing BM cells as a population of BM cells that are Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} Syndecan-1\textsuperscript{neg}. Taken together, expression of Foxn1 by cells in both the BM and the thymus is important for the generation of T cell progenitors and thymopoiesis.

**Foxn1 Expression in the Thymus**

**Regulation of Foxn1 expression in the Thymus**

Regulation of Foxn1 expression during thymic organogenesis or in the post natal thymus has not been clearly elucidated. Most of what is known about the regulation of Foxn1 comes from studies examining its expression in the skin. In the hair follicle, BMP4 and homeobox C13 (Hoxc13) can up regulate Foxn1 expression (351, 352). Signaling though the fibroblast growth factor (FGF) receptors in epidermal keratinocytes has also been shown to increase Foxn1 expression (353). However, there is no evidence to support this regulatory pathway in the regulation of Foxn1 expression in the post natal thymus. While BMP4 is expressed in the ventral region of the third pharyngeal pouch (354), direct evidence linking BMP4 to Foxn1 expression during thymic organogenesis or in the post natal thymus has not been established. In the hair follicles, Msx2 can regulate Foxn1 expression that in turn regulates Notch 1 expression (351). This pathway was
examined in the thymic stroma; however, we were unable to detect Msx2 expression in Wt or Foxn1Tg thymic stroma (data not shown). It is likely that regulation of Foxn1 expression in the thymus is different than in the skin as two Foxn1 promoters have been identified and the two promoters are differentially regulated in the thymus and in the skin (336). Therefore, it is not surprising that factors that regulate Foxn1 expression in the skin that have not been to be able to regulate Foxn1 expression in thymic epithelial cells.

Foxn1 expression in the thymus has been shown to decline with age (14, 17). In the fetal thymus, maintenance of expression of Foxn1 requires the three dimensional meshwork of TEC as it was shown that expression of Foxn1 was reduced in TEC that were obtained from enzyme-treated thymi and cultured as monolayer (76). It has been shown that Wnt signaling can up regulate Foxn1 expression in TEC in vitro, and both TEC and developing thymocytes express Wnt (355). In the aged Wt thymus, expression of the Wnt inhibitor axin increases in mTEC and promotes adipogenesis in the thymus (150). Expression of axin thus may contribute to the decline in Foxn1 expression in two ways: 1) Axin can inhibit Wnt signaling directly, resulting in decreased Foxn1 expression. 2) Axin promotes adipogenesis in the thymus which can disrupt the 3D TEC meshwork required to maintain Foxn1 expression. In aged Foxn1Tg thymi, we observed less adipose accumulation. The mechanism by which over expression of Foxn1 prevents adipogenesis in aged Foxn1Tg thymi remains to be determined. It is possible that Foxn1 negatively regulates expression of Axin thus maintains its own expression.

The targets of Foxn1 in the thymus are also not definitively indentified. However, using primers that only detect the Foxn1 transgene we were able to measure the
endogenous Foxn1 levels and determined that one target of Foxn1 is Foxn1. In Foxn1Tg thymic stroma, expression of the Foxn1 transgene resulted in an increase in endogenous Foxn1 expression, suggesting that Foxn1 is self-regulating. A DNA consensus sequence for the Foxn1 binding site has been identified (356). Five possible binding sites for Foxn1 are located upstream of the start codon. Chromatin immunoprecipitation experiments could be performed to determine whether Foxn1 regulates its own promoter directly. It is possible that regulation of the Foxn1 promoter activity by Foxn1 occurs through an unidentified intermediate protein. Our finding that expression of Foxn1 is self-regulated agrees with the previous notion that Foxn1 functions in a cell autonomous fashion and supports the finding that TEC that express high level of Foxn1 are more sensitive to changes in Foxn1 levels (17, 357). Furthermore, the same induced levels of the endogenous Foxn1 in young and aged mice suggests that down regulation of Foxn1 with age is reversible and can be manipulated to reverse expression of Foxn1 in the aged thymus.

**A Role for Foxn1 in Thymopoiesis with Age**

Our laboratory previously demonstrated that the initial decline in thymocyte number correlated with the onset in the decline in expression of Foxn1 (14). To determine if over expression of Foxn1 prevents the decline in thymopoiesis, Foxn1Tg mice were designed that express mouse Foxn1 under the control of the human keratin 14 promoter. The keratin 14 promoter was chosen because keratin 14 is expressed in epithelial and thymic epithelial progenitors (358, 359). Expression of Foxn1 mRNA in
the thymus of young Foxn1Tg was 20-fold higher than young Wt. At the protein level, the expression pattern of Foxn1 within the thymus of Wt and Foxn1Tg were similar although the intensity of Foxn1 staining was increased in Foxn1Tg. The higher intensity staining of Foxn1 in Foxn1Tg thymi is the result of translating a larger number of transcripts from both the endogenous and transgenic Foxn1 genes. Importantly, the high levels of Foxn1 did not alter the thymic architecture or the proportion of thymocyte populations, indicating normal thymopoiesis in young Foxn1Tg mice.

In agreement with previous reports, the distributions of the DN, DP, and SP thymocytes populations did not change with age in Wt mice (161, 162). This finding indicates that the decline in thymocyte number in aged Wt mice is not the result of a block in thymopoiesis, but an overall decrease in the number of developing thymocytes and consequently naïve T cell output. This was also true in aged Foxn1Tg as the frequency of each sub-population was not changed with age. However, it is possible that the kinetics at each stage of development is reduced in aged mice, contributing to the overall decrease in the number of naïve T cell produced without disrupting the distribution of DN, DP, and SP thymocytes. As previously reported, expression of Foxn1 declined in aged Wt mice (14). However expression of Foxn1 was not reduced in aged Foxn1Tg. Thus, aged Foxn1Tg mice do not demonstrate any alterations in the distribution of DN, DP, and SP thymocyte populations and provide a model for studying the effects of over expression of Foxn1 on thymic involution.

The size of the thymus is regulated by the available cortical niches for the expansion of DN cells during thymopoiesis (360). Young Foxn1Tg and young Wt had
equivalent number of thymocytes and DN, DP, SP thymocyte distribution indicating that
*Foxn1* does not increase the number or function of cortical niches that support
thymopoiesis in young mice. With age, in Wt mice there was a drastic decline in the total
number of thymocytes from 3 months to 16-19 months and from 16-19 to 20-26 months.
The total thymocyte numbers declined with age in *Foxn1*Tg, however the decline was
delayed. The total number of thymocytes in *Foxn1*Tg mice 20-26 months of age was
equivalent to Wt 16-19 months of age, suggesting that the decline in thymocytes number
was attenuated by approximately least 6 months. The higher number of thymocytes with
equal distribution of DN, DP, and SP thymocyte populations in aged *Foxn1*Tg compared
to Wt indicated a higher total number of DN cells in *Foxn1*Tg, and the size of the thymus
in aged *Foxn1*Tg was larger than aged Wt. This suggests a higher number of cortical
niches in aged *Foxn1*Tg. The total number of cortical TEC was not different between
aged Wt and aged *Foxn1*Tg; however, cTEC by themselves do not constitute a functional
niche. As previously mentioned, scattered *Foxn1^pos* cells can be detected in the cortex.
Previously, it was shown that *Foxn1^neg* cells in the thymus arise from *Foxn1^pos* cells, and
that only *Foxn1^pos* cells contribute to thymopoiesis (361). It is possible that the number
of *Foxn1^pos* cells in the cortex of Wt mice decrease with age contributing to the reduced
number of DN cells and the production of naïve T cell. If the number of *Foxn1^pos* cTEC
did not decline with age, or if the decline in *Foxn1^pos* cTEC was attenuated in aged
*Foxn1*Tg mice, then we would expect to find a larger number of DN in aged *Foxn1*Tg as
supported by our finding.
The recruitment of thymic progenitors to the thymus is also regulated by the number of available cortical niches (361). The frequency of ETP and number of ETP in young Wt and young Foxn1Tg were equivalent suggesting that over expression of Foxn1 in young mice did not increase the number of cortical niches. A reduction in the number of cortical niches with age can contribute to the decreased frequency and number of ETP found in aged mice (12). In agreement with previous reports, we detected a reduction in the frequencies and number of ETP in aged Wt mice (12). However, over expression of Foxn1 in aged mice prevented the decline in the frequency of ETP and lessened the decline in ETP number. To determine if the aged Foxn1Tg thymic environment is more efficient at the recruitment of thymic progenitors and generation of ETP adaptive transfer experiments were performed. In the adaptive transfer experiments, aged Foxn1Tg and Wt thymi showed the same frequency of the donor CD45.1pos ETP suggesting that recruitment of thymic progenitors was not enhanced in the aged Foxn1Tg. This could be explained by the fact that LSK cells from young mice were more effective in homing to the thymus. These data suggest that the aged Foxn1Tg and aged Wt thymic microenvironments are equivalent in recruiting young cells. Alternatively, young ETP maybe able to compensate some of the age associated defects in the aged Wt thymic microenvironment resulting in an ETP frequency similar to that seen in aged Foxn1Tg.

