THE ROLE OF NUCLEAR RECEPTOR COACTIVATOR AIB1/SRC3 IN NORMAL AND BREAST CANCER STROMAL FUNCTIONS

A Dissertation
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By

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The nuclear receptor coactivator amplified in breast cancer 1 (AIB1/SRC3) has a well-defined role in steroid and growth factor signaling in cancer and normal epithelial cells. Less is known about its function in stromal cells, though AIB1/SRC3 is upregulated in tumor stroma and may thus contribute to tumor angiogenesis. In addition, our lab has demonstrated previously a potential impact of AIB1/SRC3 on tumor stroma as reduced angiogenesis was observed in mammary tumors in AIB1/SRC3 knockout mice. In this study, we report for the first time the effect of AIB1/SRC3 on the function of two types of stromal cells and on keratinocytes in vitro. Endothelial cell functions including formation of tube like structures, monolayer formation, migration, proliferation and apoptosis were all inhibited by reduction in AIB1. We also observed reduction on FGF2-induced signaling of endothelial cells in vitro when AIB1/SRC3 levels were reduced. Moreover, AIB1/SRC3 did not impact fibroblast proliferation, but did impact its migration. We also observed that AIB1/SRC3 had a significant effect on keratinocyte proliferation through the up regulation of a number of cell cycle molecules. In AIB1/SRC3+/− and AIB1/SRC3−/− mice, the angiogenic responses to subcutaneous Matrigel implants
were reduced by two thirds and exogenously added FGF2 did not overcome this deficiency. Furthermore, AIB1/SRC3\(^{+/−}\) and AIB1/SRC3\(^{-/−}\) mice showed a similarly delayed healing of full-thickness excisional skin wounds indicating that both alleles were required for proper tissue repair. Analysis of this defective wound healing showed reduced recruitment of inflammatory cells and macrophages, cytokine induction and metalloprotease activity. Skin grafts from animals with different AIB1 genotype and subsequent wounding of the grafts revealed that the defective healing is attributable to local factors and not to defective bone marrow responses. Indeed, wounds in AIB1/SRC3\(^{+/−}\) mice showed reduced expression of FGF10, FGFBP3, FGFR1, FGFR2b and FGFR3, major local drivers of angiogenesis. Further, we examined the influence of AIB1/SRC3 in the epithelial and stromal cross talk in breast cancer. We reported that AIB1/SRC3 has a critical role in the stromal compartment during mammary tumor development. The loss of one allele of AIB1/SRC3 in the stroma significantly delayed tumorigenesis.
DEDICATION

I dedicate this work to my family,

who provided me with tremendous amount of help and support to make this work possible. To my parents, Dr. Abdulmohsen Al-Otaiby and Dr. Hessah AL-Faiz, for their support, guidance and taking care of my son so that I could finish my graduate work without worrying about him.

To my siblings Saad, Abdulaziz, Fawaz, Sarah, Athoug, Norah and Seba.

&

To my husband, Ziad Alzakari, for his unconditional support throughout my graduate studies.

&

To my five months old son Tariq.
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# Table of Contents

Title page ............................................................................................................................................. i
Abstract .................................................................................................................................................. iii
Dedication .............................................................................................................................................. v
Acknowledgements ........................................................................................................................... vi
Table of Contents ............................................................................................................................... vii
List of Tables ......................................................................................................................................... xi
List of Figures ....................................................................................................................................... xi

## Chapter 1: Introduction

A. SRC family of coactivators ........................................................................................................... 2
   a. SRC family Protein Structure .................................................................................................. 4
   b. SRC3 family function ............................................................................................................. 6
B. AIB1/SRC3 expression and signaling in human cancers ......................................................... 9
C. AIB1/SRC3 role in breast cancer mouse models ................................................................. 14
D. Wound healing as a stromal function model in breast cancer ........................................... 17

## Chapter 2: Methods

A. Cell lines ......................................................................................................................................... 21
B. shRNA constructs and lentivirus infection ............................................................................... 21
C. Tube formation assay ............................................................................................................... 22
D. Monolayer formation assay ..................................................................................................... 22
E. Cell migration assay ............................................................................................................... 23
F. Proliferation assays .................................................................................................................. 24
G. Apoptosis assay ................................................................. 25
H. Studies in animals ................................................................ 25
I. Matrigel plug assay .............................................................. 26
J. Wound healing assay ........................................................... 26
K. Immunohistochemistry ...................................................... 27
L. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) .. 28
M. In vivo fluorescence imaging ............................................... 29
N. Western blot analysis ......................................................... 29
O. Expression analysis ............................................................ 31
P. Skin transplants ................................................................. 32
Q. Primary tumor Epithelial and Fibroblast Cell Harvest and Culture ........................................................................... 32
R. Tumor Transplant procedure ............................................... 33
S. Data analysis and statistics .................................................. 34

Chapter 3: Results and Discussion: Role of AIB1 in Epithelial and Stromal Cell Interaction in Normal Tissues in vitro ................................................................. 35

A. Effect of AIB1/SRC-3 on endothelial cells .............................. 36
   a. AIB1/SRC3 depletion impairs endothelial cells function in vitro .... 36
   b. Effect of the reduction of AIB1/SRC3 protein levels on FGF2-induced of endothelial cell in vitro ................................................................. 47
B. Effects of AIB1/SCR-3 expression in fibroblasts in vitro .......... 49
C. Effects of AIB1/SRC3 expression in keratinocytes in vitro ......... 57
D. Discussion ............................................................................. 63
Chapter 4: Results and discussion: Role in Epithelial and Stromal Cell Interaction in Normal Tissues in vivo ................................................................. 67

A. Reduction of AIB1/SRC3 impairs endothelial cell migration in vivo .......... 68
B. Reduction of AIB1/SRC3 delays the wound healing response in vivo......... 72
C. Wound healing in transplanted skin from, and to AIB1/SRC3+/+ and AIB1/SRC3+- mice .................................................................................... 80
D. Reduction of AIB1/SRC3 delays the inflammatory and angiogenic response during wound healing ......................................................... 82
E. Driver pathways of AIB1/SRC3 effects during wound healing ............... 93
F. Discussion ............................................................................................ 97

Chapter 5: Results and Discussion: Role of AIB1/SRC3 in Epithelial and Stromal Cell Interaction in Mammary Cancer Tissues ........................................... 102

A. Altering the AIB1/SRC3 expression in mammary tumor epithelial cells (MECs) delays tumor formation in MMTV-HER2/Neu mice...................... 104
B. Altering the AIB1/SRC3 expression in the stroma has a significant impact on tumor incidence........................................................................... 108
C. Comparison of AIB1/SRC3 and HER2/Neu protein expression in the stroma and the epithelium of mammary tumors formed after transplantation of tumor MECs into host mice with different AIB1 stromal expression level.................................................................................. 115
D. A Immortalized MMTV Her2/Neu AIB1/SRC3+/+ tumor MECs............... 121
E. Discussion ............................................................................................ 125
Chapter 6: Conclusion and Future Directions

A. Conclusion

B. Clinical relevance

C. Future directions

1. Identifying new and novel signaling pathways that mediate AIB1/SRC3 action in the stroma

2. Investigate the ability of fibroblast cells to rescue the delay in tumorigenesis observed when AIB1/SRC3 levels are reduced in the stroma

3. Generation of a tissue specific knockdown of AIB1/SRC3

4. AIB1/SRC3 impacts on immune cells functions

References
LIST OF TABLES

Table 1: List of Primers used for quantitative real time PCR of signaling molecules in wound tissues........................................................................................................... 29

Table 2: mRNA expression of FGF pathway-related genes in the stroma of breast cancer and normal breast tissues........................................................................................................ 100

LIST OF FIGURES

Figure I: Diagram illustrating the structural and functional domains of SRC family .............................................................................................................................. 5

Figure II: Model illustrating AIB1/SRC3 function as a transcriptional coactivator .... 8

Figure III: AIB1/SRC3 mRNA expression in the stroma of human breast cancer relative to normal breast stroma......................................................................................... 13

Figure IV: diagram illustrating the study hypothesis.................................................... 19

Figure 1A: Western blot analysis for AIB1 protein in endothelial cells (HUVEC) ...... 36

Figure 1B and C: Tube formation in Matrigel is inhibited by reduction of AIB1........ 37

Figure 1D: Endothelial monolayer formation is inhibited by reduction in AIB1........ 39

Figure 1E and F: Repair of denuded endothelial monolayer areas by cell migration is inhibited by reduction in AIB1................................................................. 41

Figure 1G: Endothelial cell proliferation is inhibited by reduction in AIB1.......... 43

Figure 1H and I: Apoptosis in endothelial cells is inhibited by reduction in AIB1....... 44

Figure 1J: Repair of denuded endothelial monolayer areas was performed in the presence of 2 µg/ml of mitomycin C ................................................................. 45

Figure 2: Effect of the reduction of AIB1/SRC3 protein levels on FGF2-induced signaling of endothelial cells .............................................................. 47

Figure 3A: AIB1/SRC3 overexpresssion in AIB1/SRC-3 -/- murine embryonic fibroblasts (MEFs) does not alter proliferation........................................................................ 50
Figure 3B: Depletion of AIB1/SRC3 from NIH3T3 fibroblasts does not later their proliferation

Figure 3C: Western blot analysis for AIB1 48 hours post infection in Hs-27 human fibroblast cells

Figure 3D: Changes in AIB1 level do not affect proliferation of Hs-27 cells

Figure 3E and F: Reduction of AIB1 slows migration of Hs-27 cells after wounding of a confluent monolayer

Figure 4A: Western blot analysis for AIB1/SRC3 48 hours post infection in keratinocytes

Figure 4B: Keratinocyte cell proliferation is inhibited by reducing AIB1 levels

Figure 4C: Effect of reducing AIB1 on the EMT markers expression vimentin in keratinocytes

Figure 4D: Reducing AIB1/SRC3 in keratinocytes causes unregulation of the expression of p53 and cyclin-dependent kinase inhibitors p21, and p27

Figure 5A and B. Effect of AIB1/SRC3 on angiogenesis

Figure 5C. CD31 and CD14 mRNA expression in Matrigel plugs

Figure 6A and B: Skin wound analysis at day 5 after injury in AIB1/SRC3^{+/+},^{+/−}, or^{−/−} mice

Figure 6C and D: Effect of AIB1/SRC3 on skin wound healing

Figure 6E and F: Effect of AIB1/SRC3 on proliferation in the granulation tissues

Figure 6I: Effect of AIB1/SRC3 on proliferation during wound healing

Figure 6J: Immunohistochemical staining for AIB1/SRC-3 protein

Figure 6G and H: Wound healing time course in AIB1/SRC3^{+/+} and^{−/−} mice

Figure 6K: Different Healing Features of wounds in AIB1/SRC3^{+/+} vs. AIB1/SRC3^{−/−} mice
Figure 7: Wound healing in transplanted skin from and to AIB1/SRC3<sup>+/+</sup> and AIB1/SRC3<sup>+/−</sup> mice

Figure 8A: Effect of AIB1/SRC3 on the inflammatory response during wound healing

Figure 8B: Expression of marker molecules in bone marrow of AIB1/SRC3<sup>+/+</sup> and AIB1/SRC3<sup>+/−</sup> mice

Figure 8C: AIB1 mRNA expression in skin wounds

Figure 8D and E: Macrophage invasion into granulation tissue of day 5 wounds in AIB1/SRC3<sup>+/+</sup> vs. AIB1/SRC3<sup>+/−</sup> mice

Figure 8F and G: MMP activity in day 3 wounds

Figure 8H: MMP activity in wounds

Figure 9A and B: Effect of AIB1/SRC3 on wound angiogenesis

Figure 9C and D: Effect of AIB1/SRC3 on wound angiogenesis

Figure 10A: Expression of angiogenesis-related genes in day 4 wounds of AIB1/SRC3<sup>+/+</sup> versus AIB1/SRC3<sup>+/−</sup> mice

Figure 10B: Effect of AIB1/SRC3 on FGFR pathway genes

Figure 10C: Expression ratio of FGFR b and c splice isoforms in day 4 wounds of mice

Figure 11A: Diagram of how the transplant experiment that examined the effect of changes in AIB1/SRC3 levels selectively in the epithelium, was performed

Figure 11B: Reduced AIB1/SRC3 levels in the donor MECs delayed tumor incidence in MMTV-HER2/Neu AIB1/SRC3<sup>+/−</sup> host mice

Figure 12A: Diagram of experimental design of the mammary transplant experiments to determine the influence of stromal expression of AIB1/SRC3 on mammary tumor development

Figure 12B: Diagram of the experimental design of the mammary transplant experiment determining how reduction of AIB1/SRC3 in the stroma influences mammary tumor development
Figure 12C: Reducing the AIB1/SRC3 expression in the stroma has a significant impact on mammary tumor incidence................................................................. 112

Figure 12D: Diagram of the transplant experiment altering the AIB1/SRC3 levels in the stroma utilizing the AIB1/SRC3 knockout mice ........................................... 113

Figure 12E: Knock-out of AIB1/SRC3 levels in the stroma prevented tumor formation in MMTV-HER2/Neu mice ........................................................................ 114

Figure 13A: Immunohistochemical staining for AIB1/SRC-3 protein in tumors arising after transplantation of tumor MMTV-NEU/HER2 AIB1/SRC-3 +/- MECs into different AIB1/SRC3 genetic background.................................................... 116

Figure 13B: Immunohistochemical staining for Her2/Neu protein in tumors arising after transplantation of tumor AIB1/SRC-3 +/- MECs into different AIB1/SRC3 genetic background................................................................. 117

Figure 13C: Immunohistochemical staining for Her2/Neu and AIB1/SRC3 protein in tumor stroma in tumors arising after mammary gland transplantation of tumor MECs........................................................................................................ 118

Figure 13D: Tumors arising after transplantation of tumor MECs from MMTV-HER2/Neu AIB1/SRC3 +/+ mice into the same genetic background were phenotypically different from tumor arising after transplantation of tumor MECs from MMTV-HER2/Neu AIB1/SRC3 +/- mice into MMTV-HER2/NEU AIB1/SRC3 +/- background......................................................... 120

Figure 14A: Experimental design determining if immortalized MECs from mammary tumors are able to respond to changes in stromal AIB1/SRC-3 levels............ 122

Figure 14B: Immortalized MMTV Her2/Neu tumor epithelial cells can form mammary tumors after transplantation into the mouse mammary fat pad ............... 123

Figure 15: A diagram summarizing the major findings of this study .................... 133

Figure 16: Experimental design for the fibroblast rescue experiment.................. 139

Figure 17: Experimental design to generate tissue specific knockdown of AIB1/SRC3 ........................................................................................................... 141
Chapter 1: Introduction
A. SRC family of coactivators.

In Eukaryotes, gene transcription is a well-characterized and complex process that requires the presence of a variety of coregulators; such as, coactivators and corepressors. These coregulators play critical role in transcription: corepressors role is highlighted by its ability to reduce transcription, whereas coactivators play a pivotal role in promoting and enhancing gene transcription (1).

The nuclear receptor coactivator AIB1 (Amplified In Breast Cancer 1), belongs to the p160/SRC steroid receptor coactivator (SRC) family which includes SRC1 (2), SRC2 (3) and SRC3 (4). Each member of the SRC family was originally described by the steroid receptors to which they bind, interact with and activate. SRC1, the first member of the SRC family to be cloned was initially reported to interact with the progesterone receptor; however, further studies demonstrated that SRC1 interaction was not limited to progesterone receptors but interacted with almost all of the nuclear receptors (5). SRC2, which is also known as transcriptional intermediary factor 2 (TIF-2) and as glucocorticoid receptor interacting protein-1 (GRIP1), was first documented to interact with estrogen receptor (6). The discovery of AIB1/SRC3 was reported by several groups; and therefore, was given different names; such as, (ACTR/RAC3/TRAM-1/SRC3/ p/CIP). ACTR was reported as an activator of thyroid hormone and retinoid receptor (7). RAC3
was demonstrated to be a coactivator of a retinoic acid receptor (8). TRAM-1 was shown to be activator of thyroid hormone receptor. AIB1 was reported to be a gene amplified in breast cancer 1 (9). P/CIP: is the mouse homolog of AIB1 and was found through its interaction with CBP/p300 (10).
a. SRC family Protein Structure

The three members of the SRC family share several common structural domains (Figure 1). The highly conserved (60%) N-terminus possess the basic helix-loop-helix (bHLH)/Per/Arnt (PAS) domain (9). This domain was reported to be responsible for protein to DNA and protein-to-protein interactions with other proteins that contains have the same domain (11). Following by the bHLH PAS domain is the receptor interaction domain (RID). The RID contains a three LXXLL (L; leucine and X; any amino acid) motifs that facilitate the ligand dependent interaction of AIB1/SRC3 with nuclear receptors (12). The C-terminus is characterized by CBP/p300 interaction domain (CID) and histone acetyltranferase domain (HAT). The CID domain is required for AIB1/SRC3 interaction with histone acetyltransferases (HATs) such as CBP/p300 and their associated histone acetyl transferase activity (5). Moreover, AIB1/SRC3 possess its own intrinsic histone acetylation domain; where it interacts with coactivator associated arginine methyltransferase1 CARM-1(13). However the actual HAT activity of AIB1/SRC3 is much weaker than those of the CPB and p300 proteins. (14).
Figure I: Diagram illustrating the structural and functional domains of SRC family. (bHLH)/per-arnt-sim (PAS), receptor interaction domain (RID), CBP/p300 interaction domain (CID), and histone acetyltransferase domain (HAT).
b. SRC3 family function

Steroid receptors including the estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), mineralocorticoid receptor (MR), and glucocorticoid receptor (GR) are members of the nuclear receptor family. These receptors possess the ability to bind to DNA directly and control a variety of biological processes (15). In addition, nuclear receptors share similar structural domains; such as the DNA binding domain that facilitates their interaction with the DNA. The nuclear receptors require the presence of their ligand to be active; otherwise, they bind to heat shock proteins in the cytoplasm and remain inactive. Upon ligand binding to their respective hormone receptors in the cytoplasm, the whole complex changes confirmation and translocates to the nucleus, where the receptors bind to their hormone response elements in the DNA. The activated hormone receptor then recruits steroid receptors coactivators; such as, the AIB1/SRC3 to the transcription machinery. After AIB1/SRC3 interacts with the hormone receptors through its RID domain, it starts the recruitment of cofactors and other coactivators; such as, CBP/p300 and CARM1 to initiate the transcription of variable genes (Figure II) (16).

