Bmi-1 Promotes the Invasion and Metastasis and its Elevated Expression is Correlated with Advanced Stage of Breast Cancer

GUO, Baohong

A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

in

Public Health

The Chinese University of Hong Kong

September 2010

Thesis/assessment Committee

Professor Benny Zee (Chair)

Professor Hsiangfu Kung (Thesis Supervisor)

Professor Qian Tao (Committee Member)

Professor Mingliang He (Committee Member)

Professor Liwei LU (External Examiner)

論文評審委員會委員

徐仲錂教授（主席）
孔祥復教授（論文導師）
陶謙教授　（委員）
何明亮教授（委員）
呂力為教授（校外委員）

Abstract

Background

B-lymphoma Moloney murine leukemia virus insertion region-1 (Bmi-1) acts as an oncogene in various cancer such as non-small cell lung cancer, colon cancer, gastric cancer, bladder cancer and nasopharyngeal cancer (NPC). Bmi-1 not only could lead human mammary epithelial cells (HMECs) and nasopharyngeal epithelial cells (NPECs) to immortalize, but also could induce malignant transformation of human karatinocytes (HaCaT). Apart from its role in oncogensis, Bmi-1 was required for self-renewal and differentiation of stem cells. The ectopic Bmi-1 expression induced epithelial-mesenchymal transition (EMT) and enhanced the motility and invasiveness of NPECs, whereas silencing endogenous Bmi-1 expression could reverse EMT and reduce the metastatic potential of nasopharyngeal cancer cells (NPCs). Mouse xenografts studies indicated that Bmi-1 overexpression could not result in oncogenic transformation of MCF-10A cells, however, co-expression of Bmi-1 and H-Ras could induce an aggressive and metastatic phenotype with an unusual occurrence of the brain metastasis in breast cancer. However, the underlying molecular mechanism and clinical outcome of Bmi-1-mediated progression and metastasis of breast cancer were not fully elucidated.

Methods

Immunohistochemistry was performed to evaluate Bmi-1 expression in 252 breast

cancer samples. The correlations were analyzed between Bmi-1 expression and clinicopathologic parameters, including age, tumor size, lymph nodal involvement, distant metastasis, clinical stages, hormone receptor (ER, PR) and Human Epidermal Growth Factor Receptor 2 (HER-2). The overall survivals were compared by Kaplan-Meier analysis based on Bmi-1 expression. The prognosis impact of Bmi-1 expression was analyzed by univariate and multivariate cox regression analyses. Then, the levels of Bmi-1 expression were examined in immortalized HMECs and breast cancer cell lines. The effects of Bmi-1 overexpression in immortazled HMECs (76N-TERT and MCF-10A) and the role of Bmi-1 knockdown in highly metastatic breast cancer (MDA-MB-435S) cells were interpreted by wound healing assay, Boyden chamber assay, 3-D Matrigel assay and soft agar assay *in vitro* to determine the invasion, migration and transformation ability. Accordingly, the tumorigenesis and metastases caused by Bmi-1 overexpression and the ablation of Bmi-1 were further investigated by injecting cells into the fat pad of nude mice. The EMT makers and the signal pathway induced by Bmi-1 expression were tested by western blotting. Furthermore, the mRNA expression levels of Bmi-1 and E-cadherin in breast cancer tissues and adjacent non-cancerous tissues were tested by Real-time quantitative polymerase chain reaction. Immunofluorescence was adopted to identify the effect of Bmi-1 on the subcellular localization of Snail proteins in MCF-10A cells.

Results

Bmi-1 expression was significantly increased in primary cancer tissues than in matched adjacent non-cancerous tissues (*P<0.001)*. Only 35.9% (14 of 39) of adjacent non-cancerous tissues displayed high expression compared with 72.2% (182 of 252) in primary cancer tissues. Among adjacent non-cancerous tissues, no Bmi-1 staining signal was detected in 30.8% (12 in 39) samples. Only 28.2% (11 in 39) samples showed nucleus staining and the remaining 41.0% (16 in 39) samples exhibited cytoplasm staining. Of those cancer tissues, however, 75.4% (190 in 252) was stained in the nucleus and 24.6% (62 in 252) located in the cytoplasm. The elevated Bmi-1 expression was correlated with advanced clinicopathologic classifications (T, N, M) and clinical stages (*P<0.001,* respectively). A high level of Bmi-1 expression displayed unfavorable overall survival (*P<0.001*). The overall survival rate, assessed by the Kaplan-Meier method, was 85.1% (57 in 67) in low Bmi-1expression group, whereas it was only 59.9% (103 in 172) in high Bmi-1 expression group. In addition, Bmi-1 serves as a high risk for breast cancer and the relative risk increased almost four fold in patients with high Bmi-1 expression compared with that with low Bmi-1 expression by univariate Cox regression analyses. After the adjustment of the confounding factors, Bmi-1 was still found to predict the poor survival (*P=0.042*), which indicated Bmi-1 was an independent prognostic factor. The overexpression of Bmi-1 increased the mobility and invasiveness in 76N-TERT and MCF-10A, concurrent EMT-like molecular changes, the stabilization of Snail protein and the activation of Akt/GSK3β pathway. Consistent with these

observations, the repression of Bmi-1 in MDA-MB-435S remarkably attenuated the cellular mobility, invasiveness and transformation, as well as tumorigenesis and spontaneous lung metastases in nude mice. In addition, the repression of Bmi-1 reversed the EMT markers and inhibited the Akt/GSK3β/Snail pathway. However, ectopic Bmi-1 alone was not able to lead to the phenotype of HMECs. Additionally, discordant mRNA expression levels of Bmi-1 and E-cadherin were detected between primary cancer tissues and matched adjacent non-cancerous tissues. The mRNA level of Bmi-1 was strongly up-regulated in breast cancer tissues compared with paired non-cancerous tissues ($P=0.001$), whereas the mRNA level of E-cadherin was markedly down-regulated ($P=0.042$). Furthermore, there was a converse correlation between Bmi-1 and E-cadherin expression at the transcriptional level ($P=0.041$).

Conclusions

In summary, Bmi-1 contributed to the invasion and metastasis of human breast cancer and predicted worse survival.

摘要

研究背景及目的

癌基因 Bmi-1，在腫瘤的發生發展過程中，起著重要作用，非小細胞肺癌，結腸癌，胃癌，膀胱癌，鼻咽癌等很多腫瘤都高表達 Bmi-1。Bmi-1 高表達不僅能導致乳腺上皮細胞，鼻咽上皮細胞永生化，引起人角化皮膚細胞惡性轉化，還對干細胞的更新和分化起重要作用。外源性 Bmi-1，能使鼻咽上皮細胞向間葉細胞轉化，提高鼻咽上皮細胞的侵襲及浸潤能力。而 RNA 干擾不僅能降低 Bmi-1 在鼻咽癌細胞的表達，也能逆转上皮間葉轉化，降低鼻咽癌細胞的轉移能力。雖然 Bmi-1 不能致永生化的乳腺上皮細胞（MCF-10A）在裸鼠體內成瘤，但和 Ras 協同作用不僅能在裸鼠體內成瘤，而且還能導致其發生腦轉移。儘管如此，Bmi-1 是否能單獨致瘤及轉移，其分子機制及臨床預測仍然不清。

方法

首先用免疫組化法檢測 Bmi-1 在乳腺癌組織及癌旁組織的蛋白表達情況，分析 Bmi-1 與臨床病理因素之間的關係，調查 Bmi-1 與預後及生存的關係。接著分析 Bmi-1 永生化乳腺細胞株及乳腺癌細胞中的表達情況。然後建立外源性高表達 Bmi-1 的永生化乳腺細胞株（76N-TERT 和 MCF-10A），同時也用 RNA 干擾技術降低 Bmi-1 在乳腺癌細胞中的表達（MDA-MB-435S）。然後用劃痕實驗，細胞侵襲實驗，三維培養實驗，軟瓊脂擊落形成實驗檢測這些細胞的運動能力，侵襲能力和體外轉化能力。並且檢測這些細胞在裸鼠體內的成瘤能力及轉移能力。接下來檢測 Bmi-1 導致相應變化的分子機制。

結果

相比癌旁組織而言，乳腺癌組織高表達 Bmi-1($P<0.001$)。35.9% (14 of 39)的癌旁組織高表達 Bmi-1,而 72.2% (182 of 252)的癌組織高表達 Bmi-1。在癌旁組織中，30.8% (12 in 39)的組織未檢測到 Bmi-1 表達信號，28.2% (11 in 39)組織呈現核表達，41.0% (16 in 39)組織為漿表達。但是在癌組織中，75.4% (190 in 252)為核表達，其餘為漿表達。而且，Bmi-1 與腫瘤大小，淋巴結轉移，遠處轉移，臨床轉移明顯相關 ($P<0.001$)。高表達 Bmi-1 的病人生存時間較短 ($P<0.001$)，是乳腺癌的一個高危因素。低表達 Bmi-1 的病人生存率為 85.1% (57 in 67)，在高表達 Bmi-1 的病人生存率下降為 59.9% (103 in 172)。單因素危險度分析提示 Bmi-1 幾乎能增加 4 倍的患癌風險($P<0.001$)。當調整混雜因素后，多因素危險度分析提示 Bmi-1 能夠用於預後預測。外源性表達 Bmi-1 能提高 76N-TERT 及 MCF-10A 的運動和侵襲能力，但是不能引起它們惡性轉化。用 RNA 干擾技術能降低高轉移乳腺癌細胞株 MDA-MB-435S 的運動侵襲能力，使其惡性度降低。通過皮下注射 MDA-MB-435S/shBmi-12#到裸鼠的脂肪墊，能減低細胞的成瘤能力及肺轉移能力。Bmi-1 能改變 76N-TERT，MCF-10A 及 MDA-MB-435S 的細胞間葉轉化的分子表達水平。這是通過改變 Akt/GSK3β/Snail 信號通路實現的。在乳腺癌及癌旁組織中，Bmi-1 和 E-cadherin 在轉錄水平成負相關 ($P=0.041$)，在癌組織中，Bmi-1 的 mRNA 水平高 ($P=0.001$)，E-cadherin

的 mRNA 水平低(*P=0.042*)。在癌旁組織中，則相反。

結論：

Bmi-1 能促進乳腺癌的侵襲和轉移，並且能預測乳腺癌病人的生存。

Acknowledgement

My deepest gratitude goes firstly to my supervisor, Professor Hisangfu Kung. His wide knowledge and efficient organization in medical research have been of great value for me. He offered me the opportunity to resume my Ph.D study in the Chinese University of Hong Kong. His enthusiasm and interest in medical science are magnetic. I have learned not only the method of scientific thinking, but also more importantly, the optimistic outlook on life. Without his detailed and constructive comments, I did not complete the project smoothly.

I wish to express my warm and grateful thanks for my supervisor, Professor Musheng Zeng in Sun Yat-sen University, for his nice guidance, invaluable advices and constant support in the last six years. He was always there to meet and talk about the ideas. His ideals and concepts have had a remarkable influence on my entire study in the field of cancer research.

I warmly thank Professor Chunming Ding, who gave me the wonderful opportunity to pursuit my Ph.D degree in Hong Kong. His extensive discussions and interesting explorations have been very helpful for my study.

I also owe a special debt of gratitude to Prof Wenlin Huang who supplied me a good opportunity in famous universities. His enthusiasm in working and structured working attitude for his help and encouragement are beneficial to me

in the last six years.

The Chinese University offers a lot of information for young hungry minds. The close tie with world-class universities and a variety of seminars provide a golden bridge for the different minds. What I have learned at the Chinese University of Hong Kong will greatly benefit my future careers.

My warm thanks are due to the talented and hard working members of past and present for their discussions and pleasure working together. Special sincere thanks to Dennise Chan for her valuable suggestions on my thesis as well as the careful reading of the manuscript.

I am grateful to all the colleagues in Stanley Ho Centre for Emerging Infectious Diseases, the School of Public Health of the Chinese University of Hong Kong and Sun Yat-sen University Cancer center, who have provided memorable experience and previous friendship during these years.

I greatly appreciated the generous supports from Bowen, Muyi, Michael, Louise, Tommy, Ping and Wenjie. I am amazed at the confidence and help from them.

Finally, and most importantly, my deepest gratitude goes to my parents and my grandmother for their sincere love and support throughout my life. They have

spared no effort to provide the best help in difficulties. I would like express my deepest thanks to my brother, Lin, a talent professional accountant who is always one of my best counselors. I'd also like to express the immense gratitude to my other family members.

致謝

吾五歲始讀書，經二十四載，將畢業。吾幼經先外祖父教誨，背得唐詩宋詞。詩經，三字經亦能讀成誦，然不得要領。越二十一年，未曾忘。先外祖父曰，汝輩勿需理解，熟讀成誦大有裨益。先外祖父愛吾甚摯，嘗語人：“余得一孫，天資聰穎，如或珍寶。”耐其辭世，余尚童無知。今清明，赴饗祭祖，吾乃而立之年。先外祖父善書法，長敕令，能吟詩填詞。自吾有知，先外祖父必每日讀書寫字，常謂後輩，不讀書無以知天下也。然先外祖父之書籍，或在墳間火化或丟失，吾不得知。先外祖父以文行忠信，備受相鄰尊崇。先外祖父之書法，蒼勁有力，逢年過節，自撰對聯，以為饗親。雖授吾書法，耐不得其傳。吾對歷史及文學之興趣，始於先外祖父也。

先外祖母不識字，然受先外祖父影響之甚，尚能講楊家將和三國演義催吾入眠。其尊長愛幼，賢能禮達，頗得相鄰敬重。先外祖母喜種花草，每年必植花草于園中，精心培植，待花綻放，攬吾手，曰，吾之孫女，定如此花一般，吾喜之甚深。平日各子女為其備之鮮果等，必轉贈于吾。先外祖母一生最愛吾，夏日必為吾搖扇趕紋，冬日必為吾燒水泡腳。然于吾高考前夕，因病仙逝。吾有感應，詢之表兄，云，外祖母先一日離世，甚念小孫女。吾即日回家，然已蓋棺入殮，吾之傷心不可述也。吾先外祖父夫婦溫溫相守四十伍載，相敬如賓，絕不聞有爭吵尔。

吾之先祖父，定坤先生也，卒于乙未年。先祖父之生平，皆由祖母講述。只知服務于某部門，帶過兵等，余皆渺不可循。總訴一鱗片爪，余吾彌自珍重。即今追思，既無墓場，又無畫像，此憾事也。

畢業之際，特撰此文，以謝先祖。

Publications

Conference Abstract:

Bao-Hong Guo, Mu-Sheng Zeng, Hsiang-Fu Kung.

Bmi-1 plays a crucial role in the invasion and metastasis of human breast cancer. 13th Beatson International Cancer Conference: The Multiple Tiers of Gene Regulation in Cancer (Presentation).

Journal Articles:

1. **BH Guo**, X Zhang, HZ Zhang, HL Lin, Y Feng, JY Shao, WL Huang, HF Kung, MS Zeng. 2010. Low expression of Mel-18 predicts poor prognosis in patients with breast cancer. *Annals of Oncology.2010 May*. 5 *Epub ahead of print*.

2. **BH Guo**, Y Feng, R Zhang, LH Xu, MZ Li, HF Kung, LB Song, MS Zeng Bmi-1 promotes the invasion and metastasis and its elevated expression is correlated with advanced stage of breast cancer. *Molecular Cancer (Revised).*

3. QL Kong, LJ Hu, JY Cao, YJ Huang, LH Xu, Y Liang, D Xiong, S Guan, **BH Guo**, HQ Mai, QY Chen, X Zhang, MZ Li, JY Shao, CN Qian, YF Xia, LB Song, YX Zeng, MS Zeng. Epstein-Barr Virus-Encoded LMP2A Induces an Epithelial–Mesenchymal Transition and Increases the Number of Side Population Stem-like Cancer Cells in Nasopharyngeal Carcinoma. *PLoS*

Pathog. 2010 Jun 3;6(6):e1000940.

4. QL Kong, S Guan, **BH Guo**, MS Zeng. Pre-malignant nasopharyngeal epithelial cell models. *Chin J Cancer.2009 Oct;28(10):1012-5.*

5. JH Liu, LB Song, X Zhang, **BH Guo**, Y Feng, XX Li, WT Liao, MS Zeng, KH Huang. Bmi-1 expression predicts prognosis for patients with gastric carcinoma. *J Surg Oncol. 2008 Mar 1;97(3):267-72.*

Table of Contents

Abstract.....i

摘要.....v

Acknowledgements.....vii

致謝..... x

Publications.....xi

Table of Contents.....xiii

List of Tables.....xviii

List of Figures.....xx

List of Abbreviations.....xxv

Chapter 1

Introduction.....1

1.1 General Introduction.....2

1.2 Breast Cancer.....6

1.2.1 Overview of Breast Cancer.....6

1.2.2 The Known Risk Factors for Breast Cancer.....9

1.2.2.1Female.....9

1.2.2.2 Increasing Age.....10

1.2.2.3 Personal and Family History of Breast Cancer and Genetic Predisposition.....10

1.2.2.4 Hormonal Factors.....11

1.2.2.5 Clinical Factors.....11

1.2.2.6 Radiation.....12

1.2.3 The Symptoms and Diagnosis of Breast Cancer.....**15**

1.2.4 Tumor-Related Characteristics.....**16**

1.2.4.1 Pathology.....16

1.2.4.2 Tumor Size.....16

1.2.4.3 Regional Lymph Node Involvement.....16

1.2.4.4 Metastasis.....17

1.2.4.5 The Stages of Breast Cancer.....18

1.2.4.5.1 Stage 0.....18

1.2.4.5.2 Stage I.....19

1.2.4.5.3 Stage IIA.....19

1.2.4.5.4 Stage IIB.....19

1.2.4.5.5 Stge IIIA.....19

1.2.4.5.6 Stage IIIB.....21

1.2.4.5.7 Stage IIIC.....21

1.2.4.5.8 Stage IV.....21

1.2.5 Treatment for Breast Cancer.....**23**

1.2.5.1 Surgery.....23

1.2.5.2 Radiotherapy.....23

1.2.5.3 Chemotherapy.....23

1.2.5.4 Hormone Therapy.....23

1.2.5.5 Targeted Therapy.....24

1.3 The Invasion and Metastasis of Breast Cancer.....**25**

1.4 Important Role of Bmi-1 in the Development of Carcinoma.....**30**

1.4.1 Genetic Structure of Bmi-1.....30

1.4.2 The Immortalization and Transformation by Bmi-1.....33

1.4.3 The Elevated Expression of Bmi-1 in Many Carcinomas and Cancer Cell Lines.....38

1.4.4 Bmi-1 Dependence Distinguishes Stem Cell Self-renewal from Progenitor Proliferation.....43

1.5 Epithelial-Mesenchymal Transition.....**45**

1.5.1 The Epithelial-Mesenchymal Transition in Tumor Invasion and Metastasis 45

1.5.2 EMT Related Markers.....52

1.5.2.1 E-cadherin.....52

1.5.2.1.1 Loss of E-cadherin in Many Cancers.....52

1.5.2.1.2 Transcriptional Regulation of E-cadherin.....52

1.5.2.1.3 Posttranscriptional Regulation of E-cadherin.....53

1.5.2.1.4 E-cadherin Mutations.....54

1.5.2.2 β-Catenin.....56

1.5.2.3 Fibronectin and Vimentin.....58

1.5.3 Different Signaling Pathways Provide the Necessary Stimuli Triggering EMT.....59

Chapter 2 Materials&Methods.....65

2.1 Tissue Samples.....66

2.2 Immunohistochemistry in Human Breast Cancer Tissues.....67

2.3 Cell Lines.....69

2.4 Vectors, Plasmids and Virus.....70

2.5 Reverse Transcription Polymerase Chain Reaction.....71

2.6 Real-Time Quantitative PCR.....72

2.7 Western Blotting Analysis.....74

2.8 Proliferation Assay.....74

2.9 Wound Healing Assay.....74

2.10 Boyden Chamber Assay.....75

2.11 Three Dimensional Matrigel Culture.....76

2.12 Anchorage-Independent Growth in Soft Agar.....76

2.13 Confocal Immunofluorescence Microscopy.....77

2.14 Mice injections, Necropsy, Histopathology.....78

2.14 Paraffin Section Preparation.....79

2.15 Hematoxylin and Eosin (H&E) Staining.....79

2.16 Statistical Analysis.....80

Chapter 3 Results.....82

3.1 Increased Expression of Bmi-1 in Breast Cancer Tissues.....83

3.2 Correlation betweenBmi-1 expression and Clinical Aggressiveness of Breast Cancer 89

3.3 High Bmi-1 Expression was Associated with an Unfavorable Prognosis....91

3.4 Analyses of Relative Risks (RRs) Indicative of Bmi-1's Role in the Prognosis of Breast Cancer.....100

3.5 Ectopic Expression of Bmi-1 Enhanced the Mobility and Invasiveness of Immortazled HMECs.....104

3.6 The Suppression of Bmi-1 Repressed the Mobility, Invasiveness and Transformation.....112

3.7 The Repression of Bmi-1 Slows Down the Tumor Progression and Reduced the Spontaneous Lung Metastasis in Nude Mice.....116

3.8 Bmi-1 expression Induced the Expression of Mesenchymal Markers and Inhibited the Expression of Epithelial Markers.....121

3.9 Bmi-1 activated the Akt /GSK-3β/Snail Pathway.....126

Chapter 4 Discussion&Conclusion.....130

4.1 The Impact of Bmi-1 in the Invasion and Metastasis of Breast Cancer...131

4.2 The Mechanism of Bmi-1 in the Invasion and Metastasis.....152

Conclusions.....160

List of Abbreviations

ABCB1: ATP-binding cassette transporter B1

AML: acute myeloid leukemia

AP: the accelerated phase

APC: adenomatous polyposis coli

BC: blastic crisis

BDuC: bile ductular carcinoma

bHLH: basic Helix-Loop-Helix families

Bmi-1: B-lymphoma Moloney murine leukemia virus insertion region-1

B-NHL: B-cell non-Hodgkin's lymphoma

BMP-6: bone morphogenetic protein-6

BRCA1: breast cancer 1, early onset

BRCA2: breast cancer 2, early onset

BRMS1: breast cancer metastasis suppressor 1

BSA: bovine serum albumin

CDK: cyclin-dependent kinase

CDKN2A: cyclin-dependent kinase inhibitor 2A

CIS: carcinoma *in situ*

CML: chronic myeloid leukemia

c-Myc: myelocytomatosis cellular gene

CP: the chronic phase

CTEN: C-terminal tensin

DAB: diaminobenzidine

DAPI: 4' 6-Diamidino-2-phenylindole

DCIS: ductal carcinoma *in situ*

ECM: the extracellular matrix

EDTA: ethylenediaminetetraacetic acid

EGF: epidermal growth factor

EMT: epithelial-mesenchymal transition

ER: estrogen receptor

ESFT: Ewing sarcoma family of tumors

ETAR: endothelin A receptor

EZH2: enhancer of zeste homolog 2

E1A: early region 1A

FGF: fibroblast growth factor

Gal-9: galectin-9

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GATA3: trans-acting T-cell-specific transcription factor

GSK: glycogen synthase kinase

HCC: hepatocellular carcinoma

HDFs: human diploid fibroblasts

HER-2: human epidermal growth factor receptor 2

HMECs: human mammary epithelial cells

HOXA9: homeobox protein hox-A9

HOXB9: homeobox protein hox-B9

HGF: hepatocyte growth factor

HIF1α: hypoxia-inducible factor 1α

HSCs: hematopoietic stem cells

hPDMCs: human mesenchymal stem cells

HRP: streptavidin-horseradish-peroxidase

HSAECs: human small airway epithelial cells

hTERT: human telomerase reverse transcriptase

H-T-H-T: helix-turn-helix-turn

ICC: intrahepatic cholangiocarcinoma

IGF: insulin-like growth factor

ILK: integrin-linked kinase

i.v: intravenous injection

LCIS: lobular carcinoma *in situ*

LNM: lymph node metastasis

MBC: metastatic breast cancer

MDCK: Madin-Darby canine kidney

MDM2: murine double minute 2

MDS: myelodysplastic syndrome

MEFs: mouse embryonic fibroblast

MET: mesenchymal-epithelial transition

MMP-9: Matrix metallopeptidase

MRI: magnetic resonance imaging

NHOK: normal human oral keratinocytes

NF-κB: nuclear factor-κB

NPC: nasopharyngeal cancer

NPECs: nasopharyngeal epithelial cells

NSCLC: non-small cell lung cancer

NSPc1: nervous system polycomb 1

OSCC: oral squamous cell carcinomas

PBS: phosphate buffered saline

PcG: polycomb

PCR: the polymerase chain reaction

PI3K: phosphoinositol-3 kinase

PIP3: phosphatidylinositol-3, 4, 5-trisphosphate

PR: progesterone receptor

PVDF: polyvinylidene difluoride

RA: refractory anemia

RAEB: refractory anemia with excess blasts

RAEB-T: refractory anemia with excess blasts in transformation

RARS: refractory anemia with ringed sideroblasts

ROS: reactive oxygen

RR: relative risk

RTKs: receptor tyrosine kinases

SAJ: spot adherens junctions

SCID: severe combined immunodeficient

SCP: small C-terminal domain phosphatase

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

shRNAs: short-hairpin RNAs

SIP1: smad-interacting, multi-zinc finger protein

SPF: specific pathogen free

TGF-β: transforming growth factor β

TNM: tumor node metastasis

UICC: international union against cancer

VEGF-C: vascular endothelial growth factor C

YB-1: Y-box protein

ZEB1: zinc finger E-box-binding homeobox 1

Chapter 1 Introduction

1.1 General Introduction

The metastasis is frequently a fatal step in the progression of solid malignancies [1]. The invasion and metastasis are the extraordinary, distinctive features in the process of the breast cancer [2]. Albeit lymph-node involvement, large tumor size, and poorly-differentiated histo-pathological grade are commonly considered as the established prognostic markers related to the metastasis [3], distant metastasis still occurred in 20-30% of the patients with negative lymph-node involvement [4]. The identity and time of onset of the changes that endow tumor cells with these metastatic functions are largely unknown and keep a subject of debate [1]. It is believed that genomic instability generates large-scale of cellular heterogeneity within tumor populations, from which rare cellular variants with augmented metastatic abilities evolve through a darwinian selection process [1]. So far, Human Epidermal Growth Factor Receptor 2 (HER-2) [5], myelocytomatosis cellular gene (c-Myc) [6], and Homeobox protein Hox-B9 (HOXB9) [7] have emerged to predict the metastasis risk of breast cancer. Aberrant expression of these genes may induce the expression of the growth and angiogenic factors in tumors, lead to their local increase in the tumor microenvironment and favor tumor progression [7]. Recently, a new genomic test (gene-expression profiling) has been displayed to predict clinical outcome more accurately than the traditional clinical and pathological standards [8, 9]. However, it is still an open question whether or not this method will enter into the clinical routine practice for staging and grading [10]. Though these new

marks and methods has been implicated in many studies, the molecular mechanism of the invasion and metastasis of breast cancer remains far from being fully understood due to heterogeneity of the cancer and it represents a new prerequisite for developing better treatment strategies.