Using fetal thymic organ culture, aged Wt ETP showed reduced T lineage potential (12), indicating functional defects in aged ETP. Using the OP9-DL1 culture, I did not see a difference in the commitment of aged Wt ETP to T lineage or development to the DN2/DN3 stages compared to young Wt. This is likely the result of an optimized
culture system for both young and aged cells. Using this system we were unable to
determine if high levels of Foxn1 prevented the functional defects in aged ETP.
Interestingly, data from the adaptive transfer experiments indicate that the aged Foxn1Tg
thymic microenvironment may promote Rag expression and activity in ETP compared to
the aged Wt thymic microenvironment. The aged Foxn1Tg hosts generated a higher
frequency of ETP that expressed VEX than aged Wt hosts. The aged Foxn1Tg hosts had
a 20% increase in the percent of ETP that expressed VEX, indicative of Rag expression
and activity, compared to aged Wt hosts. This suggests that the Foxn1Tg
microenvironment may be more efficient in inducing Rag expression and activity and in
the initiation of TCR gene rearrangement during thymopoiesis.

A Role for Foxn1 in Maintaining Thymic Architecture with Age

Thymic involution is associated with alterations of the thymic architecture and
deposition of fat. As previously mentioned, Wnt signaling in TEC decreases with age
resulting in decreased Foxn1 expression (150, 362). In aged TEC, when Wnt4 signaling
is decreased, LAP2α expression increases (362). LAP2α expression results in an increase
in peroxisome proliferator activated receptor γ (PPARγ) which is a transcription factor
expressed in pre-adipocytes (362). It was suggested that epithelial cells transition to
mesenchymal cells, which then transition to adipocyte, and that this differentiation is a
potential mechanism of adipose deposition in the aged thymus (362). The thymus of
aged Foxn1Tg is smaller than young Foxn1Tg; however, compared to changes seen in Wt
mice, there is little fat deposition and the corticomedullary junction remains intact. The
reduced deposition of fat in aged Foxn1Tg mice may be the result of inhibition of the transition of epithelial cells to adipocytes via the EMT pathway. In the caloric restricted mice, the epithelial mesenchymal transition is blocked and adipogenesis is prevented through inhibition of PPARγ expression (154). A direct role of Foxn1 in adipogenesis remains to be determined; however, it is possible that Foxn1 negatively regulates PPARγ expression in Foxn1Tg.

Previous work has shown that reduced Foxn1 expression in the post natal thymus causes rapid thymic atrophy mainly due to severe deterioration of mTEC compartment, indicating that Foxn1 expression is critical for the maintenance of mTEC population in the postnatal thymus (17). It was also noted that the MHCIIhi mTEC display the highest proliferative rate and are responsible for generating the mTEC pool (17, 81, 363). The higher number of MHC IIpos and MHCIIhi mTEC in aged Foxn1Tg are in agreement with the above finding as there are more mTEC when Foxn1 is over expressed. The larger number of mTEC in aged Foxn1Tg is the result of a higher level of MHCIIhi mTEC that are engaged in cell cycle. The elevated number of mTEC and higher proliferation of the MHCIIhi mTEC in the aged Foxn1Tg than in Wt suggest that the presence of a larger mTEC pool may be responsible for limiting alterations in thymic architecture.

Young Foxn1Tg had a higher number of MHCIIhi and MHCIIpos mTEC compared to young Wt without increased proliferation. The mechanism that results in an increased number of mTEC in young Foxn1Tg is unknown. During thymic organogenesis, differential patterning of the thymic and parathyroid primordium could result in more epithelial cell progenitors are recruited into differentiating into thymic epithelial cells
Alterations in the patterning of the thymic primordium are unlikely to be the cause in the higher number of mTEC in young Foxn1Tg mice. The Foxn1 transgene is driven by the human K14 promoter and would not affect patterning of the thymic and parathyroid primordium as expression of the human keratin 14 promoter is not detected until 14 days post birth (359). Thymic epithelial progenitors capable of differentiating into cTEC and mTEC express K14 and reside in the corticomedullary boundary (129-131). It is possible that after the Foxn1 transgene is transcribed, a larger percent of the epithelial progenitors commit to mTEC differentiation over cTEC. However, if this were the case, then we would expect a reduced number of cTEC in young Foxn1Tg compared to young Wt, and this did not occur. It is possible that the higher number of mTEC in young Foxn1Tg is the result of altered cell cycle activity. The mTEC express the highest level of Foxn1 (17, 18). While young Foxn1Tg mTEC do not display increased cell cycle activity as measured by Ki-67, we did not determine cell death of mTEC. It is possible that high levels of Foxn1 prevent cell death in mTEC, thus resulting in an increase in their number in young Foxn1Tg compared to young Wt.

Foxn1Tg had a higher number of mTEC compared to Wt; however, the number of cTEC was equivalent to aged matched Wt. Despite the fact that Foxn1 expression can be detected in cTEC, over expression of Foxn1 does not prevent their decline in number with age. It remains to be determined how and if the number of Foxn1pos cTEC change with age and if over expression of Foxn1 affects aged Foxn1pos cTEC. It also remains to be determined if Foxn1Tg have a higher number of Foxn1pos cTEC compared to Wt.
Foxn1 and the Bone Marrow

The Effect of Foxn1 on HSC

Zipori et al. demonstrated that BM from nude mice display a reduction in cellularity and a limited protection against lethally irradiation in adaptive transferred hosts (19); a reduced number of HSC and their functions could be responsible for the observed defect. In this context, Foxn1-expressing BM cells could play a potential role in regulating HSC number through regulating cell cycle activity at several different developmental stages through out the postnatal life.

Compared to young Wt, young Foxn1Tg BM contained a higher number of HSC. I determined that the higher number was not due to an increase in cell cycle or reduced cell death of HSC. Alternatively, an established larger HSC pool could provide an explanation for our finding. It has been observed that increased HSC niche size, as determined by an increase in the numbers of osteoblasts and trabecular bone, correlates with a higher number of HSC (210, 211); however, we observed no obvious difference in the amount of trabecular bone in Foxn1Tg and Wt mice by microscopy (data not shown). During embryogenesis, the size of the HSC pool is regulated by the sry-related high mobility group box 17 (sox17) (205). At four weeks post birth, Sox17 expression is down regulated as highly proliferative fetal HSC transition to the slowly proliferating adult phenotype, establishing the adult HSC pool (205). It is possible that a larger HSC pool in Foxn1Tg BM may result from prolonged Sox17 expression during embryonic development.
In agreement with others, we found that the number of HSC in Wt BM increased from 1-4 months of age to 20-21 months of age (318, 324). It has been suggested that this increase in HSC with age is not due to increased cell cycling (345) but rather due to a block in the differentiation of HSC toward the immediate progeny MPP as their numbers are reduced with age (13). Alternatively, it was suggested that the increase in HSC number is a compensatory mechanism for the reduced number of HSC that differentiate to lymphoid lineage (324, 328). We found that with advanced age, in Wt mice (24-25 mo), the initial increase was followed by a dramatic reduction in HSC number that is coupled with increased cell death. If the increase in HSC is in fact a compensatory mechanism to increase the number of HSC that are able to differentiate to the lymphoid lineage, then the drastic decline in Wt 24 mo and older may be the result of HSC proliferative exhaustion. In Foxn1Tg 24-35 mo, the number of HSC declined; however, the total number of HSC was reduced to levels equivalent in young Foxn1Tg, suggesting that the HSC pool in aged Foxn1Tg is not exhausted even though half of the mice are older than 25 months of age. In contrast to aged Wt, we observed a higher percent of HSC from aged Foxn1Tg in S,G2/M and a reduced fraction of the cells was in G0, characteristic of cell death. We suggest that the increase in cell cycling and reduced cell death culminates in preventing proliferative exhaustion and restoring HSC number in aged mice to that found in young Foxn1Tg mice.