Furthermore, AIB1/SRC3 has been reported to predominantly function as a transcriptional coactivator. It promotes transcriptional activity of multiple nuclear receptors such as the estrogen receptor (9) as well as other
transcription factors, including E2F-1, AP-1, NFκB, and STAT6 (17-20). AIB1/SRC3 can also function to assist transcription factors to interact with other transcription cofactors; such as CBP/P300 and CARM-1 (Figure II) (21).
Figure II: Model illustrating AIB1/SRC3 function as a transcriptional coactivator.

HRE, hormone response element; HR, hormone receptor; AIB1, amplified in breast cancer 1; CBP/p300, CREB binding protein/p300; CARM-1, coactivator associated arginine methyltransferase-1.
B. AIB1/SRC3 expression and signaling in human cancers.

AIB1/SRC3 is the only member of the steroid receptor coactivator family that is reported to be amplified and overexpressed in epithelial cancer (9). Multiple studies have shown that the AIB1/SRC3 gene is amplified on chromosome 20q, a region that is often amplified in mammary cancer. The AIB1/SRC3 gene amplification was detected in 5-10% of human mammary tumors. In addition, AIB1/SRC3 mRNA and protein levels were shown to be overexpressed in 30% - 60% of breast tumors (21), and other human epithelial cancers (9, 22-26). High levels of AIB1/SRC3 mRNA or protein predict significantly worse prognosis and overall survival in breast cancer patients (21). Recently, increase AIB1/SRC3 mRNA expression has been reported to be associated with breast cancer reoccurrence (21). It is of a note that the overexpression of AIB1 was detected in both ER and PR positive and negative breast cancer (9). It was originally thought that the AIB1/SRC3 expression in mammary tumorigenesis was associated with the presence of ER and PR (27). However, further studies indicated that the amplification and overexpression of AIB1/SRC3 was detected in ER and PR negative cell lines (28). Interestingly, in ER and PR negative mammary tumors, AIB1/SRC3 expression was correlated with the expression of Her2, another oncogene in breast cancer (29).
AIB1/SRC3 expression was also intensively studied in ovarian cancer, where it was reported to be amplified in 7.4% of human ovarian tumors (30). The AIB1/SRC3 amplification in ovarian tumors is associated with poor survival rate. Interestingly, the AIB1/SRC3 expression was associated with the presence of ER and PR in these tumors. Moreover, fibroblast growth factor receptor 1 (FGFR1) amplification was reported to be associated with the amplification of AIB1/SRC3 in ovarian tumors (30).

Additionally, the AIB1/SRC3 overexpression was also reported in human prostate cancer cell lines and tumors. Clinically, the AIB1/SRC3 expression in human prostate cancer predicts worse prognosis. In addition, overexpressing AIB1/SRC3 in human prostate cancer cell lines was shown to increase prostate cancer cell proliferation, growth and survival (31). In contrast, reducing AIB1/SRC3 levels using siRNA in prostate cancer cells was associated with a reduction in Bcl-2 expression, cell growth and increased apoptosis (32). Reducing AIB1/SRC3 levels was also associated with a decrease in tumor growth in vivo (33).

AIB1/SRC3 expression was also detected in human pancreatic cancer. Initially, the overexpression and amplification of AIB1/SRC3 was detected in 6 out of 9 human pancreatic cancer cell lines (34). Our lab also reported a significant AIB1/SRC3 overexpression in COLO 357L and COLO 357SL pancreatic adenocarcinoma cell lines. Interestingly, we noticed that the
AIB1/SRC3 expression increased with an increase in disease progression (22).

Originally, AIB1/SRC3 was reported to be a coactivator of steroid receptors in breast and ovarian cancers (35). However, AIB1/SRC3 signaling in cancer can also occur independent of steroid receptors. As mentioned earlier that the AIB1/SRC3 expression was detected in ER and PR negative breast tumors (27). In addition, AIB1/SRC3 overexpression and amplification was observed in non-steroidal human cancers, such as, pancreatic (34) and hepatocellular cancers (36). Recently, AIB1/SRC3 was reported to interact with non-steroidal transcription factors, including E2F-1, AP-1, NFκB, and STAT6 (17-20). The transcription factor E2F-1 is implicated in cell cycle regulation. AIB1/SRC3 was reported to directly interact with the N-terminus region of E2F-1 and induce cell proliferation (17). Moreover, the transcription factor NFκB is involved in regulating the transcription of a set of genes that are responsible for cell growth and survival. It was reported that the phosphorylation of AIB1/SRC3 by IKK kinase, an upstream signaling molecules in the NFκB signaling cascade, lead to the transactivation of NFκB (19). It was demonstrated that AIB1/SRC3 is implicated in STAT6 signaling through its role in facilitating the interaction of CBP/P300 with STAT6 (20). AIB1/SRC3 has also been shown to be important in a diverse set of growth factor signaling pathways such as the IGF-1 and growth hormone signaling in normal mouse fibroblasts and
hepatocytes (37), IGF-1 in breast cancer epithelium (38), and also EGF and Her2 signaling in cancer epithelial cells. Additionally, the overexpression of AIB1 in the presence of Her2 was reported to be associated with tamoxifen resistance (39, 40).

Although the coactivators of the SRC family are thought of mainly as oncogenes that affect epithelial responses to external hormone, growth factor and cytokine signals (21, 41), analysis of recently published human cancer expression array data (42, 43) reveals significant increases of AIB1/SRC3 expression in the stromal compartment of human breast cancers (Figure III) suggesting a potential role of AIB1/SRC3 expression in the stromal response during malignant progression. However, there is less knowledge on the role of AIB1/SRC3 expression in the stromal compartment of mammary tumorigenesis.
Figure III: AIB1/SRC3 mRNA expression in the stroma of human breast cancer relative to normal breast stroma. Analysis of two published expression arrays of stromal tissues (42, 43) obtained from the Oncomine database (http://www.oncomine.org). Values are given as the mean ± SEM and are shown on a log₂-based scale. ***P < 0.0001.
C. AIB1/SRC3 role in breast cancer mouse models

Animal models corroborate the role of AIB1/SRC3 as an oncogene, since expression of AIB1/SRC3 under the control of the MMTV promoter in transgenic mice induced mammary hyperplasia and tumors (44) (45). The overexpression of AIB1/SRC3 in transgenic mouse model was done under the mouse mammary tumor virus long terminal repeat, MMTV-LTR, promoter. Mice who overexpressed AIB1/SRC3 exhibit enhanced epithelial cell proliferation, metastasis and an increase in mammary gland size. Moreover, tumor metastasis was detected in the uterus and ovaries. It was also reported that these mice have increased serum IGF-1 levels (45). Recently, it has been demonstrated that the overexpression of AIB1/SRC3 was associated with increased mammary stromal collagen indicating a potential role of AIB1 in the stromal-epithelial crosstalk (46).

In order to evaluate the physiological role of AIB1/SRC3 in vivo, AIB1/SRC3 knockout mice were generated by two different groups. Xu et al deleted the bHLH/PAS and RID domains and found that AIB1/SRC3−/− knockout mice exhibited reduced body growth and decreased IGF-1 levels (47). Moreover, mammary gland ductal branching was significantly impaired in the AIB1/SRC3−/− mice. It is of a note that treating the AIB1/SRC3 knockout mice with estrogen failed to rescue the IGF-1 levels or animal body
growth. Additionally, the AIB1/SRC3 knockout mice reproductive function was significantly lower than the AIB1/SRC<sup>+/+</sup> and AIB1/SRC<sup>+/−</sup> mice.

Wang et al also generated the AIB1/SRC<sup>−/−</sup> mice by deleting the RID and CBP binding domain (37). Interestingly, they reported similar findings to that of Xu et al group. IGF-1 levels were reduced as well as animal body weight. The generation of mouse embryonic fibroblasts, MEF, from these AIB1/SRC3 knockout mice revealed that AIB1/SRC3 is required for IGF-1 signaling.

Recent findings have shown that the loss of AIB1/SRC3 in MMTV/v-H-ras mice, a mouse model that develops mammary cancer at an average of 32.5 weeks, suppresses mammary gland ductal hyperplasia and tumorigenesis. Interestingly, pMAPK expression was not changed between the AIB1/SRC3<sup>+/+</sup> - ras and AIB1/SRC3<sup>−/−</sup> - ras mice. However, IGF-1 mRNA, protein, serum levels and signaling pathway were significantly reduced in the AIB1/SRC3<sup>−/−</sup> - ras mice (48).

Recently, our lab has demonstrated that AIB1/SRC3 is functionally required for HER2-mediated mammary tumorigenesis in a mouse model for breast cancer through regulation of HER2 phosphorylation and signaling (40). Generation of Her2/Neu transgenic mice with either loss of one or both copies of the AIB1/SRC3 gene resulted in reduced or complete abolition of mammary tumor development, respectively. One very interesting aspect of this study was that the tumors that arose in MMTV-HER2/Neu AIB1/SRC3<sup>+/−</sup>
mice were phenotypically different from those in MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) mice in that they were smaller, more necrotic and significantly less vascularized (40)
D. Wound healing as a stromal function model in breast cancer

Rudolf Virchow (1863) viewed tissue injury and repair as part of the malignant process (49) and tumors have been described as “wounds that will not heal” (50). Both wound healing and cancer are well-characterized processes that require defined inflammatory, proteolytic, angiogenic, tissue remodeling and regeneration responses. In the case of a healing wound, the tissue regeneration process stops once the wound has healed; however, the regeneration process does not stop in cancer tissues resulting in uncontrolled proliferation, invasion and metastasis.

At the cellular and molecular level there are many parallels between a healing wound and processes ongoing in the tumor and surrounding stroma (51). Initially, the hyperproliferation of tumor epithelial cells resemble the keratinocyte hyperproliferation activity at the edge of the wound. The hyperproliferation status of these epithelial cells in both wound healing and cancer triggers the inflammatory response. A variety of immune cells; such as, monocytes, macrophages and neutrophils get recruited to the wounded area to protect the skin from infection, to aid the healing process and to stimulate angiogenesis (52, 53). In cancer, macrophages and neutrophils also get recruited to the tumor sites, where they differentiate to either tumor-
associated macrophages (TAMs) or tumor-associated neutrophils (TANs) (54-56), which assist the tumor to grow and metastasize (57). Another hallmark of wound healing and tissue remodeling is the production of matrix metalloproteases (MMPs) predominantly by the inflammatory cells and macrophages that promote extracellular matrix breakdown (58, 59). Interestingly, the MMP activity is also a feature of tissue remodeling during tumorigenesis where it facilitates tumor growth and metastasis (60). In addition, an important component of wound healing is the formation of the new blood vessels to provide nutrition and oxygen. Likewise, angiogenesis has a critical role in tumorigensis as tumors depend on the formation of new blood vessels to grow and metastasize (61-63). Clearly, wound healing provides an ideal model to study stromal functions.
In summary, the nuclear receptor coactivator amplified in breast cancer 1 (AIB1/SRC3) has a well-defined role in steroid and growth factor signaling in cancer and normal epithelial cells. A potential impact of AIB1/SRC3 on tumor stroma was suggested from the reduced angiogenesis in mammary and thyroid tumors in AIB1/SRC3 knockout mice (40, 64). Though AIB1/SRC3 is upregulated in human tumor stroma (Figure III), less is known about the AIB1/SRC3 function in stromal cells. Therefore, we hypothesize that AIB1/SRC3 is implicated in the epithelial and stromal cell crosstalk by affecting the signaling pathways in both the epithelial and stromal cells as highlighted in (Figure IV). In this study, we investigate for the first time the role of AIB1/SRC3 in stromal function in normal and cancer tissues.

**Figure IV:** diagram illustrating the study hypothesis.

Courtesy of Dr. Anton Wellstein.
Chapter 2: Methods
A. Cell lines

Primary Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from (Lonza, Walkersville MD) and were maintained in EBM-2 media (Lonza, Walkersville MD) supplemented with 2% fetal bovine serum as recommended by the supplier (Lonza, Walkersville, MD). AIB1/SRC3\(^{-/-}\) mouse embryonic fibroblasts cells (MEF) (65), NIH3T3, human foreskin fibroblasts cells (Hs-27), and human keratinocytes cells (HaCat) were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum.

B. shRNA constructs and lentivirus infection

Control scrambled shRNA (66) was purchased from Addgene (Addgene, Cambridge, MA). AIB1 shRNA #1 (TGGTGAATCGAGACGGAAACA) and #2 (GCAGTCTATTCGTCCTCCATA) (65) were subcloned into the EcoRI and AgeI restriction sites in PLKO.1 puro (Addgene). Lentivirus production was carried out as described (67) using the recommended protocols for production of lentiviral particles with packaging plasmid (pCMV-dR8.2 dvpr) and envelope plasmid (pCMV-VSVG) (Addgene). In summary, 3 million HEK293T cells were plated in 10 cm dishes and transfected with PLKO.1 puro shRNA, envelop and packaging plasmid constructs using Fugene 6 as described by the manufacturer. The supernatant containing virus
was collected 48 hours later and frozen down in 1ml aliquots. HUVEC, HaCat, Hs-27, and NIH3t3 cells were infected as follow: cells were plated at a density of 10,000 cells/ml. Next, cells were infected with 1 ml of virus, MOI>5, in 5 ml of medium containing 8 ug/ml of polybrene. Five hours later, 5 ml fresh medium was added to the plate. After 24 hours, the medium containing the virus was removed and replaced with 10 ml fresh medium. 48 hours post infection; infected cells were selected using puromycin at a concentration of 5 ug/ml.

C. Tube formation assay

HUVECs infected with control or AIB1/SRC3 shRNA lentivirus for 48 hrs were plated in Matrigel-coated 8-well chamber slides (3x10^5 cells/well) as previously described (68). In summary, the 8-well chamber slides were coated with 50ul of matrigel (BD Biosciences, Franklin Lakes, NJ) then incubated for 20 min at 37°C to solidify the matrigel. 10,000 HUVECs were plated on top of the matrigel and were placed back in the incubator for 18 hours. Tube formation was imaged at 18 hours post plating and tube diameters was quantitated with NIH ImageJ software.

D. Monolayer formation assay

2x10^5 control or AIB1/SRC-3 shRNA infected HUVECs were plated in wells of the electrical cell-substrate impedance sensor system (ECIS)
Prior to plating HUVECs, the wells were coated with 0.1% gelatin for 1 hour. After that, the wells were washed twice with PBS, and then 200ul of EBM-2 media was added with the HUVECs cells. The wells were installed in the xCELLigence machine. The HUVECs were grown for 20 hours to form a monolayer. Analysis of the monolayer was carried out after 20 hours. Details description of the procedure are provided in (69).

E. Cell migration assay

A denuded area in control or AIB1/SRC-3 shRNA infected HUVEC confluent monolayers were generated by scraping with a micropipette tip. After viral infection, HUVEC cells were allowed to become 100% confluent. Then, a micropipette tip was used to generate a scratched area. The extent of migration was determined in the presence or absence of mitomycin C (2µg/ml) (Sigma, St. Louis, MO) by measuring the distance between the migration fronts at time 0, 24 and 48 hours. Quantification from 5 independent digitized images was performed using NIH ImageJ software. Control and shRNA infected Hs-27 cells were plated in 12 well plates. Cell culture inserts were used to generate 0.5 mm diameter, rectangular cell free spaces (Ibidi GmbH, Martinsried, Germany) were placed into the wells and removed after 24 hours. Time-lapse phase-contrast microscopy (Nikon Eclipse TE-300 inverted microscope system, Melville, NY) was used to
continuously capture images and follow migration at different time points to calculate migration rates.

F. Proliferation assays

1,000 control or AIB1/SRC-3 shRNA infected HUVECs, NIH3t3, MEFs, and HaCat cells were plated in 96 well plates. Cell proliferation was measured at 24, 48 and 72 hours with Cell Titer Glo according to the manufacturer’s instructions (Promega, Madison, WI). In brief, cells were plated in a 96 well plate in a total of 100ul medium, each cell line with its respected medium. Wells containing only medium were used to eliminate background noise. Prior to each measurement, the Cell Titer Glo substrate and buffer were first thaw at room temperature and were then mixed together. During this time, the 96 well plates were removed from the incubator and were placed at room temperature for 30 minutes. Then, 100ul of the mixture solution was added to each well. The plates were placed on a shaker for 2 min to mix the cells with the solution. The plates were then allowed to incubate at room temperature for 10 min prior to measuring the plate by a Wallac Victor 3 plate reader and software. Alternatively, cells were stained with crystal violet (0.52% crystal violet in 25% methanol). After washing the cells to remove excess stain, the plates were dried and the bound stain solubilized by the addition of 100µl of 100mM sodium citrate in
50% ethanol. Staining intensity, which is proportional to cell number, was then determined by measuring absorbance at 570 nm using a 96 well plate reader.