The polycomb (PcG) proteins constitute a global system with important roles in multi-cellular development, stem cell biology and cancer [11]. B-lymphoma Moloney murine leukemia virus insertion region-1 (Bmi-1), a member of PcG family of transcription repressors, has emerged as a Myc-cooperating oncogene in murine lymphomas [12, 13]. Bmi-1 not only can lead human mammary epithelial cells (HMECs) to bypass senescence and immortalize but also plays a key role in human breast cancer [14, 15]. Bmi-1 expression has a significant correlation with the axillary lymph node metastasis in invasive ductal breast cancer [16]. These findings suggest that Bmi-1 could be involved in the carcinogenesis and metastasis of breast cancer. Although growing evidences have shown that Bmi-1 expression favored the poor prognosis [17, 18], opposing results have recently emerged [19, 20]. Bmi-1 protein is only detected in 25% African breast cancer patients and is associated with a low histological grade [19]. Meanwhile, higher Bmi-1 mRNA expression has been observed in the early stage patients without lymph node metastasis [21]. On the contrary, the up-regulation of Bmi-1 has been showed to have a relationship with the invasion of nasopharyngeal carcinomas and predict the poor survival [22]. In colon

cancer and gastric cancer, Bmi-1 expression is significantly correlated with nodal involvement, distant metastasis and clinical stages [23-25]. Furthermore, metastatic melanoma tissues and cell lines show much higher expression of Bmi-1 than primary melanoma tissues and cell lines [26]. While knockdown of Bmi-1 is able to decrease the invasiveness of cervical cancer cells and gastric cells [5, 27]. These findings suggest that Bmi-1 expression contributes to increased aggressive behaviors of cancer cells. The overexpression of Bmi-1 can promote epithelial-mesenchymal transition (EMT) of nasopharyngeal epithelial cells (NPECs), whereas Bmi-1 knockdown can reverse EMT and deduce the metastasis of nasopharyngeal cancer cells (NPCs) [28]. Although Bmi-1 overexpression could not result in oncogenic transformation of MCF-10A cells, Bmi-1, together with H-Ras, has induced an aggressive and metastatic phenotype with an unusual occurrence of brain metastasis in breast cancer [29]. In spite of foregoing, very few studies have been focused on the molecular mechanism and clinical outcome of Bmi-1 in the invasion and metastasis of breast cancer.

The metastasis of cancer is a complex and multistep process, including a series of successive and dynamic events with the alteration of the morphology and biological function [30]. After acquiring EMT ability, cancers are prone to metastasize and establish secondary tumors at distant sites [31, 32]. In EMT, epithelial cells can acquire mesenchymal-like properties with increasing cellular

motility and/or lost epithelial-like properties with decreasing intercellular adhesion [33, 34]. The loss of E-cadherin is a hallmark to the invasive phase of cancer and it can be repressed by certain dominant transcriptional factors, such as Snail, Zinc finger E-box-binding homeobox (ZEB), Twist, and basic Helix-Loop-Helix families (bHLH) [35-37]. Snail-induced EMT is an important breakthrough to metastasis, providing new insights into the molecular mechanisms of tumor invasion [38, 39]. Moreover, Snail expression is associated with E-cadherin repression and metastasis in breast cancer cells, as well as in other cancer cells [40-44]. Apart from it, numerous agents are involved in EMT progress of breast cancer, such as Six1, Y-box protein (YB-1) and the miRNA-200 families [45-47]. Therefore, it is important to understand whether Bmi-1 can regulate EMT in the progression and metastasis of breast cancer.

1.2 Breast Cancer

1.2.1 Overview of Breast Cancer

Breast cancer is the most common cancer affecting women after skin cancer, with a lifetime risk of up to 10.4% in 2002 in U.S.A (http://www.iarc.fr/en/Publications/PDFs-online/World-Cancer-Report/World-Cancer-Report). Breast cancer, like other cancers, occurs because of an interaction between the environment and a defective gene. The number of cases worldwide has significantly increased since the 1970s and a phenomenon partly attributes to the modern lifestyles. More than half of the cases are in developed regions such as Europe (27.3% of cancers in women) and North America (31.3%) (Figure 1.2.1) [2]. However, the mortality of breast cancer remains stable or decreases in the last 10-15 years in many industrialized countries in spite of the increasing incidence. These decreases may come from earlier treatment, earlier detection and increased awareness [48, 49]. Although about 40,170 women were expected to die because of breast cancer in the U.S.A. in 2009, there are about 2.5 million women who have survived breast cancer in the U.S.A in 2008 (http://www.breastcancer.org). About 5-10% of breast cancers are caused by gene mutations inherited from parents, however, about 90% patients are due to genetic abnormalities that happen as a result of the aging process and life in general (http://supportpink.org/). Incidence rates of breast cancer in most part of China are currently low compared with those in developed countries, however, Hong Kong, Beijing, Shanghai and Guangzhou have already seen dramatic rises

of the incidence and breast cancer is now becoming the most common women cancer in these regions [50]. From1998 to 2002, rates were 81 per 100,000 in Canada, 41 per 100000 in Hong Kong, 31 and 35 per 100 000 respectively in Guangzhou City and Shanghai, urban areas in Mainland China, and 15 per 100 000 in rural areas in Mainland China [51]. These increases have been supposed to link to rapid changes in lifestyle risk factors and reproductive behavior. In China the average birth rate fell from 5.9 births per woman in 1970 to 2.9 in 1979 to 1.7 in 2004 [52].

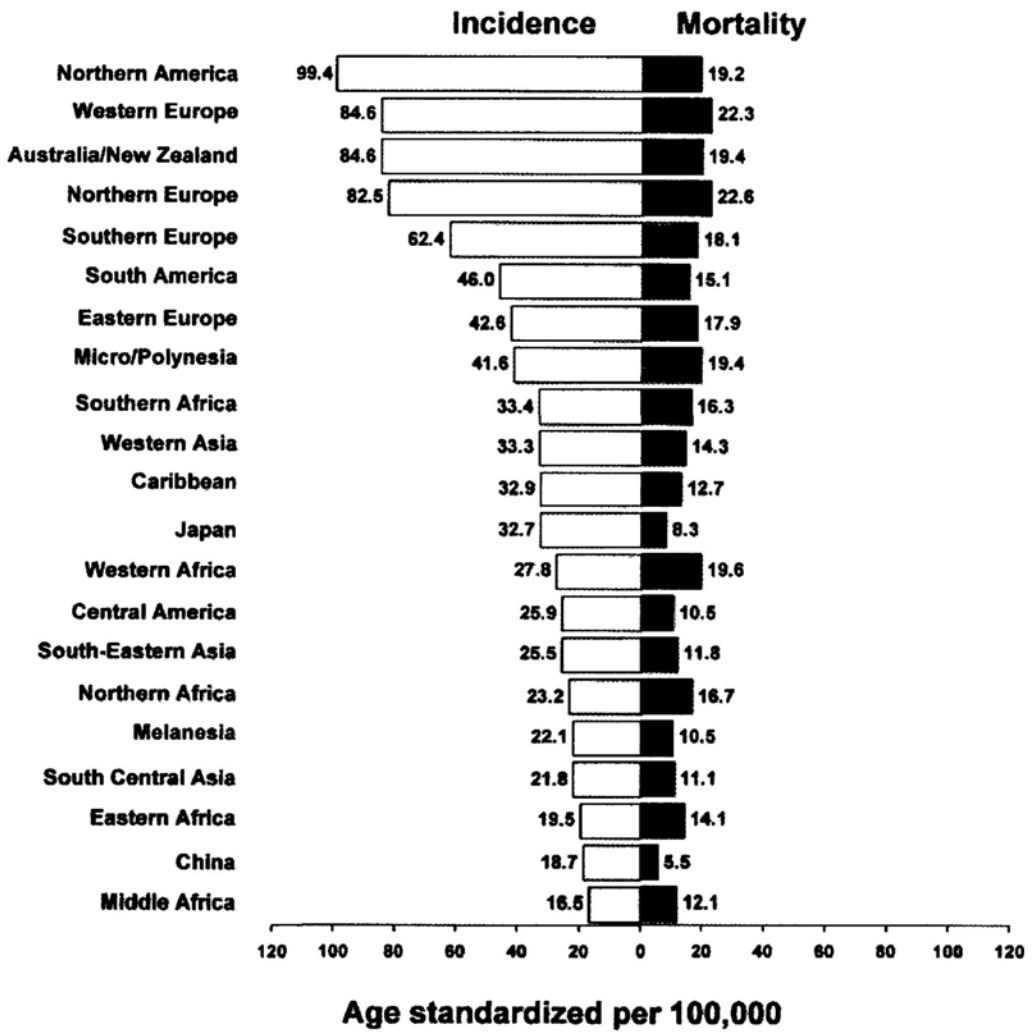

Figure 1.2.1. Breast cancer incidence and mortality rates per 100,000 by region or country. The very favorable survival in the more affluent developed regions and poor survival in some developing regions results in the differences in mortality rates worldwide being much less marked than for incidence. The estimated mortality rates in Africa and the Pacific (Micronesia and Polynesia), for example, are not greatly inferior to those in Europe [49, 53].

1.2.2 The Known Risk Factors for Breast Cancer

The risks for breast cancer depend upon several factors, such as sex [54], age [55], family history [56], age at first full-term pregnancy [57], early menarche [58], late menopause [59] and breast density [60]. However, there are other modifiable factors associated with the increased breast cancer risk, such as postmenopausal obesity [61], use of combined estrogen and progestin menopausal hormones [62], alcohol consumption [63] and physical inactivity [64]. Some risk factors, like early menarche, late menopause, obesity and hormone use, directly increase the lifetime exposure of breast tissue to circulating ovarian hormones, whereas others, such as higher socioeconomic status, are correlated with reproductive behavior and/or other factors. Established risk factors for breast cancer are listed in Table 1.2.2.1.

1.2.2.1 Female

Being female is the well established primary cause of breast cancer. In 2009, an estimated 192,370 women were expected to be diagnosed as new cases of invasive breast cancer in the U.S.A, along with 62,280 new cases of non-invasive (*in situ*) breast cancer. Breast cancer is about 100 times more common in women than in men, although males tend to have poorer outcomes due to delays in diagnosis (http://www.breastcancer.org). Less than 1% of all new breast cancer cases occur in men, however, there has been an increased incidence of male breast cancer during the last few years.

1.2.2.2 Increasing Age

Besides being female, age is the most important risk factor for breast cancer. Table 1.2.2.2 shows the risk of a woman diagnosed as breast cancer at different ages in the whole population. Approximately 77% of breast cancer cases occur in women over 50 years of age. While breast cancer is less common at a young age (*i.e.,* in their thirties), younger women tend to have more aggressive breast cancers than older women, which may explain why survival rates are lower among younger women (Table 1.2.2.2) [65].

1.2.2.3 Personal and Family History of Breast Cancer and Genetic Predisposition

The risk in the other breast is increased after getting breast cancer in one breast. Women with a family history of breast cancer, especially in a first-degree relative (mother, sister, or daughter), are at increased risk of developing breast cancer [66]. Having one first-degree relative with breast cancer approximately doubles a woman's risk and having 2 first-degree relatives increases her risk about 5-fold [67].

An estimated 5% to 10% of all breast cancers arise in the setting of inherited mutations in *BRCA1* (breast cancer 1, early onset) or *BRCA2* (breast cancer 2, early onset) [68]. Germ-line mutations in the *BRCA1* and *BRCA2* genes in women confer a 10- to 20-fold higher risk of breast cancer than the risk of

women without these mutations [69, 70]. However, another study which compared patients from *BRCA1*, *BRCA2* and non- *BRCA1/2* families as well as sporadic cases did not confirm the prognostic role of *BRCA1/2* [71]. It is believed that the interaction between environmental and heritable factors shared by women within a family leads to the occurrence of breast cancer [72].

1.2.2.4 Hormonal Factors

Reproductive hormones are shown to be the risk for breast cancer by having an effect on cell proliferation, DNA damage and promotion of cancer growth. ER^+PR^+ (estrogen receptor positive and progesterone receptor positive) is a stronger increased risk for breast cancer than ER^-PR^- [73-77].

1.2.2.5 Clinical Factors

High breast tissue density, which is a mammographic indicator of the amount of glandular tissue relative to fatty tissue in the breast, is a strong independent risk factor for the development of breast cancer. The risk with the highest levels of breast density increased 4- to 6-fold compared with the least dense breasts [78-80].

Certain benign breast conditions are more closely linked to breast cancer than others [81-84]. Depending on how they affect this risk, benign breast conditions are often subdivided into three general groups: non-proliferative lesions,

proliferative lesions without atypia and proliferative lesions with atypia [85-87]. The relative risk associated with atypia was 4.24, as compared with a relative risk of 1.88 for proliferative changes without atypia and 1.27 for nonproliferative lesions [82].

1.2.2.6 Radiation

It is well established that exposure to ionizing radiation increases a woman's risk for breast cancer [88]. Much data on the relationship between radiation exposures and subsequent breast cancer development are derived from atomic bomb survivors and women who received medical exposures either for diagnostic or therapeutic purposes [89, 90].

Table 1.2.2.1 Factors that increase the relative risk for breast cancer in women

Relative Risk	Factor
>4.0	Female
	Age (65+ vs. <65 years, although risk increases across all ages until age 80)
	Certain inherited genetic mutations for breast cancer (BRCA1 and/or BRCA2)
	Two or more first-degree relatives with breast cancer diagnosed at an early age
	Personal history of breast cancer
	High breast tissue density
	Biopsy-confirmed atypical hyperplasia
2.1-4.0	One first-degree relative with breast cancer
	High-dose radiation to chest
	High bone density (postmenopausal)
1.1-2.0	
Hormones	Late age at first full-term pregnancy (>30 years)
	Early menarche (<12 years)
	Late menopause (>55 years)
	No full-term pregnancies
	Never breastfed a child
	Recent oral contraceptive use
	Recent and long-term use of estrogen and progestin
	Obesity (postmenopausal)
Other factors	Personal history of endometrial or ovarian cancer
	Alcohol consumption
	Height (tall)
	High socioeconomic status
	Ashkenazi Jewish heritage

It comes from breast cancer facts & figures 2009-2010 (American Cancer Society, *www.cancer.org*.)

Table 1.2.2.2 Estimated new female breast cancer cases and deaths by age, U.S.A, 2009

Age	In Situ Cases	Invasive Cases	Deaths
Younger than 45	6,460	18,640	2,820
45 and older	55,820	173,730	37,350
Younger than 55	24,450	62,520	8,890
55 and older	37,830	129,850	31,280
Younger than 65	40,940	120,540	17,200
65 and older	21,340	71,830	22,970

Data source: Estimated cases are based on 1995-2005 incidence rates from 41 states as reported by the North American Association Central Cancer Registries, representing about 85% of the US population. Estimated deaths are based on data from US Mortality Data, 1969-2006. The table comes from breast cancer facts & figures 2009-2010 (American Cancer Society, *www.cancer.org.*).

1.2.1 The Symptoms and Diagnosis of Breast Cancer

No typical symptoms are observed when the tumor is small. Therefore, it is very important for women to follow recommended screening guidelines for detecting breast cancer at an early stage before symptoms develop. When the size of breast cancer can be felt, a painless mass of the tumor could be found. Sometimes, the cancer can spread to underarm lymph nodes and cause a lump or swelling, before the original breast tumor is large enough to be felt. Less common signs and symptoms include breast pain or heaviness, persistent changes to the breast such as swelling, thickening, or redness of the breast's skin and nipple abnormalities such as spontaneous discharge, erosion, inversion, or tenderness (breast cancer facts & figures 2009-2010).

Mammography, an x-ray picture of tissues inside the breast, can detect very early breast tumors, when they are too small to be felt. In fact, 85-90% breast cancers are able to be detected by screening at very early stage, when they are relatively easy to cure [91]. A breast magnetic resonance imaging (MRI), as a complementary test, reveals more details of breast health than a mammogram [92]. MRI can differentiate the diseased tissue from the normal tissue. However, MRI test is much more expensive and not as widely available. A biopsy, the removal of tissue to search cancer cells, is the only way to confirm if cancer is present [93, 94]. Then, the tissue or fluid removed from breast will be checked by pathologist.

1.2.4 Tumor-Related Characteristics

1.2.4.1 Pathology

Most breast cancers are derived from the epithelium lining of the ducts or lobules. Carcinoma *in situ* (CIS) is an early form of carcinoma defined by the absence of invasion of surrounding tissues, whereas invasive carcinoma is defined as the presence of invading the surrounding tissues. Many forms of invasive carcinoma originate from a CIS lesion.

1.2.4.2 Tumor Size

Tumor size is one of the main distinguishing prognostic indicators [95, 96], even after 20 years of follow-up (Figure 1.2.4.2) [97, 98]. Tumor Size is divided into four classes: T1 is from 0 - 2 centimeters (cm), T2 is from 2-5 cm, T3 is greater than 5cm, T4 is a tumor of any size that has broken through the skin, or is attached to the chest wall. A larger tumor is often related to more positive lymph nodes, thus their interaction further influences the survival of breast cancer [99].

1.2.4.3 Regional Lymph Node Involvement

Regional lymph node involvement is a valuable indicator for long-term survival (Figure 1.2.4.3) [97, 99]. The risk of mortality is increased about 4-8 times in patients with node involvement than those without nodal involvement [100]. Prognosis for patients with 10 or more involved axillary nodes showed 70% more deaths at 10 years than for those with 1-3 involved nodes [99, 101]. The

early stages and application of systemic treatment has conferred the improvement of the survival of node-positive patients [101].

1.2.4.4 Metastasis

The patients with metastasis generally exhibit the short survival time [102, 103]. The most common places that breast cancer spreads to are the bones, the liver and the lungs. A patient may be found to have a distant metastasis when the initial breast cancer is found, or months to years later.

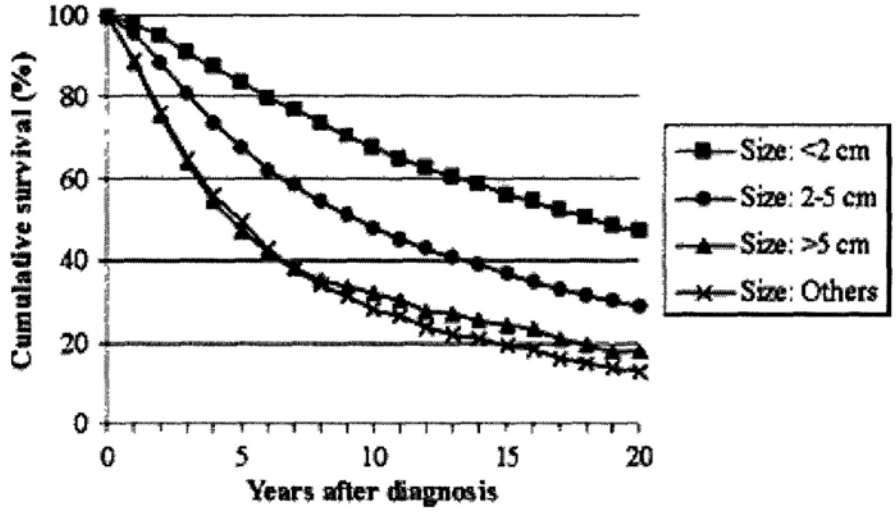

Figure 1.2.4.2 Cumulative survival proportion of breast cancer patients diagnosed in southern Netherlands in 1970-1994 and followed until 2004, according to tumor size (based on pathological diagnosis) [104].

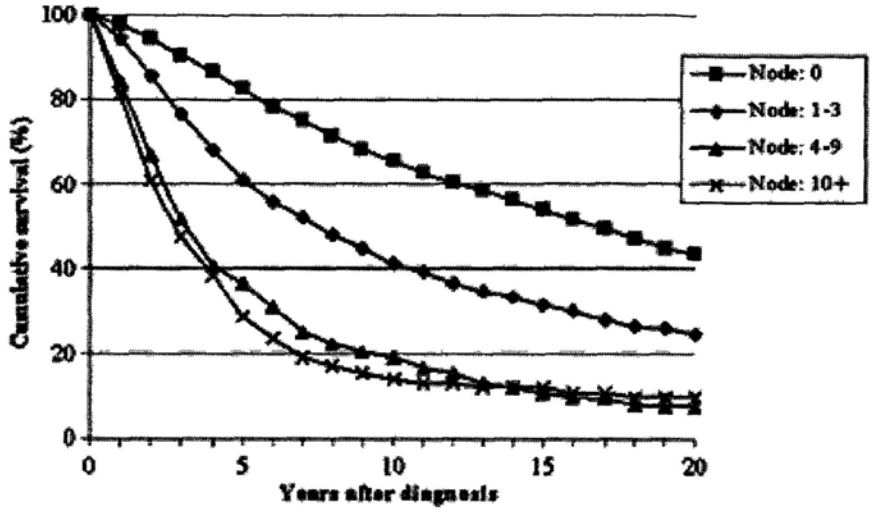

Figure 1.2.4.3 Cumulative survival proportion of breast cancer patients diagnosed in southern Netherlands in 1970-1994 and followed until 2004, based on nodal status (based on pathological diagnosis) [104].

1.2.4.5 The Stages of Breast Cancer

Tumor node metastasis (TNM) staging system consists of the information on primary tumor size, involvement of regional lymph node and the presence of distant metastasis. Stages 0, I and II are considered to be the early stage of breast cancer.