A mechanism by which Foxn1 expression affects cell cycle activity of HSC remains to be elucidated. RT-PCR revealed that Foxn1 is not expressed in HSC or MPP, again suggesting cell-cell interaction between the Foxn1pos cells and HSC play a critical role.
role in homeostasis of HSC with age. Recently, it has been shown that HSC express the
E3 ligase Itch and HSC in Itch deficient mice display increased cell cycling, suggesting
that Itch negatively regulated HSC development and function (364). The increase in cell
cycling of HSC in Itch deficient mice resulted in an increase in the numbers of HSC and
MPP (364). These data are similar with our observation in BM of aged Foxn1Tg mice,
but not young Foxn1Tg mice. While it remains to be determined if expression of Itch
increases with age, it is possible that with age Foxn1-expressing BM cells negatively
affect Itch expression, leading to HSC proliferation and a larger number of HSC and
MPP. The cyclin dependent kinase inhibitor p16\(^{\text{INK4a}}\) which controls the G1 check point
is expressed in aged but not young HSC and contributes to proliferation and number of
HSC observed in aged mice (365). Conversely, aged p16\(^{\text{INK4a}^{-/-}}\) mice have an increase in
the number of HSC due to increased cell cycling and with decreased cell death as
determined by BrdU labeling (365). It is possible that Foxn1\(^{\text{pos}}\) cells in the BM
environment affect age-associated changes in p16\(^{\text{INK4a}}\) expression in HSC, thus resulting
in the proliferation of HSC in responding to stress with advanced age. In contrast, the
number of HSC in Wt mice are exhaustedly depleted with advanced age.

Signaling through c-kit receptor is important for HSC survival (279, 280). The
level of c-kit on embryonic and adult HSC is equivalent, thus its levels are not affected
with age (206, 278, 366); however, the sensitivity of the response of c-kit to its ligand
SCF decreases from fetal to adult HSC (366). Although it was not significantly different,
the MFI expression level of c-kit on aged Foxn1Tg HSC appears higher than young
Foxn1Tg and aged Wt. Eight out of ten HSC from aged Foxn1Tg mice had a higher level
of c-kit expression compared to the average seen on aged Wt HSC. Whether this expression level of c-kit on aged Foxn1Tg HSC has a physiological effect remains to be determined. If the sensitivity of c-kit to SCF decreases with age in Wt mice, then HSC cell death would increase. Indeed, I found more cell death in HSC from aged Wt mice. A compensatory mechanism commonly found in responding to a decrease in receptor signaling is to up regulate expression of the receptor; I found that expression of c-kit increase in HSC from our aged Foxn1Tg. If up regulation of c-kit resulted in increased signaling, then this would result in increased survival which also was observed in aged Foxn1Tg HSC. Because signaling through c-kit is also required to maintain HSC self-renewal and proliferation, the increase in expression of c-kit may also contribute to the increase in HSC proliferation seen in aged Foxn1Tg HSC (367-369).

A Role for Foxn1 in Hematopoiesis

Based on cell surface phenotype, young Foxn1Tg have a larger HSC pool compared to Wt; however, the number of functional HSC as determined by the CFU assay in young Foxn1Tg was equivalent to young Wt. The number of HSC in Foxn1Tg 1-4 months of age was 2-fold higher than Wt as determined by flow cytometry. However, the number of functional HSC that gave rise to multipotent progenitors (CFU-GEMM) as determined by methylcellulose cultures was not different between young Wt and Foxn1Tg. This is likely due to a limited and small number of active HSC that is required to maintain hematopoiesis. As seen in young and aged CTP as well as aged HSC, high levels of Foxn1 can affect their cell cycle activity. Thus, it is possible that in
young *Foxn1Tg* a larger percent of the HSC pool are in the quiescent state. In our experiments, PI staining was used to determine the cell cycle activity of HSC in Wt and *Foxn1Tg* mice. Identification of quiescent HSC in G0 can not be separated from HSC in G1 using propidium iodine. In the future, HSC in G0 and G1 can be separated using Ki-67 to determine the percent of quiescent HSC (Ki-67 negative) in young *Foxn1Tg* BM.

While the number of functional HSC is similar between young Wt and *Foxn1Tg*, with age the number of functional HSC declined in Wt but not in *Foxn1Tg*. The initial larger HSC pool may act as a reservoir, extending the maintenance of hematopoiesis in aging mice. It is estimated that each long term repopulating HSC replicates only 5 times in the life span of a mouse (370). With a larger HSC pool, the number of times each HSC from *Foxn1Tg* replicates for the same number of months as in a Wt mouse would be less, thus exhaustion of HSC would occur much later in life or that age-associated proliferative exhaustion is attenuated. This notion corroborates the previous observation that increased longevity was associated with a larger HSC pool (325). Our data suggest that HSC exhaustion in Wt BM can be seen in mice 24-25 months of age; however, the functional decline can be seen even earlier as the number of GEMM colonies generated in aged Wt methylcellulose cultures 21 months of ages are not different than at 25 months of age. While longevity studies have not been preformed on *Foxn1Tg* mice, we collected data from numerous mice in our two colonies that are healthy and between 25 to 35 months of age.

Aged *Foxn1Tg* HSC also do not experience an age associated decline in the ability to commit to T lineage when cultured on the OP9-DL1 stromal cell line. While
the percent of cells that expressed CD90 did not change with age in Foxn1Tg, the percent
of young Foxn1Tg HSC that expressed CD90 was less than young Wt. This lower
percent of cells that express CD90 in young Foxn1Tg may not result from decreased T
lineage commitment, but of increased self renewal. HSC from Foxn1Tg may have
increased expansion when cultured on OP9-DL1. Notch signaling in vitro was shown to
be important for HSC self renewal (218, 219). Cells harvested from culture on the OP9-
DL1 were not stained for HSC cell surface markers to determine if HSC from Foxn1Tg
expanded in vitro. The expression level of the notch receptors on HSC from Foxn1Tg
and Wt have yet to be examined; however, it is possible that HSC from Foxn1Tg expand
and proliferate more than Wt HSC.

The aged Foxn1Tg microenvironment not only has a higher number of functional
HSC, but the aged BM microenvironment promotes the generation of MPP from donor
HSC. The increased generation of MPP in aged Foxn1Tg is not due to an intrinsic effect
in HSC. This difference in the generation of MPP from donor Wt and Foxn1Tg HSC was
abolished when sub-lethally irradiated hosts were used, reinforcing the notion that the
increase in generation of MPP in aged Foxn1Tg is an environmental effect.

The number of MPP in the BM of Foxn1Tg at advanced age was equivalent to
mice 1-4 months of age. MPP give rise to myeloid progenitors as well as CLP which are
the B cell progenitors. The total number and various lineages of myeloid progenitors
were not different between Foxn1Tg and Wt mice. However, Foxn1Tg had a lower
number of CLP and subsequently a reduced number of B-lineage cells compared to aged
matched Wt mice, suggesting that Foxn1 expression affects B lymphopoiesis (Fig. 72).
Figure 72: MPP differentiation into ETP and CLP in aged Foxn1Tg mice. Aged Foxn1Tg mice have decreased CLP numbers, with a higher number of ETP compared to aged Wt. Foxn1 may work at the level of the MPP to inhibit CLP / B lineage development and priming toward ETP and T lineage development.
Furthermore, although the number of CLP in young Foxn1Tg was less than Wt, over expression of Foxn1 did not prevent the decline in CLP with age, indicating that the effect of Foxn1 with age is T lineage specific. Because the number of MPP is not reduced with age but the generation of CLP is reduced, Foxn1 expression in the BM may limit the development of MPP to CLP, thus providing a larger pool of the MPP as precursors for ETP (Fig. 72). If this were true, then we would expect a higher number of ETP in Foxn1Tg, which is in agreement with our finding that aged Foxn1Tg thymus have a higher number of ETP than aged Wt. This scenario would also result in increased production of T cells which we also saw in the aged Foxn1Tg mice compared to aged Wt.

The increase in MPP development toward ETP over CLP may be the result of priming MPP toward T lineage or alternatively, of inhibiting B lineage development (Fig. 73). T lineage commitment requires Notch signaling (44, 45). It is possible that Foxn1pos cells in the BM express Notch ligands and thus prime MPP to differentiate to T lineage as opposed to B lineage. It is also possible that through cell-cell contact, Foxn1pos cells alter the combinatory transcription factor codes in MPP required for B lineage commitment and development. The transcription factor Ikaros is required for the generation of CLP (310); however, Ikaros null mice retain normal levels of ETP (82). Whether over expression of Foxn1 in the BM environment results in decreased Ikaros expression in MPP leading to reduced CLP development in Foxn1Tg BM remains to be determined. It has also been shown that mice deficient in the fms-like tyrosine kinase 3 ligand (Flt3L) or its receptor have reduced numbers of CLP and pro B cells, resulting in decreased B
Figure 73: Skewing of MPP in Foxn1Tg mice toward T lineage or away from B lineage development. Foxn1$^{\text{pos}}$ cells in the BM may express Notch ligands priming MPP towards T lineage resulting in a higher number of ETP in the thymus. Alternatively, Foxn1$^{\text{pos}}$ cells may negatively regulate IL7 secretion, Ikaros expression in MPP, or reduced Flt3 signaling all of which are required for CLP and B lineage development.
Possible mechanism in which Foxn1\textsuperscript{pos} cells in BM promote T lineage development

Possible mechanisms in which Foxn1\textsuperscript{pos} cells in BM inhibit B lineage development
lymphopoiesis (284-286). Furthermore, signaling through both the Flt3 and IL7 receptor enhances B lineage commitment and differentiation (371). Preliminary data from our laboratory showed no differences in the mRNA levels of Flt3L or IL7 in the BM of Wt and Foxn1Tg mice. Interestingly, we find a higher level of CD135 (Flt3 receptor) on MPP in Foxn1Tg mice compared to Wt. Whether the increase in CD135 expression is a compensatory mechanism for reduced Flt3 signaling or whether high expression of CD135 promotes T lineage development instead of B lineage development needs to be examined.