G. Apoptosis assay

Apoptosis in control or AIB1/SRC-3 shRNA infected HUVEC (2x10^5 cells) was determined by Annexin V-FITC staining, according to the manufacturer’s instructions (Trevigen, Gaithersburg, MD), 48 hours after infection. Basically, HUVEC cells were trypsinized and 2x10^5 cells were resuspended in 500ul of 1X binding buffer. 5ul of Annexin V-FITC and Propidium Iodide were added to the cells. The tubes were incubated at room temperature for 5 min prior to quantification by flow cytometry.

H. Studies in animals

The generation of mice with different MMTV-Neu AIB1/SRC-3 genotypes (+/+,+/-,-/-) is as previously described (40). Briefly, the MMTV-Neu transgenic mice were purchased from the Jackson Laboratory. The AIB1 knockout mice were obtained from Dr. Bert O’Malley’s laboratory (Baylor College of Medicine, Texas) (47). The two mouse strains were interbred to generate the MMTV-Neu AIB1/SRC-3 mice with three different genotypes (+/+,+/-,-/-). All animal studies in this dissertation were performed on the MMTV-Neu AIB1/SRC-3 genotypes (+/+,+/-,-/-) mice. In this dissertation, the
use of these mice will be referred to as the AIB1/SRC3 mice. Animal experiments were reviewed and approved by the Georgetown Animal Care and Use Committee (GUAUC).

I. Matrigel plug assay

Growth factor depleted Matrigel (0.5 ml; BD Biosciences, Franklin Lakes, NJ) with and without FGF2 (10 µg/ml) was injected subcutaneously into the abdomen of 3-4 month old AIB1/SRC3 \textsuperscript{+/+}, AIB1/SRC3 \textsuperscript{+/-}, and AIB1/SRC3 \textsuperscript{-/-} mice. Five days later the Matrigel plugs were harvested and 5 µm sections of formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin (H&E). The number of endothelial cell nuclei was counted in 10 random fields at 40x magnification.

J. Wound healing assay

A dermal biopsy punch (3 mm diameter; Miltex Inc., Bethpage, NY) was used to create four, full-thickness skin wounds through the skin and panniculus carnosus muscle in anesthetized animals (3-4 month old males) and left to heal. After wounding, animals were euthanized and wounded tissues were harvested at the times indicated. Histological sections were cut rectangular to the skin surface across the wound. Serial paraffin-embedded tissue sections (5 µm) were stained with H&E and analyzed by three
observers blinded to the design (46). Photographs of open wound areas at
different times (1-8 days) after injury were quantified using NIH ImageJ
software. The distance between the epithelial tips was measured in day 5
wound microphotographs using NIH ImageJ software, with the following
formula: (wound diameter – length of epithelial tongues).

K. Immunohistochemistry

Immunohistochemical analyses of wounds sections for PCNA
(Sigma, F4/80 (AbD Serotec, Raleigh, NC), VEGF-A (Santa Cruz
Biotechnology, Santa Cruz, CA), and AIB1 (Cell Signaling, Danvers, MA)
were performed on wound sections, as previously described (40). Briefly,
wounded tissues were first fixed in 10% formalin. Serial paraffin-embedded
tissue sections (5 µm) were obtained for IHC analyses. The wound tissues
were deparaffinized by incubating the slides at 60°C. The slides were then
placed in xylene two times for 8 minutes to complete the deparaffinization
process. Next, the slides went through several alcohol washes (100%, 95%,
90%, and 70%) for rehydration purposes and were boiled for 10 minutes for
antigen retrieval. Slides were allowed to cool at room temperature for 20
minutes. Tissue sections were then incubated with 3% hydrogen peroxide for
10 minutes. Then, slide sections were blocked for one hour and were
incubated with the primary antibodies overnight at 4°C. The primary
antibodies were detected using the DAKO Envision Plus HRP system
(DAKO Cytomation) and DAB (Vector). The sections were then counterstained with hematoxylin (Polysciences) for 45 seconds to stain the nuclei. After hematoxylin, the slides were washed with distilled water and were dehydrated using several concentrations (70%, 90% and 100%) of ethanol. At the end, slides were mounted using Clearmount (Zymed). F4/80, VEGF-A and PCNA positive cells were counted in 5 to 10 non-overlapping visual fields.

**L. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)**

Total RNA from wounded tissues and bone marrow was extracted using the RNeasy Fibrous Mini Kit and RNeasy Mini Kit (Qiagen, Valencia, CA), respectively, according to the manufacturer’s instructions. cDNA was synthesized with the iScript cDNA synthesis kit, according to the manufacturer’s protocol (Biorad Laboratories, Hercules, CA). qRT-PCR was performed in an iCycler iQ (BioRad) using the iQ SYBR Green Supermix (BioRad) under the following conditions: 95°C for 3 min followed by 40 cycles (95°C for 20 sec, 60°C for 30 sec and 72°C for 40 sec). PCR primers are listed in Table 1.
<table>
<thead>
<tr>
<th></th>
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<th>Reverse primer</th>
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<td>AACGTGGACTTCACCGCACAGCATT</td>
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<td>GCAACTTACCACAGACGTTTGTCTTAT</td>
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<td>mAIB1</td>
<td>AGTGGACTAGCGAAAGCTCT</td>
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Table 1: List of Primers used for quantitative real time PCR of signaling molecules in wound tissues.
M. *In vivo* fluorescence imaging

2 nmol of a broad range fluorescence activatable substrate (MMPsense 750Fast) (Perkin Elmer, Waltham, MA) for matrix metalloproteases (MMPs) 2, 3, 7, 9, 12, 13 that is optically silenced upon injection was injected into the mouse tail vein one day after wounding. *In vivo* fluorescence imaging was performed from days 2 to 8 after wounding on a Maestro *In vivo* Imaging System (Perkin Elmer) [setting, excitation wavelength = 641-681 nm, emission wavelength = 700-950 nm]. Spectral analysis of the images was done with the Maestro software by unmixing the pure spectrum of the agent from the auto fluorescence of the mice before injection. The fluorescence signal (photon flux/cm²/s) of areas around each wound was quantified and averaged among the four wounds.

N. Western blot analysis

Western blots were performed as described⁷. Basically, 48 hours post HUVEC, HaCat, Hs-27, and MEFs infection with control or AIB1/SRC-3 shRNA, cell were harvested and were lysed using NP40 lysis buffer. The cells were then incubated on ice for 30 minutes. Cells were then centrifuged at max speed for 15 minutes to eliminate cells debris. The protein concentration in the lysis supernatant was measured using the Bradford protein assay (Bio-Rad). The supernatant then was boiled in SDS-PAGE buffer for 5 minutes at 95°C. Proteins were separated by SDS-PAGE
electrophoresis using 4-20% tris-glycine gels at 120 V for approximately two hours. Separated proteins were then transferred to a Polyvinylidene fluoride (PDVF) membrane using the I-blot machine (Invitrogen). The membrane was blocked with 3% milk/PBST for one hour and was washed three times with PBST. The membrane was incubated with the primary antibodies overnight at 4°C and signals were detected with the respective HRP-conjugated secondary antibodies. Western blots for AIB1, p21, BIM, phospho MAPK, MAPK, phospho Akt, Akt, phospho p38 and p38 (Cell Signaling), were performed with the respective rabbit polyclonal antibodies. Immunoblot analyses for human actin were performed with a respective mouse monoclonal antibody (Chemicon International, Temecula, CA).

O. Expression analysis

The expression profile of 84 angiogenesis related genes was determined using a 96-well format Mouse Angiogenesis RT² Profiler PCR array (SABiosciences, Frederick, MD) according to the manufacturer’s instructions. Purification of total RNA prepared from 4-day wounds and cDNA preparation is described under “Quantitative Real Time Polymerase Chain Reaction”. qRT-PCR was performed with the RT² SYBR Green qPCR Master Mixes (SABiosciences).
P. Skin transplants

Full-thickness skin was taken from the ears of AIB1/SRC3\(^{+/+}\) and AIB1/SRC3\(^{+/-}\) mice (donors) and was transplanted onto the back of AIB1/SRC3\(^{+/+}\) as well as AIB1/SRC3\(^{+/-}\) mice (recipients), respectively (70). At day 9 post grafting, the transplants had healed and a 3 mm diameter dermal biopsy punch (Miltex) was used to create full-thickness skin wounds through the grafted skin in anesthetized animals (3-4 month old males) and wounds were allowed to heal. Photographs of open wound areas at different times after wounding were quantified using NIH ImageJ software.

Q. Primary tumor Epithelial and Fibroblast Cell Harvest and Culture

The MMTV-Neu AIB1/SRC3\(^{+/+}\) mice develop mammary tumors around 9 months old, whereas The MMTV-Neu AIB1/SRC3\(^{+/-}\) mice develop mammary tumors around 15 months (40). Once mammary tumors formed and reached 1 cm, the tumors were harvested and immediately were placed in a tube containing DMEM/F12 (Invitrogen) medium with 50\(\mu\text{g/ml}\) gentamicin (Invitrogen). Tumor tissues were then minced using razor blades for approximately 20 minutes. After mincing, the tumor tissues were then incubated with 1mg/ml collagenase solution (Sigma-Aldrich) for one hour at 37\(^\circ\text{C}\). Then, the tubes were centrifuged at 1000x for 10 minutes. The supernatant, which included the collagenase solution, was removed. The pellets, comprised of epithelial and fibroblast cells, were washed four times
using DMEM/F12 medium to eliminate any fibroblast contamination. After the fourth wash, the cell pellets were resuspended in Ham’s F12 medium (Invitrogen) supplemented with 10% FBS, 10ng/ml EGF (Roche), 50µg/ml gentamicin (Invitrogen), 4µg/ml insulin, and 1µg/ml hydrocortisone (Sigma-Aldrich). The cells were then plated in 10cm dishes coated with collagen for 15-20 minutes to minimize fibroblast contamination (fibroblasts will stick to the collagen plates). The medium containing the epithelial cells was then removed from the 10cm dishes and plated in low adherent dishes overnight. The next day cells were pelleted down and resuspended in HESS medium (Invitrogen). Cells were then used for the transplant procedure.

**R. Tumor Transplant procedure**

Tumor mammary epithelial cells from the AIB1/SRC3 \(^{+/+}\) and AIB1/SRC3 \(^{+/-}\) were generated as previously described in section Q. AIB1/SRC3 \(^{+/+}\) and AIB1/SRC3 \(^{+/-}\) mice that are 2 to 3 months old were used as transplant recipients of tumor cells injected into their mammary fat pads. The mice remained under anesthesia for the duration of the procedure. First, the abdomen of the mice were shaved using hair clippers. Then, the shaved skin was cleaned with alcohol and iodine to minimize contaminations. The skin nearest the #4 mammary gland was lifted up using forceps (all equipment was sterilized 24 hours prior to the transplant procedure), and scissors were used to generate a small opening in order to
inject the tumor cells into the fat pad of the #4 mammary gland. One million cells in a 10 microliter volume was injected into the fat pad using a 100 microliter Hamilton syringe. The opening was then closed using wound clips; one to two clips were utilized per cut. The tumor cell transplantation was repeated on the contralateral side. The mice were placed under a heat lamp for 10 min and were given food and water to recover after anesthesia. One week after the transplant procedure, the wound clips were removed and tumor formation monitoring started. The mice were monitored three days a week for palpable tumors and were recorded as a survival curve. Once the tumors reached 1 cm, tumors were harvested for H8E analysis, IHC, western blot and qPCR analysis.

**S. Data analysis and statistics**

Experiments were performed three times unless noted in the figure legend. Prism 5 (Graphpad Inc) was used for statistical analysis and for non-linear regression analysis. ANOVA was used for multiple comparisons and t-tests for paired comparisons. Statistical significance was defined as $P$ values $<0.05$ unless stated otherwise.
Chapter 3. Results and Discussion: Role of AIB1 in Epithelial and Stromal Cell Interaction in Normal Tissues \textit{in vitro}
To address the contribution of AIB1/SRC3 to stromal responses, we first assessed the impact of AIB1/SRC3 depletion on endothelial cell and fibroblast cell functions.

A. Effect of AIB1/SRC-3 on endothelial cells

a. AIB1/SRC3 depletion impairs endothelial cells function in vitro

To determine the impact of AIB1/SRC3 on endothelial cells function, endogenous AIB1/SRC3 was depleted by infecting endothelial cells (HUVEC) with lentiviral vectors expressing shRNAs targeting two distinct sequences in AIB1/SRC3 (AIB1 #1 or #2) or scrambled shRNA (control) (Figure 1A).

![Western blot analysis for AIB1 protein in endothelial cells (HUVEC).](image-url)
Endothelial cells infected with a scrambled control shRNA or AIB1/SRC3 shRNA #1 and #2 48 hours post infection. The blots are representative of three experiments.

Endothelial cells ability to sprout in response to appropriate cues was examined using a tube formation assay in collagen (68). Endothelial cells were depleted of AIB1/SRC3 by shRNA silencing. Reducing AIB1/SRC3 levels disrupted the ability of endothelial cells (HUVEC) to form tubes like structure and reduced the average diameter of the tubes (Figures 1B and C). Interestingly, AIB1/SRC3 shRNA #1 showed a more profound impact in comparison to shRNA#2, which suggest that the impact level is dose-dependent effect for the

Figure 1B and C: Tube formation in Matrigel is inhibited by reduction of AIB1. (B)

Representative images of tubes like structure
expression level of AIB1/SRC3. One of the hallmarks of endothelial cells is their ability to form the barrier layer between the blood stream and parenchyma to allow the exchange of substances between blood and tissues (71). Therefore, the impact of AIB1/SRC3 depletion on the ability of endothelial cells to form monolayers with tight junctions was monitored using electric cell impedance sensing (ECIS) as described (69). Endothelial cells (HUVEC) infected with a scrambled control shRNA or AIB1/SRC3 shRNA #1 and #2 and were plated in electrical cell impedance wells. The electrical resistance of endothelial cells was monitored at 15 sec intervals over a twenty hour time period. Endothelial monolayer resistance dropped by >50% in both AIB1/SRC3 shRNAs as shown in (Figure 1D) when compared to control shRNA cells. These results suggest a reduction in barrier function of endothelial cells when AIB1/SRC3 levels were depleted.
Figure 1D: Endothelial monolayer formation is inhibited by reduction in AIB1. Electrical resistance of endothelial cells infected with a scrambled control shRNA or AIB1/SRC3 shRNA #1 and #2 monolayers formed on microelectrodes was monitored at 15 sec intervals over a twenty hour time period. Resistance is shown relative to three hours after cell plating and is representative of one of three independent experiments in duplicate wells.
Beyond this steady-state barrier function of endothelial cells, the repair of an endothelial monolayer \textit{in vitro} can be used to assess the potential impact on the repair of endothelia \textit{in vivo}. To study the impact of reducing AIB1 levels on the ability of endothelial to migrate and repair, an \textit{in vitro} scratch assay was utilized. Endothelial cells were infected with a scrambled control shRNA and AIB1/SRC3 shRNA #1. Forty-eight hours post infection, the cells were allowed to grow until they form a monolayer. A scratch in the middle was made using a pipet tip. The endothelial cells migration was then measured at 24 hours and 48 hours. Depletion of AIB1/SRC3 in endothelial cells resulted in a delay of migration towards the denuded area compared to control cells (Figures 1E and F). The data suggests a strong impact of AIB1/SRC3 on endothelial cells ability to migrate and close a wounded area \textit{in vitro}.
Figure 1E and F: Repair of denuded endothelial monolayer areas by cell migration is inhibited by reduction in AIB1.

(E) Representative images of denuded areas at 24 and 48 hours with the white line indicating the migration front of endothelial cells infected with a scrambled control shRNA or AIB1/SRC3 shRNA #1. Size bar, 0.1 mm. (F) Quantitation of the closure of the denuded area. Mean ± SEM of one of two independent experiments done in triplicate. **, P<0.001 vs. control.

We further decided to examine the impact of reducing AIB1/SRC3 levels on endothelial cells proliferation using two distinct assays, which are Cell Titer
Glo and Crystal Violet assays. To Reduce AIB1/SRC3 levels, endothelial cells (HUVECs) were infected with a scrambled control shRNA or AIB1/SRC3 shRNA #1 and #2. The endothelial cells were then plated in 96 well plates to examine their proliferation. Cell proliferation was measured at day 1, 2 and 3 after plating the same number of cells at day 0 using Cell Titer Glo, while cell proliferation was measured at day 1, 2, 3, and 4 after plating the same number of cells at day 0 using the Crystal Violet assay. Depletion of AIB1/SRC3 in endothelial cells resulted in a decrease of cell proliferation compared to control cells using the Cell Titer Glo (Figures 1G Left). In addition, similar results were found using the Crystal Violet assay (Figures 1G Right).