1.2.4.5.1 Stage 0

Stage 0 is used to describe non-invasive breast cancers, such as DCIS (ductal carcinoma *in situ*) and LCIS (lobular carcinoma *in situ*). In stage 0, there is no evidence of cancer cells or non-cancerous abnormal cells breaking out of the part of the breast in which they started, or getting through to invade neighboring

normal tissue.

1.2.4.5.2 Stage I

Tumor is 2 cm or smaller in size and has not spread outside the breast. (Figure 1.2.4.5.2)

1.2.4.5.3 Stage IIA

1. No tumor is found in the breast, but cancer is found in the axillary lymph nodes.
2. Tumor is 2 cm or smaller in size and has spread to the axillary lymph nodes.
3. Tumor is 2-5 cm in size but has not spread to the axillary lymph nodes.

1.2.4.5.4 Stage IIB

1. Tumor is 2-5 cm in size and has spread to the axillary lymph nodes
2. Tumor is larger than 5 cm but still confined to the breast (Figure 1.2.4.5.4)

.

1.2.4.5.5 Stage IIIA

1. No tumor is found in the breast but has spread to the axillary lymph nodes that are attached to each other or to other structures.
2. The tumor is 5 cm or smaller in size and has spread to the axillary lymphonodes that has clumped together or sticked to other structures.
4. The tumor is larger than 5 cm in size and has spread to the axillary

lymphonodes that may or may not be attached to each other or to other structures.

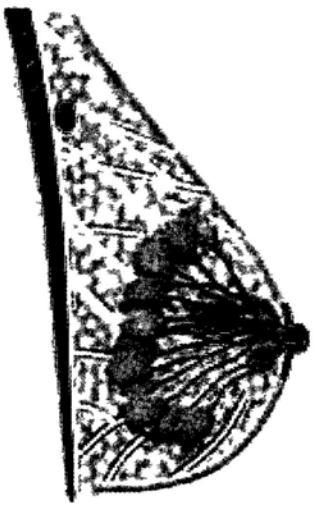

Figure 1.2.4.5.2 Stage Ⅰ: Picture comes from robin of pink (A Novartis Oncology Program)

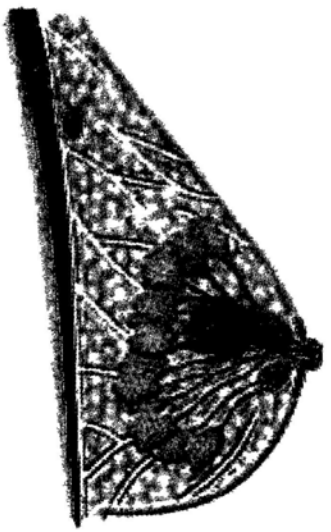

Figure 1.2.4.5.4 Stage ⅡB: Picture from ribbon of pink (A Novartis Oncology Program)

1.2.4.5.6 Stage IIIB

The tumor has spread to tissue near the breast (the skin or chest wall including the ribs and chest muscles) (Figure 1.2.4.5.6).

The tumor has spread to lymph nodes within the breast area or under the arm.

1.2.4.5.7 Stage IIIC

1. There may be no sign of cancer in the breast or it may be any size and may spread to the chest wall and/or the skin of the breast, if there is a tumor.
2. The cancer has spread to lymph nodes above or below the collarbone.
3. The cancer may have spread to axillary lymph nodes or to lymph nodes near the breastbone.

1.2.4.5.8 Stage IV

Tumor has spread to other organs of the body, most often the bones, lungs, liver, or brain (Figure 1.2.4.5.8)

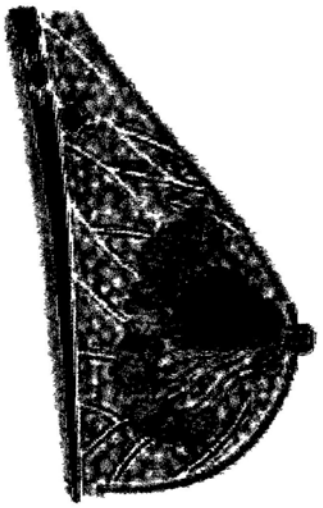

Figure 1.2.4.5.6 Stage ⅢB: Picture from ribbon of pink (A Novartis Oncology Program)

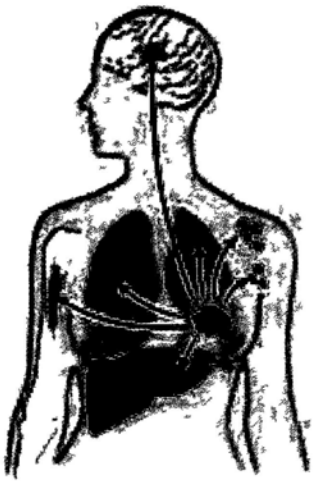

Figure 1.2.4.5.8 Stage Ⅳ: Picture from ribbon of pink (A Novartis Oncology Program)

1.2.5 Treatment for Breast Cancer

1.2.5.1 Surgery

Surgery is the most common treatment for breast cancer. It includes Breast-sparing surgery and Mastectomy. Breast-sparing surgery (breast-conserving surgery) is an operation to remove the cancer but not the breast. Mastectomy is an operation to remove the entire breast (or as much of the breast tissue as possible).

1.2.5.2 Radiotherapy

Radiotherapy is the cancer treatment by using high-energy rays. It is often used after breast surgery, as it can destroy breast cancer cells that remain in the area and lowers the risk of the recurrence.

1.2.5.3 Chemotherapy

Chemotherapy is usually used for stage Ⅱ-Ⅳ disease through a vein (intravenous) or as a pill to kill cancer cells. Chemotherapy can be used to shrink a tumor down before surgery, to reduce the chance of spreading or recurrence after surgery, or as treatment for breast cancer that has spread or recurred.

1.2.5.4 Hormone Therapy

Hormone therapy can be used to treat some types of breast cancer with the presence of hormone receptor. They can decrease the levels of hormones, affecting the growth of some breast cancer cells. Hormone therapy, used before or after surgery, has been proven to reduce the risk of the occurrence of breast cancer. Tamoxifen, an antagonist of the estrogen receptor in breast tissue, can prevent the original breast cancer from returning and the development of new cancers in the other breast [105]. As a treatment for metastatic breast cancer, Tamoxifen can slow or stop the growth of cancer cells present in the body [106].

1.2.5.5 Targeted Therapy

Targeted therapy, a type of medication blocking the growth of cancer cells, can interfere with specific targeted molecules needed for carcinogenesis and tumor growth [107]. Monoclonal antibodies, such as Trastuzumab (Herceptin), are used to treat cancer cells with the HER-2 mutation. Herceptin and Tykerb both attach to the HER-2 receptors inside cancer cells by blocking their reception of growth signals, preventing the cell division and slowing the progress of cancer.

1.3 The Invasion and Metastasis of Breast Cancer

For a malignant tumor to metastasize, tumor cells must go through a series of sequential steps collectively referred to as the 'metastatic cascade'. These steps are: (1) detachment ('loosing up') of cells from primary tumor; (2) invasion and migration through the extracellular matrix; (3) intravasation into the vasculature (blood vessels and lymphatic vessels); (4) evasion of the host immune responses; (5) extravasation from the vasculature to new sites; and (6) growth at the new location and forming metastatic deposit [32]. Invasion and metastasis caused the majority of cancer deaths are both the characteristics of breast cancer. Axillary lymph node status is the most important prognostic factor in breast cancer. The prognosis decreases as the number of tumor-positive lymph nodes increases [108]. The five-year relative survival is 98.1% in local invasive breast cancer patients, while it is only 26% in patients with distant metastases [109]. The unpredictable clinical behavior of metastatic breast cancer (MBC) reflects the biological heterogeneity of the disease [109]. Effective treatment of breast cancer metastases is hampered by a poor understanding of the mechanisms involved in the formation of these secondary tumor deposits [110]. A growing body of evidences underlie that tumor-induced lymphangiogenesis is a predictive indicator of metastasis to lymph nodes and might also be a target for the prevention of metastasis [111, 112].

The development of modern technologies has enabled scientists to further classify breast tumors by measuring the differences in thousands of biological pathways simultaneously. Recent findings indicate that Epidermal Growth Factor (EGF) signal pathway is an important mediator of bone metastasis in breast cancer by increasing tumor cell proliferation and by indirectly engaging bone stromal cell in metastasis-promoting activities [113]. The overexpression of integrin-linked kinase results in an invasive, metastatic phenotype in breast cancer model system both *in vivo* and *in vitro*, thus, implicating a role for integrin-linked kinase in oncogenic transformation, angiogenesis and metastasis [114]. CXC chemokines of 4q13 region have an enhanced expression in metastatic sites [115]. More and more evidences have showed that HER-2 can also increase the invasiveness and targeted lung metastases of breast cancer though upregulation of the chemokine receptor (CXCR4) expression [116, 117]. The expression of large intervening non-coding RNAs (lincRNAs) termed HOTAIR is increased in primary breast tumors and metastases, and HOTAIR expression level in primary tumors is shown to be a powerful predictor of eventual metastasis and death [110]. MicroRNAs (miRNAs) are increasingly implicated in the regulation of metastasis. They act as oncogenes and promote the metastasis of breast cancer cell by targeting multiple tumor/metastasis suppressor genes including miR-21 and miR-10b [9, 100, 118]. The C-terminal tensin-like (*CTEN*) gene is a member of the *Tensin* gene family involved in cell migration and localized at focal adhesion sites. The CTEN expression and C

alpha kinase 1 (PICK1) are significantly associated with poor prognostic variables including larger tumor size, higher histological grade and axillary nodal involvement [119] [120]. The RhoA GTPase is crucial in numerous biological functions and is also linked to cancer metastasis. The RhoA transcription is orchestrated by the Myc-Skp2-Miz1-p300 transcriptional complex. Deficiency of this complex result in impairment in RhoA expression, cell migration, invasion and breast cancer metastasis, recapitulating the phenotypes observed in RhoA knockdown. However, RhoA restoration rescues the defect in cell invasion. The overexpression of the Myc-Skp2-Miz1 complex is found in metastatic human cancers and is correlated with RhoA expression [121]. The high level of the galectin-7 expression in breast cancer cells is also reported to drastically increase their ability to metastasize to the lungs and bones [122]. In addition, Vascular Endothelial Growth Factor C (VEGF-C) can promote intralymphatic spread of breast metastases in the lung and formation of tumor emboli in the pulmonary arteries [123].

The invasion and metastasis of breast cancer can occur when some proteins show low expression. Homeobox protein Hox-A9 (HOXA9) is identified as a gene frequently downregulated in human breast cancers and tumor cell lines. The reduced HOXA9 transcript levels are associated with tumor aggression, metastasis and patient mortality [124]. On the contrary, GATA3 (Trans-Acting T-cell-Specific Transcription Factor) is able to inhibit breast cancer metastasis through the reversal of EMT [125]. Bone morphogenetic protein-6 (BMP-6) is

recognized as an inhibitor of the EMT through rescuing E-cadherin expression in breast cancer. It may function as an anti-metastasis factor by a mechanism involving the transcriptional repression of miR-21 in breast cancer [126]. Breast cancer metastasis suppressor 1 (BRMS1) can up-regulate miR-146, which suppresses breast cancer metastasis. Therefore, modulating the levels of miR-146a or miR-146b could have a therapeutic potential to suppress breast cancer metastasis [127]. $P27^{Kip1}$ protein is a cyclin-dependent kinase (CDK) inhibitor which has been reported to be associated with invasion, metastasis and angiogenesis in malignant tumors. Up-regulation of $p27^{Kip1}$ can remarkably inhibit the invasion of the breast cancer cells, in part due to the depressed expression of Matrix metallopeptidase (MMP-9) [128]. Additionally, Galectin-9 (Gal-9) can suppress tumor metastasis by blocking adhesion to endothelium and extracellular matrices [129].

Several clinico-histopathological parameters are considered to be strong predictors of metastasis, however, they fail to accurately classify breast tumors according to their clinical behavior and to predict the disease recurrence [130]. Gene expression profiling enables scientists to understand the heterogeneous nature of breast cancer on a genomic level. Several gene expression profiles for breast cancers have emerged in the initial studies and appear to be generally concordant in their ability to predict poor outcome. Of these profiles, the Oncotype Dx and Mammaprint assays are the best validated and are commercially available [131]. Gene expression profiling may identify tumors

that are likely to metastasize, and even perhaps the location of the metastases [132, 133]. Most of these markers identified by genome-wide microarray can be detected in the primary tumors, which can potentially lead to the ability to identify patients at the time of diagnosis who are at high risk for lymph node metastasis, and allow for early intervention or more suitable adjuvant treatments [130, 134, 135]. However, it is still an open question whether or not this method will enter into clinical routine practice for staging and grading [10]. Although a lot of studies have highlighted the possible involvement of certain genes in breast cancer progression and metastasis, the mechanism of breast cancer progression remains unclear. Therefore, there is significant interest in developing strategies to antagonize these genes' function, and an opportunity to interfere with metastasis that lead to most death in breast cancer patients.

1.4 Important Role of Bmi-1 in the Development of Carcinoma

PcG family proteins, the well-known epigenetic gene silencers and assembling into polycomb repressive complexes (PRCs), play a crucial role in the self-renewal and differentiation of stem cells and cancer development [11, 136]. *Bmi-1*(B-lymphoma Moloney murine leukemia virus insertion region-1), the first identified PcG gene, is involved in the transcriptional repression of *hox* gene and affects stem cell self-renewal, embryonic development, cell proliferation and cancer progress [13, 15, 137].

1.4.1 Genetic Structure of Bmi-1

The *Bmi-1* gene localizes on human chromosome 10p11.23, a known region involved in translocations in various leukemias, and extends over 4.9 kb comprising at least 10 exons and 9 introns [138]. Human *Bmi-1* cDNA is 3203 base pairs in length and shows 86% identity to that of the mouse nucleotide sequence. Human proto-oncogene, *Bmi-1* encodes a 44-46 kDa protein which consists of 326 amino acids and shares 98% identity to the amino acid sequence of the mouse Bmi-1 protein [139] (Figure 1.4.1). The Bmi-1 protein contains a conserved RING finger (RF) domain at the NH_2- terminus, which is required for its ability to cooperate with c-Myc in tumorigenesis and regulate cell proliferation [140]. Moreover, the deletion of the RING finger domain of Bmi-1 may lead to dominant negative activity of the mutant Bmi-1 [141], as

overexpression of the ΔRF mutant of Bmi-1 induces p16INK4A expression and premature senescence, whereas the expression of the wild type Bmi-1suppresses p16INK4A expression and bypasses senescence in HDFs [141]. The RING finger is required for proper subnuclear localization of Bmi-1 [142]. Because it clearly differs from classical DNA-binding zinc finger in terms of sequence homology and tertiary structure [143], Bmi-1 RING finger is suggested to play a role in protein-protein interactions rather than protein-DNA interactions [142]. Bmi-1 also contains a conserved centrally located helix-turn-helix-turn (H-T-H-T) motif [13], which is required for transcriptional repression but not transformation in rat embryo fibroblasts [144]. Nevertheless, RING and HTHT domains are both required for telomerase activity and immortalization by downregulating P16INK4A tumor suppressor [14, 22, 141, 142]. A proline/serine rich region (P/S) is contained at the carboxyl terminus of Bmi-1 protein. This region probably implicates rapid protein degradation [142] and it may function as a negative regulatory domain of Bmi-1[145]. Bmi-1 also contains two putative nuclear localization signals (NLS), NLS1 (amino acid residues 92-95) and NLS2 (amino acid residues 232-235). Of these two, only NLS2 appears to be functional in targeting Bmi-1 to the nucleus in mouse and human cells [144] [14].

Figure 1.4.1 The structure of Bmi-1. The RF box represents the RING finger domain at the NH_2-terminal; the HTH box represents the putative helix-turn-helix-turn region; P/S represents the proline/serine rich region at the carboxyl terminus of the Bmi-l protein; the small region (non-dark region) indicates nuclear localization signals of the Bmi-l protein.

1.4.2 The Immortalization and Transformation by Bmi-1

The telomerase complex maintains the telomere length, which is required for an unlimited cellular proliferation. Telomerase is unactivated in normal human somatic cells, while it is activated in 90% tumors [146]. A less stringent requirement for the loss of $p16^{INK4A}$ expression seems to characterize telomerase immortalization of the human fibroblasts. Besides the $p16^{INK4A}$ inactivation, a large body of evidences show that the long term propagation in culture of telomerase immortalized cells is frequently associated with the activation of cancer pathways, such as the overexpression of the proto-oncogenes Myc and Bmi-1 [147, 148], resistance to growth inhibition induced by transforming growth factor β [149], and loss of p53 function [150]. Combination of hTERT (human telomerase reverse transcriptase) and human papillomavirus type 16 E6/E7, Bmi-1 induces the prolongation of the life span of bone marrow stromal cells without affecting their neurogenic potential [151]. Alternatively, Bmi-1 with hTERT can result in efficient immortalization of human small airway epithelial cells (HSAECs), cementoblast progenitor cells and human mesenchymal stem cells (hPDMCs) without crisis or growth arrest [152-154]. Exogenous Bmi-1 expression can not cause normal human oral keratinocytes (NHOK) to immortalize except extending the replicative life span. On the contrary, co-overexpression of Bmi-1 and HPV-16 E6 can induce the immortalization of NHOK [155]. Notably, Bmi-1 overexpression can induce the immortalization of mouse embryonic fibroblast (MEFs) by down-regulating the

expression of p16 ^{INK4A}/p19^{ARF} [156]. In addition, Bmi-1overexpression can also lead to activation of hTERT, induction of telomerase activity, and immortalization of HMECs and NPECs [14, 22]. However, Bmi-1 was not overexpressed in hTERT-immortalized HMECs, suggesting that Bmi-1 functions upstream of hTERT [14].

The acquisition of immortality is an essential step in the process of malignant transformation. Most human *in vitro* transformation models do require some viral oncogenes in order to induce the phenotype of malignant transformation [157-159]. Combined expression of the adenovirus E1A (Early region 1A), Ha-RasV12, and MDM2 (Murine Double Minute 2) can convert normal human cells into malignant cells [157]. The combination of Ras and c-Myc enable the human diploid fibroblasts (HDFs) to form tumors in nude mice [158]. Bmi-1 can contribute to lymphomagenesis in the T and B cell lineages and collaborate with the *myc* gene in tumor development [160]. Bmi-1 enhances the motility and invasiveness of NPECs, although it could not lead to the transformation of NPECs [161]. Furthermore, Bmi-1 can induce the malignant transformation of human skin keratinocytes (HaCaT) *in vitro* and *in vivo* by downregulation of differentiation related factor (KRT6), p16^{INK4A} and p14^{ARF} to promote cell cycle progression, and by decreasing E-cadherin to increase cell mobility [162]. Although it does not significantly affect the proliferation or the survival of Ewing sarcoma family of tumors (ESFT) cells, Bmi-1 actively promotes

anchorage-independent growth *in vitro* and tumorigenicity *in vivo*. Bmi-1 promotes the tumorigenicity of both p16 wild-type and p16-null cell lines, demonstrating that the mechanism of Bmi-1 oncogenic function in ESFT is, at least in part, independent of CDKN2A (Cyclin-Dependent Kinase Inhibitor 2A) repression and in part mediated through modulation of adhesion pathways [163]. The roles of Bmi-1 in HaCaT and ESFT strongly support that Bmi-1 is indispensible for the transformation of these cells. Nevertheless, Bmi-1 overexpression alone is not sufficient for oncogenic transformation of MCF-10A cells, MEFs, neural progenitor cells and human neuroblastoma cell line (SHEP1) [29, 156, 164, 165]. However, Bmi-1 co-overexpression with activated H-Ras is able to transform MCF-10A cells and MEFs *in vitro* by transformation assays and *in vivo* by mouse model [29, 156]. Importantly, Bmi-1 and H-Ras co-overexpression in MCF-10A cells also induce EMT, while, Bmi-1 inhibits senescence and permits the proliferation of cells that express high levels of Ras [166]. Bmi-1 and Ras can promote breast carcinogenesis by deregulation of multiple growth-regulatory pathways by $p16^{INK4A}$-independent mechanisms [166]. Conversely, they promote MEFs to form tumor by $p16^{INK4a}$ and $p19^{ARF}$ repression [156]. Similarly, Bmi-1 knockdown using RNA interference approach results in the reduction in transformed phenotype of MCF-7 cells and down-regulation of Akt/PKB activity [167]. These findings suggest that Bmi-1 play an important role in carcinogenesis, but the ability of Bmi-1 alone leading to transformation depend on the cell specificity (Figure 1.4.2.1).

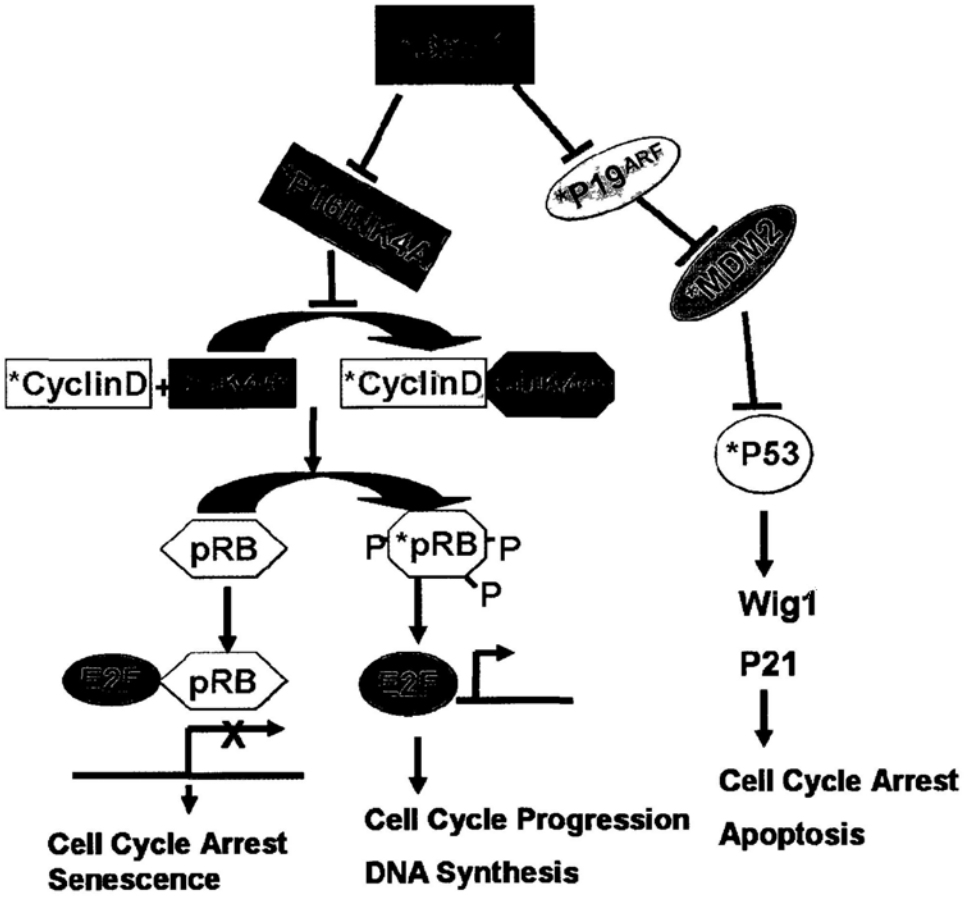

Figure 1.4.2 Regulation of cell cycle, apoptosis, and senescence by Bmi-1. In normal stem cells, *p16*^{INK4A} and *p19*^{ARF} genes are repressed in a Bmi-1-dependent manner. In the absence of *p16*^{INK4A}, the cyclin D/CDK4/6 complex can phosphorylate pRb, allowing the E2F-dependent transcription that leads to cell cycle progression and DNA synthesis. In addition, MDM2-mediated p53 degradation causes the low p53 levels in the absence of $p19^{ARF}$, thus preventing cell cycle arrest and apoptosis. The absence of Bmi-1 relieves the repression of the *INK4A* locus, resulting in the expression of $p16^{INK4A}$ and $p19^{ARF}$. $p16^{INK4A}$ inhibits binding of cyclin D to CDK4/6, resulting in inhibition of the kinase activity. This leads to a hypophosphorylated pRb, which then can bind E2F and inhibit E2F-dependent transcription, resulting in

cell cycle arrest and senescence. $p19^{ARF}$ inhibits MDM2, which mediates ubiquitin-dependent degradation of p53, thus leading to accumulation of p53 protein in the cell. This leads to induction of various p53 target genes involved in cell cycle arrest and apoptosis [168]. Proteins affected by high and low levels of Bmi-1 are shown by black and red arrows, respectively.*Sites of frequent mutations associated with cancer.