**Role for Foxn1 in the Development of CTP and CIP**

CTP are T cell progenitors that are capable of contributing to the peripheral T cell pool (269-271). In the BM of Foxn1 mutated nude mice CTP are generated but display reduced cell cycling, resulting in a decline in development of CIP (275). Our data showed that over expression of Foxn1 enhanced proliferation of CTP in both young and aged mice; although it is not known how cell cycling of CTP is affected. The notion that Foxn1 functions in an autonomous fashion suggests that cell-cell contact may be responsible for the increase in CTP proliferation. Since over expression of Foxn1 does not affect cell cycle activity of CIP, the higher number of CIP found in young Foxn1Tg is due to the increase in CTP proliferation and differentiation to CIP. The higher ratio of CIP to CTP in Foxn1Tg suggests that Foxn1 expression promotes the generation of CIP from CTP. This was confirmed in our adaptive transfer experiments in which a higher ratio of donor CIP to CTP was found in aged Foxn1Tg hosts compared to Wt hosts. The
difference in the generation of CIP from CTP between Wt and Foxn1Tg was abolished when donor cells were transferred to a sub-lethally irradiated host, suggesting that the cells responsible for the generation of CIP may be sensitive to irradiation. My findings are not the first to demonstrate that irradiation affects the outcomes of donor cells in the aged hosts (13). Irradiation results in increased cytokine production and increased proliferation of HSC to replenish hematopoietic cells killed by irradiation treatment. It is possible that the elevated cytokine levels affects the proliferation of CTP and the generation of CIP from CTP in a manner in which differences in the environment are no longer obvious. It is also possible that irradiation of Foxn1Tg mice results in death of the cells that promote the generation of CIP, thus eliminating the effect seen in non-irradiated Foxn1Tg hosts.

In the Wt BM, the number of CTP and CIP increase with age. Although it is a controversial issue, it was previously suggested that the generation of T cells from the CTP and CIP pathway is thymic independent (269, 270, 274). If CTP and CIP develop independently from the thymus, then the increase in the number of CTP and CIP with age may be a compensatory mechanism for thymic involution. With age, the development of MPP is reduced. Development of T cells from MPP requires homing to and development within the thymus. As the thymus involutes, aged mice may take advantage of a thymic independent pathway to maintain some level of T cell production (Fig. 74).

The development of T cell from CTP is inhibited by the presence of mature TCR α/β+ T cells (273). Although the total number of mature peripheral T cells is not reduced with age, the distribution of naïve and memory T cells changes as the number of naïve T
Figure 74: Age-associated alterations in T lineage progenitors within the BM of Wt mice. HSC give rise to MPP as well as CTP. Both MPP and CTP have the potential to develop into T cells through different pathways. With age, the MPP pathway is reduced while the CTP pathway is enhanced.
cells is reduced due to thymic involution, and the number of memory T cells increases via homeostatic proliferation (147). If the production of CTP is inhibited by naïve T cells, then we would expect that the number of CTP increases with age as thymic output of naïve T cells is reduced. Indeed, we found that CTP numbers increase with age in Wt mice, but the increase was 2-fold less in our Foxn1Tg mice. The lower magnitude of the increase in CTP could be due to a higher level of naïve T cells produced in the aged thymus of Foxn1Tg (347). Because the contribution of T cells developed from CTP progenitors has not been demonstrated, it is not clear how the increase in the number of CTP and CIP with age affects the peripheral T cell pool and its function; however, this potential effect is limited in the Foxn1Tg mice.

**Expression and Regulation of Foxn1 in the Bone Marrow**

Changes in various T lineage subsets and HSC observed in Foxn1Tg compelled us to search for Foxn1 expressing cells in the BM of Foxn1Tg and Wt mice. Foxn1pos cells were found located not adjacent to trabecular bone but were instead found within the central marrow and either immediately adjacent to or within an estimated 3 cell length distance to sinusoids. Thus, Foxn1pos cells appear associated with the previously described vascular niche rather than the endosteal niche. The quiescent long-term HSC reside within an endosteal niche, while the short-term HSC are located within the vascular niche adjacent to sinusoids; HSC in this niche are readily proliferating and differentiating into MPP (208). Based on the location of the Foxn1pos cells in the BM, we speculate that Foxn1 expressing cells within a vascular niche may contribute to the
proliferation of HSC thus, contributing to the maintenance of higher number of MPP with age.

Morphologically, Foxn1pos cells have a large amount of cytoplasm and a prominent nucleus, resembling plasma cells in morphology and size. However, it is unlikely that plasma cells express Foxn1. Plasma cells express the epithelial cell adhesion molecule EpCAM (349, 350); however, plasma cells also express Syndecan-1 (350). Since the Foxn1pos cells are a large subset of the Linneg/low EpCAMpos that do not express Syndecan-1, it is unlikely that the Foxn1pos cells are plasma cells. Linneg/low EpCAMpos cells that were negative for Syndecan-1 were sorted from the BM of Foxn1Tg mice and stained for Foxn1. Approximately 68% of the EpCAMpos Linneg/low Syndecan-1neg cells expressed Foxn1 in aged Foxn1Tg BM. To verify that the presence of Foxn1pos cells in the BM is not due to ectopic expression of Foxn1 in Foxn1Tg mice, EpCAMpos Linneg/low Syndecan-1neg cells from aged Wt BM were sorted and stained for Foxn1. Approximately 45% of Linneg/low EpCAMpos Syndecan-1neg cells isolated from aged Wt BM were positive for Foxn1. To further confirm the phenotype of Foxn1-expressing cells within the BM, I electronically sorted cells of identical phenotype from Foxn1cre-lacZ mice 24 months of age and found that these cells were positive for the bacterial β-galactosidase. This demonstrated that the endogenous Foxn1 promoter is active in a population of Linneg/low EpCAMpos Syndecan-1neg cells. Furthermore, the morphology of Foxn1pos cells in the BM from Foxn1cre-lacZ was the same as that seen in Wt and Foxn1Tg.
Previous experiments by Takaki et al. suggested that the BM contains precursors to TEC (372). In these experiments, BM cells from a GFP-H2b donor mouse (B6 background) was transferred directly into the BM of lethally irradiated hosts (372). After 8 weeks, GFP<sup>pos</sup> donor cTEC and mTEC could be identified in the thymus of host mice (372). TEC progenitor in the thymus express Foxn1, thus it is possible that any TEC progenitor in the BM would also express Foxn1. Signals that would require TEC progenitors in the BM to migrate to the thymus were not examined. However, it is unlikely that the Foxn1<sup>pos</sup> cells indentified in the BM are TEC precursors. TEC are CD45<sup>neg</sup> and in contrast, the Foxn1<sup>pos</sup> cells in the BM are CD45<sup>pos</sup>.

Recently, it was identified that non-myelinating Schwann cells reside in the BM within the HSC niche (373). It was shown that the non-myelinating Schwann cells produce TGF-β that is responsible for maintaining HSC in quiescent state (373). Morphologically these non-myelinating Schwann cells have a lot of cytoplasm and appear similar to the Foxn1<sup>pos</sup> cells detected using immunohistochemistry; however, the Foxn1<sup>pos</sup> cells are larger in size then the non-myelinating Schwann cells. Whether the Foxn1<sup>pos</sup> cells are a neuronal cell or neuroendocrine cell remains to be determined, but it is thought provoking as regulation of the immune system by the nervous system has been reported (374).

In young Wt BM the frequency of Foxn1<sup>pos</sup> cells in sternum sections were low, and while higher in young Foxn1<sup>Tg</sup> compared to young Wt, the number of Foxn1<sup>pos</sup> cells per sternum section was still rare. Despite the low number of Foxn1<sup>pos</sup> cells in young Wt and Foxn1<sup>Tg</sup> BM, a clear role for Foxn1 in hematopoiesis was demonstrated as the
number of CLP are significantly reduced in young FoxnTg, and young FoxnTg have a higher frequency of CTP in cell cycle. It is possible that Foxn1<sup>pos</sup> cells are precursors that give rise to more mature cells that no longer express Foxn1 but expand in number and that this larger population is responsible for affecting hematopoiesis. It is also possible that the small population of Foxn1<sup>pos</sup> cells affects hematopoiesis directly. Expression of Foxn1 in the BM promotes the development of T cell progenitors but as we can see the number of B cell progenitors is drastically reduced. A large population of Foxn1<sup>pos</sup> cells could completely inhibit B lineage development in the BM and the size of the Foxn1<sup>pos</sup> population would need to be tightly regulated. If the Foxn1<sup>pos</sup> cells are within the vascular niche interacting directly with MPP to influence T lineage vs. B lineage fate, then a large number of Foxn1<sup>pos</sup> cells would not be needed. Rather, a small number of Foxn1<sup>pos</sup> cells located in the functionally appropriate niche that interacts with MPP could influence the cell fate decision as MPP themselves are a small population within the BM.