Another mechanism, which can explain the decrease in number of proliferating cells, is apoptosis; therefore, we wanted to determine whether reducing AIB1/SRC3 levels on endothelial cells would increase the percentage of cell death using Annexin V assay. AIB1/SRC3 levels were depleted in endothelial cells (HUVECs) through infecting the cells with a scrambled control shRNA or AIB1/SRC3 shRNA #1 and #2. The endothelial cells were then plated
Figure 1G: Endothelial cell proliferation is inhibited by reduction in AIB1. (Left) Endothelial cells infected with a scrambled control shRNA or AIB1/SRC3 shRNA #1 and #2 were plated in 96 well plates to examine their proliferation using Cell Titer Glo. Mean ± SEM of one of three independent experiments done in triplicate. ***, P<0.0001 vs. control. (Right) Cell proliferation measured by crystal violet assay. Mean ± SEM of one of two independent experiments done in triplicate. ***, P<0.0001 vs. control.
increase in the percentage of dead cells compared to control cells (Figures 1I). The data supports the decrease in proliferation observed when AIB1/SRC3 levels were knockdown. Since reducing AIB1/SRC3 levels impacted endothelial cells proliferation, the scratch assay was repeated in the presence of mitomycin C. The experiment was designed in the same way the initial scratch assay was performed, but this time

**Figure 1H and I: Apoptosis in endothelial cells is inhibited by reduction in AIB1.** *(H)* Flow cytometry analysis with annexin V-FITC/PI double staining of HUVEC cells 48 hrs after infection with a control shRNA or AIB1/SRC3 shRNA. The percentage of cells in late apoptosis is indicated. *(I)* Annexin V positive cells 48 hours after AIB1/SRC3 depletion. Mean ± SEM of three independent experiments. *, P<0.05 vs. control.
we added mitomycin C to the cells to inhibit proliferation. Interestingly, the delay in scratch closure was not due to lack of proliferation of the endothelial cells since the same result was obtained in the presence of mitomycin C (Figure 1J). The results conclude that AIB1/SRC3 does effect endothelial cell migration.

Taken altogether, these data suggest that AIB1/SRC3 is critical for endothelial-specific functions, which include tube, monolayer formation, proliferation and apoptosis.

![Figure 1J: Repair of denuded endothelial monolayer areas was performed in the presence of](image)
2 µg/ml of mitomycin C. Repair of denuded endothelial monolayer areas was performed in the presence of 2 µg/ml of mitomycin C. (left) Representative images of denuded areas at 24 and 48 hours with the black line indicating the migration front of HUVEC cells. Size bar, 0.1mm. (Right) Quantitation of the closure of the denuded area. Mean ± SEM of one of two independent experiments done in triplicate. **, P<0.001 vs. control.
b. Effect of the reduction of AIB1/SRC3 protein levels on FGF2-induced signaling of endothelial cells in vitro.

Since AIB1/SRC3 reduction had significant effects on endothelial cells functions, we wanted to investigate its impact on distinct changes in FGF2-induced signal transduction in endothelial cells (HUVEC) in vitro. AIB1/SRC3 levels were depleted in endothelial cells (HUVECs) through infecting the cells with a scrambled control shRNA or AIB1/SRC3 shRNA #1 and #2. Forty-eight hours post infection; the cells were starved from growth factors for 24 hours. After that, the cells were either treated with FGF2 for 10 min or were untreated. Then, the cells were harvested for western blot analysis. The 18-fold induction of phospho-MAPK by exogenously added FGF2 was unchanged after AIB1/SRC3 knockdown, whereas the 4-fold phospho-AKT and 3-fold phospho-p38 inductions were reduced (Figure 2).

![Figure 2: Effect of the reduction of AIB1/SRC3 on FGF2-induced signaling of endothelial cells in vitro.](image)
**AIB1/SRC3 protein levels on FGF2-induced signaling of endothelial cells.** HUVEC cells were transduced with shRNA for either control or AIB1/SRC-3 and non-transduced cells were eliminated by 5 mg/ml puromycin treatment. After 4 hours of growth factor depletion in EBM-2 medium, cells were stimulated with or without 10 ng/ml FGF-2 for 10 minutes and cell lysates analyzed by Western blotting. Densitometry values are normalized to no FGF treatment for each shRNA treatment. The same pattern of results was achieved in two separate experiments.

Thus, the loss of AIB1/SRC3 in endothelial cells *in vitro* appears to affect signal transduction targets downstream of the FGF receptor pathway. Altogether, these findings indicate that the loss of AIB1/SRC3 negatively impacts key drivers of the FGF pathway in endothelial cells.
B. Effects of AIB1/SRC-3 expression in fibroblasts in vitro

To further examine the role of AIB1/SRC3 in other stromal cells, different fibroblast cell lines were utilized. First, we utilized mouse embryonic fibroblasts cells -/- (MEF) in which AIB1 is knocked out (65). These cells do not express any endogenous levels of AIB1/SRC3. AIB1/SRC3 was introduced into these cells by infecting with a lentiviral vector expressing FLAG tagged AIB1/SRC3. To confirm AIB1 overexpression, a western blot of cell extracts was performed (Figure 3A bottom). Next, we examined the effect of overexpressing AIB1/SRC3 on MEF cells compared with the control knocked out cells.
Figure 3A: AIB1/SRC3 overexpression in AIB1/SRC-3 /- murine embryonic fibroblasts (MEFs) does not alter proliferation. Proliferation of MEFs cells infected with a control vector or flagged tagged AIB1/SRC3 was measured 48 hours post infection at day 2, 4 and 6. Top: Cell Proliferation measurement using Cell Titer Glo. Bottom: Western blot for AIB1 protein and actin cell extracts showing the overexpression of AIB1 post infection.
proliferation using Cell Titer Glo assay. Cell proliferation was measured at day 2, 4 and 6 after plating the same number of cells at day 0. The proliferation of the MEF cells was unaffected due to the overexpression of AIB1/SRC3 (Figure 3A top).

We also utilized another mouse cell lines NIH3T3, which express endogenous levels of AIB1/SRC3, to determine the impact of reducing the AIB1/SRC3 on fibroblast proliferation. NIH3T3 cells were infected with a scrambled control shRNA or mAIB1/SRC3 shRNA #1 and #2. Cell proliferation was measured at day 1, 2, 3, 4, and 5 after plating the same number of cells at day 0 using Cell Titer Glo assay. The proliferation of the NIH3T3 cells was unchanged when AIB1/SRC3 levels were depleted as indicated in (Figure 3B).
Figure 3B: Depletion of AIB1/SRC3 from NIH3T3 fibroblasts does not later their proliferation. Proliferation of NIH3T3 cells infected with a control shRNA or AIB1/SRC3 shRNAs #1 or #2 was measured 48 hours post infection over 5 days period. Mean ± SEM of two independent experiments done in triplicate. ANOVA analysis was used to compare the results. No significant difference was detected.

Next, we examined the impact of AIB1/SRC3 on Hs-27 fibroblast cells which are a human fibroblast cell lines (Hs-27). Endogenous AIB1/SRC3 was depleted by infecting these cells with lentiviral vectors expressing
shRNAs targeting two distinct sequences in AIB1/SRC3 (AIB1 #1 or #2) or scrambled shRNA (control). After 48 hours cell extracts were prepared and a western blot analysis showed reduced protein expression of AIB1/SRC3 (Figure 3C).

![Western blot analysis for AIB1 48 hours post infection in Hs-27 human fibroblast cells.](image)

**Figure 3C: Western blot analysis for AIB1 48 hours post infection in Hs-27 human fibroblast cells.**

Fibroblast cells infected with a scrambled control shRNA or AIB1/SRC3 shRNA #1 and #2 48 hours post infection. The blots are representative of two experiments.

We then decided to study the impact of reducing AIB1/SRC3 levels on human Hs-27 cell proliferation using Cell Titer Glo assay. After lentiviral
infection as described in Figure 3C, the fibroblast cells were plated in 96 well plates and cell proliferation was measured at day 1, 2 and 3 after plating the same number of cells at day 0 using Cell Titer Glo. Depletion of AIB1/SRC3 in fibroblast cells did not affect cell proliferation compared to control cells (Figures 3D).

Figure 3D: Changes in AIB1 level do not effect proliferation of Hs-27 cells. Fibroblast cells infected with a scrambled control shRNA or AIB1/SRC3 shRNA #1 and #2 were plated in 96 well plates to examine their proliferation using Cell Titer Glo. Mean ± SEM of one of three independent experiments done in triplicate.
Since we found no effects in AIB1 levels on fibroblast proliferation we next determined the impact of reducing AIB1/SRC3 levels on the ability of fibroblast to migrate and repair. For this an *in vitro* scratch assay was utilized. Fibroblast cells were infected with a scrambled control shRNA and AIB1/SRC3 shRNA #1. The fibroblast cells (Hs-27) were plated in 12 well plates. Cell culture inserts were used to generate a 0.5 mm diameter scratch; the fibroblast cells migration to fill the scratch was then measured at 6, 12 and 24 hours. Depletion of AIB1/SRC3 in fibroblast cells resulted in a significant delay of migration towards the denuded area compared to control cells (Figures 3E and F). The data suggests that although AIB1 does not affect fibroblast proliferation, it has a strong influence of AIB1/SRC3 on fibroblast cells ability to migrate and close a wounded area *in vitro.*
Figure 3E and F: Reduction of AIB1 slows migration of Hs-27 cells after wounding of a confluent monolayer. E. Representative microphotographs of the wounded area at different times after wounding. The white line represents the migration front of the cells. F. Closure of the denuded area as shown by the % closure against time. Data are expressed as mean ± SEM of one of two independent experiments done in triplicate. ***, P<0.0001 vs. control (ANOVA).

In conclusion, proliferation of mouse or human fibroblasts was unaffected by the depletion or overexpression of AIB1/SRC3; however, the motility of human fibroblasts in a scratch assay was impaired by AIB1/SRC3 loss. These data suggest distinct roles of AIB1/SRC3 in different stromal cell types.
C. Effects of AIB1/SRC3 expression in keratinocytes in vitro

The Riegel lab has previously demonstrated that AIB1 is rate limiting for breast epithelial proliferation (65), and we next assessed the impact of AIB1/SRC3 on other epithelial cells using the human keratinocytes cell line, HaCat. Endogenous AIB1/SRC3 was depleted by infecting human keratinocytes cells (HaCat) with lentiviral vectors expressing shRNAs targeting two distinct sequences in AIB1/SRC3 (AIB1 #1 or #2) or scrambled shRNA (control). A western blot analysis showed reduced protein expression of AIB1/SRC3 (Figure 4A).

**Figure 4A**: Western blot analysis for AIB1/SRC3 48 hours post infection in keratinocytes. HaCat cells infected with a scrambled control shRNA or AIB1/SRC3 shRNA #1 and #2 48
hours post infection. The blots are representative of three experiments.

We also examined the impact of reducing AIB1/SRC3 levels on human keratinocytes proliferation using the Cell Titer Glo assay. After shRNA infection the keratinocytes cells were then plated in 96 well plates to examine their proliferation. Cell proliferation was measured at day 1, 2 and 3 after plating the equivalent numbers of cells from the various treatments at day 0. Depletion of AIB1/SRC3 in HaCat cells resulted in a decrease of cell proliferation compared to control cells (Figure 4B).

Figure 4B: Keratinocyte cell proliferation is inhibited by reducting AIB1 levels. HaCat cells infected with a scrambled
control shRNA or AIB1/SRC3 shRNA #1 and #2 were plated in 96 well plates to examine their proliferation using Cell Titer Glo. Mean ± SEM of one of three independent experiments done in triplicate. **, P<0.001, ***, P<0.0001 vs. control.

Furthermore, we wanted to investigate the impact of reducing AIB1/SRC3 levels in keratinocytes on epithelial/mesenchymal transition, (EMT), status of these epithelial cells. Therefore, HaCat cells were infected with a scrambled control shRNA or AIB1/SRC3 shRNA #1 and #2. Forty-eight hours post infection; the cells were harvested for western blot analysis of EMT markers; such as E-cadherin and vimentin.
Figure 4C: Effect of reducing AIB1 on the EMT markers  

expression vimentin in keratinocytes. HaCat cells infected with a scrambled control shRNA or AIB1/SRC3 shRNA #1 and #2 were harvested for western blot analysis of EMT markers. The blots are representative of three experiments.

The western blots data revealed that there was no major change in vimentin expression when AIB1/SRC3 levels were reduced in keratinocytes (Figure 4C).

We next examined the effect of AIB1/SRC3 depletion in controlling p53 and cyclin-dependent kinase inhibitors p21, and p27 in keratinocytes. After
AIB1/SRC3 levels were knocked down in HaCat cells, the cells were harvested for Figure 4D: Reducing AIB1/SRC3 in keratinocytes causes unregulation of the expression of p53 and cyclin-dependent kinase inhibitors p21, and p27. HaCat cells infected with a scrambled control shRNA or AIB1/SRC3 shRNA #1 and #2 were harvested for western blot analysis of EMT markers. The blots are representative of three experiments.
western blot analysis of p53, p21 and p27 protein expression. The data showed an increase in p53 expression when AIB1/SRC3 was reduced, which was accompanied by an increase in cyclin-dependent kinase inhibitors p21 and p27 as shown in (Figure 4D). Overall, the data suggest that reduction of AIB1/SRC3 inhibits keratinocytes proliferation and this is most likely related to increases in p53, p21 and p27 expression.
D. Discussion

Prior to this study most examination of AIB1/SRC3 function in vitro had been performed on cancer epithelial cells (9, 22-26). Here we report for the first time the effect of AIB1/SRC3 on the function of two types of stromal cells and on keratinocytes in vitro. Endothelial cell functions including formation of tube like structures, monolayer formation, migration, proliferation and apoptosis were all inhibited by reduction in AIB1. We also observed reduction on FGF2-induced signaling of endothelial cells in vitro when AIB1/SRC3 levels were reduced. In addition, we have demonstrated that AIB1/SRC3 affect the function of another stromal cell i.e. fibroblasts. AIB1/SRC3 did not impact fibroblast proliferation, but did impact its migration. Furthermore, AIB1/SRC3 had a significant effect on keratinocyte proliferation through the up regulation of a number of cell cycle molecules.

All of the data gathered to date on the role of AIB1/SRC3 has focused on its role as an oncogene exerting its influence through phenotypic changes in the mammary epithelium (9, 22-26). Additionally, previous reports indicated that reducing the AIB1/SRC3 levels in epithelial cells inhibits responses to steroid hormones, IGF-1, EGF and heregulin (21, 41). These pleiotropic effects are expected given that AIB1/SRC3 is not only a coactivator of nuclear receptors but also a coactivator of a diverse group of other transcription factors (21). Therefore, the keratinocytes data were not surprising to us given the data published already on the role of AIB1/SRC3
in impacting epithelial cells function. However, it is interesting to observe that the impact the AIB1/SRC3 had on epithelial cells in cancer cells also applies to normal cells.

We report in this study a novel and unknown function of AIB1/SRC3 in controlling endothelial cell phenotypes \textit{in vitro}. Endothelial cells are known to control angiogenesis, a physiological process that involves the formation of new capillaries from previously existing blood vessels. This complex process requires endothelial cells to proliferate, form monolayers and migrate (72). Reducing AIB1/SRC3 levels impacted the endothelial cells ability to form tube like structures and migrate. Also, endothelial cell proliferation and apoptosis was affected. These results suggest an influential role of AIB1/SRC3 in controlling the cellular functions important for angiogenesis.

We also show that the loss of AIB1/SRC3 negatively impacts key drivers of the FGF pathway. Our lab and others have reported that reducing AIB1/SRC3 levels in the epithelium significantly reduces pAKT levels (45), (73), (38). It is interesting to note the same observation in a stromal cell line, endothelial cells, where we report a reduction in pAKT and pJNK levels when AIB1/SRC3 levels were knocked down in HUVEC. It was not observed before that AIB1/SRC3 reduce the level of pAKT and pJNK in normal cells. An unexpected result was that reducing AIB1/SRC3 levels did not significantly impact or alter the phosphorylation status of pMAPK. This
could be due to the fact that pAKT was phosphorylated through a different signaling pathway; such as, the PI3 kinase pathway. We also document a reduction in FGFR-1 levels when AIB1 levels were depleted. AIB1 and FGFR-1 expression were reported previously to correlate in a number of breast cancer tissues (30). Furthermore, in earlier work we had found that AIB1 impacts transcription of a secreted FGF-binding protein (FGFBP1) (74) that can control angiogenesis (75, 76). In addition, the FGF pathway is known to be a major driver of angiogenesis (77). Therefore, the data suggest that AIB1/SRC3 controls angiogenesis through the FGF signaling pathway.

Fibroblast cells are known to be responsible for the production of extracellular matrix (ECM), tissue regeneration and collagen production (78) (79) (80). In addition, extracellular matrix and collagen are important for the recruitment of endothelial cells into the stroma (81). Therefore, the lack of correct fibroblast function impairs the stroma to function accurately. In this dissertation, we report that AIB1/SRC3 impacted fibroblast migration, which suggests that a reduction in AIB1/SRC3 could delay collagen and ECM production in the stroma of breast tissues or in the granulation tissue of a wound; which facilitate tumor formation and/or poor wound healing.