CDK4/6: Cyclin-Dependent Kinase 4/6

E2F is a group of genes that codifies a family of transcription factors (TF) in higher eukaryotes.

1.4.3 The Elevated Expression of Bmi-1 in Many Carcinomas and Cancer Cell Lines

The modulation of Bmi-1 is observed in many tumor tissues. There are increasing evidences that dysregulated expression of Bmi-1 contributes to cancer development. The elevated Bmi-1 expression has been found in a variety of human carcinomas, including mantle cell lymphomas [169], non-small cell lung cancer (NSCLC) [170], B-cell non-Hodgkin's lymphoma (B-NHL) [171], colorectal cancer [172], prostate cancer [17], nasopharyngeal carcinoma (NPC) [22], hepatocellular cancer [173], bladder cancer [174], gastric cancer [24], tongue cancer [175], ovarian carcinoma [176], chronic myeloid leukemia (CML) [177] and breast cancer [16]. In many carcinomas, the overexpression levels of Bmi-1 shows converse correlations with clinicopathologic characteristics, such as larger tumor size in NPC and bladder cancer [22, 174], ascending histological grade in ovarian cancer, B-NHL and glioma [171, 176, 178], advanced clinical stage in ovarian cancer, colon cancer and bladder cancer [23, 174, 176, 179], the increasing depth of invasion in colon cancer and bladder cancer [23, 174]. The high Bmi-1 expression not only implies shorter overall survival time of patients, but also it is a significant and independent prognostic parameter in HCC [173], ovarian carcinoma [176], NPC [22], colon cancer [23], oligodendroglial tumor [180], acute myeloid leukemia (AML) [181], glioma [178], and pancreatic cancer [182]. In addition, the recurrence often occur in patients with high Bmi-1 expression in tongue cancer [175], bladder cancer [174] and breast cancer [18].

Additionally, Bmi-1 has been shown to be involved in the tumor progression and metastasis of invasive ductal breast cancer [16] and colon cancer [23]. Strong positive signals with more intense staining patterns are observed in the invading fronts than in the central portions of primary invasive ductal breast cancers [16]. A high level of Bmi-1 expression is significantly correlated with lymph node metastases [16, 23] and distant metastasis [23]. These findings suggest that Bmi-1 could be involved in the metastasis of breast cancer and colon cancer.

Interestingly, the increased expression of Bmi-1 is incremental depending upon the magnitude of colon cancer progression from normal tissue, colon cancer, to lymph node metastasis (LNM) [23]. Furthermore, the level of Bmi-1 expression in chronic myeloid leukemia (CML) is significantly higher in the chronic phase (CP) than in controls and further increased during the course of the disease progression (control--5.66%; CP--36.93%; the accelerated phase (AP) and blastic crisis (BC)--76.41%) [177]. Bmi-1 is also useful as a novel molecular marker for predicting progression of myelodysplastic syndrome (MDS). The higher positivity rate of Bmi-1 is preferentially seen in refractory anemia with excess blasts (RAEB), RAEB in transformation (RAEB-T), and MDS-AML compared with refractory anemia (RA) and RA with ringed sideroblasts (RARS) [183]. These findings suggest that Bmi-1 may contribute to an increased aggressive behavior of cancer cells and give new insight into the understanding of its molecular mechanisms and serve as the potential targets for future

carcinoma treatment. Therefore, the examination of Bmi-1 protein expression is potentially valuable in prognostic evaluation of carcinoma.

On the other hand, Bmi-1 expression is an early event in the progress of cancer. Bmi-1 is upregulated in hepatocellular carcinoma (HCC) with ATP-binding cassette transporter B1 (ABCB1), as a new potential target for Bmi-1, and in particular it is highly expressed in early and well differentiated HCC [184]. The level of Bmi-1 is markedly enhanced in mild epithelial dysplasia, carcinoma *in situ* and oral squamous cell carcinomas (OSCC) compared with that of NHOK. This indicates that Bmi-1 expression can occur at a very early stage in oral carcinogenesis [185]. Low-, intermediate-, and high- grade B-NHLs are also associated with increased co-expression of the Bmi-1 and EZH2 PcG proteins. The extent of Bmi-1/EZH2 (Enhancer of Zeste Homolog 2) coexpression correlates with clinical grade and the presence of Mib-1/Ki-67 expression, suggesting that irregular expression of Bmi-1 and EZH2 is an early event in the formation of B-NHL [171]. In addition, Bmi-1 expression is higher in intestinal metaplasia than in gastric cancer, indicating that Bmi-1 expression is involved in the mechanism that determines malignant potential, and may play a role in the occurrence and development of gastric cancer [186]. Although Bmi-1 mRNA expression is found to be elevated in cancerous tissues in comparison to non-cancerous tissues obtained from identical patients, Bmi-1 mRNA is highly expressed even in the early clinical stages of breast cancer [21].

The elevated expression of Bmi-1 is also seen in many cancer cell lines, except the presence in tissues and plasma of breast cancer. The invasiveness and colony formation of gastric cancer -AGS cells are decelerated by Bmi-1 knockdown [48, 187]. Furthermore Bmi-1 can modulate EMT and lead to the stabilization of Snail, a transcriptional repressor associated with EMT, via modulation of PI3K/Akt/GSK-3β signaling [161]. Bmi-1 knockdown in human medulloblastoma DAOY cells results in the loss of clonogenic survival, anchorage-independent growth, and suppression of tumor formation in nude mice [188]. Neuroblastoma development in MYCN transgenic mice, an animal model for the human disease, is also associated with a marked increase in the levels of Bmi-1 expression. However, down-regulation of Bmi-1 impairs the ability of neuroblastoma cells to grow in soft agar and induce tumors in immunodeficient mice [165].

On the contrary, no difference in the expression patterns and stainings of Bmi-1 is observed in tumor cells and surrounding noncancerous cells in hepatocellular carcinoma [173]. In renal clear cell carcinomas Bmi-1 is a differentiation marker that is lost in carcinomas with high malignancy rather than an oncogene involved in tumor progression [189]. The loss of Bmi-1 seems to be associated with an aggressive phenotype in endometrial carcinomas. Bmi-1 expression is significantly weaker in tumors with vascular invasion, deep myometrial infiltration, and loss of ER or PR [190]. In melanomas, the low level of Bmi-1

expression predicts poor survival. The loss of Bmi-1 expression is also associated with features of aggressive tumors, such as increased tumor cell proliferation, presence of necrosis and increased expression of both N-cadherin and β-integrin, indicating a more invasive and mesenchymal phenotype [191]. Choi *et al* has also reported that Bmi-1 expression in tumor tissues is associated with favorable prognosis in breast cancer patients. In addition, this correlation of Bmi-1 expression with favorable overall survival is maintained in patients underwent uniform chemotherapy, regardless of undergoing adjuvant chemotherapy [20]. Bmi-1 protein is only detected in 25% African breast cancer patients and associated with a low histological grade [19]. Meanwhile, higher Bmi-1 mRNA expression has been observed in patients with the early stages and without lymph node metastasis [21].

1.4.4 Bmi-1 Dependence Distinguishes Stem Cell Self-renewal from Progenitor Proliferation

Apart from its role in oncogenesis, Bmi-1 has been shown to be required for self-renewal of hematopoietic stem cells and neuronal stem cells [15, 192-194]. An emerging concept in the field of cancer biology is that a rare population of 'tumor stem cells' exists among the heterogeneous group of cells that constitute a tumor. This concept indicates that stem cell function (whether normal or neoplastic) might be defined by a common set of critical genes. Forced expression of Bmi-1 is able to enhance symmetrical cell division of hematopoietic stem cells (HSCs) and leukaemic stem cells, mediate a higher probability of inheritance of stemmness through cell division. Correspondingly, forced expression of Bmi-1, but not the other PcG genes, can lead to a striking expansion of multipotential progenitor *in vitro* and marked augmentation of the repopulating capacity of HSC and leukaemic stem cells *in vivo*. However, loss-of-function analyses reveal that among PcG genes, the absence of Bmi-1 is preferentially linked with a profound defect in HSC self-renewal and leukaemic stem cells [15, 192]. Furthermore, Bmi-1 is required to promote neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the $p16^{INK4a}$ and $p19^{ARF}$ senescence pathways [164, 193]. Bmi-1 plays a key role in the self-renewal of stem cells in the peripheral and central nervous systems but not in their survival or differentiation [194]. Thus, Bmi-1 dependence distinguishes stem cell self-renewal from restricted progenitor

proliferation in the central and peripheral nervous systems tissues [194].

In addition, evidences show that Bmi-1 regulates self-renewal of normal and cancer stem cells in breast, and that modulation of Bmi-1 expression in mammosphere-initiating cells alters mammary development in a humanized nonobese diabetic-severe combined immunodeficient (SCID) mouse model [195, 196]. A lot of evidence support that Bmi-1 can mediate the Hedgehog signaling. Hedgehog signaling components are highly expressed in normal human mammary stem/progenitor cells cultured as mammospheres and these genes are down-regulated when cells are induced to differentiate. Activation of Hedgehog signaling increases mammosphere-initiating cell number and mammosphere size, whereas inhibition of the pathway results in a reduction of these effects [195]. Thus, Bmi-1 is necessary for the maintenance of breast cancer stem cells.

1.5 Epithelial-Mesenchymal Transition

1.5.1 The Epithelial-Mesenchymal Transition in Tumor Invasion and Metastasis

During embryonic development, EMT has been observed to underlie a variety of tissue remodeling events including mesoderm formation, neural crest formation, cardiac valve formation and secondary palate formation [197]. EMT also is involved in some fibrosing conditions subsequent to tissue injury [198-200]. Mesoderm formation and neural crest development represent the key EMT programs that occur during early embryonic development, resulting mesenchymal and neural crest cells maintain oliogopotentiality, and enabling them to further differentiate into various cell types. In contrast, heart valve development and secondary palate formation occur in relatively well-differentiated epithelial cells that are destined to become defined mesenchymal cell types. The latter two processes, which occur in well-differentiated epithelia, raise the possibility that EMT may also be induced under certain physiological or pathological conditions in adult tissues, including tumor invasion and metastasis processes that will be discussed below.

The concept of the multistep carcinogenesis in favor of the tumor progression being a stepwise accumulation of genetic alterations has been observed in several tumor types. Local invasion can be considered as an initial and essential step in the malignancy of carcinomas, leading to the generation of usually fatal

distant metastasis. Tumor invasion appears to be controlled by a coordinated series of cellular and molecular processes that enable tumor cells to dissociate and migrate from primary tumor [201]. Metastasis occurs when genetically unstable cancer cells adapt to a tissue microenvironment that is distant from primary tumor. This process involves both the selection of traits that are advantageous to cancer cells and the concomitant recruitment of traits in the tumor stroma that accommodate invasion by metastatic cells [202]. After acquiring the EMT ability, cancers are prone to metastasize and establish secondary tumors at distant sites [31, 32]. The changes in cell adhesion and migration during tumor invasion are reminiscent of an important developmental process termed EMT, a process that also has an active role in other stages of the metastatic cascade such as intravasation [33, 202, 203]. The first step to invasion relies on looser cell-cell contacts at the tumor leading edge.

EMT is a highly dynamic, multistep process requiring the accomplishment of some key events. Cells undergoing EMT are characterized as losing their epithelial morphology, reorganizing their cytoskeleton and acquiring a motile phenotype through the up- and down-regulation of several molecules including tight and adherent junction proteins and mesenchymal markers [204] (Figure 1.5.1.1 and 1.5.1.2). Morphology often changes in EMT. Cells lose their baso-apical polarization and acquire front-rear polarization, which are both necessary for cell migration. By EMT, carcinoma cells with higher motile ability

are able to invade by acquiring characteristics similar to embryonic mesenchymal cells, thereby allowing penetration of the stroma surrounding the initial neoplastic focus (Figure 1.5.1.2). Similarly, EMT can also facilitate intravasation of tumor cells into blood or lymph vessels, thus leading to dissemination to distant sites (Figure 1.5.1.1). As embryonic EMT is often followed by the reverse process, mesenchymal-epithelial transition (MET), EMT could be a transient phenomenon during progression of many carcinomas. Therefore, tumor cells would re-acquire epithelial characteristics once they accomplish the invasion step [205].

Exposure of the tumor cells to growth factors present in the interstitial compartment could activate tyrosine or serine/threonine kinase receptors on the epithelial basal surface, thereby initiating cell dissociation and stimulating EMT. Similarly, direct contact of tumor cells with the interstitial-type collagens of the stroma could also concur to promote EMT [206]. EMT can be prompted by various intrinsic signals (e.g. gene mutations) as well as extrinsic signals (e.g. Growth Factor Signaling). Although the molecular bases of EMT have not been completely elucidated, a lot of signal molecules potentially involved have been identified. Among the growth factors known to induce EMT are Transforming Growth Factor β (TGF-β) [207], Hepatocyte Growth Factor (HGF) [208], members of EGF family [209], Insulin-like Growth Factor (IGF) [210], Fibroblast Growth Factor (FGF) [201, 211] and Slug [212]. Recently, changes in

the composition of the extracellular matrix (ECM) are also able to induce EMT, as shown for collagen I and hyaluronan [213].

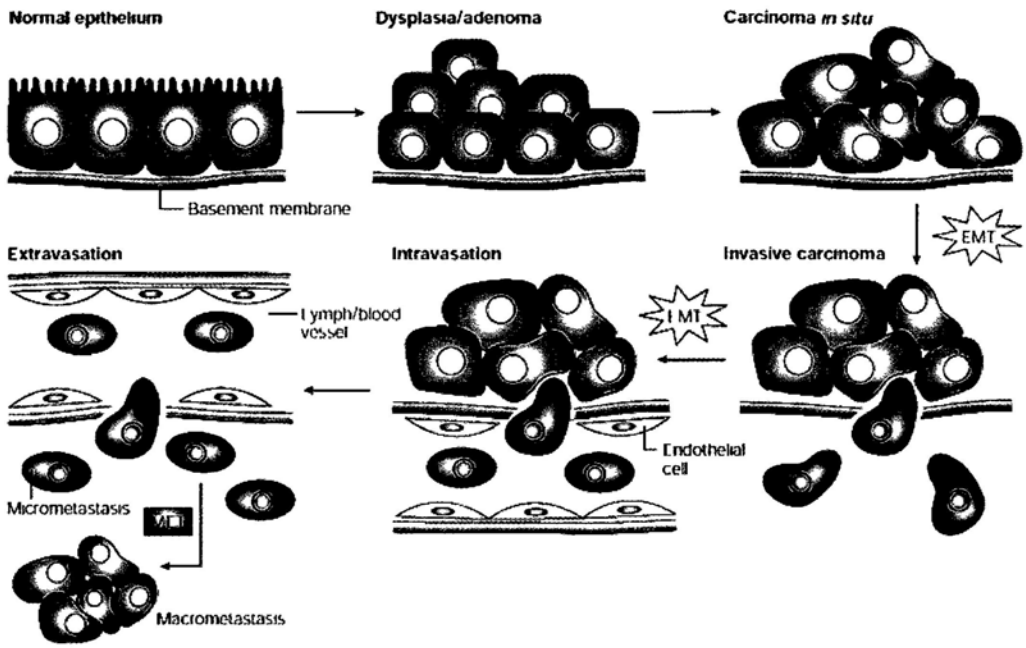

Figure 1.5.1.1 EMT contributes to cancer progression. Progression from normal epithelium to invasive carcinoma goes through several stages. The invasive carcinoma stages involve epithelial cells losing their polarity and detaching from the basement membrane. The composition of the basement membrane also changes, altering cell-ECM interactions and signaling networks. The next step involves EMT and an angiogenic switch, facilitating the malignant phase of tumor growth. Progression from this stage to metastatic cancer also involves EMT, enabling cancer cells to enter the circulation and exit the blood stream at a remote site, where they may form micro- and macro-metastases, which may involve MET and thus a reversion to an epithelial phenotype [33, 214].

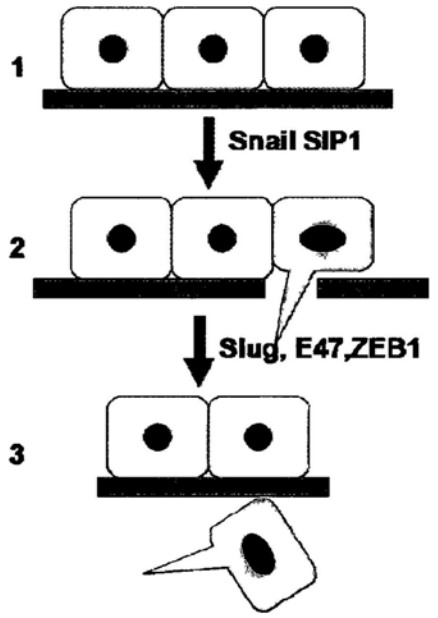

Figure 1.5.1.2 EMT process. (1) At first, tumor cell express E-cadherin, are cohesive and stably immotile. EMT inducers act on these cells and make them competent for invasion by repressing E-cadherin transcription and by enhancing their migratory potential. Therefore, these cells detach from their neighbors, and migrate into the underlying stroma. (2) Snail and SIP1 (Smad-interacting, multi-zinc finger protein) would play a role in the first step, leading to the initiation of the process through the down-regulation of E-cadherin and making tumor cells receptive to further EMT-inducing stimuli. (3) Other repressors, such as Slug, E47 and possibly ZEB1, might contribute to the maintenance of the invasive mesenchyme-like phenotype by keeping repressed E-cadherin transcription, and promoting ECM degradation and cell migration [199].

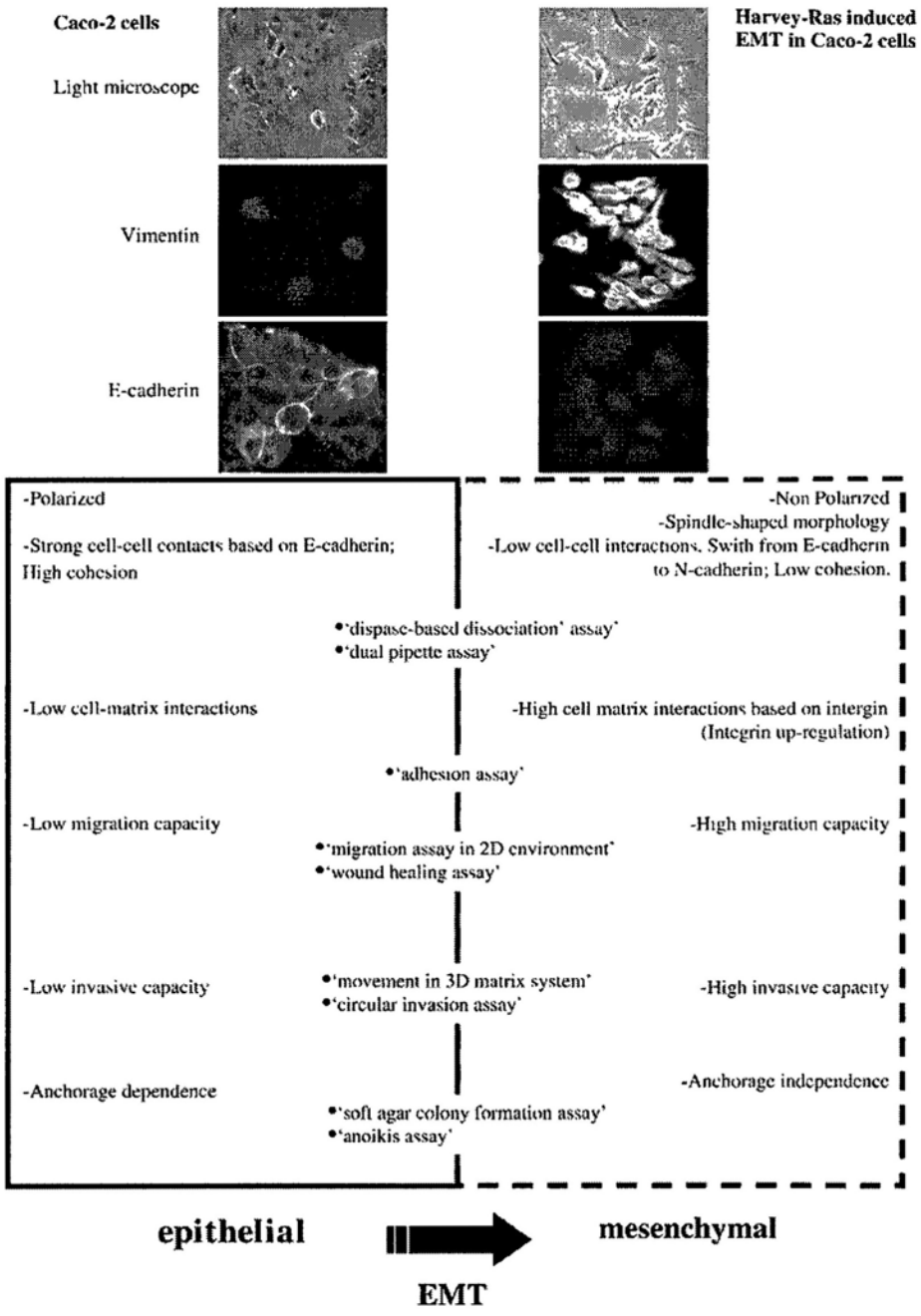

Figure 1.5.1.3 The characteristics of epithelial-mesenchymal transition.

Images show the particular cell morphology of Harvey-Ras induced EMT in the intermediate adenoma Caco-2 colorectal cell line. Immunofluorescence analysis of Vimentin and E-cadherin are adapted from [215, 216]. The table summarises cells properties that are modified during EMT (on each side of the table) and indicates some *in vitro* methods to identify them (in the center of the table).

1.5.2 EMT Related Markers

1.5.2.1 E-cadherin

1.5.2.1.1 Loss of E-cadherin in Many Cancers

E-cadherin is the prototype family member of classical cadherins, the single-span transmembrane glycoproteins that interact in a calcium-dependent, homophilic manner with E-cadherin on neighboring cells [204]. The functional loss of E-cadherin (encoded by *CDH1*), is considered a key step in the progression of cancer and it is also a fundamental event in EMT [217, 218]. E-cadherin is the main adhesion molecule of epithelia [219], and loss of E-cadherin is linked to poor prognosis, tumor progression and metastasis in many human epithelial cancers, such as breast cancer [220] and colorectal cancer [221]. E-cadherin acts as a tumor suppressor against invasion and metastasis, and its function is abolished during the malignant progression of most carcinomas by a variety of mechanisms [222].

1.5.2.1.2 Transcriptional Regulation of E-cadherin

Detailed analyses of the human E-cadherin promoter have identified E-box elements are responsible for its transcriptional repression in non-E-cadherin-expressing mesenchymal cells [197]. The zinc-finger transcription factors directly bind to the E-boxes of the E-cadherin promoter and repress transcription of E-cadherin, including Slug [223], Snail [38, 39], ZEB1 [224] and ZEB2 [225]. More recent studies have found that ZEB1, Snail, and Slug are capable of repressing the transcription of several polarity factors,

including Crumbs3 and Lgl2 [226, 227], indicating their roles in suppressing critical components of epithelial cell traits. In addition to these zinc-finger transcription factors that have a high affinity for the E-box elements of the E-cadherin promoter, transcription factors belonging to other families also regulate EMT in culture and during development. E47, a widely expressed bHLH transcription factor with lower affinity, has been shown to repress E-cadherin transcription in Madin-Darby canine kidney (MDCK) cells directly by binding to E-boxes in the E-cadherin promoter [228].

1.5.2.1.3 Posttranscriptional Regulation of E-cadherin

E-cadherin-mediated cell-cell adhesion complexes are anchored to the actin cytoskeleton via its cytoplasmic domain and β-Catenin [204]. Thus, the formation of E-cadherin-mediated cell-cell adhesion fundamentally modulates the organization of cytoskeleton. A lot of findings expand E-cadherin's functional repertoire beyond its adhesive functions and emphasize the critical role of E-cadherin as a regulator of signaling complexes. Accumulating evidences suggest that receptor and non-receptor tyrosine kinase, such as Met and Src, can phosphorylate both β-Catenin and the short cytoplasmic tail of E-cadherin that are associated with adherens junctions. Phosphorylation of E-cadherin promotes its binding to a Hakai E3-ligase. Ubiquitinated β-Catenin is degraded in the proteasome, while ubiquitinated E-cadherin-Hakai complexes are internalized to endosomes and thereafter recycled back to the cell surface or

degraded in lysosomes. Therefore, activation of tyrosine kinases in carcinoma cells could lead to rapid disruption of cell adhesion and a scattering phenotype [229, 230].