Immunohistochemistry analysis of Wt and FoxnTg sternums suggest that the number of cells that express Foxn1 increase with age. The frequency of Lin<sup>neg/low</sup> EpCAM<sup>pos</sup> cells in the BM which contain the Foxn1<sup>pos</sup> cells is low and difficult to sort in young Wt and FoxnTg BM. This is supported by the rare presence of Foxn1<sup>pos</sup> cells seen in young BM using immunohistochemistry. The frequency of Lin<sup>neg/low</sup> EpCAM<sup>pos</sup> BM cells is higher in aged Wt and FoxnTg compared to their young counterparts, and the presence of Foxn1<sup>pos</sup> cells in the BM of aged Wt and FoxnTg BM were readily detectable using immunohistochemistry assay. Thus, with age it is likely that as the
number of Lin\textsuperscript{neg/low} EpCAM\textsuperscript{pos} increases, so does the number of Foxn1\textsuperscript{pos} cells within this subset.

Expression of Foxn1 in the thymus and the skin is well documented; however, expression of Foxn1 in other organs was not previously investigated. Data from other laboratories have suggested a potential role for Foxn1 in the BM (19, 275); however, direct evidence of Foxn1 expression in the BM was lacking. Using quantitative RT-PCR, we demonstrated that Foxn1 was expressed in the BM of Wt mice; contrary to the thymus, expression of Foxn1 did not change with age. Foxn1 was expressed 98-fold higher in the BM of Foxn1Tg and also did not decline with age. As previously reported in the thymus, expression of the Foxn1 transgene resulted in an increase in the level of endogenous Foxn1. Also in agreement with data from the thymus, the increase in the level of endogenous Foxn1 was equivalent in young and aged Foxn1Tg BM. These data support the previous notion that Foxn1 is auto-regulated. While the direct targets of Foxn1 in the BM remain to be elucidated, and in agreement with the thymus, one target of Foxn1 is itself.

In the thymus, the endogenous level of Foxn1 increased 100-fold when the transgene was expressed. In the BM, the endogenous Foxn1 level was only increased 20-fold. This may be due to a different Foxn1 promoter being active in the BM versus the thymus; it remains to be determined which endogenous promoter is active in the BM. While expression of Foxn1 in the thymus decreased with age, expression of Foxn1 in the BM remained the same. This suggests that the control of Foxn1 expression in the BM is regulated by a different mechanism than in the thymus. A contributing factor to the
decline in Foxn1 expression with age in the thymus is the decline in the number of Foxn1 expressing mTECs. With age, the number of Foxn1<sup>pos</sup> cells in the BM does not decline. In fact our immunohistochemistry staining data suggests the opposite. More Foxn1<sup>pos</sup> cells were detected in the BM of both aged Wt and Foxn1Tg compared to their young counterparts. Since the expression level of Foxn1 mRNA does not change with age it is possible that differences result from the regulation of translation of Foxn1 transcripts or Foxn1 protein turnover. Currently, very little is known about the regulation of Foxn1 at the mRNA level and nothing is known about the post translational regulation of Foxn1. While the level of Foxn1 mRNA is equivalent in young and aged mice, our data suggest that post translational regulation of Foxn1 may change with age.

**Concluding Remarks**

**Putting it all Together**

This dissertation identified a novel role for Foxn1 in the thymus and in the BM in promoting thymopoiesis with age. A novel population of Foxn1<sup>pos</sup> cells in the BM was identified that I speculate to promote the development of T cell progenitors. The increased generation of T cell progenitors occurs by regulating the cell cycle activity of HSC that give rise to MPP. While MPP have multilineage potential, the development of B lineage progenitors from MPP is reduced in Foxn1Tg mice, resulting in a larger MPP pool that can differentiate toward T lineage. This results in a larger number of ETP found in the thymus of aged Foxn1Tg mice. Not only do aged Foxn1Tg thymi have a higher number of ETP, but the proper thymic architecture required for the development
of naïve T cell from ETP is not affected in aged Foxn1Tg thymi. Over expression of Foxn1 minimizes the age associated changes in thymic architecture seen in Wt thymi by regulating the cell cycle activity of MHCII\(^{hi}\) mTEC. Figure 75 summarizes how over expression of Foxn1 in the BM and thymus promotes thymopoiesis with age. Data presented in this dissertation suggest that manipulation of Foxn1 expression in the aged BM and thymus can be utilized to promote the development of T cells.

**Significance**

With advances in health care, the average human life span is being extended, resulting in a larger population of the elderly. Even with advances in health care, the elderly are highly susceptible to illness and diseases, especially to new emerging diseases such as SARS, West Nile virus, and H1N1 otherwise known as swine flu. Decreased production of naïve T cells with age contributes significantly to the reduced immune response in the elderly. Understanding the mechanisms that promote T cell development in aged individuals would provide new strategies and therapeutic interventions to more effectively fight infections. In addition to the elderly, individuals that are immunocompromised due to chemotherapy or HIV infection would also benefit from such therapeutic approaches to promote T cell production. My dissertation lays the foundation that stimulates future discovery of molecular and cellular mechanisms that can be captured to manipulate Foxn1 expression in the BM and the thymus, thus promoting T lineage development in the context of improving general health in the
elderly and of restoring T cell production in patients who undergo chemotherapy or radiation therapy for the treatment of hematologic malignancy diseases.
**Figure 75**: Foxn1<sup>pos</sup> cells are located in close proximity to sinusoids in the BM. The sinusoids are part of the vascular niche where HSC are proliferating and differentiating into MPP. In the BM of aged *Foxn1Tg* mice, there is an increase in HSC proliferation and the aged *Foxn1Tg* BM microenvironment promotes the generation of MPP from HSC contributing to the maintained number of MPP. Over expression of Foxn1 inhibits development of CLP from MPP, resulting in a larger MPP pool that can leave BM and seed the thymus becoming an ETP. Within the aged *Foxn1Tg* thymus ETP encounter an intact thymic microenvironment that promotes the development of naïve T cells. This intact environment is in part the result of higher mTEC proliferation and a larger mTEC pool in aged *Foxn1Tg* thymi.
CHAPTER VIII
MATERIALS AND METHODS

C57Bl/6, H2-VEX, and Foxn1cre-LacZ mice

Young and aged C57Bl/6 (Wt) mice were purchased from Harlan or through contract with the NIA. BM cells from Foxn1cre-lacZ mice were a gift from Dr. Vishwa Deep Dixit (Pennington Biomedical Research Center, Baton Rouge, LA). H2-SVEX mice were a gift from Dr. Rachel Gerstein (University of Massachusetts, Worcester, MA). Both male and female mice were used; comparisons were only made between the same sexes unless otherwise noted. Mice were sacrificed early in the morning, between 6 am and 9 am. All animal work was performed according to protocols approved by Loyola University Stritch School of Medicine IACUC committee.

**Foxn1 transgenic mice (Foxn1Tg)**

The mouse Foxn1 cDNA fragment (2.1Kb) from nucleotide positions 78 to 2255 (NM_008238.1) was cloned into the BamH1 site of the human keratin-14 promoter expressing cassette pG3ZK14 (Dr. Fuchs, Rockefeller University, New York, NY) (K14-Foxn1). The K14-Foxn1 expression construct was sequenced to confirm the correct orientation and its open reading frame. The 5.5- kb NarI-VspI fragment of the K14-Foxn1 construct was used to generate transgenic mice on the B6D2F1 background using standard protocol. Transgenic founders (line 60 and 5) were first identified by Southern
blot analysis and subsequently by PCR analysis of tail genomic DNA using Sigma’s Extract-N-AMP tissue PCR kit (Sigma, St. Louis, MO). Transgenic founders were identified using primers specific for rabbit β-globin, wbnβ-globin intron-Foxn1 (5’ end) and Foxn1-human K14 polyA (3’ end) junctions (Fig. 76). Founders were back crossed to the B6 background for 15 generations. Transgene copy numbers were determined by quantitative PCR using primers specific for exon 3 of Foxn1 gene. Two Foxn1Tg colonies, line 5 with 10-12 copies and line 60 with 4-5 copies of the transgene were maintained at Loyola University Medical Center vivarium. Data from both lines were pooled as no difference was observed between line 5 and line 60.