A possible explanation to the fibroblast data is that AIB1/SRC3 control the transcription of a particular set of genes that are known to be involved in migration. As an example, AIB1/SRC3 is known to be coactivator to a number of transcription factors including AP-1 and PEA3.
Furthermore, it was previously reported that AIB1/SRC3 directly regulates matrix metalloproteinase (MMP)-2 and MMP-13 transcription through its coactivation of AP-1 and PEA3 (82). Therefore, we speculate that AIB1/SRC3 may contribute to fibroblast migration through its regulation of MMP-2 and MMP-13 transcription. Interestingly, there appears to be some cell type specific effects as the differential changes in the endothelial cell implicate AIB1/SRC3 in regulating a different set of genes in the endothelial cell. This may suggest that in the endothelial cell AIB1/SRC3 is acting to coregulate a different, yet to be determined, set of genes modulating differentiation, proliferation and apoptosis, highlighting the cell type specific effects of AIB1/SRC3.

Results from this chapter suggest a significant impact of AIB1/SRC3 on angiogenesis, which is required in many physiological and pathological conditions; such as, wound healing and tumor formation (83). Therefore, in the next chapter we explored in depth the effect of AIB1 on angiogenesis using two in vivo models; matrigel plug and wound healing.
Chapter 4. Results and discussion: Role of AIB1 in Epithelial and Stromal Cell Interaction in Normal Tissues \textit{in vivo}
In the previous chapter, we demonstrated a defect in endothelial cells function when AIB1/SRC3 levels were reduced suggesting a significant role of AIB1 in angiogenesis. In addition, we have reported earlier that angiogenesis is reduced in mammary tumors of MMTV-HER2/Neu mice when comparing AIB1/SRC3^{+/-} and AIB1/SRC3^{+/-} genotype mice, whereas the AIB1/SRC3^{-/-} mice did not form mammary tumors at all (40). Therefore, in this chapter, we investigated in depth the angiogenic response when AIB1/SRC3 levels were reduced in mice using matrigel plug and wound healing assays.

**A. Reduction of AIB1/SRC3 impairs endothelial cell migration**

*in vivo*

To investigate the effect of AIB1/SRC3 genotype on angiogenesis, we utilized a Matrigel plug assay. Matrigel plug assay is the standard method to use to study angiogenesis *in vivo* (84). In this assay, Cold liquid Matrigel is placed subcutaneously in the mice abdomen. Upon injection, the matrigel plug solidifies and induces vascularization (84). We then can monitor neoangiogenesis by increased mRNA expression of an endothelial marker CD31 and loss of inflammatory marker CD14 within the plug (Figure 5C). An important feature about this assay is that it allowed us to look at the effect of AIB1/SRC3 on stromal cells without the influence of an epithelial cell.
We took two to three months old AIB1/SRC3+/+, AIB1/SRC3 +/- and AIB1/SRC3 -/- mice and subcutaneously injected them with Matrigel plug either with or without the present of FGF2. The plugs were harvested at day 5 post injection and were analyzed using H&E staining. Histological analysis and quantitation of the relative number of endothelial cells after a fixed time showed that endothelial cell invasion into the plugs of AIB1/SRC3+/- and AIB1/SRC3 -/- mice were significantly reduced when compared to those of AIB1/SRC3 +/- mice (Figures 2A and B, upper panels). The induction of angiogenesis in this assay is driven by locally released growth factors due to the subcutaneous wound caused by the Matrigel injection. We further tested whether supplementation of the Matrigel plugs with an excess of FGF2 would restore angiogenesis to similar levels across the different AIB1/SRC3 genotypes. Addition of FGF2 induced a significant, 5.5-fold angiogenic response in the AIB1/SRC3 +/- mice relative to baseline levels. However, AIB1/SRC3 +/- and AIB1/SRC3 -/- mice still showed significantly reduced angiogenesis even with the added FGF2 (Figures 5A and B, lower panels). We conclude that the loss of AIB1/SRC3 results in a reduced capacity to mount an angiogenic response to a subcutaneous injury that cannot be rescued by an excess of FGF2. Also, it appears that the loss of a single AIB1/SRC3 allele, i.e. in the AIB1/SRC3 +/- mice, reduces the angiogenic response by 70 - 90% (Figure 5B).
Figure 5A and B. Effect of AIB1/SRC3 on angiogenesis.
Neangiogenesis in subcutaneous Matrigel plugs injected subcutaneously into AIB1/SRC3^{+/+}, ^{+/−} or ^{−/−} mice was monitored. 
(A): Representative H&E-stained cross sections at 4x and 10x magnification of Matrigel plugs without (top panels) and with (bottom panels) added FGF2 (10 µg/ml) at 5 days after implantation. The size bar is 0.25 mm. (B): Number of endothelial cell nuclei in the plugs from 10 high-power fields per plug. Mean ± SEM (n = 3-5 animals per group). ***, P<0.0001 vs. AIB1/SRC3^{+/+}; ns, P>0.05, AIB1/SRC3^{+/−} vs. ^{−/−}. 
Figure 5C. CD31 and CD14 mRNA expression in Matrigel plugs. Quantitation of CD31 and CD14 mRNA by quantitative real-time PCR of mRNA prepared from Matrigel plugs 3 to 7 days after implantation. Values were normalized to CD31 or CD14 expression levels in the plugs on day 3. Values are given as the Mean ± SEM (n =3 animals per time point and genotype).
B. Reduction of AIB1/SRC3 delays the wound healing response in vivo.

From our previous data, which showed a defective neoangiogenic response in AIB1/SRC3 knockout mice, we hypothesized that AIB1/SRC3 might impact on tissue repair processes. Therefore, to test this hypothesis, we explored the healing of full-thickness skin wounds of AIB1/SRC3\(^{+/+}\), AIB1/SRC3\(^{+-}\) and AIB1/SRC3\(^{-/-}\) mice. In mammals, wound healing is a well-characterized process that requires defined inflammatory, proteolytic, tissue remodeling and angiogenic responses (58.). Therefore, we took AIB1/SRC3\(^{+/+}\), AIB1/SRC3\(^{+-}\) and AIB1/SRC3\(^{-/-}\) mice, and wounded these mice using a dermal biopsy punch. We allowed the skin to heal for five days; and then, we examined the healing process at day 5. We found that five days post wounding skin wounds from AIB1/SRC3\(^{+-}\) and AIB1/SRC3\(^{-/-}\) mice showed a macroscopic greater wound opening and distinctly inflamed appearance when compared to AIB1/SRC3\(^{+/+}\) mice. The latter showed more complete healing of the lesions with no evidence of residual inflammation (Figure 6A). Analysis of the digitized images of the wound areas using image J software revealed a significant wound closure delay in the AIB1/SRC3\(^{+-}\) and AIB1/SRC3\(^{-/-}\) mice with wound openings of 5.4±1.3 and 4.9±0.9 mm\(^2\) respectively, versus 1.4±0.1 mm\(^2\) in the AIB1/SRC3\(^{+/+}\) mice (Figures 6A and B).
Figure 6A and B: Skin wound analysis at day 5 after injury in AIB1/SRC3 \(^{+/+}\), \(^{+/-}\) or \(^{-/-}\) mice. (A) Macroscopic images and (B) quantitation of the open wound area of full-thickness dorsal skin wounds. Mean ± SEM (n=3-11 animals per genotype). **, P<0.001; ***, P<0.0001 vs. control.

We further examined the histology of these wounds and measured the distance between the epithelial tips as indicated by the black arrows under the incisional injury scab using Image J software. The data indicated that AIB1/SRC3\(^{+/-}\) and AIB1/SRC3\(^{-/-}\) mice had a significantly reduced epithelial closure relative to the AIB1/SRC3\(^{+/+}\) animals (0.9±0.06 and 0.98±0.085 mm vs. 0.57±0.03 mm, respectively; Figures 6C and D).
Figure 6C and D: Effect of AIB1/SRC3 on skin wound healing

(C) Representative H&E-stained sections of excisional skin wounds at day 5 after wounding Size bar = 0.2 mm. (D) Quantitation of wound re-epithelialization, as described under “Materials and Methods”. Black arrowheads = wound edges; black arrows = tips of epithelial tongues. Mean ± SEM (n = 3-5 animals per group). **, P<0.001 vs. control.

Next, we stained the granulation tissue and the hyperproliferative epithelium at the wound edge for proliferating cell nuclear antigen, PCNA, a marker of proliferation (Figure 6E) and (Figure 6I, left). The proliferation index showed a significantly lower number of proliferating cells of AIB1/SRC3+/− and AIB1/SRC3−/− mice relative to AIB1/SRC3+/+ animals (Figure 6F and 6I right).

Figure 6E and F: Effect of AIB1/SRC3 on proliferation in the granulation tissues. (E) Proliferating cell nuclear antigen (PCNA) staining (brown) of proliferating cells (white

74
arrowheads) in the wound granulation tissue. Size bar, 0.1 mm.

\((F)\) Quantitation of the PCNA-positive nuclei. Proliferation index = PCNA positive cell nuclei per 100 cells. Mean ± SEM (n = 3-4 animals per group). *, P<0.05; **, P<0.001 vs. control.

![Figure 6I: Effect of AIB1/SRC3 on proliferation during wound healing.](image)

**Figure 6I: Effect of AIB1/SRC3 on proliferation during wound healing.** (Left) Proliferating cell nuclear antigen (PCNA) immunostaining of proliferating keratinocytes in the hyperproliferative epithelium (he) of skin wounds of AIB1/SRC3\(^{+/+}\) and AIB1/SRC3\(^{+-}\) mice on day 4 after wounding. Dotted lines depict the delimitation between the he and the granulation tissue (g). Scale bars: 0.2 mm. (Right) Quantitation of PCNA-positive nuclei. Values are given as the mean ± SEM (n =3 animals per group). **P=0.001 versus control.

It is of note that AIB1/SRC3 protein levels measured by immunohistochemistry were reduced significantly in the healthy skin and
granulation tissue of the AIB1/SRC3\textsuperscript{+/−} mice relative to the controls and were not detectable in AIB1/SRC3\textsuperscript{−/−} mice (Figure 6J). Still, the extent of defective wound healing in the AIB1/SRC3\textsuperscript{+/−} and AIB1/SRC3\textsuperscript{−/−} mice showed no significant difference (Figures 6B, D and F), indicating that the loss of one AIB1/SRC3 allele was sufficient to maximally impede the wound healing response. Since homozygous AIB1/SRC3\textsuperscript{−/−} mice have reduced reproductive function and viability (47) in contrast to heterozygous AIB1/SRC3\textsuperscript{+/−} mice, we utilized the AIB1/SRC3\textsuperscript{+/−} mice for further in-depth analyses of the wound healing response.

**Figure 6J:** Immunohistochemical staining for AIB1/SRC-3 protein. Mouse skin tissue sections and wound granulation tissue from AIB1/SRC3\textsuperscript{+/+}, AIB1/SRC3\textsuperscript{+/−} and AIB1/SRC3\textsuperscript{−/−} mice. Scale bars: 0.2 mm (top); 0.1 mm (bottom).
Furthermore, we performed an intensive analysis of wound closure of AIB1/SRC3\(^{+/+}\) and AIB1/SRC3\(^{+-}\) mice. Wound healing of AIB1/SRC3\(^{+/+}\) mice and their control AIB1/SRC3\(^{+/+}\) littermates was followed over an 8-day time period (Figure 6G). Differences in wound closure became apparent by day 4 after wounding and was sustained throughout the healing process. In fact, day 8 wounds from AIB1/SRC3\(^{+/+}\) mice exhibited an almost complete closure, whereas those from AIB1/SRC3\(^{+-}\) mice still showed a 40\% opening (Figure 6H). Histological analysis revealed well progressing healing over time that was characterized by mature granulation tissue, continuous wound contraction and re-epithelialization in the controls. This was accompanied by

**Figure 6G and H: Wound healing time course in AIB1/SRC3\(^{+/+}\) and \(^{+-}\) mice.** (G) Macroscopic images of wounded skin from AIB1/SRC3\(^{+/+}\) and AIB1/SRC3\(^{+-}\) mice and (H) quantitation of the open wound area relative to day 0 is shown.

***, P<0.0001 vs. control.
superficial neutrophils at the base of the scab, migrating and proliferating spindled fibroblasts and macrophages, gradual increase of infiltrating endothelial cells with subsequent neoangiogenesis and collagen deposition from the base throughout the whole wound area. The different healing features are indicated in (Figure 6K). In contrast, wounds from AIB1/SRC3\(^{+/−}\) mice showed a significantly slower healing process with poorer wound contraction, delayed fibrin breakdown and very little collagen deposition. Immature granulation tissue was mainly present at the periphery of the wound, with fewer infiltrating cells and delayed neoangiogenesis (Figure 6K).

Taken together, the data suggest a significant impact of AIB1/SRC3 in wound healing as poor wound healing was associated with low levels of AIB1/SRC3 in mice.
Figure 6K: Different Healing Features of wounds in AIB1/SRC3+/- vs. AIB1/SRC3+/- mice. Representative H&E-stained sections of excisional skin wounds from AIB1/SRC3+/- and AIB1/SRC3+/- mice at different times after wounding (4, 6, and 8 days). Magnified areas shown on the right are indicated. Scale bars: 0.2 mm. s, scab; e, epithelium; n, polymorphonuclear neutrophils; g, granulation tissue; asterisks denote fibrin clots; arrowheads point to capillaries.
C. Wound healing in transplanted skin from, and to AIB/SRC3+/- and AIB1/SRC3 +/- mice.

To directly examine the local contribution of the altered AIB1/SRC3 genotype on the wound, we transplanted skin from AIB1/SRC3+/- and AIB1/SRC3+/- mice onto back of AIB1/SRC3+/- or AIB1/SRC3+/- mice, respectively. Within two weeks these grafts healed-in and full-thickness excisional wounds were then generated in the center of the grafts using a dermal biopsy punch. Image analysis of the wounds was used to assess the impact of the recipient and donor genotype on wound closure (Figure 7). It should be noted that in this crossover study, each host animal carried grafts from both donor genotype AIB1/SRC3+/- and AIB1/SRC3+/- mice. It was quite striking that wounds in skin grafts from AIB1/SRC3+/- donors healed significantly better than grafts from AIB1/SRC3+/- donors irrespective of the recipient host genotype. This suggests that AIB1/SRC3 mostly affects locally acting drivers rather than cells or soluble factors in the circulation of the host or bone marrow cells induced and recruited during wound healing.
Figure 7: Wound healing in transplanted skin from and to AIB1/SRC3^{+/+} and AIB1/SRC3^{+-} mice. Full-thickness skin grafts from AIB1/SRC3^{+/+} and AIB1/SRC3^{+-} mice (donors) were grafted onto the backs of AIB1/SRC3^{+/+} (A) and AIB1/SRC3^{+-} (B) mice (recipients), respectively. Day 9 after grafting, a dermal biopsy punch (3-mm diameter) was used to generate full-thickness skin wounds through the center of the grafted skin in anesthetized animals (3 to 4 months old), and they were left to heal. Representative images of the grafts and wounds on day 3 are shown. Open wound areas were quantified daily for 5 days.
Values are given as the mean ± SEM of the wound area ($n = 3$ animals per genotype). ***$P < 0.0001$ AIB1/SRC3$^{+/+}$ versus AIB1/SRC3$^{+/−}$. 
D. Reduction of AIB1/SRC3 delays the inflammatory and angiogenic response during wound healing.

The inflammatory response is a characteristic of the early stages of wound healing (85). Normal skin tissue day 0 and wounded skin tissue at day 1 and day 4 from AIB1/SRC3\(^{+/−}\) and AIB1/SRC3\(^{+/+}\) mice were harvested for mRNA analysis. Consistent with the notion of rapid recruitment of inflammatory cells such as monocytes and macrophages which express CD14\(^+\) and CD16\(^+\) markers, one day after wounding there was a >300-fold increase in CD14 and a >50-fold increase in CD16 expression in the wounds of AIB1/SRC3\(^{+/+}\) mice, as compared to non-wounded skin, day 0 (Figure 8A). In contrast, increases in CD14 and CD16 levels in wounds from AIB1/SRC3\(^{+/−}\) mice were significantly smaller than those of AIB1/SRC3\(^{+/+}\) mice. In day 4 wounds, the levels of CD14 and CD16 decreased, and were no longer different between AIB1/SRC3\(^{+/+}\) and AIB1/SRC3\(^{+/−}\) mice (Figure 8A). This suggests that the impact of AIB1/SRC3 on inflammatory cell recruitment occurs during the initial response to injury.
Figure 8A: Effect of AIB1/SRC3 on the inflammatory response during wound healing. (A): CD14 and CD16 mRNA expression in wounds and bone marrow at day 0, 1 and 4 post injury. Mean ± SEM (n=3 animals per time point and genotype); **, P<0.001; ***; P<0.0001.

This difference in inflammatory response could be due to changes in the complement of immune and endothelial progenitor cells in the bone marrow of the different AIB1/SRC-3 genotypes. Therefore, we harvested the bone marrow from AIB1/SRC3^{+/+} and AIB1/SRC3^{+/−} mice and analyzed the expression levels of a diverse set of hematopoietic, mesenchymal stem cell and pro-inflammatory markers. However, the data showed no significant
differences between bone marrow of AIB1/SRC3\textsuperscript{+/+} and AIB1/SRC3\textsuperscript{+/-} mice (Figure 8B).