1.5.2.1.4 E-cadherin Mutations

Genomic alterations of the *CDH1* gene, causing loss-of-function of E-cadherin, have been identified in a variety of tumors. In breast cancer, somatic E-cadherin mutations are reported exclusively for sporadic lobular subtype, however not for breast cancers of other histopathological subtypes [231]. Furthermore, endometrial and ovarial carcinomas are found to burden somatic mutations of the *CHD1* gene, as well as in primary gastric cancers and gastric cancer cell lines [232]. In addition, germline mutations of the *CHD1* gene are also discovered in patients, predisposed to suffer from diffuse gastric cancer [233-235]. Another means to loose E-cadherin expression in tumor cells is CpG-island hypermethylation of the *CDH1* gene promoter [236, 237]. Aberrant methylation of 5'CpG islands in the promoter region of *CDH1* was reported for different types of mammary carcinomas and increased with malignant progression [238, 239]. Methylation are found in thyroid, hepatocellular, and prostate cancer, as well as in laryngeal cancer and colorectal cancer [240, 241]. The gain of mesenchymal N-cadherin expression plays a crucial role in EMT, besides the loss of epithelial E-cadherin. Cadherin switch leads to a drastic change in the adhesive properties of a cell, as it loses its affinity for epithelial

neighbors and gains affinity for mesenchymal cells, such as fibroblasts or vascular endothelial cells. N-cadherin is upregulated during progression of breast cancer where its expression correlates with poor prognosis [242]. The characterization of E-cadherin regulation during malignant progression has provided important insights into the molecular mechanisms implicated in tumor invasion [201]. Taken together, the loss of functional E-cadherin, and thus of adherens junction-mediated cell-cell contacts, enables the first step of metastasis: local invasion and dissemination of cancer cells from the main tumor mass.

1.5.2.2 β-Catenin

In addition to E-cadherin, Catenins has been found to be inactivated in human cancer [243]. In normal fibroblasts and endothelial cells, β-Catenin staining is limited to cytoplasm and/or cell membranes, and it is strictly membranous in normal epithelial cells. However, β-Catenin appears to be mainly localized in the nuclear in mesenchymal cells [244]. β-Catenin accumulation in the nucleus, which is often associated with loss of E-cadherin expression, correlates with susceptibility to enter into an EMT and acquisition of an invasive phenotype [33, 245]. The budding tumor cells accumulate β-Catenin in the nucleus, which is a direct factor for prognosis and thus of utmost clinical importance [246, 247] (Figure 1.5.2.2.1). When β-Catenin is released in the cytosol, it is phosphorylated in a complex containing the adenomatous polyposis coli (APC) protein and GSK3β (Glycogen Synthase Kinase), responsible for leading β-Catenin to degradation through the ubiquitin–proteasome system [248]. GSK3β phosphorylation allows β-Catenin to accumulate in the cytoplasm and translocate to the nucleus [249].

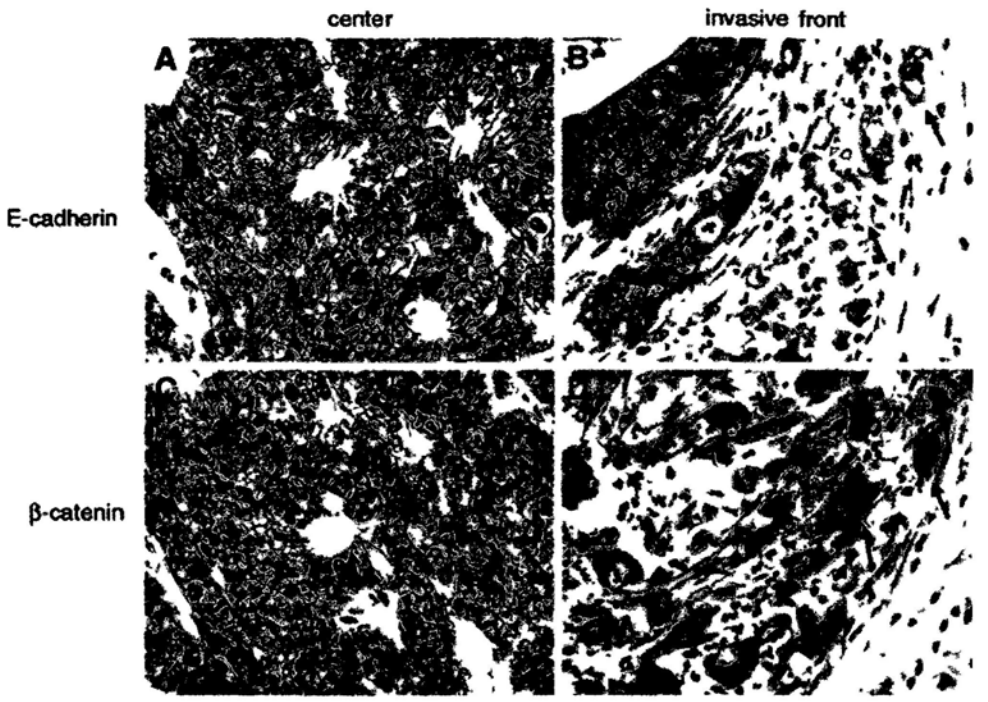

Figure 1.5.2.2 Nuclear accumulation of β-Catenin at the tumor invasion front is associated with EMT. Heterogenous expression of E-cadherin (A and B) and β-Catenin (C and D) in human colorectal adenocarcinomas. Serial sections of central (A and C) and invasive (B and D) areas of the same tumor. Note membranous staining of β-Catenin and E-cadherin in central, differentiated tumor areas, characterized by tubular structures, built up by more or less polarized epithelial tumor cells (arrows in A, C). Dedifferentiation towards a mesenchyme-like phenotype of tumor cells at the invasive regions, with loss of membranous E-cadherin and β-Catenin, but cytoplasmic E-cadherin and nuclear and cytoplasmic β-Catenin (arrows in B, D) [33, 250].

1.5.2.3 Fibronectin and Vimentin

Fibronectin plays a major role in the adhesion of many cell types. The extent of cell adhesion *in vitro* is related not only to the ability of the cells to interact with matrix-bound fibronectin, but also to the synthesis or lack of synthesis of fibronectin by the cells, and to the lack of deposition of synthesized fibronectin into an insoluble matrix surrounding the cells [251]. Vimentin is a mesenchymal marker most commonly associated with EMT. Its elevated and aberrant expression correlates well with up-regulated migration and invasion by cancer cells, coupled with down-regulated epithelial marker expression as expected with EMT [252, 253]. Vimentin is selectively expressed in aggressive breast cancer cell lines, where it correlates with EMT-like changes such as declined expression of cytokeratins and up-regulation of other mesenchymal markers [253, 254].

1.5.3 Different Signaling Pathways Provide the Necessary Stimuli Triggering EMT

The complexity of the interactive downstream effective pathways increases with the diversity of signals inducing EMT. Among the candidates which are engaged by TGF-β-induced EMT are the small GTPases RhoA and Rac1 [255, 256], Ras [257], phosphoinositol-3 kinase (PI3K) [258], integrin-linked kinase (ILK) [259], and the Jagged1/Notch signaling pathway [207]. Phosphorylation and dephosphorylation are key phenomena in intracellular signaling and it is known that the elevated expression and/or activation of tyrosine kinases are/is a molecular feature of the migratory phenotype implicated in EMT/invasion. The activation of the receptor tyrosine kinases subsequent to specific ligand binding initiates a series of cytoplasmic transduction events such as activation of Ras/MAPK, PI3K/Akt, Rho/Rac and Src pathways. Upon growth factor binding, the receptor tyrosine kinases dimerise and autophosphorylate on tyrosine residues which, in turn, act as binding sites for proteins that contain SH_2 domains. Then, several SH_2-containing adaptor molecules such as Grb2 and Shc, or SH_2-containing signaling proteins such as PI3K and Src can be recruited to the cytoplasmic domain of the receptor tyrosine kinase, thereby activating the respective downstream pathways [206]. In various human cancer cell lines and in mouse tumor models, the activation of the TGF-β pathway at the later stages of tumor progression has been shown to promote EMT in carcinoma cells, which allows these cells to invade the ECM in culture and to spread to distant

organs in mice [257, 260]. In various tumor cell lines, TGF-β signaling induces Snail, Slug, and SIP1, which may then proceed to repress the expression of E-cadherin, thereby causing a loss of cell-cell adhesion [197]. The ILK, which interacts with the cytoplasmic domain of β-Integrins, is an intracellular serine-threonine kinase with transducing function that acts downstream of both ECM stimuli. Activated ILK can directly phosphorylate downstream targets such as Akt and GSK-3β. The overexpression of ILK promotes the translocation of β-Catenin to the nucleus, formation of β-Catenin-LEF-1 complexes and induction of EMT [261]. With increasing interest in microRNAs, miR-200 and miR-205 have been recently shown to play an important role in TGF-β-induced EMT by modulating the function of ZEB1 (δEF1) and ZEB2 (Sip1), the transcriptional repressors of E-cadherin gene expression [262]. These findings shed light into a miRNA-mediated regulatory pathway that influences EMT in a developmentally and pathologically relevant setting. Such complexity of interactive signaling upstream and downstream of the induction of EMT also explains why EMT is not a simple matter of changes in a cell's adhesive capabilities or its cytoskeletal organization, it rather represents a fundamental reprogramming of almost every aspect of a cell's biology [204]. Together, these data suggest that in response to various inductive signals, EMT-inducing transcription factors may serve as major signaling mediators of the EMT program to promote metastasis.

The Snail families of transcriptional repressors regulate various aspects of EMT during embryonic development as well as the participation in tumor progression. Snail expression has been detected in different invasive carcinoma and melanoma cell lines and, importantly, in invasive regions of squamous cell carcinomas and dedifferentiated ductal breast carcinomas and hepatocarcinomas [34, 222]. Snail binds to E-box consensus sequences in the E-cadherin promoter with the help of local modifications of chromatin [263]. In addition to being tightly regulated at the transcriptional level, Snail factors undergo posttranslational modifications that control their nuclear localization or degradation. These modifications include phosphorylation by PAK and GSK3β, dephosphorylation by SCP (Small C-terminal domain Phosphatase), and lysine oxidation by LOXL2 [201, 264]. Selective phosphorylation by GSK3 leads to export of Snail from the nucleus and to its destruction via the ubiquitin-proteasome pathway [40].

Though EMT processes are documented in many cancer cell models *in vitro*, the significance of EMT and metastasis induced by Bmi-1 during breast cancer progression and even its relevance in human cancer tissues has remained a matter of debate. Systemic spread has been detected from early lesions in HER-2 transgenic mice and in human ductal carcinoma, suggesting that metastasis is not necessarily a late event in tumor progression [263, 265]. The present study focuses on the expression patterns and roles of Bmi-1 in breast

cancer tissues and cells in order to investigate the nature of Bmi-1 in the metastasis of breast cancer. We demonstrate that Bmi-1 is not only increased in breast cancer tissues compared with adjacent non-cancerous tissues but also associated with clinical features, such as tumor size, lymph node involvement, distant metastasis and clinical stages. The high Bmi-1 expression predicts the unfavorable prognosis of the patients and may serve as a high risk factor for breast cancer. Furthermore, we also shed light on the biological impact of Bmi-1 on invasive and metastatic properties of breast cancer cells. The Bmi-1 overexpression raises mobile and invasive properties in immortalized HMECs, concurrent EMT-like molecular changes, the stabilization of Snail and the activation of the Akt/GSK3β pathway. Consistent with these observations, repression of Bmi-1 in highly metastatic breast cancer cells remarkably declines the cell mobility and invasion, as well as tumorigenesis and lung metastases in nude mice. In addition, the repression of Bmi-1 reverses the EMT markers and inhibits the Akt/GSK3β pathway. Taken together, this study provides evidences for the aggressive and metastatic properties of breast cancer withBmi-1 expression.

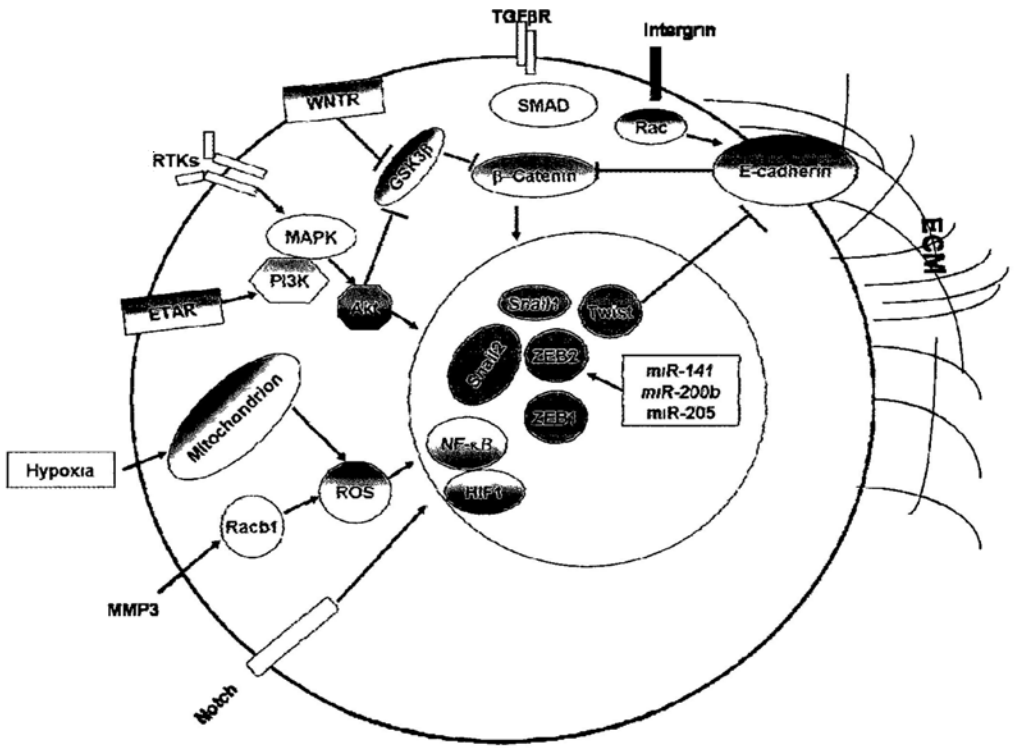

Figure 1.5.3 Signalling networks regulating epithelial-mesenchymal transitions [266]. Selected signalling pathways and some of their downstream effects and interactions are depicted. Receptor tyrosine kinases (RTKs), transforming growth factor-β (TGFβ), Notch, endothelin A receptor (ETAR), integrins, Wnt, hypoxia and matrix metalloproteinases (MMPs) can induce EMTs through multiple different signalling pathways, and the relative importance of each of these may depend on the particular cellular context. EMTs and mesenchymal-epithelial transitions (METs) are associated with dramatic changes in the cytoskeleton and extracellular matrix (ECM) composition and attachment that act together to alter cell morphology. EMT-inducing signals can lead to the disruption of tight junctions and desmosomes through protein phosphorylation (for example PAR6A

phosphorylation by TGF-β signalling) or by repressing protein levels (for example ZEB1 represses plakophilin 3. EMT also results in the dramatic reorganization of the ECM as many EMT-inducing factors upregulate the expression of ECM proteins (such as fibronectin and collagens), proteases (such as MMPs) and other remodelling enzymes (such as lysyl oxidase). Hypoxia, RAC1B activation and activation of certain kinase pathways (such as Akt) may lead to increased mitochondrial production of reactive oxygen species (ROS) that elicit pleiotropic effects, including activation of hypoxia-inducible factor 1α (HIF1α) and nuclear factor-κB (NF-κB), signalling and inactivation of glycogen synthase kinase 3β (GSK3β). Besides the interaction among the various signalling pathways, there is also extensive crosstalk among the EMT-inducing transcription factors and the microRNAs (miRNAs) regulating them. E-cadherin: epithelial Catherin

H/E(Spl): Hairy and Enhancer of split

WNTR: Wnt receptor.

Chapter 2 Materials&Methods

2.1 Tissue Samples

Paraffin-embedded breast cancer samples were obtained from 252 Chinese female patients first diagnosed as breast cancer in 1999-2001 at Cancer Center, Sun Yat-sen University, Guangzhou, China. Of the 252 breast cancer samples, 39 matched adjacent non-cancerous tissues were obtained from the above mentioned patients. Among them, 239 cases had the follow-up records and the median follow-up time was 51.1 months. Clinical follow-up after diagnosis and treatment was based on periodic visits (every 3 months during the first year, every 6 months during the second year, and then yearly until relapse in Sun Yat-sen University). Overall survival (OS) was calculated from the date of diagnosis and to the date of death or last follows up. Clinical and pathologic factors were evaluated including age, TNM classifications, clinical stage, presence of steroid receptors and HER-2. Only 159 cases were tested for the HER-2 expression, while others were not. 147 cases were tested for the ER, PR and HER-2 expression. 13 cases missed the record of age, survival time and survival status, but showed the TNM classification, clinical stages as well as the presence of steroid receptors and HER-2 expression. In order to use these clinical materials for research purposes, prior patients' consent and approval from the Institute Research Ethics Committee were obtained. The observation period was from 1999 to 2006. The clinical stages of all the patients were classified according to the 2002 TNM staging of UICC (International Union against Cancer).

2.2 Immunohistochemistry in Human Breast Cancer Tissues

Commercially available antibody against Bmi-1 (at the dilution 1:100, clone 229F6, Upstate Biotechnology, Lake Placid, USA) was used as the primary antibody. Immunohistochemical kit (SP-9002 Mouse SP Kit) was obtained from Zhongshan Golden Bridge Biotechnology Co. Ltd. (Beijing, China). The 4 μm paraffine-embedded sections of breast cancer were deparaffinized with xylene, rehydrated, and treated with 3% hydrogen peroxide (Zhongshan Golden Bridge Biotechnology Co. Ltd.) in methanol (Sango biotech(shanghai) Co.Ltd) for 10 min to quench the endogenous peroxidase activity. Subsequently, sections were submerged and microwaved in ethylenediaminetetraacetic acid (EDTA, pH 8.0, Zhongshan Golden Bridge Biotechnology Co. Ltd.) for antigenic retrieval. Then the slides were allowed to cool down at room temperature. One percent Bovine Serum Albumin (BSA), (Sigma Aldrich, Inc. St Louis, Missouri, USA) was used to block the non-specific binding for 30 min at room temperature, followed by the incubation of the sections with the primary antibody (BD, Transduction Laboratories, Lexington, UK) overnight at 4°C. After washing with phosphate buffered saline (PBS) (Zhongshan Golden Bridge Biotechnology Co. Ltd.), sections were incubated with biotinylated secondary antibody (Zhongshan Golden Bridge Biotechnology Co. Ltd.) for 15 min at 37°C, further followed by incubation with the streptavidin-horseradish-peroxidase (HRP) complex (Zhongshan Golden Bridge Biotechnology, Beijing, China). The sections were then immersed in 3'-Diaminobenzidine (DAB) (Zhongshan Golden Bridge

Biotechnology, Beijing, China) and counterstained with 10% Mayer's hematoxylin (Zhongshan Golden Bridge Biotechnology, Beijing, China), dehydrated, and mounted in crystal mount (Zhongshan Golden Bridge Biotechnology, Beijing, China). To minimize variations in the immunopositive cells, all sections were stained in DAB (Zhongshan Golden Bridge Biotechnology, Beijing, China) for the same period of time. Two pathologists, blinded to the clinical outcome, scored the results of the staining independently. The positive criteria were estimated as before [167]. For each sample, positive cells were quantified as a percentage of the total number of tumor cells in 4 low power field (40X) and assigned to one of five categories: $\leq$5% of the cells (1 point), 6% to 35% of the cells (2 points), 36% to 70% of the cells (3 points), $\geq$71% of the cells (4 points). The staining intensity was subclassified as negative staining (1 point), weak staining (2 points), moderate staining (3 points) and strong staining (4 points). A final score was then calculated by multiplying the above two scores. If the final score was >4, Bmi-1 expression was considered high, otherwise, Bmi-1 expression was considered low. If majority cells of cancer tissue and non-cancer tissue showed the nucleus staining , we defined Bmi-1 was localized in nuleus. Otherwise, it was cytoplasm staining.

Antibodies for ER, PR and HER-2 from Zhongshan Golden Bridge Biotechnology Co. Ltd (Beijing, China) were used. Protein expressions of ER, PR and HER-2 were also determined by immunohistochemistry. Measurements

of ER, PR and HER-2 were routinely performed as previously described [267]. For evaluation of ER and PR, the fraction of positively staining nuclei was categorized into negative (0-1%), weak (2-10%), medium (11-75%) or high (76-100%). For statistical analyses, variables were dichotomized into 'negative' or 'positive' using the clinically established cut-off for ER and PR at 10% positive nuclei. HER-2 expression was evaluated according to a standard protocol. The protocol categorizes tumors into four groups: grade 0, lack of staining in all tumor cells or membrane staining in less than 10% of the tumor cells; grade 1, weak with not circumferential membrane staining in more than 10% of the tumor cells; grade 2, intermediate with circumferential membrane staining in more than 10% of the tumor cells; and grade 3, intense and circumferential staining in more than 10% of the tumor cells [268]. Patients were categorized as having HER-2-positive if their tumor samples exhibited either gene amplification by fluorescent in situ hybridization technique (FISH) [269] or overexpression (3+) by IHC.

2.3 Cell Lines

Immortalized HMECs (76N-TERT and MCF-10A) and radiation-transformed cells (76R-30) were cultured in Keratinocyte-SFM medium (Invitrogen, Grand Island, NY) supplemented with bovine pituitary extract. MDA-MB-435S cells were maintained in DMEM/F12 (Gibco, U.S.A) supplemented with 10% fetal calf serum (Hyclone, U.S.A). SK-BR-3, ZR-75-30 and BCAP-37 cells were

grown in RPMI 1640 (Invitrogen, U.S.A) with 10% fetal calf serum.

2.4 Vectors, Plasmids and Virus

The Human pMSCV-Bmi-1 and Bmi-1 short hairpin RNA (shRNAi) constructs were generated according to the protocols as described [22, 270]. Bmi-1 was amplified by the polymerase chain reaction (PCR) using cDNA from human breast cancer tissues. Wild type Bmi-1 cDNAs were cloned into the pBabe-puro retroviral vector (pB0) obtained from Prof. Vimla Band (Eppley Institute for Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, USA). Bmi-1 short-hairpin RNAs (shRNAs) were designed and cloned in the retroviral vector pRS (retro-super) obtained from Oligoengine Company (Seattle, WA). The sequences of shRNAi were as follows:

shBmi-1 1# GUUCACAAGACCAGACCAC and shBmi-1 2# GACCAGACCACUACUGAAU [270].

Infectious virus was produced by transfecting retroviral vectors and the pIK packaging vector into 293T cells [271]. Virus-containing supernatants were centrifuged to remove cell debris and stored at -80°C. The retroviral supernatants were quick thawed at 37°C and used to infect cells for three sequential infections, each for 4 h with the presence of Polybrene (4 μg/ml) [14]. Retrovirus expressing Bmi-1 was produced and transfected into 76N-TERT and MCF-10A cells. The plasmid of shBmi-1 was introduced into MDA-MB-435S cells, which

showed strong ability of metastasis [272]. pMSCV and pRS scrambled RNAi were used as control vectors. All retrovirus infected cells were maintained under Puromycin (Sigma Aldrich, Inc. St Louis, Missouri, USA) selection and used as stable cells.