**Isolation of thymocytes from thymic stroma**

Thymi were removed and placed into a 6 well dish containing 5mL of RPMI supplemented with 5% FCS, 10mM hepes, 100 units of penicillin and 100μg of streptomycin (Invitrogen, Grand Island, NY). Thymocytes were removed from thymic stoma by gentle teasing with a wire mesh and syringe plunger. Thymocytes were washed in 2% BSA/PBS and resuspended for cell count. Thymocytes were counted in 2% acetic acid and trypan blue using a hemocytometer.

**Isolation of BM cells**

Tibias and femurs were removed and placed in to 5mL of RPMI supplemented with 5% FCS, 10mM hepes, and 100 units of penicillin and 100μg of streptomycin (Invitrogen). Bone marrow was flushed from bones using a 21 gauge needle with RPMI
Figure 76: A-B: Design and specificity of primers used for genotyping mice. A. Design of primers used to genotype mice. Green arrows represent Foxn1D primers. Black arrows denote either rabbit β-globin or whnβ-globin that detect the 5’ end of the transgene. Red arrows denote Foxn1-human K14 polyA that recognizes the 3’ end of the transgene. B. Representative gel from one transgenic mouse from line 5 and one from line 60 as well as a Wt mouse.
A

5'

Exon

3'

5'

Foxn1 cDNA 78-2255

3'

Rabbit β-Globin

Human K14 Promoter

1 2 3 4 5 6 7 8 9

Human K14 Poly A

B

Foxn1D 150bp

Wbnβ-blobin 400bp

Rabβglobinwhn 800bp

βglobin Foxn1K14polyA 366bp

Ln 5 Ln 60 Wt
media. Bone marrow plugs were separated by gently pipetting and the cells were washed, resuspended, and counted as for thymocytes.

**Digestion of thymus and isolation of thymic epithelial cells**

Thymi were removed, injected with 0.2 mL of 0.05mg/ml Liberase TM (Roche, Indianapolis, IN) and incubated at room temperature for 8 minutes before dispersing by gentle teasing and pipetting. Digestion was continued at 37°C for 35 minutes with the addition of DNAse (0.2 mg/ml, Roche). After 20 minutes in 37°C cells were gently pipetted, and incubated for another 15 minutes. Thymic stromal cells were released mechanically and enriched using a 50%-25% step percoll (Sigma) gradient (unpublished protocol from TD Logan and Dr. Avinash Bhandoola, University of Pennsylvania). Cells were centrifuged at 900g for 30 minutes with no brake, which separated the cells into three layers. The top layer of the percoll gradient contained adipocytes, the middle interface contained the stromal cells, and the pellet at the bottom contained the thymocytes. The stromal layer was isolated and washed in PBS followed by staining with CD45, MHCII, EpCAM, and Ly51 for 30 minutes. After staining with cell surface molecules, stromal cells were fixed using ebioscience fixation permeabilization kit (ebioscience, SanDiego, CA). Cells were then washed and incubated with Ki-67 for 30 minutes on ice. Total number of TEC was calculated from the frequencies obtained from flow cytometric analysis and total number of isolated thymic stromal cells counted with a hemocytometer.
Separation of BM Cells using Percoll

BM cells were isolated as mentioned above. Cells were washed and layered onto a 50%-25% step percoll (Sigma) gradient. Cells were centrifuged at 900g for 30 minutes at 10°C with no break on the centrifuge. Cells from the three layers were then washed in PBS twice, prior to lysing in Trizol (Sigma). The top layer was adipose, the middle interface contained the stromal cells, and the pellet contained hematopoietic cells.

Following RNA isolation, cDNA was synthesis using the Invitrogen’s SuperScript II synthesis kit according to manufacture’s protocol. RT-PCR was used to determine which layer of BM cells expresses Foxn1.

Quantification of Foxn1 mRNA levels in thymic stroma and BM

Thymic stroma samples were homogenized in Trizol reagent (Sigma) or total BM cells were lysed in Trizol. Total RNA was isolated using according to manufacturer’s protocol. Isolated RNA was then treated with DNase using Ambion DNA-free kit and 0.5-2 µg of RNA was used for cDNA synthesis using the Invitrogen’s SuperScript II synthesis kit. Quantitative RT-PCR was performed on an Applied Biosystems 7100. To differentiate the expression levels of the endogenous and transgene Foxn1 in Foxn1Tg mice, two sets of primer were designed. The first primer set recognize both the endogenous (Foxn1\textsuperscript{Endo}) and the transgene (Foxn1\textsuperscript{Tg}) Foxn1 transcripts; this primers set was used to determine the total levels of Foxn1 (Foxn1\textsuperscript{Total}). The second primer set only detects the Foxn1 transgene (Foxn1\textsuperscript{Tg}). The levels of endogenous Foxn1 mRNA were then calculated by subtracting transgenic Foxn1 levels from total Foxn1 levels (Foxn1\textsuperscript{Endo})
= Foxn1$^{\text{Total}}$ – Foxn1$^{\text{Tg}}$). Sequences for the above primers are presented in Table V. Expression of target genes were normalized to the house keeping gene *Gapdh*. Levels of *Foxn1* expression were extrapolated from a standard curve constructed with five known concentrations ranging from 10 copies/µl to 100,000 copies/µl using the K14-Foxn1 construct or plasmid with the mouse *Gapdh* fragment. Levels of *Foxn1* were expressed as copy numbers/µg RNA.

**Quantitative RT-PCR analysis of *Foxn1* in different BM populations**

Sorted HSC, MPP, CD45$^{\text{pos}}$ BM cells, and CD45$^{\text{neg}}$ BM cells were lysed in Trizol (Sigma) and RNA was isolated according to manufacture’s instructions. Isolated RNA was then treated with DNase using Ambion DNA-free kit and 0.5-2 µg of RNA was used for cDNA synthesis using the Invitrogen’s SuperScript II synthesis kit. PCR was preformed using an Applied Biosystems thermal cycler. PCR amplification was performed in 1.2mM MgCl₂, 0.125mM dNTPs, 0.25µM primers, and with 0.0576 units/µl Taq polymerase (ABI Grand Island, NY). PCR reactions were denatured in 95°C for 4 minutes and each amplification cycle consisted of 3 steps; denatured for 95°C for 15 seconds, annealed at 55°C for 20 seconds, and elongated at 72°C for 40 seconds. A total of 40 amplification cycles were used. Reactions were held at 4°C after the 40th cycle. PCR products were analyzed by gel electrophoresis in 1.2% agarose gel. Gels were stained with ethidium bromide (Sigma) for 5 minutes and then de-stained for 10 minutes in distilled water prior to visualization.
Table V.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward</th>
<th>Reverse</th>
<th>Reference</th>
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<td>TTCCTCAAGGGCAACCACAT</td>
<td>TGTCCACAGGGATCTCCTCAA</td>
<td>436F 586R</td>
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<tr>
<td><em>Whnβ-blobin</em></td>
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<td>TGTGCAGTGCCCTGGATGAAG</td>
<td>445F 845R</td>
</tr>
<tr>
<td><em>Rabβglobinwhn</em></td>
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<td>GTGTTCCCTGCTGGGATGAAG</td>
<td>322F 1169R</td>
</tr>
<tr>
<td><em>βglobin</em></td>
<td>GCTTGAGCTATGCCCAACATC</td>
<td>CGAGAATTATGCAACTCAGATAATGAAG</td>
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</tr>
<tr>
<td><em>Foxn1K14polyA</em></td>
<td>GACCTTGGGACAGACCTGGATGACAANM_008238.1</td>
<td>1663F 1867R</td>
<td></td>
</tr>
<tr>
<td><em>Foxn1</em>endo</td>
<td>ACAGCATCTCTACATCTGGATGACAANM_008238.1</td>
<td>1663F 1867R</td>
<td></td>
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<tr>
<td><em>Foxn1</em>endo set #2</td>
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<td>1663F 1867R</td>
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<tr>
<td><em>Foxn1Tg</em></td>
<td>CTTGAGCTATGCCAACATCAGGAGGAGG</td>
<td>2537F 2845R</td>
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<tr>
<td><em>Gapdh</em></td>
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<td>CATCCTGCACACCAAGAGG</td>
<td>M32599 303F 566R</td>
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<tr>
<td><em>musHprt</em></td>
<td>AGCAGTACAGCCTCCTAGTCTTG</td>
<td>TGGCCCTACATCTTGCTTTGCTTTG</td>
<td>484F 699R</td>
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<tr>
<td><em>Notch 1</em></td>
<td>TTCGTGCTCTCTGTCTTTTGG</td>
<td>TCCTCAAACCGGAACCTTGT</td>
<td>5279F 5529R</td>
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<tr>
<td><em>Notch 3</em></td>
<td>GATTTCCCATACCCACTTCCG</td>
<td>TCTTCATTCCCAGTGATCTTG</td>
<td>4923F 5179R</td>
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</table>

List of primers sequences used for RT-PCR.
Quantification of HSC, MPP, CLP, ETP, CTP and CIP frequency and total cell number

Isolated thymocytes or BM cells (1-2 x10^6 cells) were incubated with anti-mouse CD16/32 in a total volume of 50μL for 20 min. Cells were then stained with a cocktail of biotinylated or FITC-conjugated lineage specific antibodies followed by antibodies to specific cell surface makers as presented in Table VI. If a biotin lineage cocktail was used cells were stained with streptavidin-FITC. For HSC, MPP, and CLP, cells were stained for 30 minutes with a cocktail containing CD117, CD127, Sca-1, and CD135. For ETP cells were stained with a cocktail containing CD117, CD44, CD25, and CD127. For CTP and CIP cells were stained with a cocktail containing CD90 and CD2. The frequency of HSC, MPP, CLP, CTP, and CIP were determined using flow cytometry and the total number was calculated by multiplying the frequency by the number of thymocytes or BM cells isolated.