**Figure 8B: Expression of marker molecules in bone marrow of AIB1/SRC3\textsuperscript{+/+} and AIB1/SRC3\textsuperscript{+/-} mice.** Hematopoietic, mesenchymal stem cell and pro-inflammatory marker mRNA expression in bone marrow of AIB1/SRC3\textsuperscript{+/+} and AIB1/SRC3\textsuperscript{+/-} mice Mean ± SEM (n= 3 mice per group).

It is noteworthy that AIB1/SRC3 mRNA levels in the wounds of AIB1/SRC3\textsuperscript{+/+} mice were upregulated two fold one day after wounding and returned to control levels after four days, whereas no such changes were
found in the wounds of AIB1/SRC3+/− mice (Figure 8C). AIB1 mRNA in the wounds of AIB1/SRC3+/− mice were reduced by 80% relative to the peak levels in AIB1/SRC3+/+ mice, and AIB1 protein levels in the wound also corroborate this observation (Figure 6J). This might explain why the delayed wound healing response in the homozygous AIB1/SRC3−/− mice and the heterozygous AIB1/SRC3+/− mice was indistinguishable (see Figures 6A to F).

**Figure 8C: AIB1 mRNA expression in skin wounds.**
Quantitative RT-PCR for AIB1 in skin wounds of AIB1/SRC3+/+ or AIB1/SRC3+/− mice 0 to 4 days after wounding. Values are given as the mean ± SEM (n =3 animals per time point and genotype); **, P<0.001; ***, P<0.0001.
Consistent with the impact of AIB1/SRC3 silencing on the local inflammatory response, wounds from AIB1/SRC3^{+/−} mice showed a significant reduction by 55% in F4/80 staining of mature macrophages relative to AIB1/SRC3^{+/+} mice (Figures 8D and E), whereas the analysis of bone marrow in the AIB1/SRC3^{+/+} vs AIB1/SRC3^{+/−} mice did not show a difference in F4/80 levels (Figure 8B).

**Figure 8D and E: Macrophage invasion into granulation tissue of day 5 wounds in AIB1/SRC3^{+/−} vs. AIB1/SRC3^{+/+} mice.** (D) Staining of granulation tissue for F4/80 positive macrophages (white arrowheads). Size bar, 0.1 mm. (E) Number of F4/80-positive macrophages invading the granulation in 5 non-overlapping visual fields. Mean ± SEM (n=3-4 animals per group). **, P<0.001 vs. control.
Another hallmark of wound healing and tissue remodeling is the production of matrix metalloproteases (MMPs) predominantly by the inflammatory cells and macrophages that promote extracellular matrix breakdown (58) (59). MMP activity is also a feature of tissue remodeling during tumorigenesis (60). We therefore examined, by in vivo imaging, the overall activity of MMPs (MMP 2, 3, 7, 9, 12, 13) in the wounds by monitoring an MMP activatable fluorescent substrate that was injected intravenously. Peak levels of MMP activity were observed in AIB1/SRC3\(^{+/+}\) mice three days after wounding with a continuous decrease until day six (Figure 8F and 8H). In contrast, in the AIB1/SRC3\(^{+/−}\) mice, the MMP activity did not increase above the initial levels and progressively decreased. Also, at all time points the MMP activity was lower in AIB1/SRC3\(^{+/−}\) than in the AIB1/SRC3\(^{+/+}\) mice (Figure 8H). These findings were corroborated by an expression survey of wound healing related genes. A significant decrease in MMP9 mRNA expression in day 4 wounds of AIB1/SRC3\(^{+/−}\) versus AIB1/SRC3\(^{+/+}\) mice was observed (Figure 10A). Altogether, these data support the notion of compromised immune cell infiltration and a consequently slower extracellular matrix remodeling by MMPs in wounds from AIB1/SRC3\(^{+/−}\) mice as a contributing factor to the defective wound healing seen at the macroscopic level.
Figure 8F and G: MMP activity in day 3 wounds. (F) Representative images of fluorescent activatable MMP substrate. Four wounds per animal with an approximate average diameters of 3 mm are visible (see Figure 6A). The color represents signal intensity ranges from red (highest activity) to blue (lowest activity); (G) quantitation of the MMP activity. Mean ± SEM (n=4 animals per group). *, P<0.05.
Figure 8H: MMP activity in wounds. Quantitation of the fluorescence signal of MMP activatable fluorescence agent measured in wounds from AIB1/SRC3\(^{+/+}\) and AIB1/SRC3\(^{+/-}\) mice 2 to 6 days after wounding. Values are given as the mean ± SEM (\(n = 4\) animals per group).

Consistent with a direct effect of AIB1/SRC3 on endothelial cell function, the number of infiltrating capillaries from the normal tissues into the wound granulation tissue of AIB1/SRC3\(^{+/-}\) mice was significantly reduced relative to AIB1/SRC3\(^{+/+}\) mice (Figures 9A,B) and this decrease in neoangiogenesis was consistent with a reduction in VEGF-A immunoreactivity in the same tissues (Figures 9C and D).
**Figure 9A and B: Effect of AIB1/SRC3 on wound angiogenesis.** (A) Representative H&E-stained sections of the granulation tissue from excisional skin wounds from of AIB1/SRC3^{+/+} and AIB1/SRC3^{+-} mice at day 5 after wounding. White arrowheads = capillaries. Size bar, 0.1 mm. (B) Quantitation of the number of neocapillaries across the wound.

**Figure 9C and D: Effect of AIB1/SRC3 on wound angiogenesis** (C) Immunostaining of granulation tissue for microvessels with an anti VEGF-A antibody. Size bar, 0.1 mm.
(D) Quantitation of VEGF-A positive neocapillaries. *, P<0.05; **, P<0.001 vs. control; Mean ± SEM from 3-5 animals per genotype group.
E. Driver pathways of AIB1/SRC3 effects during wound healing

To explore which signaling pathways are impacted by AIB1/SRC3 during wound healing, we surveyed the expression of a set of known genes involved in wound healing and angiogenesis. Overall comparison of gene expression patterns in wounds from AIB1/SRC3<sup>+/+</sup> vs. AIB1/SRC3<sup>+-/</sup> mice surveying 84 known angiogenic modulator genes did not show significant changes above 2-fold for the majority of genes represented on the array, including prominent angiogenic factors, such as FGF1 and FGF2. Noteworthy examples of genes with differential expression between wounds of AIB1/SRC3<sup>+/+</sup> and AIB1/SRC3<sup>+-/</sup> mice, were the macrophage-derived cytokine CXCL2 and HIF1-alpha (Figure 10A).

![Figure 10A: Expression of angiogenesis-related genes in day 4 wounds of AIB1/SRC3<sup>+/+</sup> versus AIB1/SRC3<sup>+-/</sup> mice.](image-url)
Confirmatory analysis of a gene expression survey in day 4 wounds using the mouse angiogenesis RT2 Profiler PCR array. Quantitative real-time PCR results relative to actin are shown. *$P$ <0.05 comparing wounds across genotypes.

Since AIB1/SRC3 knock-out mice failed to fully respond to FGF2 stimulation in the Matrigel assay (Figure 5A), we hypothesized that FGF signaling molecules could be likely drivers of the differential wound healing response of AIB1/SRC3$^{+/+}$ vs. AIB1/SRC3$^{+/−}$ mice. Also, in earlier work we had found that AIB1 impacts transcription of a secreted FGF-binding protein (FGFBP1) (74) that can control angiogenesis (75, 76) wound healing (86) (87) and vascular permeability (88). Therefore, we assessed mRNA expression of FGF7, FGF10, FGF receptors (FGFR1-4) and FGFBP1 and FGFBP3 (89, 90) (91) in 4-day wounds. Interestingly, wounds from AIB1/SRC3$^{+/−}$ mice showed a significantly lower expression of FGF10, FGFBP3 and FGFR1 and 3, compared to AIB1/SRC3$^{+/+}$ controls (Figure 10B).
Figure 10B: Effect of AIB1/SRC3 on FGFR pathway genes.

mRNA expression (qRT-PCR) of FGF7, FGF10, FGFBP1, FGFBP3, FGFR1 to FGFR4 in day 4 skin wounds. Mean ± SEM (n=3; *, P<0.05; **, P<0.001 vs. control). Note that mice, in contrast to other vertebrates, lack the FGFBP2 gene (75, 76).

Also, the expression ratio of FGFR2b to c splice isoforms was changed in favor of the b-isoform in wounds from AIB1/SRC3+/+ mice, whereas the FGFR1 and 3 isoform ratios were not impacted (Figure 10C). FGF10 preferentially signals through FGFR2b (92) and the impact of the increased FGF10 expression in the wounds of AIB1/SRC3+/+ mice will thus be enhanced together with a difference in overall levels of FGFR1 and 3 in favor of wounds from AIB1/SRC3+/+ mice. The phenotypic effects of
AIB1/SRC3 reduction were also reflected in distinct changes in FGF2-induced signal transduction in endothelial cells \textit{in vitro}.

Altogether, these findings indicate that the loss of AIB1/SRC3 negatively impacts key drivers of wound healing along the FGF pathway.

\textbf{Figure 10C:} Expression ratio of FGFR b and c splice isoforms \textbf{in day 4 wounds of mice.} mRNA expression of the b and c isoforms of FGFR1 to FGFR3 was analyzed by quantitative RT-PCR. The ratio of the b/c isoforms is shown. Values are given as the mean ± SEM (\(n = 3\) animals per time point and genotype). *\(P < 0.05\).
F. Discussion

In this chapter, we report that AIB1/SRC3 has a profound impact in angiogenesis and wound healing. In the Matrigel plug experiments, neoangiogenesis in AIB1/SRC3$^{+/c}$ and AIB1/SRC3$^{-/-}$ mice were significantly lower than the control AIB1/SRC3$^{+/+}$ mice. In addition, adding FGF2 exogenously was not able to rescue the angiogenic response in these animals. Moreover, we show a significant impact of AIB1/SRC3 in wound healing as poor wound healing was associated with low levels of AIB1/SRC3 in mice. We further demonstrated that the loss of AIB1/SRC3 negatively impacts key drivers of wound healing along the FGF pathway.

It should be noted that despite these defects in neoangiogenesis and tissue repair in adult knockouts animals described in this chapter, AIB1/SRC3$^{-/-}$ mice are viable at birth and have no discernible vascular phenotype. However, fertility and the number of offspring-per-birth is low. This could be due to poor uterine implantation of embryos that requires invasion into the uterine lining and the recruitment of uterine blood vessels (37, 47). AIB1/SRC3 is known to affect epithelial proliferation (Chapter 3 discussion) and we did observe a reduced number of proliferating keratinocytes in the wounds in AIB1/SRC3$^{+/c}$ mice (Figure 6E, F and I). This reduced epithelial proliferation could indirectly impact on stromal cell function and provide a further mechanism of reduced wound closure and delayed re-epithelialization.
Quite surprising to us was the fact that the wound healing response is already maximally impacted in heterozygous AIB1/SRC3\(^{+/-}\) mice and not further affected by a complete loss of the AIB1/SRC3 gene. Interestingly, whereas wounds on AIB1/SRC3\(^{-/-}\) mice showed no differential expression of AIB1/SRC3 mRNA over time, steady-state AIB1/SRC3 mRNA was upregulated in wounds of AIB1/SRC3\(^{+/-}\) mice, resulting in a 5-fold expression difference at peak levels during healing. This suggests a positive feedback of AIB1/SRC3 expression in the injury site as well as a threshold expression level needed to engage physiologic repair processes in the adult.

On the other hand, upregulation of AIB1/SRC3 in breast cancer stroma (Figure III) may reflect a wound healing stromal response, given that there are overlapping pathways between cancer and healing wounds (49, 50) and an activated wound response signature indicates poor outcome in breast cancers (93). Since loss of one AIB1/SRC3 allele delays development of MMTV-HER2/Neu induced tumors (40), it is tempting to speculate that similar threshold mechanisms seen in wound healing are involved in limiting the tumorigenesis, possibly due to the reduced AIB1/SRC3 in the tumor stroma. All this data suggest that AIB1/SRC3 is a haploinsufficiency oncogene meaning we need both alleles in order to have a functional gene.

The reduced response of wounds in AIB1/SRC3\(^{+/-}\) mice was evident at different levels including histology, gene expression level and overall metalloprotease activity. This was accompanied by reduced recruitment of
inflammatory cells, such as macrophages and monocytes, to the healing wound site evidenced by the smaller rise of CD14 and CD16 inflammatory cell markers as well as cytokines IL1-beta and CXCL2 that are produced by inflammatory cells in wounds.

Interestingly, we also observed reduced angiogenesis in wound tissues, which was supported by reduction in VEGF-A staining. Another interesting finding is the significant reduction in HIF-1A mRNA expression in the AIB1/SRC3+/− wound tissues (Figure 10A), which could provide a possible explanation to the reduction in angiogenesis data. HIF-1A was reported to be a key regulator of angiogenesis and embryogenesis (94). In addition, p300 is known to be a transcriptional coactivator for HIF-1A (7) (95). Moreover, AIB1/SRC3 play a critical role in stimulating p300 to the transcription machinery. This may suggest that in the wound tissues AIB1/SRC3 is acting to modulating angiogenesis through its role in p300 recruitment (96).

The functional and expression analysis suggests that major drivers in the FGF pathway (97) require AIB1/SRC3 to modulate neoangiogenesis and wound healing. FGFR1 and FGFR3 as well as the FGFR2b ligand, FGF10 (92) were significantly reduced in the wounds of the AIB1/SRC3+/− animals relative to the +/+ controls. Both FGF7 and 10 are known to be involved in wound re-epithelialization and angiogenesis (58) and are typically produced by stromal cells to act predominantly on the epithelial cell FGFR2b-isoform
Interestingly, the relative expression of the FGFR2b isoform was significantly lower in the wounds of the AIB1/SRC3<sup>+/−</sup> mice making this a further indicator as well as driver of the delay in epithelial closure. The largest change in the comparison of wounds from AIB1/SRC3<sup>+/−</sup> and control mice was the 16-fold difference in expression of the secreted FGFBP3 (Figure 10E). This secreted FGF binding protein was shown earlier to interact with FGF1 and FGF2 and to potentiate FGF2-dependent vascular permeability and angiogenesis (88) (91). Of note is that FGF-pathway genes monitored in the wound healing studies and those reported to be expressed in breast cancer stroma showed parallel changes (Table 2) suggesting these as common target(s) of AIB1/SRC-3 in the stromal compartment and shared drivers of healing wounds and malignancies proposed much earlier (49).

<table>
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<th>gene</th>
<th>FGF7</th>
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<th>FGF1R</th>
<th>FGF2R</th>
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<td>$10^{-4}$</td>
<td>$10^{-4}$</td>
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**Table 2: mRNA expression of FGF pathway-related genes in the stroma of breast cancer and normal breast tissues.** The expression ratios of cancer (n = 53) versus normal tissues (n = 6) are given as well as the respective p values for the comparison. NS, $P > 0.05$. The original expression data were obtained by Oncomine and published in (42).
All the data we gathered in the normal tissues *in vitro* and *in vivo* show a profound role of AIB1/SRC3 in several cell types in the normal stroma in a physiological response to a wound. At the cellular and molecular level there are many parallels between a healing wound and processes ongoing in the tumor and surrounding stroma (51). Some 150 years ago Rudolf Virchow (1863) viewed tissue injury and repair as part of the malignant process (49) and tumors have been described as “wounds that will not heal” (50). An important component of wound healing is the formation of new blood vessels that is controlled by a well-orchestrated set of different drivers that can be dysregulated in tumors (61-63). Therefore, we wanted to further investigate the impact of altering the AIB1/SRC3 levels in the stroma in tumor tissues. In the next chapter, we altered the AIB1/SRC3 levels in the stroma utilizing AIB1/SRC3\(^{+/+}\), AIB1/SRC3\(^{+/-}\) and AIB1/SRC3\(^{-/-}\) mice as different stromal background for transplantation experiments. Then, we next examined breast tumor formation when AIB1/SRC3 levels were altered in the stroma.
Chapter 5. Results and Discussion: Role of AIB1/SRC3 in Epithelial and Stromal Cell Interaction in Mammary Cancer Tissues.
We have previously demonstrated that AIB1/SRC3 is functionally required for HER2-mediated mammary tumorigenesis in a mouse model for breast cancer. This occurs through regulation of HER2 phosphorylation and signaling (40). Generation of a Her2/Neu transgenic mice with either loss of one or both copies of the AIB1/SRC3 gene resulted in reduced or complete abolition of mammary tumor development, respectively. One very interesting aspect of this study was that the tumors that arose in MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) mice were phenotypically different from those in MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) mice in that they were smaller, more necrotic and significantly less vascularized (40). This data indicated to us that the role of AIB1/SRC3 in breast cancer was not confined to mammary epithelium proliferation but also had major effects on the surrounding stromal and in epithelial-stromal crosstalk in mammary tissue. Since the MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) mice had reduced AIB1/SRC3 in all tissues, it was not clear from gross examination of the tumors that the reduction of AIB1 affects the epithelial secreted factors that influence the stroma or whether the loss of AIB1/SRC3 in the stroma itself has a direct effect on the signaling; thus, altering the stromal and epithelial cell interactions in these tumors. In addition, analysis of human breast cancer stromal gene expression versus normal stromal gene expression indicated that AIB1/SRC3 was highly unregulated (Figure III). Therefore, we investigated the impact of reducing AIB1/SRC3 levels in the epithelial or stromal compartment on mammary
tumorigenesis utilizing a transplant experiment. All experiments presented in this chapter were performed on the transplanted tumors.