2.5 Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from fresh tissues and cell lines by Trizol reagent (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. About 5-10 x 10^6 cells or 30 mg tissue was homogenized in 1ml Trizol and incubated at room temperature for 10 min to permit the complete dissociation of protein complexes. After 0.2ml chloroform (Sango biotech(shanghai) Co.Ltd) was added to the each tissue, the mixture was shaken vigorously for 15 sec and sat at room temperature for another 3 min and centrifuged at more than 12,000 g for 15min at 4°C. Following the centrifugation, the mixture was separated into a lower red phonel-chloroform, an interphase and a colorless upper aqueous phase. The aqueous phase containing RNA was transferred to the new centrifuge tube, precipitated by 0.5ml isopropyl alcohol (Sango biotech(shanghai) Co.Ltd), incubated at room temperature for 10min and centrifuge at no more than 12,000g for 10 min at 4°C. The RNA precipitate, often invisible before centrifuge, formed a gel-like pellet on the side and bottom of the tube. After removing the supernatant, the RNA pellet was washed twice with 75% ethanol (Sango biotech(shanghai) Co.Ltd), air-dried and re-dissolved with RNase-free

water (Invitrogen, Grand Island, NY). The quality and quantity of total RNA were determined by measuring the absorbance at 260/280mm by NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

1.0 milligram of total RNA treated with DNAase (QIAGEN Comany) was used for cDNA synthesis with random hexamers. Full-length open reading frame of Bmi-1 was amplified by PCR from cDNA samples of cancer tissues and cell lines. Genes were amplified by PCR from cDNA (primers as listed in Table 4). The products were analyzed by agarose gel electrophoresis and confirmed by appropriate size and/or sequencing.

2.6 Real-Time Quantitative Polymerase Chain Reaction

Real-time quantitative PCR was carried out using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Reactions were performed by triplicate repeats in two independent experiments. The geometric mean of the GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) housekeeping gene was used as an internal control to normalize the variability in expression levels. Quantitative PCRs were performed with an SYBR Green Supermix Kit (ABI) according to the manufacturer's protocol. Cycling conditions were as follows: initial denaturation (10 min at 95°C) followed by 42 cycles of denaturation (10 sec at 95°C), annealing (20 sec at 58°C), and elongation (1 min at 72°C). ΔΔCt (the comparative Ct) represented the

difference in cycle thresholds between Bmi-1 and GAPDH. Standard curves were generated on the basis of the linear relationship between the first cycle number at which the fluorescence signal significantly increased (Ct value) and the logarithm of the starting quantity. The gene expression was analyzed using the relative quantification $2^{-\Delta\Delta CT}$ method [273]. The primers were provided in Table 2.6.1.

Table 2.6.1 List of Primers

	Gene	Forward primer	Reverse primer
RT-PCR	*Bmi-1*	CTGGTTGCCCATTGACAGC	CAGAAAATGAATGCGAGCCA
	GAPDH	AGCCGTTCGGAGGATTATTCG	CTTCTCCTCAGCAGCCAGAG
Real-time PCR	*Bmi-1*	CTGGTTGCCCATTGACAGC	CAGAAAATGAATGCGAGCCA
	E-cadherin	GAACAGCACGTACACAGCCCT	GCAGAAGTGTCCCTGTTCCAG
	GAPDH	GACTCATGACCACAGTCCATGC	AGAGGCAGGGATGATGTTCTG

2.7 Western Blotting Analysis

Cells and tissues were harvested by lysis buffer and centrifuged for 15 min at 500g. Proteins were heat inactivated, separated by SDS-PAGE gel (Sigma Aldrich, Inc. St Louis, Missouri, USA) and followed by trans-blotting onto equilibrated polyvinylidene difluoride (PVDF) (Millipore company Billerica, MA) membrane. Then, the membranes were blocked by incubation in 5% non-fat milk (Royal Numico N.V.) for 1 hour at room temperature and probed with mouse anti-Bmi-1, anti-E-cadherin, anti-β-Catenin, anti-Fibronectin and anti-Vimentin antibodies (BD, Transduction Laboratories, Lexington, UK), as well as rabbit anti-p-GSK, anti-t-GSK (Cell Signaling Technology, Inc. USA), anti-Snail (Abcam, Cambridge Science Park, Cambridge, UK), anti-p-Akt (Santa Cruz Biotechnology, CA, USA)

and goat anti-t-Akt (Santa Cruz Biotechnology, CA, USA) antibodies. After primary antibodies were incubated at 4°C overnight and washed by TBST for three times, the membranes were replaced with second antibody/HRP conjugate (Sigma Aldrich, Inc. St Louis, Missouri, USA) for 45 min at room temperature. After three washes with TBST, the members were developed with detection reagent. The membranes were stripped and reprobed with mouse anti-α-Tubulin (Sigma Aldrich, Inc. St Louis, Missouri, USA) to confirm equal loading of the samples.

2.8 Proliferation Assay

$1*10^5$ cells were plated on the P60 plate. Every 24 h, cells were trypsinized and counted at light microscope for at least three times until the sixth day. Experiments were repeated a minimum of three times.

2.9 Wound Healing Assay

Cells were seeded in six-well plates and cultured under permissive conditions until 80% confluence, which is important for cells to be starved without becoming confluent during the 24 hour starvation period. Then the medium containing serum was removed and the cells were gently rinsed with PBS to remove the serum. After starving for 24 h in medium without EGF or FBS and removing media from cells, the monolayer confluent cells were lightly and quickly scratched with a pipette tip produce a straight line. The wound healing assays were performed in growth factor-free medium, which further excluded any effect due to a potential

proliferation difference. The loosely cells were removed with PBS washing and the wound area was free of cells. The images of the open gap were captured microscopically at the beginning and at regular intervals during cell migration to close the wound, and compared the images to quantify the migration rate of the cells at a 200 x magnification. The migration activity was calculated by the number of cells entering into rectangle. Experiments were repeated a minimum of three times.

2.10 Boyden Chamber Assay

This assay measures the ability of cells to invade a thin film of Matrigel matrix overlying a membrane containing 8-μm pores. Matrigel resembles the complex extracellular environment found in many tissues. Matrigel (BD Biosciences, USA) was thawed at 4°C and diluted at desired working concentration in serum free-cold cell culture media. The upper chamber of 24-well transwell was coated with 100 microliter Matrigel and air-dried at 37°C incubation at least 4 to 5 h for gelling. 2 x 10^4 cells were seeded in the medium deprived of EGF or FBS in the top chamber (BD Biosciences, USA), whereas the medium with EGF or FBS was added to the bottom chamber. With appropriate time cultivation depending on the invasiveness of the cells, cells were removed on the topside of the filter by scrubbing twice with cotton tipped swab moistened with no FBS medium. Then, the chambers were fixed with 4% paraformaldehyde (Sango Biotech (shanghai) Co.Ltd) and stained with hematoxylin. The number of cells in random ten fields

of view was randomly enumerated at 200 x magnification for each filter. Three independent experiments were performed and the data are presented as the mean ± SD.

2.11 Three Dimensional Matrigel Culture

Three-dimensional (3D) epithelial culture models are widely used to promote a physiologically relevant microenvironment for the study of normal and aberrant epithelial organization [274]. Acinar structures grown in Matrigel can be monitored for invasive potentiality. Development of multicellular protrusion and development of interacinar bridges were observed as indicators of invasive behavior [275]. 100 microliter Matrigel was evenly coated on the bottom of a 24-well plate with a thin layer and polymerized at 37°C incubation. Cells were suspended in the growth medium containing 2% Matrigel and plated onto the pre-coated surface. These cells were cultivated in 37°C incubation for two weeks and the morphologic phenotype alteration was monitored at 200 x magnification every second day. Experiments were repeated a minimum of three times.

2.12 Anchorage-Independent Growth in Soft Agar

The soft agar assay was used to determine the ability of anchorage dependent growth. Noble agar (Sigma Aldrich, Inc. St Louis, Missouri, USA) at 1.3% and 0.66% was melted in microwave and cooled to 50°C in water bath to maintain the

liquid phase. Equal volumes (1ml) of 2 x medium and 1.3% agar as the basal layer were thoroughly mixed, poured to the 60-mm dish (BD Corp) and incubated at 37°C to solidify. Cells were kept in a single suspension in 2 ml of growth medium, which was the mixture containing equal volumes of 2 x medium and 0.66% agar, and plated onto the bottom layer. The cells were fed every two days with 1 ml medium. Colonies were photographed and counted in ten fields of view randomly at 200 x magnification using light microscopy. Each experiment was done in triplicates.

2.12 Confocal Immunofluorescence Microscopy

For the immunofluorescence experiments to detect the localizations of Snail protein, cells were seeded onto the glass slides which sit on the 24-well plate for 24 h, washed twice with PBS, fixed in freshly prepared 4% paraformaldehyde (Sango Biotech (shanghai) Co.Ltd) on ice for 20 min. The cells were washed twice for 3 min with PBS and permeabilized with 0.5% Triton X-100 (Sango Biotech (shanghai) Co.Ltd) for 5 min at room temperature. After blocking with BSA (Sigma Aldrich, Inc. St Louis, Missouri, USA), cells were stained with the anti-Snail primary antibody (Abcam, Cambridge Science Park, Cambridge, UK) followed by the incubation with FITC-conjugated anti-rabbit IgG (eBioscience, San Diego, USA). To visualize the nucleus, 4' 6-Diamidino-2-phenylindole (DAPI) staining (eBioscience, San Diego, USA) was also done, as previously described [276]. Immunofluorescence was detected by fluorescence microscopy

(Olympus, Japan).

2.13 Mice injections, Necropsy, Histopathology

The ability to form tumors and metastasize was measured by injecting the Bmi-1-repression cells into nude mice. Mice were bred and maintained under specific pathogen free (SPF) conditions in the Department of Animal Center, Cancer Center, the Chinese University of Hong Kong, as approved by the China Care Committee Institute. Ten nude mice, which were four to six weeks old, female, young, and healthy, were used for each group randomly. Each mouse was injected in the fat pad with $1x10^6$ MDA-MB-435S control cells and MDA-MB-435S/ shBmi-1 cells in 0.1ml PBS solution with a 27-gauge needle [277]. However, $2x10^7$ MCF-10A control cells and MCF-10A/ Bmi-1 cells were injected in the fat pad. Tumor growth was measured by caliper, and tumor volume was calculated with the formula of length* width^2 * 0.5 [278, 279]. All the mice were euthanized by CO_2 asphyxiation on the sixth week after injection. The primary tumor, brain, heart, bone, liver and lymph nodes of each mouse were removed, weighed, and embedded into 10% paraffine (Sango Biotech (shanghai) Co.Ltd). Each tissue was chopped into small pieces and total protein was extracted to detect Bmi-1 expression from the primary xenografts. Each section from the primary xenografts and lung tissues was subjected to Hematoxylin and Eosin staining (Zhongshan Golden Bridge Biotechnology, Beijing, China) according to standard protocols for histological examination and

metastasis evaluation. The nodes of lung metastasis were quantified by counting metastasis lesions in ten sections with 10 μm. Data were collected by counting the total numbers of metastasis lesions from ten sections. The sections of primary tumors and lung lesions were used to detect the expression of Bmi-1, β-Catenin, Fibronectin by immunochemistry method, as described before in our study.

2.14 Paraffin Section Preparation

Tissues were fixed with 5-10 times of tissue volume of 10% formalin for 24 hours at room temperature in order to prevent any deterioration and/or degradation of cells. Then, the tissues were dehydrated with graded alcohols for at least 1 hour at different concentration (from 70%, 80%, 90% to 100%) in order to remove and replace all water with alcohol, embedded with xylene for 1 hour for two times and transferred with equal volumes of paraffin wax mixture at 56-58 °C for 2 hour. Finally, the tissues were infiltrated into wax for at least 3 hour. Paraffin blocks were trimmed as necessary and cut at 10 um. Paraffin ribbon were placed in water bath at about 40-45 °C and mounted onto slides.

2.15 Hematoxylin and Eosin (H&E) Staining

Paraffin mouse tissue sections were deparaffinized with xylene, rehydrated in graded alcohols and rinsed in deionized water. After blotting excess water, the slides were stained with hematoxylin for 5 min at room temperature and

followed by rinsing in running tap water. Then, the undesirable colouration was selectively removed by controlled leaching in alcoholic acidic solution (1 ml concentrated hydrochloric acid + 400 ml 70% ethanol) (Zhongshan Golden Bridge Biotechnology, Beijing, China). The differentiation step was arrested by returning to running tap water to achieve blueing up in a short period of time. After rinsing in running tap water and blotting excess water, the slides were stained with eosin for 1 min. Finally, the sections were dehydrated with graded alcohols, kept in xylene overnight to get rid of the remaining water and mounted with coverslips.

2.16 Statistical Analysis

Chi-Square test was employed to evaluate the differences in Bmi-1 expression between the two categories of tissues. For assessment of the correlation between clinical features and Bmi-1 expression in breast cancer, P values were calculated by using the Chi-Square test or the Fisher's exact test. Relative risks (RRs) of death associated with Bmi-1 expression and other predictive variables were estimated by using the univariate and multivariate Cox proportional hazards model. Overall survival curve was plotted using the Kaplan-Meier survival analysis and compared by the log-rank test. Result variations for the chamber invasion assays, soft agar assay, tumor volume, tumor weight and lung metastasis lesions in mice, described as mean ± BD, were assessed by the two-tailed Student's t test. A value of $P < 0.05$ was considered significant (two

tailed) by using SPSS 16.0.

Chapter 3 Results

3.1 Increased Expression of Bmi-1 in Breast Cancer Tissues

The median age of the study population at diagnosis was 47 years (range 26-78). The percentage of positive cells and staining intensity of Bmi-1 expression related to clinicopathologic features were described in Table 3.1.1. In order to reveal the role of Bmi-1 in aggressiveness and metastasis of breast cancer, immunohistochemistry was first performed to measure Bmi-1 expression in breast cancer tissues and adjacent non-cancerous tissues. Bmi-1 expression was significantly increased in the primary cancer tissues compared with the matched adjacent non-cancerous tissues (χ^2=20.237, ***$P<0.001$, Table 3.1.2). Only 35.9% (14 of 39) of matched adjacent non-cancerous tissues displayed high Bmi-1 expression and the remaining tissues (64.1%, 25 of 39) were scored as none or low expression of Bmi-1(Figure 3.1 A, B). However, as many as 72.2% (182 of 252) of the cancer tissues were defined as high Bmi-1 expression (Figure 3.1 C, D) and the remaining tissues (27.8%, 70 of 287) were defined as low Bmi-1 expression. Interestingly, more intensive staining was observed in carcinoma *in situ* compared with the adjacent lobular glandules hyperplasia (Figure 3.1 E, F, G).

Among adjacent non-cancerous tissues, no Bmi-1 staining signal was detected in 30.8% (12 in 39) samples. Only 28.2% (11 in 39) samples showed nucleus staining and the remaining 41.0% (16 in 39) samples exhibited cytoplasm staining. Of those cancer tissues, however, 75.4% (190 in 252) was stained in the nucleus and 24.6% (62 in 252) located in the cytoplasm. As a result, Bmi-1

protein seems to localize in the nucleus of the majority of breast cancer cells and in the cytoplasm of the most non-cancer cells (Figure 3.1).

Table 3.1.1 Percentage of positive cells and staining intensity of Bmi-1 expression related to clinicopathologic features

	Percentage of Positive Cells				Staining Intensity			
	1	2	3	4	1	2	3	4
	N	N	N	N	N	N	N	N
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Age								
≤45 year	11	38	20	30	5	33	35	26
	(11.1)	(38.4)	(20.2)	(30.3)	(5.1)	(33.3)	(35.4)	(26.3)
>45 year	16	40	21	64	5	46	54	36
	(11.3)	(28.4)	(14.9)	(45.4)	(3.5)	(32.6)	(38.3)	(25.5)
T Classification								
T1	8	21	0	16	5	23	8	9
	(17.8)	(46.7)	(0.0)	(35.6)	(11.1)	(51.1)	(17.8)	(20.0)
T2	16	36	23	60	4	45	52	34
	(11.9)	(26.7)	(17.0)	(44.4)	(3.0)	(33.3)	(38.5)	(25.2)
T3	4	16	14	18	2	9	26	15
	(7.7)	(30.8)	(26.9)	(34.6)	(3.8)	(17.3)	(50.0)	(28.8)
T4	0	7	5	8	0	5	9	6
	(0.0)	(35.0)	(25.0)	(40.0)	(0.0)	(25.0)	(45.0)	(30.0)
N Classification								
N0	18	35	6	31	6	44	24	16
	(20.0)	(38.9)	(6.7)	(34.4)	(6.7)	(48.9)	(26.7)	(17.8)
N1	10	35	28	59	5	30	57	40
	(7.6)	(26.5)	(21 .2)	(44.7)	(3.8)	(22.7)	(43.2)	(30.3)
N2	0	6	6	10	0	5	10	7
	(0.0)	(27.3)	(27.3)	(45.5)	(0.0)	(22.7)	(45.5)	(31.8)
N3	0	4	2	2	0	3	4	1
	(0.0)	(50.0)	(25.0)	(25.0)	(0.0)	(37.5)	(50.0)	(12.5)
M Classification								
M0	27	67	30	83	10	72	79	46
	(13.0)	(32.4)	(14.5)	(40.1)	(4.8)	(34.8)	(38.2)	(22.2)
M1	1	13	12	19	1	10	16	18
	(2.2)	(28.9)	(26.7)	(42.2)	(2.2)	(22.2)	(35.6)	(40.0)
Clinical Stage								
I	3	14	0	6	2	16	2	3
	(13.0)	(60.9)	(0.0)	(26.1)	(8.7)	(69.6)	(8.7)	(13.0)
II	21	33	6	34	7	41	30	16
	(22.3)	(35.1)	(6.4)	(36.2)	(7.4)	(43.6)	(31.9)	(17.0)
III	3	20	24	43	1	15	47	17
	(3.3)	(22.2)	(26.7)	(47.8)	(1.1)	(16.7)	(52.2)	(30.0)
IV	1	13	12	19	1	10	16	18

	(2.2)	(28.9)	(26.7)	(42.2)	(2.2)	(22.2)	(35.6)	(40.0)
ER Presence								
Negative	19	37	23	42	8	39	42	32
	(15.7)	(30.6)	(19.0)	(32.7)	(6.6)	(32.2)	(34.7)	(26.4)
Positive	9	43	19	60	3	43	53	32
	(6.9)	(32.8)	(14.5)	(45.8)	(2.3)	(32.8)	(40.5)	(24.4)
PR Presence								
Negative	16	30	23	38	5	33	40	29
	(15.0)	(28.0)	(21.5)	(35.5)	(4.7)	(30.8)	(37.4)	(27.1)
Positive	12	50	19	64	6	49	55	35
	(8.3)	(34.5)	(13.1)	(44.1)	(4.1)	(33.8)	(37.9)	(24.1)
HER-2 Presence								
Negative	9	12	8	22	4	17	14	16
	(17.6)	(23.5)	(15.7)	(43.1)	(7.8)	(3.3)	(27.5)	(31.4)
Positive	12	30	16	50	5	34	38	31
	(11.1)	(27.8)	(14.8)	(46.3)	(4.6)	(31.5)	(35.2)	(28.7)

Four categories of the percentage of positive cells: 1: ≤5%; 2: 6%-35%; 3: 36%-70%; 4: ≥71%.

Four categories of the staining intensity: 1: negative; 2: weak; 3: moderate; 4: strong.

Table 3.1.2 Difference of Bmi-1 expression between breast cancer tissues and adjacent non-cancerous tissues

	Bmi-1 Expression				χ^2	*P-value*
	Low		High			
	N	(%)	N	(%)		
Tissues					20.237	*<0.001*
Non-cancer	25	(64.1)	14	(35.9)		
Cancer	70	(27.8)	182	(72.2)		

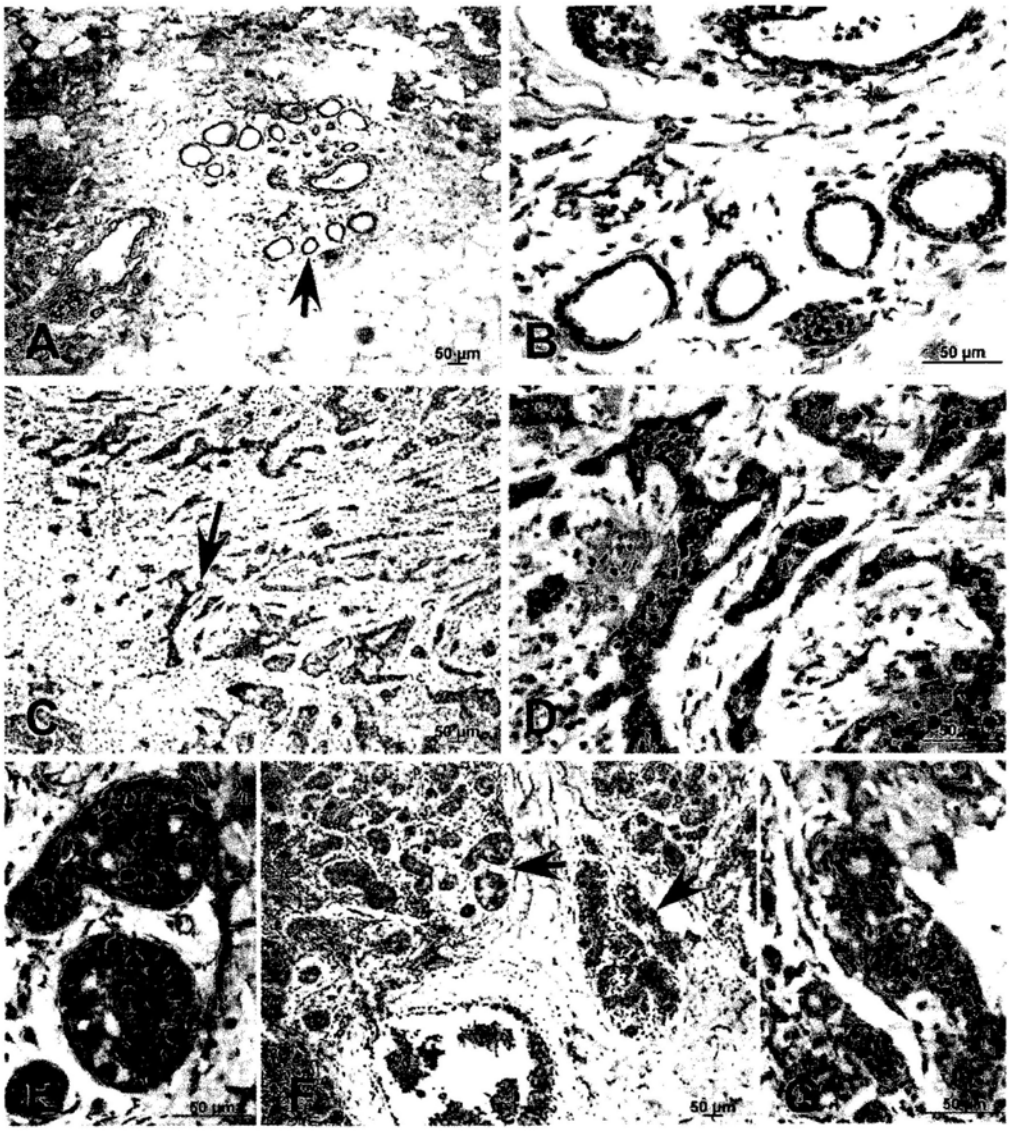

Figure 3.1 Increased expression of Bmi-1 in breast cancer tissues.

(A, B) Bmi-1 showed none or weak staining in the adjacent non-cancerous tissue. **(E, F)** Strong Bmi-1 staining was detected in the invasive breast cancer tissue. **(E, F, J)** Carcinoma *in situ* showed high Bmi-1 expression and nuclear staining, whereas the adjacent lobular glandules hyperplasia showed the low expression and cytoplasmic staining (100 x &400 x).

3.2 Correlation between Bmi-1 Expression and Clinical Aggressiveness of Breast Cancer

We further examined possible correlations between Bmi-1 expression profiles and patients' clinicopathologic characteristics. As revealed in Table 3.2, Bmi-1 expression rate was higher in patients with larger tumor size (T1: 42.2%; T2: 74.1%; T3: 82.7%; T4: 100.0%; *P<***0.001*), lymph node involvement (N0: 46.7%; N1: 84.8%; N2: 100%; N3: 75.0%; *P<***0.001*), distant metastasis (M0: 67.1; M1: 95.6; *P<***0.001*), and advanced clinical stages (Ⅰ :30.4%; Ⅱ : 48.9%; Ⅲ: 95.6%; Ⅳ: 95.6%; *P<***0.001*) by analyzing 252 primary breast cancer cases. These observations suggested a correlation between increased Bmi-1 expression and clinical progression in breast cancer. On the other hand, the correlations were not evident between Bmi-1 expression profiles and other clinical/laboratory features, including age (*P=0.551*), ER (*P=0.217*), PR (*P=0.837*), and HER-2 (*P=0.400*).