Electronic sorting of ETP

Thymocytes were first enriched for CD117 positive cells using the EasySep procedure (Stem Cell Technologies Vancouver, Canada) according to manufactures protocol. Briefly, thymocytes were incubated with Fc block followed by incubation with an anti-mouse CD117 PE antibody. Thymocytes were then incubated with an anti-PE antibody attached to magnetic beads. A magnet was used to positively enrich for CD117^{pos} cells. Following positive selection, thymocytes were incubated with a lineage positive cocktail (CD3, CD8, B220, DX5, Gr-1, CD11b, and Ter119) conjugated to FITC
Table VI.

<table>
<thead>
<tr>
<th>Population</th>
<th>Cell Surface Markers</th>
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<tr>
<td>Lin</td>
<td>CD3, CD8, B220, CD11b, DX5, Gr-1, Ter119</td>
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<tr>
<td>ETP</td>
<td>Lin$^{\text{neg}}$ CD117$^{\text{pos}}$ CD44$^{\text{int}}$ CD25$^{\text{neg}}$ CD127$^{\text{neg}}$</td>
</tr>
<tr>
<td>HSC</td>
<td>Lin$^{\text{neg}}$ CD117$^{\text{pos}}$ Sca-1$^{\text{pos}}$ CD135$^{\text{neg}}$</td>
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<tr>
<td>MPP</td>
<td>Lin$^{\text{neg}}$ CD117$^{\text{pos}}$ Sca-1$^{\text{pos}}$ CD135$^{\text{pos}}$</td>
</tr>
<tr>
<td>CLP</td>
<td>Lin$^{\text{neg}}$ CD117$^{\text{low}}$ Sca-1$^{\text{low}}$ CD135$^{\text{pos}}$ CD127$^{\text{pos}}$</td>
</tr>
<tr>
<td>CTP</td>
<td>Lin$^{\text{neg}}$ CD90$^{\text{pos}}$ CD2$^{\text{neg}}$</td>
</tr>
<tr>
<td>CIP</td>
<td>Lin$^{\text{neg}}$ CD90$^{\text{pos}}$ CD2$^{\text{pos}}$</td>
</tr>
<tr>
<td>cTEC</td>
<td>CD45$^{\text{pos}}$ MHCII$^{\text{pos}}$ EpCAM$^{\text{pos}}$ Ly51$^{\text{pos}}$</td>
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<tr>
<td>mTEC</td>
<td>CD45$^{\text{pos}}$ MHCII$^{\text{pos}/\text{hi}}$ EpCAM$^{\text{pos}}$ Ly51$^{\text{neg}}$</td>
</tr>
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</table>

Cell surface identification of population using flow cytometry.
followed by CD44 AF750, CD25 APC, and CD127 PECY7. ETP were identified and sorted as $\text{Lin}^{\text{neg}} \text{CD44}^{\text{hi}} \text{CD117}^{\text{pos}} \text{CD25}^{\text{neg}}$ and $\text{CD127}^{\text{neg}}$.

**Electronic Isolation of HSC, MPP, CTP, and CIP**

All antibody concentration were the same as staining of $1-2 \times 10^6$ cells, however the volume was increased to $150 \mu\text{L}$. Total bone marrow cells were incubated with CD16/32 Fc block for 20 minutes followed by a lineage biotin or lineage FITC cocktail for 30 minutes. Cells were washed twice in $2\text{mL}$ of OptiMEM media supplemented with 10%FCS, 10mM Hepes, 100 units of penicillin and 100$\mu\text{g}$ of streptomycin. For CTP and CIP populations, cells were stained with CD90 and CD2. For HSC and MPP cells were stained with CD117, Sca1, and CD135. Cells were sorted according to cell surface molecules as listed in Table VII.

**Cell cycle analysis**

Electronically sorted progenitors (HSC, MPP, CTP, and CIP) were washed in PBS and fixed in 70% ethanol containing 15% FBS and 15% PBS overnight. Cells were then washed 2 times in PBS and re-suspended in $250\mu\text{l}$ of a PI solution containing 0.5mg/mL propidium iodine, 0.1mM EDTA, and 0.05mg/mL RNase A at room temperature for one hour prior to flow cytometry analysis.
<table>
<thead>
<tr>
<th>CD</th>
<th>Clone</th>
<th>Name</th>
<th>Company</th>
<th>Dilution used</th>
<th>Fluorochrome</th>
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<td>CD2</td>
<td>RM2-5</td>
<td>LFA-2</td>
<td>eBioscience</td>
<td>1 to 5</td>
<td>PE</td>
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<td>CD3e</td>
<td>145-2c11</td>
<td>TCR</td>
<td>eBioscience</td>
<td>1 to 5</td>
<td>FITC, biotin</td>
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<td>CD4</td>
<td>GK1.5</td>
<td>co-receptor for helper T cell</td>
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<td>1 to 10</td>
<td>PE</td>
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<td>CD8a</td>
<td>53-6.7</td>
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<td>eBioscience</td>
<td>1 to 10</td>
<td>APC, FITC</td>
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<tr>
<td>CD25</td>
<td>PC61.5</td>
<td>Alpha chain of IL2 receptor</td>
<td>eBioscience</td>
<td>1 to 8</td>
<td>APC</td>
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<tr>
<td>CD11b</td>
<td>MI70</td>
<td>Mac-1</td>
<td>eBioscience</td>
<td>1 to 10</td>
<td>FITC, biotin</td>
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<td>CD16/32</td>
<td>2.4G2</td>
<td>Fc receptor block</td>
<td>eBioscience</td>
<td>1 to 5</td>
<td>FITC, biotin</td>
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<td>CD19</td>
<td>ID3</td>
<td>Component of B cell co-receptor</td>
<td>eBioscience</td>
<td>1 to 10</td>
<td>PE</td>
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<tr>
<td>CD44</td>
<td>IM7</td>
<td>Pgp-1, Hyaluronate receptor</td>
<td>eBioscience</td>
<td>1 to 20</td>
<td>AF780</td>
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<tr>
<td>CD45</td>
<td>30-F11</td>
<td>Leukocyte common antigen</td>
<td>BD Pharmingen</td>
<td>1 to 20</td>
<td>PE</td>
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<td>A20</td>
<td>Protein tyrosine phosphatase</td>
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<td>1 to 20</td>
<td>AF780</td>
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<tr>
<td>CD45.2</td>
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<td>BD Pharmingen</td>
<td>1 to 20</td>
<td>PERCP</td>
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<tr>
<td>CD49b</td>
<td>DX5</td>
<td>Integrin alpha 2</td>
<td>eBioscience</td>
<td>1 to 5</td>
<td>FITC, biotin</td>
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<td>Thy1</td>
<td>eBioscience</td>
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<td>CD117</td>
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<td>c-kit / Steel factor receptor</td>
<td>eBioscience</td>
<td>1 to 5</td>
<td>PE, APC</td>
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<td>IL7 receptor alpha</td>
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<td>1 to 5</td>
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<tr>
<td>CD135</td>
<td>A2F10</td>
<td>Flt3/ Fms-like tyrosine kinase 3</td>
<td>eBioscience</td>
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<td>PE</td>
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<tr>
<td>CD138</td>
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<td>Syndecan-1</td>
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<td>PE</td>
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<td>CD326</td>
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<td>EpCAM</td>
<td>eBioscience</td>
<td>1 to 5</td>
<td>APC</td>
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<td>GR1</td>
<td>RB6-8c5</td>
<td>Ly-6G</td>
<td>eBioscience</td>
<td>1 to 10</td>
<td>FITC, biotin</td>
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<td>Ki67</td>
<td>B56</td>
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<td>AF488</td>
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<td>LY51</td>
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<td>BP1/6C3</td>
<td>BD Pharmingen</td>
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<td>Ter119</td>
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<td>Ly-6A/E</td>
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<td>Stem cell antigen 1</td>
<td>eBioscience</td>
<td>1 to 5</td>
<td>PECY5, PECY7</td>
</tr>
</tbody>
</table>

Antibodies used for cell surface marker listing company, dilution, and color of fluorochrome.
**Flow cytometers and antibodies**

Samples were analyzed on FACSCanto II (BD San Jose, CA) or sorted using a FACSaria (BD San Jose, CA). Analysis was performed using FlowJo 7.6.1 (Treestar Ashland, OR). All antibodies were purchased from eBioscience or BD Pharmigen. Antibody information is summarized in Table VI.