**A. Altering the AIB1/SRC3 expression in mammary tumor epithelial cells (MECs) delays tumor formation in MMTV-HER2/Neu mice.**

The majority of the previous published data on the AIB1/SRC3 function in tumorigenesis has focused on its role in the epithelium. The data presented in Chapters 3 & 4 strongly suggests that AIB1/SRC3 has an additional role in stromal cell function in pathological processes such as wound healing. The role of the tumor stroma in the development and progression of neoplasms is gaining more recognition. In fact AIB1/SRC3 is elevated in tumor stroma versus normal stroma (Figure III). In this next series of experiments, we begin to define the relative roles of AIB1 in the epithelia and stromal compartments in mammary carcinogenesis. For this, we first investigated tumor formation when the levels of AIB1/SRC3 were altered in the epithelium. We have reported earlier that MMTV-HER2/Neu AIB1/SRC3<sup>+/−</sup> mice formed tumors at a delayed rate compared to MMTV-HER2/Neu AIB1/SRC3<sup>++</sup> mice, whereas the MMTV-HER2/Neu AIB1/SRC3<sup>−/−</sup> mice did not form mammary tumors at all (40). Therefore, we isolated tumor MECs from MMTV-HER2/Neu AIB1/SRC3<sup>++</sup> tumors and
MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) tumors as described in the methods chapter, which represent two types of tumor mammary epithelial cells with two different AIB1/SRC3 levels. A western blot of the level of AIB1/SRC3 in these tumor types was published previously (40). We then transplanted these tumor MECs into the mammary fat pad of MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) mice and we monitored tumor formation (Figure 11A).

Figure 11A: Diagram of how the transplant experiment that examined the effect of changes in AIB1/SRC3 levels selectively in the epithelium, was performed. MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) MECs labeled in black or MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) MECs labeled in red were transplanted into the
The presence of palpable mammary tumors was documented against time and is shown in a Kaplan Meier plot (Figure 11B). The MMTV-HER2/Neu AIB1/SRC3+/+ mice that received MMTV-HER2/Neu AIB1/SRC3+/- tumor MECs developed palpable mammary tumors at a delayed rate relative to the MMTV-HER2/Neu AIB1/SRC3+/+ mice that received MMTV-HER2/Neu AIB1/SRC3+/+ tumor MECs (Figure 11B).

Figure 11B: Reduced AIB1/SRC3 levels in the donor MECs delayed tumor incidence in MMTV-HER2/Neu AIB1/SRC3+/+ host mice. Kaplan-Meier
plot of tumor free incidence comparing MMTV-HER2/Neu AIB1/SRC3<sup>+/+</sup> mice that received 1x10<sup>5</sup> MMTV-HER2/Neu AIB1/SRC3<sup>+/+</sup> tumor MECs and MMTV-HER2/Neu AIB1/SRC3<sup>+/+</sup> mice that received 1x10<sup>5</sup> MMTV-HER2/Neu AIB1/SRC3<sup>+-/-</sup> tumor MECs into the mammary fat pad. (n =8 fat pad per group).

***P=0.0001 versus control.
B. Altering the AIB1/SRC3 expression in the stroma has a significant impact on tumor incidence.

We further investigated the influence of AIB1/SRC3 on epithelial/stromal cross talk by next altering the AIB1/SRC3 levels in the stroma. We altered the AIB1/SRC3 levels in the stroma utilizing MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\), MMTV-HER2/Neu AIB1/SRC3\(^{+-}\) or MMTV-HER2/Neu AIB1/SRC3\(^{-/-}\) mice as different stromal background for transplantation experiments. We isolated tumor MECs from MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) mice as described previously described. We transplanted these tumor MECs into the mammary fat pad of the three different AIB1/SRC3 genotype mice. A summary of the experiment steps is shown in (Figure 12A).

In the first experiment, we transplanted the MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) tumor MECs into the mammary fat pad of MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) or MMTV-HER2/Neu AIB1/SRC3\(^{+-}\) mice backgrounds. We then monitored tumor formation as highlighted in (Figure 12B). The presence of palpable mammary tumors was documented against time and is shown in a Kaplan Meier plot (Figure 12C). The MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) tumor MECs that were transplanted into the mammary fat pad of MMTV-HER2/Neu AIB1/SRC3\(^{+-}\) mice developed palpable mammary tumors at a significantly delayed rate (***p<0.0001) relative to the MMTV-HER2/Neu
AIB1/SRC3\textsuperscript{+/+} mice that received MMTV-HER2/Neu AIB1/SRC3\textsuperscript{+/+} tumor MECs (Figure 12C).

In the second experiment, we transplanted the MMTV-HER2/Neu AIB1/SRC3\textsuperscript{+/+} tumor MECs into the mammary fat pad of MMTV-HER2/Neu AIB1/SRC3\textsuperscript{+/+} and MMTV-HER2/Neu AIB1/SRC3\textsuperscript{-/-} host mice and we then monitored tumor formation as highlighted in (Figure 12D). The presence of palpable mammary tumors was documented against time and is shown in a Kaplan Meier plot (Figure 12E). The MMTV-HER2/Neu AIB1/SRC3\textsuperscript{+/+} tumor MECs that were transplanted into the mammary fat pad of MMTV-HER2/Neu AIB1/SRC3\textsuperscript{-/-} did not develop tumors, whereas all the MMTV-HER2/Neu AIB1/SRC3\textsuperscript{+/+} mice that received MMTV-HER2/Neu AIB1/SRC3\textsuperscript{+/+} tumor MECs developed tumors in approximately 21 days (Figure 12E).

Taken together, these data indicate a pivotal role of AIB1/SRC3 expressed in the stroma compartment on the development of mammary tumors.
**Figure 12A:** Diagram of experimental design of the mammary transplant experiments to determine the influence of stromal expression of AIB1/SRC-3 on mammary tumor development. MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) MECs were transplanted into the mammary fat pad of the three different mice genotypes with altered stromal background: MMTV-HER2/Neu...
AIB1/SRC3<sup>+/+</sup>, MMTV-HER2/Neu
AIB1/SRC3<sup>+/-</sup> and MMTV-HER2/Neu
AIB1/SRC3<sup>-/-</sup> mice.

Figure 12B: Diagram of the experimental design of the mammary transplant experiment determining how reduction of AIB1/SRC-3 in the stroma influences mammary tumor development. MMTV-HER2/Neu AIB1/SRC3<sup>+/+</sup> tumor mammary MECs labeled in black were transplanted into two different stromal backgrounds MMTV-HER2/Neu AIB1/SRC3<sup>+/+</sup> mice labeled in black or MMTV-HER2/Neu AIB1/SRC3<sup>-/-</sup> mice labeled in red.
Figure 12C: Reducing the AIB1/SRC3 expression in the stroma has a significant impact on mammary tumor incidence. A Kaplan-Meier plot of tumor free incidence comparing MMTV-HER2/Neu AIB1/SRC3+/+ mice that received $1 \times 10^6$ MMTV-HER2/Neu AIB1/SRC3+/+ tumor MECs (black line) and MMTV-HER2/Neu AIB1/SRC3+/+ mice that received $1 \times 10^6$ MMTV-HER2/Neu AIB1/SRC3+/+ tumor MECs (Red line). ($n = 8$ fat pad per group). ***$P=0.0001$ versus control.
Figure 12D: Diagram of the transplant experiment altering the AIB1/SRC3 levels in the stroma utilizing the AIB1/SRC3 knockout mice. MMTV-HER2/Neu AIB1/SRC3^{+/+} tumor MECs labeled in black were transplanted into two different stromal backgrounds MMTV-HER2/Neu AIB1/SRC3^{+/+} mice labeled in black and MMTV-HER2/Neu AIB1/SRC3^{-/-} mice labeled in red.
Figure 12E: Knock-out of AIB1/SRC3 levels in the stroma prevented tumor formation in MMTV-HER2/Neu mice. A Kaplan-Meier plot of tumor free incidence comparing tumor development in MMTV-HER2/Neu AIB1/SRC3+/+ mice that received 1x10^6 MMTV-HER2/Neu AIB1/SRC3+/+ tumor MECs (black line) or MMTV-HER2/Neu AIB1/SRC3−/− mice that received 1x10^6 MMTV-HER2/Neu AIB1/SRC3+/+ tumor mammary epithelial cells (Red line). (n =8 fat pad in MMTV-HER2/Neu AIB1/SRC3+/+, n =4 fat pad in MMTV-HER2/Neu AIB1/SRC3−/−). ***P=0.0004 versus control.
C. Comparison of AIB1/SRC3 and Her2/Neu protein expression in the stroma and the epithelium of mammary tumors formed after transplantation of tumor MECs into host mice with different AIB1/SRC3 stromal expression levels.

Next we examined the differences in the protein levels of AIB1/SRC3 protein expression in the different tumor backgrounds examined in the previous sections. Using immunohistochemistry analysis, AIB1/SRC3 levels were measured in tumor tissues arising after transplantation of MMTV-HER2 neu MECs AIB1/SRC3+/+ MECs into the mammary fat pad of MMTV-HER2/Neu AIB1/SRC3+/+, MMTV-HER2/Neu AIB1/SRC3+/− and MMTV-HER2/Neu AIB1/SRC3−/− mice. Interestingly the AIB1/SRC3 protein levels were reduced in the tumors arising in the host MMTV-HER2/Neu AIB1/SRC3+/− mice relative to the MMTV-HER2/Neu AIB1/SRC3+/+ mice (Figure 13A) and the AIB1/SRC3 protein expression were not detectable in the MMTV-HER2/Neu AIB1/SRC3−/− mice (Figure 13A), which lack discernible tumor development.
Figure 13A: Immunohistochemical staining for AIB1/SRC-3 protein in tumors arising after transplantation of tumor MMTV-NEU/HER2 AIB1/SRC-3 \(^{+/+}\) MECs into different AIB1/SRC3 genetic background. Mouse mammary tumor tissues sections from MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\), MMTV-HER2/Neu AIB1/SRC3\(^{+-}\) recipient mice and skin tissues from MMTV-HER2/Neu AIB1/SRC3\(^{-/-}\) recipient mice were stained for AIB1/SRC3. Scale bars: 0.2 mm.

Our laboratory has previously reported that in MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) mice the HER2 /neu and phosphor-HER 2 levels were very high in the mammary tumors whereas after one allele loss of AIB1/SRC-3, the levels of HER2 dropped dramatically (40). Therefore we also looked into how the changes in AIB1/SRC-3 in the epithelial or stromal tissue altered HER2/Neu levels in the tumors. We performed immunohistochemistry
analysis to look at the protein expression of Her2/Neu in tumor tissues from MMTV-HER2/Neu AIB1/SRC3^{+/+} and MMTV-HER2/Neu AIB1/SRC3^{+-} mice (Figure 13B).

Figure 13B: Immunohistochemical staining for Her2/Neu protein in tumors arising after transplantation of tumor AIB1/SRC-3^{+/+} MECs into different AIB1/SRC3 genetic background. Mouse mammary tumor tissues sections from MMTV-HER2/Neu AIB1/SRC3^{+/+} and MMTV-HER2/Neu AIB1/SRC3^{+-} mice that received the transplanted MMTV-HER2/Neu AIB1/SRC3^{+/+} tumor MECs were stained for Her2/Neu. Scale bars: 0.2 mm.
It is of note that Her2/Neu protein levels measured by immunohistochemistry were only detected in the epithelium compartment in tumors arising after transplantation of tumor MECs, whereas AIB1/SRC3 protein expression was detected in both the epithelial and the stromal compartment in tumors arising after transplantation of tumor MECs as indicated by the black arrow in (Figure 13C). Also of note was that the HER2 levels were similar in the epithelial tumor cells irrespective of the stromal background, implying that reduction of AIB1 in the stroma does cause parallel reductions in HER2 expression in the tumor epithelium.

Figure 13C: Immunohistochemical staining for Her2/Neu and AIB1/SRC3 protein in tumor stroma in tumors arising after mammary gland transplantation of tumor MECs. Mouse mammary
tumor tissues sections from MMTV-HER2/Neu AIB1/SRC3+/+ mice that received the transplanted tumor mammary epithelial cells were stained for Her2/Neu and AIB1/SRC3. Arrows indicate stromal staining of AIB1. Scale bars: 0.2 mm.

We have previously reported that angiogenesis is reduced in mammary tumors of MMTV-HER2/Neu AIB1/SRC3+/− mice when compared to MMTV-HER2/Neu AIB1/SRC3+/+ mice (40). Angiogenesis is also reduced in thyroid tumors with reduced AIB1/SRC-3 levels (64). Therefore, we wanted to examine the angiogenic profile of tumors arising after transplantation of tumor MECs from different AIB1/SRC3 genetic background. Interestingly, we noted that tumors arising after transplantation of tumor MECs from MMTV-HER2/Neu AIB1/SRC3+/− mice into a +/- background displayed similar necrotic phenotype and similar angiogenesis profile as observed in the tumors arising in the original MMTV-HER2/Neu AIB1/SRC3+/+ mice (40) and in (Figure 13D). In contrast, AIB1/SRC3+/+ tumors, in the AIB1/SRC3+/− background lacked significant angiogenesis and had high levels of necrosis (Figure 13D).
Figure 13D: Tumors arising after transplantation of tumor MECs from MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) mice into the same genetic background were phenotypically different from tumor arising after transplantation of tumor MECs from MMTV-HER2/Neu AIB1/SRC3\(^{+/-}\) mice into MMTV-HER2/NEU AIB1/SRC3\(^{+/+}\) background. Representative H&E-stained sections of tumors arising after transplantation of tumor MECs from MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) and MMTV-HER2/Neu AIB1/SRC3\(^{+/-}\) mice. Blood vessel (BV) is indicated in the (left panel) and area of Necrosis (N) is indicated in the (right panel). Scale bars: 0.2 mm.
D. A Immortalized MMTV Her2/Neu AIB1/SRC3\textsuperscript{+/-} tumor MECs.

All the previous transplantation experiments were performed with MECs prepared from tumors and then injected directly into the mammary fat pad of mice. This method requires a significant number of mice per experiment and is open to quite a degree of experimental variation due to varying levels of recovery of MECs populations and contamination with other cell types. In addition, these MECs also undergo senescence after passaging; and thus, cannot be expanded easily necessitating fresh cells to be prepared directly from mice for each experiment. For future experiments, we wanted to determine if immortalized MECs could be used for transplantation experiments. In particular we wished to determine if immortalized MECs could also be responsive to changes in the AIB1 levels in the stroma that we observed with native preparations. Therefore, we were looking for a method to immortalize MECs from mouse mammary tumors and tissue. Recently, an interesting method was reported to successfully immortalize primary human keratinocytes (98). In this method, the epithelial cells were treated with a ROCK inhibitor and were grown in the presence of irradiated fibroblast cells. Therefore, we adopted the same technique and treated the mouse tumor mammary epithelial cells with a ROCK inhibitor; (Y-27632); and the cells were allowed to grow in the presence of irradiated fibroblasts. When these cells reached passage 7, we transplanted $1 \times 10^6$ tumor
mammary epithelial cells into the mammary fat pad of two different AIB1/SRC3 genotype mice: MMTV-HER2/Neu AIB1/SRC3^{+/+} and MMTV-HER2/Neu AIB1/SRC3^{+/+} mice and monitored tumor formation as highlighted in (Figure 14A). The presence of palpable mammary tumors was documented against time and is shown in a Kaplan Meier plot (Figure 14B). Similar to the results we obtained with freshly prepared MECs, the MMTV-HER2/Neu AIB1/SRC3^{+/+} immortalized tumor mammary epithelial cells that were transplanted into the mammary fat pad of MMTV-HER2/Neu AIB1/SRC3^{+/+} mice developed palpable mammary tumors at a significantly delayed rate relative to the MMTV-HER2/Neu AIB1/SRC3^{+/+} mice that received MMTV-HER2/Neu AIB1/SRC3^{+/+} immortalized tumor mammary epithelial cells (Figure 14B).

**Figure 14A:** Experimental design determining if immortalized MECs from

- ROCK inhibitor immortalized MMTV-HER2/Neu AIB1/SRC3^{+/+} Epithelial mammary tumor cells
- MMTV-HER2/Neu AIB1/SRC3^{+/+}

- ROCK inhibitor immortalized MMTV-HER2/Neu AIB1/SRC3^{+/+} Epithelial mammary tumor cells
- MMTV-HER2/Neu AIB1/SRC3^{+/+}
mammary tumors are able to respond to changes in stromal AIB1/SRC-3 levels.

Immortalized passage 7 MMTV-HER2/Neu AIB1/SRC3+/+ tumor MECs labeled in black were introduced into two different stromal backgrounds MMTV-HER2/Neu AIB1/SRC3+/+ mice labeled in black or MMTV-HER2/Neu AIB1/SRC3+/- mice labeled in red.