Table 3.2 Correlation betweenBmi-1 expression and the clinicopathologic features of breast cancer

	Bmi-1 Expression				Total		χ^2	*P-value*
	Low		High					
	N (%)		N (%)		N (%)			
Age							0.36	*0.551*
≤45 year	26	(26.3)	73	(73.7)	99	(41.2)		
>45 year	42	(29.8)	99	(70.2)	141	(58.8)		
T Classification							30.92	*<0.001*
T1	26	(57.8)	19	(42.2)	45	(17.9)		
T2	35	(25.9)	100	(74.1)	135	(53.6)		
T3	9	(17.3)	43	(82.7)	52	(20.6)		
T4	0	(0.0)	20	(100.0)	20	(7.9)		
N Classification							46.45	*<0.001*
N0	48	(53.3)	42	(46.7)	90	(35.7)		
N1	20	(15.2)	112	(84.8)	132	(52.4)		
N2	0	(0.0)	22	(100.0)	22	(8.7)		
N3	2	(25.0)	6	(75.0)	8	(3.2)		
M Classification							14.87	*<0.001*
M0	68	(32.9)	139	(67.1)	207	(82.7)		
M1	2	(4.4)	43	(95.6)	45	(17.9)		
Clinical Stage							82.06	*<0.001*
I	16	(69.6)	7	(30.4)	23	(9.1)		
II	48	(51.1)	46	(48.9)	94	(37.3)		
III	4	(4.4)	86	(95.6)	90	(35.7)		
IV	2	(4.4)	43	(95.6)	45	(17.9)		
ER Presence							1.53	*0.217*
Negative	38	(31.4)	83	(68.6)	121	(48.0)		
Positive	32	(24.4)	99	(75.6)	131	(52.0)		
PR Presence							0.04	*0.837*
Negative	29	(27.1)	78	(72.9)	107	(42.5)		
Positive	41	(28.3)	104	(71.7)	145	(57.5)		
HER-2 Presence							0.71	*0.400*
Negative	17	(33.3)	34	(66.7)	51	(32.1)		
Positive	29	(26.9)	79	(73.1)	108	(67.9)		

3.3 High Bmi-1 Expression was Associated with an Unfavorable Prognosis

The characteristics of the patients relevant to overall survival were shown in the Table 3.3. As expected, clinicopathologic classifications (T, N, M) and clinical stages were found to be important prognostic indicators in breast cancer (*P<***0.001,* respectively). The presence of PR also appeared to have a clinical prognostic value (*P=**0.002*), but age (*P=0.466*), ER (*P=0.272*) and HER-2 (*P=0.406*) did not. After the first year of follow-up, OS (Overall Survival) was 97.1% (232 in 239); After the second year, OS was 86.6% (207 in 239); after the third year, OS was 77.0% (184 in 239); after the fourth year, OS was 71.1 % (170 in 239) and after the fifth year, OS was 49.4 % (118 in 239).

As shown in Table 3.3, Bmi-1 expression displayed a significant correlation with patients' survival status (*P<***0.001*). The median follow up period was 51.10 months (range, 4-78). At the time of analyzing Bmi-1 expression, 33.1% (79 of 239) had died. The overall survival rate, assessed by the Kaplan-Meier method, was 85.1% (57 in 67) in low Bmi-1 expression group, whereas it was only 59.9% (103 in 172) in high Bmi-1 expression group (Table 3.3, Figure 3.3.1, *P<***0.001*). Now that there was strong association between Bmi-1 expression status and the clinicopathologic parameters, the overall survival could be further distinguished based on Bmi-1 expression and adjusting status for clinicopathologic parameters. Because small number of tissues showed low Bmi-1 expression in T3/4 classification (N=9), N2/3 classification (N=2), M1

classification (N=2) and clinical stage III/IV (N=6) (Table 3.2), the overall survivals were not analyzed stratified by them. In addition, only 7 samples with stage I exhibited high Bmi-1 expression (Table 3.2). There was only one patient died of breast cancer, no plot can be drawn for these data in stage I subgroup. As shown in Figure 3.3.2, the significantly different outcome based on Bmi-1 expression was compared in patient subgroups with T1 (*P=**0.006*) and T2+3+4 (*P=*0.034*). When the tumor was less than 2cm (T1 classification), the survival rate was 96.0% (24 in 25) in low expression panel alien to 63.2% (12 in 19) in high expression panel. Similarly, the survival rate was 78.6% (33 in 42) in low expression panel alien to 59.5% (91 in 153) in high expression panel when the tumor was larger than 2cm (T2+3+4 classification). However, no obvious difference was seen based on Bmi-1 expression compared in N0 (*P=0.061*) and N1+2+3 (*P=0.248*) subgroups (Figure 3.3.3). When the patients with N0 classification were analyzed, the survival rate was 91.3% (42 in 46) in low expression group and 74.4% (29 in 39) in high expression group. However, when the patients with N1+2+3 classification were analyzed, the survival rate was 71.4% (15 in 21) in low expression group and 55.6% (74 in 133) in high expression group. In our study, 26.7% (52 of 195) patients were died in M0 panel when we analyzed these samples and patients showed longer survival time with low Bmi-1 expression (Figure 3.3.4 A, *P=*0.018*). The survival rate was 84.8% (56 in 66) in low Bmi-1 expression panel contrary to 67.4% (87 in 129) in high Bmi-1 expression panel. The similar result was found in patients with

stage Ⅱ/Ⅲ/Ⅳ according to Bmi-1 expression (Figure 3.3.4 B, *P=**0.009*). The survival rate was 80.8% (42 in 52) in low Bmi-1 expression panel contrary to 58.8% (97 in 165) in high Bmi-1 expression panel.

Then we carried out the subset analysis on the basis of hormones receptor and HER-2 status. Interestingly, using the subset analysis, the impact on the outcome associated with high Bmi-1 expression was still more unfavorable in both positive and negative ER group (Figure 3.3.5, A, *P=**0.003*; B, *P=*0.041*). When patients showed negative ER, the survival rate was 84.2% (32 in 38) in low expression panel and 53.8% (43 in 80) in high expression panel. Similarly, the survival rate is obviously different in positive ER subgroup. The survival rate was 86.2% (25 in 29) in low expression panel and 65.2% (60 in 92) in high expression panel. Similar results were shown for positive and negative PR groups (Figure 3.3.6, A, *P=**0.010*; B, *P=*0.028*). When patients showed negative PR, the survival rate was 79.3% (23 in 29) in low expression panel contrary to 47.4% (36 in 76) in high expression panel. Likewise, the survival rate was 89.5% (34 in 38) in low expression panel contrary to 69.8% (67 in 96) in high expression panel at positive PR subgroup. However, the overall survival was not obviously different based on Bmi-1 expression in HER-2 negative subgroup (Figure 3.3.7 A, *P=0.701*), although the outcome was much better with low Bmi-1 expression in HER-2 positive subgroup (Figure 3.3.7 B, *P=*0.018*). At the time of Bmi-1 analysis, 17.6% (3 in 17) died with low Bmi-1

expression and 26.7% (8 in 30) died with high Bmi-1 expression in HER-2 negative subgroup. However, the survival rate was 88.9% (24 in 27) in low expression panel and 63.0% (46 in 73) in high expression panel in the HER-2 positive subgroup. Consistent with previous data of the tested patients [47], 20.4% cases (30 of 147) displayed a prominent triple negative phenotype (TNP, ER^-, PR^-, and $HER\text{-}2^-$). The overall survival rate in the TNP subgroup was 70% in low expression group (7 in 10), whereas it was 75% (15 in 20) in high expression group. The outcome was not significantly different between high and low Bmi-1 expression patients (Figure 3.3.8, $P=0.483$). Taken together, these results suggest Bmi-1 expression in breast cancer tissues could help to evaluate the prognosis of breast cancer patients.

Table 3.3 Distribution of the patients' characteristics by survival status

	Survival Status				Survival Time	χ^2	*P-value*
	Alive		Dead		(Mean±SD)		
	N	(%)	N	(%)	(Months)		
Age						0.531	*0.466*
≤45	63	(64.3)	35	(35.7)	51.24±18.07		
>45	97	(68.8)	44	(31.2)	51.00±18.79		
T						22.070	*<0.001*
T1	36	(81.8)	8	(18.2)	54.85±15.28		
T2	93	(71.5)	37	(28.5)	54.47±16.31		
T3	26	(56.5)	20	(43.5)	46.88±19.68		
T4	5	(26.3)	14	(73.7)	29.56±20.58		
N						25.791	*<0.001*
N0	71	(83.5)	14	(16.5)	57.27±12.49		
N1	80	(62.5)	48	(37.5)	49.76±19.04		
N2	6	(28.6)	15	(71.4)	34.82±23.02		
N3	3	(60.0)	2	(40.0)	48.94±24.09		
M						19.531	*<0.001*
M0	143	(73.3)	52	(26.7)	53.30±17.00		
M1	17	(38.6)	27	(61.4)	41.35±21.55		
Stage						28.705	*<0.001*
I	21	(95.5)	1	(4.5)	60.61±5.77		
II	68	(77.3)	20	(22.7)	55.21±13.98		
III	54	(63.5)	31	(36.5)	49.43±20.58		
IV	17	(38.6)	27	(61.4)	41.35±21.55		
ER						1.208	*0.272*
Negative	75	(63.6)	43	(36.4)	49.97±18.55		
Positive	85	(70.2)	36	(29.8)	52.46±18.35		
PR						9.790	*0.002*
Negative	59	(56.2)	46	(43.8)	48.33±19.23		
Positive	101	(75.4)	33	(24.6)	53.27±17.63		
HER-2						0.692	*0.406*
Negative	36	(76.6)	11	(23.4)	54.07±16.22		
Positive	70	(70.0)	30	(30.0)	51.61±18.49		
Bmi-1						13.827	*<0.001*
Low	57	(85.1)	10	(14.9)	55.34±14.00		
High	103	(59.9)	69	(40.1)	49.45±19.72		

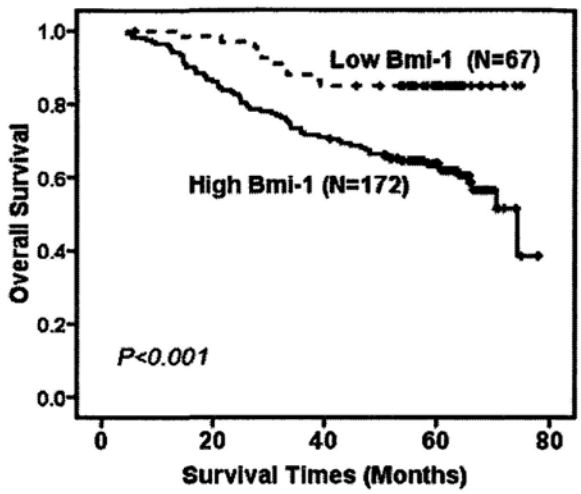

Figure 3.3.1 Influence of Bmi-1 expression on the overall survival. The cumulative overall survival exhibited a significant difference based on Bmi-1 expression assessed by the Kaplan-Meier curves in primary breast cancer tissues (***P=0.001*)

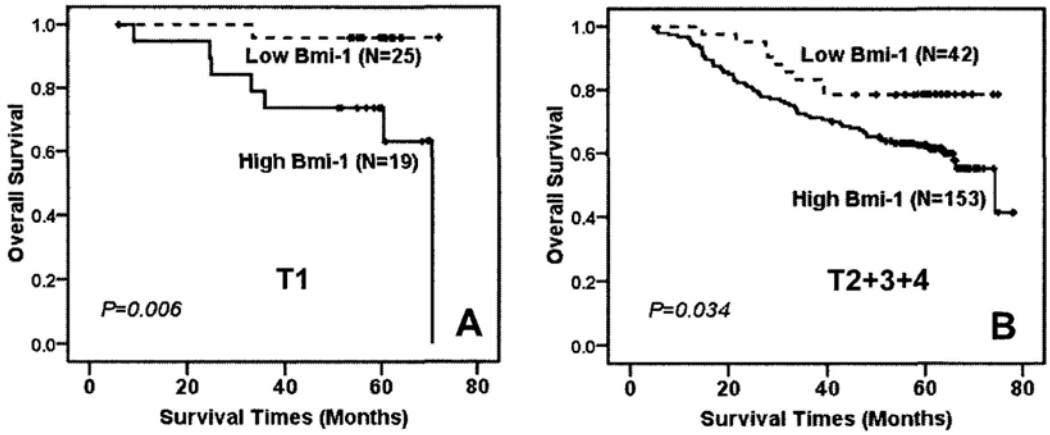

Figure 3.3.2 Kaplan-Meier analysis showed the overall survival of breast cancer patients categorized according to tumor size and the levels of Bmi-1 expression. Significant difference in overall survival of patients between high Bmi-1 expression and low Bmi-1 expression was found in T1 (**A**, ***P=0.006*) and T2+3+4 (**B**, *P=*0.034*) subgroups.

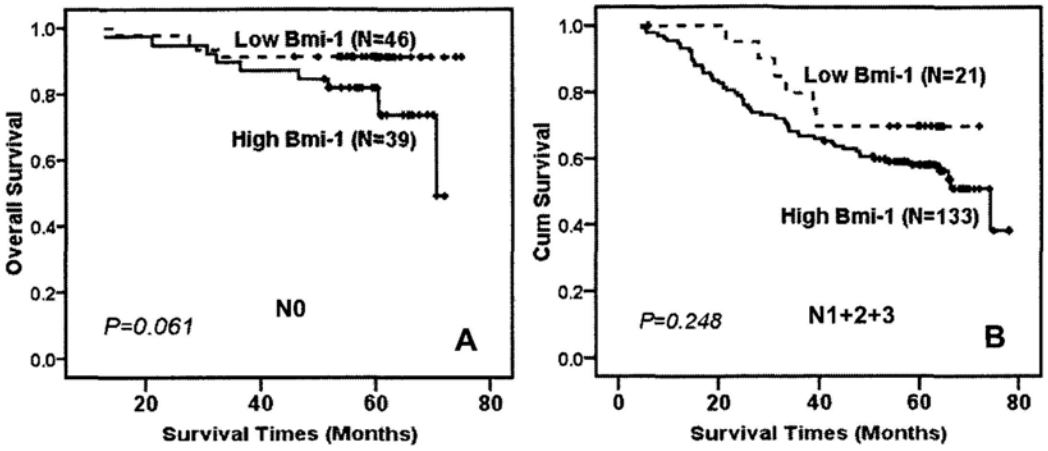

Figure 3.3.3 Kaplan-Meier analysis showed the overall survival of breast cancer patients categorized by lymphonode involvement and the status of Bmi-1 expression. No obvious difference was seen according Bmi-1 expression compared in N0 (**A**, *P=0.061*) and N1+2+3 (**B**, *P=0.248*) patients subgroups.

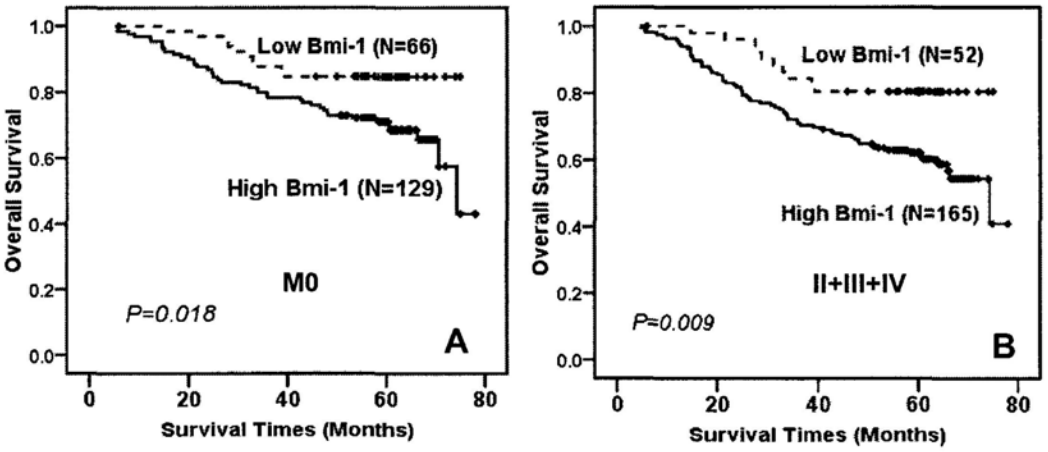

Figure 3.3.4 Kaplan-Meier overall survival curves of breast cancer patients stratified by Bmi-1 expression status in distant metastasis and clinical stage. In M0 **(A)** panel and clinical stage II+III+IV **(B)** panel, the overall survival was significantly shorter in high Bmi-1 expression group than that with low expression (**A**, **P=0.018*; **B**, *P=**0.009*).

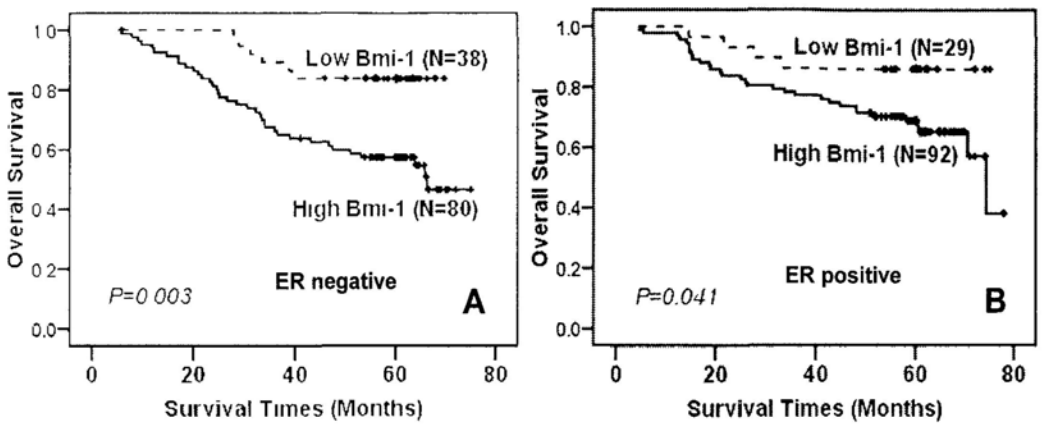

Figure 3.3.5 Kaplan-Meier overall survival curves of breast cancer patients stratified by Bmi-1 expression expression level in ER absence (A) and presence (B). High Bmi-1 expression was correlated with unfavorable prognosis irrelevant of the ER status (**A**, ***P=0.003*; **B**, **P=0.041*).

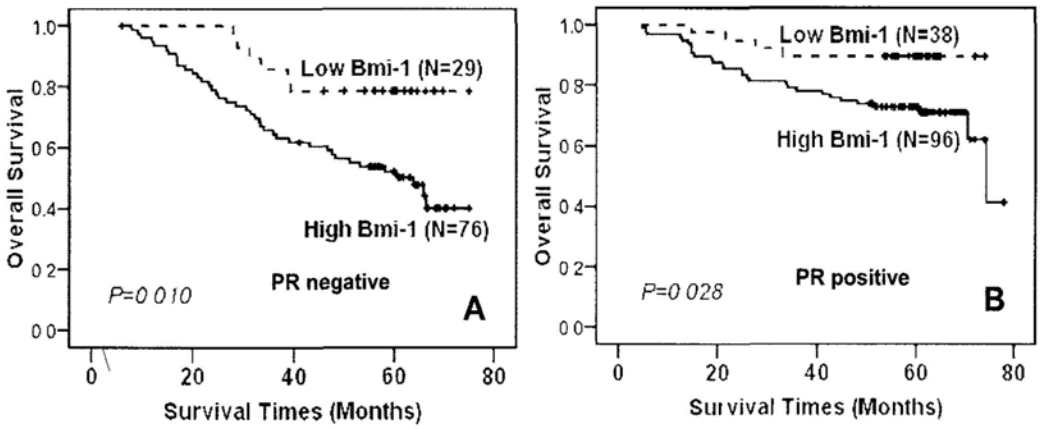

Figure 3.3.6 Kaplan-Meier overall survival curves of breast cancer patients stratified by Bmi-1 expression status in PR absence (A) and presence (B). High Bmi-1 expression was correlated with unfavorable prognosis irrelevant of PR status (**A**, ***P=0.010*; **B**, **P=0.028*).

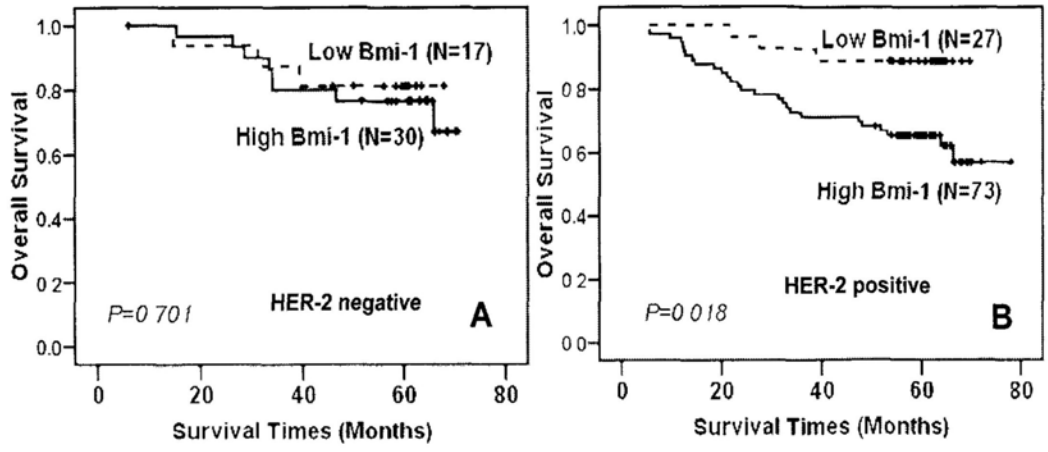

Figure 3.3.7 Kaplan-Meier overall survival curves of breast cancer patients stratified by Bmi-1 expression status in HER-2 absence (A) and presence (B). The survival curves were significantly different in HER-2 positive panel (**B**, **P*=0.018) but not in HER-2 negative panel (**A**, *P*=0.701) according to Bmi-1 expression.

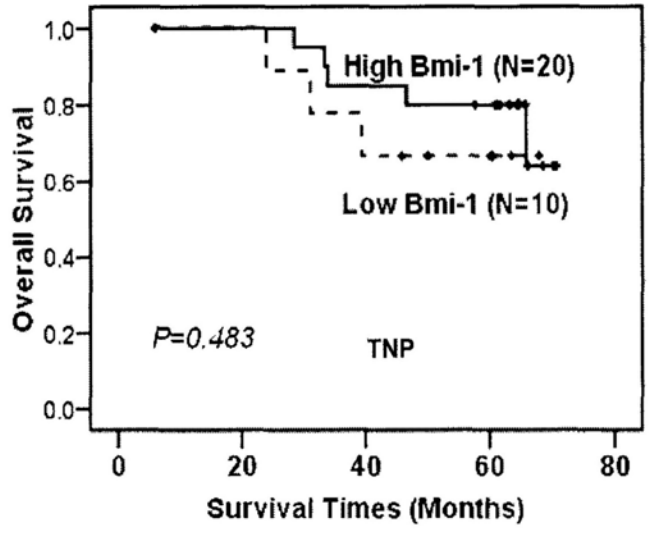

Figure 3.3.8 Overall survival curves of breast cancer patients stratified by Bmi-1 expression status in patients with TNP. No difference in the overall survival curve was observed according to Bmi-1 expression in TNP subgroup.