**OP9 Cell Cultures**

Sorted ETP, MPP, and HSC were cultured in a 24 well plate with 10,000 previously seated OP9-DL1 (GFP) cells (Gift from Juan Carlos Zuniger Plucker, University of Toronto). Cultures were supplemented with 10ng/mL each of SCF, Flt3L, and IL7 (Peprotech, Rocky Hill, NJ). Fresh cytokines and media were added every 3-4 days. Upon confluency cells were split as needed. ETP and MPP cultures were harvested after 2 weeks. HSC cultures were harvested after 3 weeks. T lineage commitment was measures by expression of CD90, and differentiation to the DN2 or DN3 among the CD90 positive cells was measured by expression of CD25 using flow cytometry.

**Colony forming assay methylcellulose cultures**

BM cells from Wt and Foxn1 Tg were isolated and washed in PBS. Ten thousand nucleated BM cells were cultured in 1.5mL methylcellulose media (Stem Cell Technologies, Vancouver Canada) in a 35mM dish. Samples were cultured in duplicate and after 9 days the total number and types of colonies were counted and averaged.
CFU-G, CFU-M, CFU-GM, CFU-E, and CFU-GEMM were distinguished based on morphology. The frequency of each CFU per 10,000 cells was multiplied by the total number of nucleated cells isolated to calculate the total number of each progenitor in two tibia and two femurs.

**Non-irradiated adaptive transfer**

BM cells from CD45.1 H2-SVEX mice were isolated and stained with a lineage positive cocktail conjugated to FITC followed by CD117 APC and Sca-1 PECY5. LSK cells were electronically sorted and 8,000-16,000 cells were transferred retro-orbitally into non-irradiated Wt and Foxn1 Tg (CD45.2) hosts 17-21 months of age. Isoflurane was used to anesthetize mice. After 10 weeks the frequency of donor HSC, MPP, CTP, and CIP in the BM were determined using flow cytometry. Flow cytometry was used to determine the frequency of donor ETP as well as the percent of donor ETP that expressed VEX.

**Irradiated adaptive transfers**

Sub-lethally irradiation of age Wt and Foxn1 Tg (CD45.2) hosts 17-21 months of age were performed 24 hours prior to adaptive transfer. Mice were irradiated with 900 rads from a cesium source at the Hines VA hospital. LSK cells from CD45.1pos H2-SVEX mice were isolated as and retro-orbitally transferred into host as mentioned for non-irradiated adaptive transfers. After 10 weeks the host mice were sacrificed and BM
cells from the tibia and femur were used to determine the frequency of donor CTP, CIP, HSC, and MPP using flow cytometry.

Immunofluorescence and hematoxylin and eosin (H&E) staining of thymi

Acetone-fixed frozen thymi sections (4-5 μm) in OCT media were used for immunofluorescent studies. Anti-Foxn1 antibody was raised in rabbits against a Foxn1 peptide (amino acid 303-324, exon 6). Sections were first blocked with Super Block (ScyTek Logan, UT) and then incubated with either rabbit anti-mouse Foxn1 (IgG, 100-150 μg/ml), rabbit anti-mouse keratin 5 (0.1μg/ml, Covance), rat anti-mouse keratin 8 (0.221 μg/ml, DSHB), rabbit IgG (Invitrogen), or rat IgG (BD Biosciences). Sections were then washed and incubated with either goat anti-rabbit IgG-FITC, goat anti-rabbit IgGAF488, or goat anti-rat IgG- AF546 (F(ab)2’, 10 μg/ml, Invitrogen). Sections were analyzed using a Zeiss confocal microscope. For H&E staining, thymic tissues were fixed in formalin and then embedded in paraffin.

Immunohistochemistry of sternums for Foxn1

Sternums removed from Wt and Foxn1Tg mice were fixed for 48 hours in Zamboni solution (4% paraformaldehyde with picric acid) and decalcified in 15% sucrose containing 2% acetic acid for 72 hours. For staining, 5 μm paraffin sections were deparaffinized using xylene and rehydrated in PBS. Antigen retrieval was performed with Dako’s Target Retrieval Citrate Buffer (pH 6, Carpinteria, CA) with steam at 89°C for one hour. Sections were then treated with 3% hydrogen peroxide for 10 minutes.
Sections were blocked by incubating with human serum (Invitrogen) for 30 minutes, followed by superblock (ScyTek) for 20 minutes, anti mouse CD16/32 (5ng/ml, eBioscience) for 10 minutes, and finally with 2% BSA in PBS for 10 minutes. Rabbit anti-mouse Foxn1 (Santa Cruz Technologies, Santa Cruz, CA H-270) was used at 2μg/ml and incubation was performed overnight at 4°C. To visualize positive cells, biotinylated donkey anti-rabbit IgG (6μg/ml Jackson Immuno Research Laboratories, West Grove, PA) followed by streptavidin-HRP or Dako’s labeled streptavidin biotin kit was used. Sections were developed with 3-Amino-9-ethylcarbazole (AEC) and counterstained with hematoxylin.

**Identification of Foxn1⁺ cells within the sorted Lin⁻neg/low EpCAM⁺pos BM cells**

Electronically sorted Lin⁻neg/low EpCAM⁺pos or Lin⁻neg/low EpCAM⁺pos Syndecan-1⁻neg cells were cyto-centrifuged on to Superfrost plus microscope slides (Fisher Scientific, Hanover Park, IL) at approximately 500 cells per slide. Cells were fixed in acetone at -20°C for 5 minutes and stored at room temperature. Prior to staining, slides were rehydrated in PBS for 5 minutes and then permeabilized using 0.1% NP-40 for 18 minutes at room temperature. Cells were blocked as described for tissue sections and stained with rabbit anti-mouse Foxn1 (Santa Cruz Technologies H270 2μg/mL) or rabbit anti-E.coli β-galactosidase (Immunology Consultant Laboratory, Portland OR, RGAL-45A-Z 4μg/ml). Purified rabbit IgG was used as control. Visualization of positive cells was detected as mentioned for tissue sections.
For data in which the affect both age and over expression of *Foxn1* were examined, a two way ANOVA was performed. Two way ANOVA analyses determine if there was an age affect, an affect of over expression of *Foxn1*, and if over expression of *Foxn1* interacted with the aged affect. For the two way ANOVA, p values less than or equal to 0.05 were considered significant. If significance was reported using the two way ANOVA analysis, the Bonferroni t-test was used for pair wise comparisons. A summary of the two way ANOVA analysis is presented in Table I. Student t-test was also used for comparisons between groups in which the populations were normally distributed. For non-normal distributed populations the Mann-Whitney U test was used for comparisons. All statistical tests were performed using Sigma Stat 2.03. Significant p values using student’s t test or Mann-Whitney U are presented in the figures.
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VITA

Erin C. Zook was born on April 14, 1981 in Elgin, Illinois to Barbara Bocage and Rodney Zook. Erin received her secondary education at Elk Grove High School in Elk Grove Village, Illinois. In August of 1999, Erin entered the University of Illinois in Champaign-Urbana majoring in Biology. During her college education, Erin worked in the laboratory of Dr. Kimberly Hughes investigating genes associated with aging in *Drosophila melanogaster*. Upon graduation, Erin spent a year in the AmeriCorps working with the American Red Cross of Greater Chicago; she taught health and safety classes to underserved areas around Chicago.

In August of 2004, Erin joined the laboratory of Dr. Phong T. Le at Loyola University Medical Center as a research assistant where she studied alterations in thymic function with age. In January of 2006, Erin was accepted into the graduate program in the Department of Cell Biology, Neurobiology, and Anatomy. She later joined Dr. Le’s laboratory. Erin continued to work on aging in the thymus; her focus was on the transcription factor *Foxn1* and its roles in preventing aging of the thymus and restoring thymic function. Erin was the recipient of the pre-doctoral fellowship awards from the Immunology and Aging training grant (2009-2010) and the Experimental Immunology training grant (2010-2012). Erin will be pursuing a postdoctoral position.