Figure 14B: Immortalized MMTV Her2/Neu tumor epithelial cells can form mammary tumors after transplantation into the mouse mammary fat pad.
A Kaplan-Meier plot of tumor free incidence comparing MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) mice that received \(1 \times 10^6\) MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) immortalized tumor MECs (black line) or MMTV-HER2/Neu AIB1/SRC3\(^{+/-}\) mice that received \(1 \times 10^6\) MMTV-HER2/Neu AIB1/SRC3\(^{+/-}\) immortalized tumor MECs (Red line). \((n = 8\) fat pad per group). ***\(P=0.0004\) versus control.
E. Discussion

Several histopathology and molecular studies illustrate that tumor formation and progression consist of a complex cellular signaling between the epithelial tumor cells and the stromal compartment (99). Understanding and identifying new and novel signaling pathways involved in the communication between these two compartments may provide new therapeutic targets. In this chapter, we examined the influence of AIB1/SRC3 in the epithelial-stromal cross talk in breast cancer. Ultimately, we altered the AIB1/SRC3 levels in the stroma using MMTV-HER2/Neu AIB1/SRC3^{+/+}, MMTV-HER2/Neu AIB1/SRC3^{+-}, and MMTV-HER2/Neu AIB1/SRC3^{-/-} mice as different stromal backgrounds for transplantation of tumor MECs that had wild type levels of AIB1/SRC3. Overall the data suggested a critical role of AIB1/SRC3 in the stromal compartment during mammary tumor development. The loss of one allele of AIB1/SRC3 in the stroma significantly delayed tumorigenesis as shown in the Kaplan-Meier plots.

There are several published studies that highlighted variable roles of AIB1/SRC3 in tumorigenesis. For example, one study showed that the loss of AIB1/SRC3 in mice induces the development of B-cell lymphomas tumor development (100). Whereas the Riegel lab has demonstrated that the loss of AIB1/SRC3 in mice prevented tumorigenesis in MMTV-Her2/Neu mice (40). In addition, all of the data gathered to date on the role of AIB1/SRC3 has focused on its role as an oncogene exerting its influence through
phenotypic changes in the mammary epithelium (9, 22-26). Here we report for the first time the impact of the loss of AIB1/SRC3 in the stroma compartment on mammary carcinogenesis. The exact mechanisms or pathways involved when AIB1/SRC3 levels were reduced in the stroma compartment are yet to be determined. However by analogy to the stromal signaling we observe in wound healing (see Chapter 3), a possible novel pathway, not previously linked to AIB1/SRC3 action that might contribute to the role of AIB1/SRC3 is the Fibroblast Growth Factor (FGF) pathway. We have demonstrated that the loss of AIB1/SRC3 negatively impacts key drivers of the FGF pathway in the endothelial cells \textit{in vitro} (chapter 3). Therefore, AIB1/SRC3 could impact mammary tumorigenssis through the FGF signaling pathway in the stroma of MMTV-Her2/Neu tumors.

Another possible mechanism is through the AIB1/SRC3 regulation of collagen production. Fibroblast cells are known to be responsible for the production of extracellular matrix (ECM), tissue regeneration and collagen production (78-80). We reported earlier that AIB1/SRC3 impacted fibroblast migration \textit{in vitro} (chapter 3), which suggests that a reduction in AIB1/SRC3 could delay collagen and ECM production in the stroma of breast tissues. Moreover, the loss of AIB1/SRC3 was reported to be associated with decreased liver fibrosis (101), whereas overexpressing AIB1/SRC3 was associated with increase mammary stromal collagen (46). All these data highlight a possible mechanism for AIB1/SRC3 contribution to mammary
tumorigenesis through its role in regulating fibroblast function.

In addition, the histological examination of the tumors that arise after transplantation from MMTV-HER2/Neu AIB1/SRC3\(^{+/−}\) and MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) mice revealed that tumors arising after transplantation of tumor MECs from MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) mice were phenotypically different from tumor arising after transplantation of tumor MECs from MMTV-HER2/Neu AIB1/SRC3\(^{+/−}\) mice. The MMTV-HER2/Neu AIB1/SRC3\(^{+/−}\) tumors exhibited areas that were necrotic, whereas the MMTV-HER2/Neu AIB1/SRC3\(^{+/+}\) tumors displayed a vascularized tumor phenotype. Moreover, we also observed reduced angiogenesis in MMTV-HER2/Neu AIB1/SRC3\(^{+/−}\) wound tissues (chapter 4), which was supported by a significant reduction in VEGF-A staining and in HIF-1A mRNA expression in the wound tissues (Figure 10A). This may suggest that AIB1/SRC3 is acting to modulate tumorigenesis by regulating angiogenesis via the stroma of MMTV-Her2/Neu tumors.

The concept that cancer cells rely on cross talk with the surrounding tissues to grow is not a novel idea; Steven Paget first described it over a century ago in his famous ‘seed and soil’ theory. The data presented in this chapter highlight the importance of having AIB1/SRC3 in the stroma (microenvironment) in order for tumor MECs to grow. Interestingly, we did not observe any changes in HER2 expression in the transplant experiment into the AIB1/SRC3\(^{+/−}\) background in which tumor growth is significantly
inhibited thus suggesting that the oncogene alone in the epithelium is not sufficient to drive tumorigenesis but also requires stromal signals. Moreover, the inability of AIB1/SRC3 knockout mice to form mammary tumors after transplanting tumor MECs provide critical evidence to the soil hypothesis. Basically, the soil needs to possess the appropriate factors in order for tumors to grow; in this case, the soil requires the presence of AIB1/SRC3 in order for the stroma to function accurately. Perhaps inducing inactivation or degradation of AIB1/SRC-3 loss or loss of AIB1 induced signaling in stroma would be a therapeutic approach in breast cancer and thus, inhibit tumor progression. In this regard, recently it has been shown that there are beneficial aspects of developing drugs that target the stroma and this approach can inhibit tumor progression (102, 103).

In this chapter, we also showed for the first time the ability to immortalize tumor MECs using a ROCK inhibitor. Remarkably, these immortalized tumor MECs provided us with similar results to what we obtained using freshly prepared tumor MECs. The underneath mechanism behind the ROCK inhibitor is still unknown and further research is needed to track the exact targets of this inhibitor. However, the preliminary data shown in this chapter indicate that this method could be a very useful tool in cancer research.
Chapter 6. Conclusion and Future Direction
A. Conclusion

The stroma plays a critical role in many physiological and pathological diseases; such as wounding, pregnancy and cancer. In addition, the stroma response can be triggered positively to respond to a wound or negatively to promote tumor growth (104) (105). Emerging data demonstrates that the stroma plays a profound role in the development and metastasis of human breast cancer. In this study, we report for the first time that the nuclear receptor coactivator AIB1/SRC3 has a pivotal role in the stroma of normal and cancer tissues utilizing several in vitro and in vivo models (Figure 15). The tumor microenvironment consists of various cells including endothelial cells, fibroblasts and immune cells (106). Here, we have shown that AIB1/SRC3 plays a critical role in the proliferation, migration, differentiation and monolayer formation of endothelial cells, and reported that AIB1/SRC3 impacted fibroblast migration (Chapter 3). The growth of tumor epithelial cells induces an inflammatory response, which is considered the characteristic of the early phases of tumor development (107). The inflammatory cells then invade the tumor stroma to promote tumor growth. We illustrated that reducing AIB1/SRC3 levels reduced the recruitment of inflammatory cells in response to wounds (Chapter 4). Furthermore, the induction of immune cells into the tumor site initiates one of the important physiological and pathological processes in the stroma, which is angiogenesis (108). We evaluated the AIB1/SRC3 effect on angiogenesis using two in vivo
models, matrigel plug and wound healing. We observed a reduction in angiogenesis and neoangiogenesis when AIB1/SRC3 levels were reduced (Chapter 4). There are several key molecules that are important in breast cancer progression; such as MMPs, which enable the breakdown of ECM to facilitate tumor growth and metastasis (58, 59). We showed a reduction in MMP activity when AIB1/SRC3 levels were reduced (Chapter 4). We also investigated the role of AIB1/SRC3 in impacting mammary tumorigenesis utilizing the transplant procedure (Chapter 5). The loss of one allele of AIB1/SRC3 in the stroma significantly delayed mammary tumorigenesis. Overall, the data indicate a critical role of AIB1/SRC3 in the stromal compartment during mammary tumor development (Chapter 5). The loss of one allele of AIB1/SRC3 in the stroma significantly delayed tumorigenesis as shown in the Kaplan-Meier plots (Chapter 5). Furthermore, the field of tumor microenvironment is facing many challenges, one of which is the identification of new pathways that are implicated in the tumor stroma. Recent data demonstrated that altering signaling pathways in the stroma prevented tumorigenesis. Recently, the activation of Notch1 signaling pathway in stromal fibroblasts was reported to inhibit melanoma tumor growth in vivo (109). In addition, the activation of PI3K signaling pathway was shown to enhance tumor inflammatory response and growth (110). In this study, we reported for the first time that AIB1/SRC3 modulates stromal cell responses through crosstalk with the FGF and PI3K signaling pathways.
(Chapter 3 and Chapter 4). Therefore, the future goal of this study is to identify novel mechanisms and signaling pathways that are unique to the stroma compartment of mammary tumorigensis and are controlled directly or indirectly by AIB1/SRC3. Summary of all the results presented in this dissertation is shown in (Figure 15).
Figure 15: A diagram summarizing the major findings of this study.
B. Clinical relevance

Our findings indicate that AIB1/SRC3 plays a major role in wound healing as poor wound healing was associated with reduced levels of AIB1/SRC3 in mice (Chapter 4). There are various diseases that are associated with chronic wounds such as diabetes. Therefore, the discovery or development of new agents that can help promote wound healing is of great benefits to many patients. The results in this dissertation could assist in the process of developing a therapeutic agent containing AIB1/SRC3 that can be applied to non-healing wounds. Further studies addressing the role of AIB1/SRC3 in wound healing in humans is necessary to fully evaluate the role of AIB1/SRC3 in the human wound healing responses.

In regards to breast cancer, AIB1/SRC3 is overexpressed in a large number of human breast cancers and is associated with significantly worse prognosis and endocrine resistance. In this study, we determined the influence of AIB1/SRC3 in the stroma surrounding the epithelial tumor. Although it is difficult to target endogenous nuclear proteins like AIB1/SRC3 for therapy, my expectation is identifying new and novel signaling pathways that mediate AIB1/SRC3 action in the stroma will be much more amenable to targeting either by inhibiting secretion of factors that control stromal function or by blocking critical cell surface receptors and their subsequent cell signaling pathways. Interruption of novel AIB1/SRC3 driven epithelial/stromal cross talk pathways represents a new avenue for therapeutic targeting
in breast cancer that emphasizes the emerging role of the stroma in the
development and metastasis of human breast cancer. Knowledge in this area
will lead to new therapeutic paradigms especially for women whose primary
tumor has high levels of AIB1/SRC3.
C. Future directions:

1. Identifying new and novel signaling pathways that mediate AIB1/SRC3 action in the stroma

The transplant experiment data indicated to us that the role of AIB1/SRC3 in breast cancer was not confined to mammary epithelium proliferation but also had major effects on the surrounding stromal and epithelial crosstalk in mammary tissue. The identification of novel AIB1/SRC3 driven epithelial and stromal cross talk pathways represents a critical future step to better understand the communication between the two compartments. Therefore, we would harvest the tumors that arise after transplantation of MMTV-HER2/Neu AIB1/SRC3+/+ tumor MECs from MMTV-HER2/Neu AIB1/SRC3+/− and MMTV-HER2/Neu AIB1/SRC3+/+ mice. After harvesting these tumors, we would isolate RNA in order to perform Affymetrix microarrays to identify genes differentially expressed between the two AIB1/SRC3 stromal backgrounds. We would then validate these newly identified genes by q-RT-PCR. Knowledge in this area will lead us to identify the exact mechanisms exerted by AIB1/SRC3 in the stromal compartment of mammary tumorigenesis.
2. Investigate the ability of fibroblast cells to rescue the delay in tumorigenesis observed when AIB1/SRC3 levels are reduced in the stroma.

We have shown that reducing the AIB1/SRC3 in the stroma compartment of mammary tumorigenesis significantly delayed tumor incidence as indicated in (Figure 12C and E). Therefore, it would be important to examine the role of a stromal cell type; such as the fibroblast in rescuing the phenotype observed in order to have a better understanding of the AIB1/SRC3 effect in the stroma. Fibroblast cells are considered to be one of the major cell components in the mammary stroma. Moreover, recently fibroblast cells have received more attention as a critical stromal cell type that is capable of promoting tumor growth through its communication with various cell types including epithelial and other stromal cells and through its production of various growth factors and cytokines (81). In addition, it is relatively easy to harvest fibroblast cells from the MMTV Her2/Neu AIB1/SRC3+/−, whereas it is rather difficult to harvest the endothelial cells.

Our results show that The MMTV Her2/Neu AIB1/SRC3+/− mice exhibit a much delayed tumor incidence in comparison to the control mice MMTV Her2/Neu AIB1/SRC3+/+ after transplanting the same number of MMTV Her2/Neu AIB1/SRC3+/+ tumor MECs. A possible experiment to address the ability of fibroblast cells harvested from MMTV Her2/Neu
AIB1/SRC3<sup>+/−</sup> to reduce the difference in tumor incidence between the two different AIB1/SRC3 stromal backgrounds is by mixing the tumor mammary epithelial and fibroblast cells generated from MMTV Her2/Neu AIB1/SRC3<sup>+/−</sup> as shown in (Figure 16). First, we would harvest both cell types as indicated in the methods’ section, and grow them separately overnight. After that, we would mix these two cell types together prior to transplanting them into two different AIB1/SRC3 stromal backgrounds: MMTV Her2/Neu AIB1/SRC3<sup>+/−</sup> and MMTV Her2/Neu AIB1/SRC3<sup>++</sup>. The hypothesis is that having the fibroblast from the MMTV Her2/Neu AIB1/SRC3<sup>+/−</sup> mice transplanted into the mammary fat pad of MMTV Her2/Neu AIB1/SRC3<sup>++</sup> would be able to reduce the difference in tumor incidence between those mice and the MMTV Her2/Neu AIB1/SRC3<sup>+/−</sup> mice. This experiment will help us in defining whether the fibroblast cells are the key regulators in the role of AIB1/SRC3 in mammary stroma.
Figure 16: Experimental design for the fibroblast rescue experiment. The diagram highlights the mixing of two different cell types, epithelial and fibroblast harvested from MMTV Her2/Neu AIB1/SRC3<sup>+/+</sup> tumors prior to transplanting them into two different AIB1/SRC3 stromal backgrounds: MMTV Her2/Neu AIB1/SRC3<sup>+/+</sup> and MMTV Her2/Neu AIB1/SRC3<sup>+-</sup>.
3. Generation of a tissue specific knockdown of AIB1/SRC3.

Our data suggest that AIB1/SRC3 is critical in the stroma of breast cancer. However, we did not identify which stromal cell type is contributing to these results. Therefore, it is necessary to examine the AIB1/SRC3 role in the tumor microenvironment of breast cancer. To rigorously evaluate the role of AIB1/SRC3 in breast cancer stroma in the future, we would need to generate mice with a stromal cell specific deletion of AIB1/SRC3; and then, examine mammary tumorigenesis. In this case, we would produce mice that no longer express AIB1/SRC3 in fibroblast cells or endothelial cells in vivo. The Cre/ lox system is a well characterized system used to specifically knockout a target gene in one cell type (111). Basically, we would generate a mouse that has the AIB1/SRC3 gene surrounded by two loxP sites as indicated in (Figure 17) and we would then cross the mouse with a mouse that has the cre gene under a tissue specific promoter of only one cell type (Figure 17); such as the fibroblast specific promoter, FSP1 (99) or endothelial specific promoter Tie-1(112) . The resulting mouse would express AIB1/SRC3 in all tissues except where cre is expressed (Figure 17). There are several disadvantages of this system in that it is expensive and time consuming (111); however, developing mice with a mesenchymal-specific cre would allow us to thoroughly examine the role of AIB1/SRC3 in stroma and better identify new signaling pathways controlled by AIB1/SRC3 in the stroma. In addition, it will provide strong and novel evidence to the
importance of AIB1/SRC3 in the stroma compartment of mammary tumorigenesis.

**Figure 17:** Experimental design to generate tissue specific knockdown of AIB1/SRC3. The diagram illustrates the process of generating mice with a mesenchymal-specific cre.
4. AIB1/SRC3 impacts on immune cells functions.

Inflammatory cells play a major role in promoting cancer growth and metastasis (107). These inflammatory cells get recruited to the tumor site to stimulate angiogenesis (53). Moreover, once macrophages and neutrophils get triggered to the tumor sites these cells differentiate to either tumor-associated macrophages (TAMs) or tumor-associated neutrophils (TANs) (54-56), which assist the tumor to grow and metastasize, and cause therapy relapse (57). Therefore, understanding the mechanisms or pathways involved in the recruitment of inflammatory cells to the tumor stroma could lead to new therapeutic targets for breast cancer. Recently, the PI3K pathway has been implicated in the inflammatory response (113). Interestingly, we reported for the first time in this study that AIB1/SRC3 impacted pAKT status in a stromal cell (Chapter 3). We have also demonstrated that AIB1/SRC3 impacted inflammatory cells (CD14 and CD16: markers for monocytes, macrophages and neutrophils) recruitment to the wounded sites (Chapter 4) indicating a possible explanation to the delayed wound healing observed in mice with reduced level of AIB1/SRC3. In addition, we observed reduced macrophage infiltration in wounds with low level of AIB1/SRC3. All these data provide a rational future step to examine the role of AIB1/SRC3 in immune cells functions, and to identify novel pathways that control immune cells recruitment.
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