3.4 Analyses of Relative Risks (RRs) Indicative of Bmi-1's Role in the Prognosis of Breast Cancer

In our analyses, we defined a relative risk of 1.000 as the baseline for factors including age (≤45 years), T1, N0, M0, clinical stage I, the low level of Bmi-1 expression and the absence of ER, PR and HER-2. To determine if Bmi-1 could serve as a risk factor with clinical usefulness, Cox regression proportional hazard analyses were used to examine the relative risk. As shown in Table 3.4.1, univariate Cox regression analyses revealed that high level of Bmi-1 was related to a significantly increased risk of death in breast cancer patients (****P=0.001*). The relative risk increased almost four fold in patients with high Bmi-1 expression compared with that with low Bmi-1 expression (RR=3.979, 95% Confidence Interval: 1.534-5.875) (Table 3.4.1). As expected, large tumor size (T3, RR=2.841, 95%CI: 1.251-6.454, **P=0.013*; T4, RR=7.822, 95%CI: 3.294-18.861, ****P<0.001*), lymph node involvement (N1, RR=2.597, 95%CI: 1.431-4.713, *P=***0.002*; N2, RR=7.621, 95%CI: 3.667-15.836, ****P<0.001*), distant metastasis (M1, RR=3.309, 95%CI: 1.903-4.853, ****P<0.001*) and advanced clinical stage (III, RR=9.750, 95%CI: 1.330-71.481, **P<0.025*;Ⅳ, RR=20.065, 95%CI: 2.724-147.736, ***P=0.003*) were also significant unfavorable prognostic factors. However, the presence of PR was a favorable prognostic factor (RR=0.051, 95%CI: 0.327-0.800, ***P=0.003*), although age (*P=0.624*), the presence of ER (*P=0.223*) and HER-2 (*P=0.398*) did not predict the survival.

With the aid of multivariate Cox regression analyses, Bmi-1 still claimed the trend of high relative risk in breast cancer (RR=1.708, 95%CI: 1.213-3.087) and the difference was significant (*P=0.042*). Moreover, advanced clinical stage (III, RR=6.322, 95%CI: 0.832-48.209, **P=0.075*; Ⅳ, RR=12.948, 95%CI: 1.688-99.333, **P=0.014*) remained unfavorable prognosis. PR was also found as a potential prognostic factor by multivariate Cox regression analyses (Positive, RR=0.539, 95%CI: 0.344-0.845, ***P=0.007*) (Table 3.4.2).

Table 3.4.1 Univariate Cox regression analysis of potential prognostic parameters for breast cancer patients

	RR	95% CI	*P-value*
Age			
≤45 year	1.000		
>45 year	1.057	0.846-1.321	*0.624*
T Classification			
T1	1.000		
T2	1.534	0.713-3.301	*0.274*
T3	2.841	1.251-6.454	*0.013*
T4	7.822	3.294-18.861	*<0.001*
N Classification			
N0	1.000		
N1	2.597	1.431-4.713	*0.002*
N2	7.621	3.667-15.836	*<0.001*
N3	2.846	0.646-12.551	*0.167*
M Classification			
M0	1.000		
M1	3.039	1.903-4.853	*<0.001*
Clinical Stage			
I	1.000		
II	5.429	0.728-40.465	*0.099*
III	9.750	1.330-71.481	*0.025*
IV	20.065	2.724-147.736	*0.003*
ER Presence			
Negative	1.000		
Positive	0.758	0.486-1.183	*0.223*
PR Presence			
Negative	1.000		
Positive	0.511	0.327-0.800	*0.003*
HER-2 Presence			
Negative	1.000		
Positive	1.347	0.675-2.689	*0.398*
Bmi-1 Expression			
Low	1.000		
High	3.979	1.534-5.875	*0.001*

RR: Relative Risk; CI: Confidence Interval

Table 3.4.2 Multivariate Cox regression analysis of potential prognostic factors for breast cancer patients

	RR	95% CI	*P-value*
Clinical Stage			
I	1		
II	4.378	0.584-32.793	*0.151*
III	6.322	0.832-48.209	*0.075*
IV	12.948	1.688-99.333	*0.014*
PR Presence			
Negative	1		
Positive	0.539	0.344-0.845	*0.007*
Bmi-1 Expression			
Low	1		
High	1.708	1.213-3.087	*0.042*

RR: Relative Risk; CI: Confidence Interval

3.5 Ectopic Expression of Bmi-1 Enhanced the Mobility and Invasiveness of Immortalized HMECs

The cellular mobility and invasiveness are indispensable for the metastasis of cancer. As Bmi-1 expression was correlated with larger tumor size, lymph node involvement, distant metastasis and advanced clinical stage in the breast cancer tissues, we hypothesized that Bmi-1 may regulate the progression of breast cancer. To probe this hypothesis, we investigated Bmi-1 expression in breast cancer cells and immortalized HMECs. As shown in Figure 3.5.1, it was low in immortalized HMECs (76N-TERT and MCF-10A), whereas it was abundant in all detected breast cancer cell lines, including SK-BR-3, ZR-75-30, BCAP-37, and MDA-MB-435S. The radiation-transformed cell line (76R-30) showed the moderate expression. Then, immortalized HMECs (76N-TERT and MCF-10A) with ectopic expression of Bmi-1 were stably established to measure the role of Bmi-1 in cancer progression (Figure 3.5.2). In this study, Bmi-1 did not affect the proliferation of immortalized HMECs (Figure 3.5.3). Notably, Bmi-1 did not alter the morphology of 76N-TERT and MCF-10A cells, even in the long time culture (Figure 3.5.4). Interestingly, the overexpression of Bmi-1 could advance the wound healing process, which closed a "wound" scratch into a confluent epithelial monolayer much faster (Figure 3.5.4). Concurrently, Boyden chamber assay showed that Bmi-1 overexpression increased the ability of cellular invasion compared with the control (Figure 3.5.5). The pool populations of cells expressing Bmi-1 and Vectors were studied for transformed phenotype using

soft agar and Matrigel assays. The 3-D Matrigel assay indicated that the induction of Bmi-1 failed to transform the morphology of immortalized HMECs. No irregular branched structures indicative transformed phenotypes were observed, except normal spherical acini (Figure 3.5.6). To further confirm transformation potentiality, HMECs derived cells were seeded in soft agar. Cells expressing either Bmi-1 or vector did not exhibit anchorage-independent growth (Figure 3.5.7). These observations depicted that Bmi-1 did promote cellular mobility and invasiveness, however it was not sufficient to transform immortalized HMECs by itself.

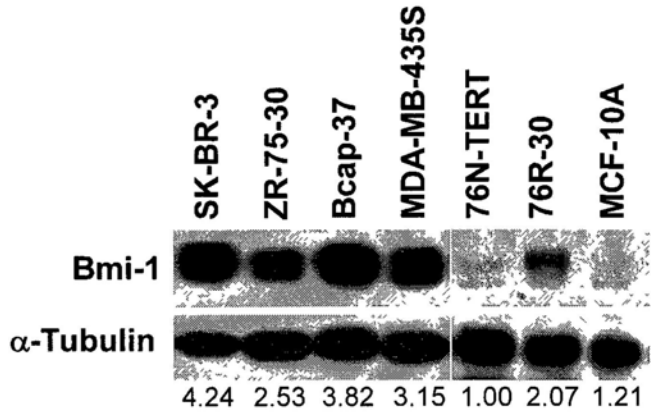

Figure 3.5.1 Endogenous expression of Bmi-1 was detected in cells by immunoblotting. Bmi-1 expression was low in 76N-TERT and MCF-10A cells, moderate in 76R-30 cells and abundant in all detected breast cancer cells, including SK-BR-3, ZR-75-30, BCAP-37 and MDA-MB-435S. Anti-α-Tubulin was the loading control.

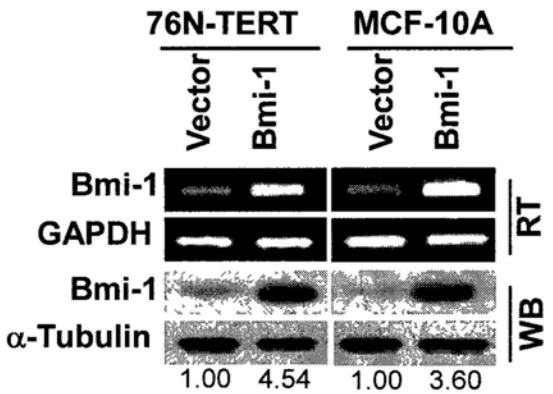

Figure 3.5.2 Overexpression of Bmi-1 in transfected immortalized HMECs was confirmed by RT-PCR and immunoblotting. GAPDH and anti-α-Tubulin were used as loading controls, respectively.

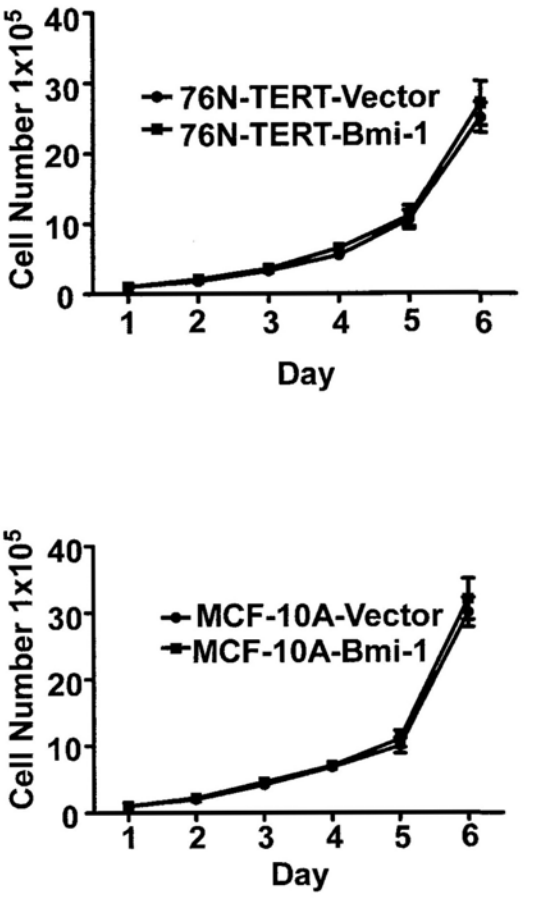

Figure 3.5.3 Bmi-1 did not affect the proliferation of HMECs

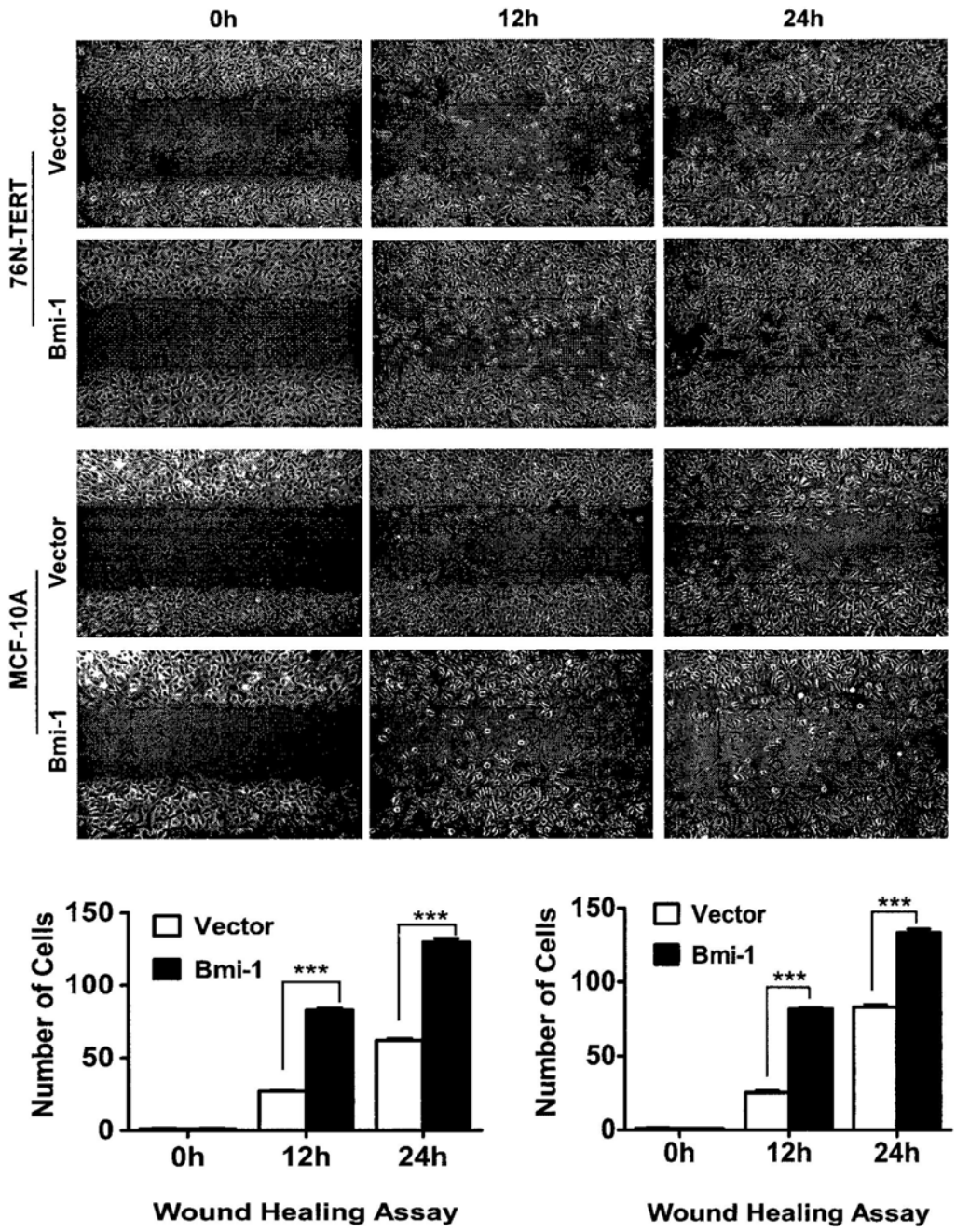

Figure 3.5.4 Overexpression of Bmi-1 could advance wound healing process compared with control. The wound gap was monitored at 0, 12, and 24 hours as the cells moved and filled the damaged area in serum-free medium (200 x, ***$P<0.001$).

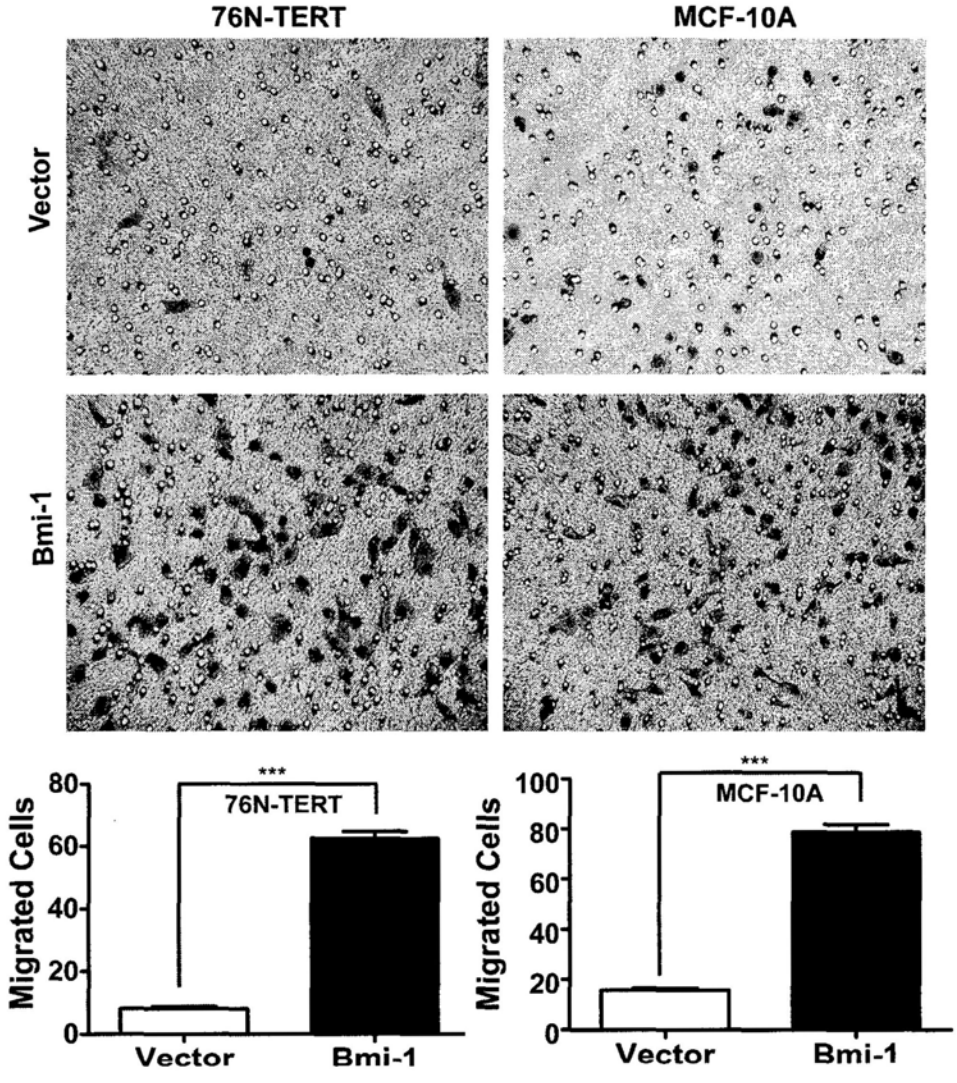

Figure 3.5.5 Overexpression of Bmi-1 increased the ability of cellular invasiveness compared with the control. The invasive properties were analyzed by the invasion assay using a Matrigel coated Boyden chamber and scored under light microscope (200 x). The data was plotted as an average number of cells per field of view (***$P<0.001$).

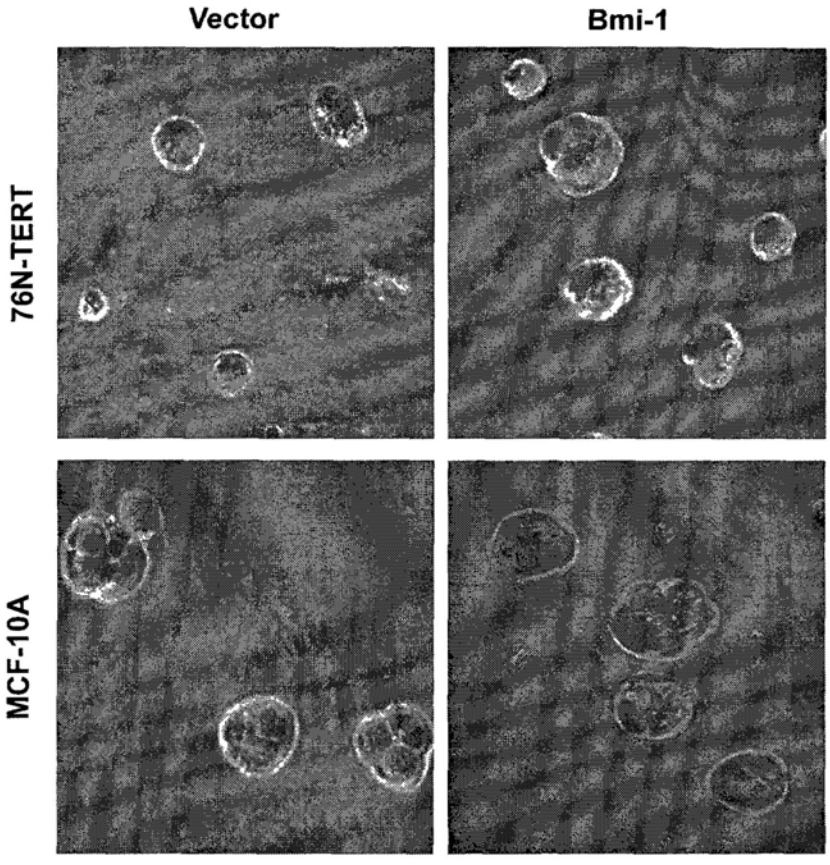

Figure 3.5.6 Bmi-1 failed to transform immortalized HMECs in 3-D Matrigel culture. Bmi-1-mediated immortalized HMECs was retained the spherical architecture. Cells were photographed (200 x) at the 7^{th} day.

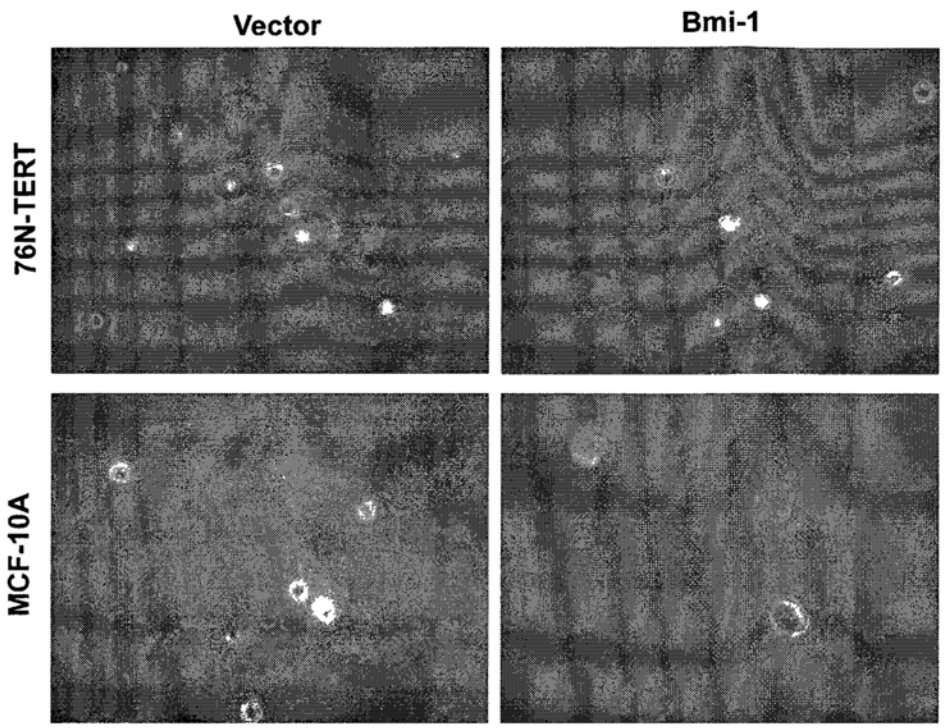

Figure 3.5.7 Bmi-1-derived 76N-TERT and MCF-10A cells did not exhibit the anchorage -independent growth ability. Cells were grown in soft agar to determine the independent growth ability. Cells were photographed (200 x) at 14^{th} day.

3.6 *Suppression of Bmi-1 Repressed the Mobility, Invasiveness and Transformation*

To further identify the role of Bmi-1 in the progression of cancer, short hairpin RNA for Bmi-1 was generated to repress Bmi-1 expression stably and efficiently in the MDA-MB-435S cell line, a highly metastatic breast cancer cell line with high Bmi-1 expression [272] (Figures 3.6.1). Bmi-1 knockdown did not alter the proliferation of MDA-MB-435S (Figures 3.6.2). Both the Boyden chamber invasion assay and the scratch wound healing assay revealed that mobility and invasiveness of MDA-MB-435S cells were dramatically hampered by the ablation of Bmi-1 (Figure 3.6.3 and 3.6.4). The Bmi-1 repression even caused the disappearance of the highly disorganized, irregular branched structures in Matrigel culture (Figure 3.6.5). In addition, the colonies in soft agar were less in frequent and smaller in size, which indicated that the depletion of Bmi-1 caused the markedly inhibition of their anchorage-independent ability (Figure 3.6.5). Our results suggest that the repression of Bmi-1 could decrease cellular mobility, invasiveness and transformation.

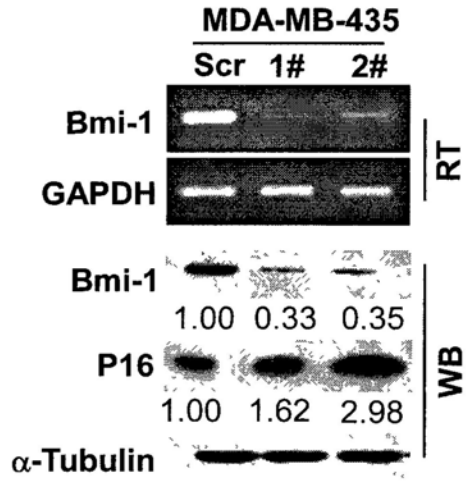

Figure 3.6.1 Bmi-1 expression was dramatically decreased by RNA interference in the MDA-MB-435S cell line. Bmi-1 expression was confirmed by RT-PCR and immunoblotting. GAPDH and anti-α-Tubulin were used as loading controls, respectively.

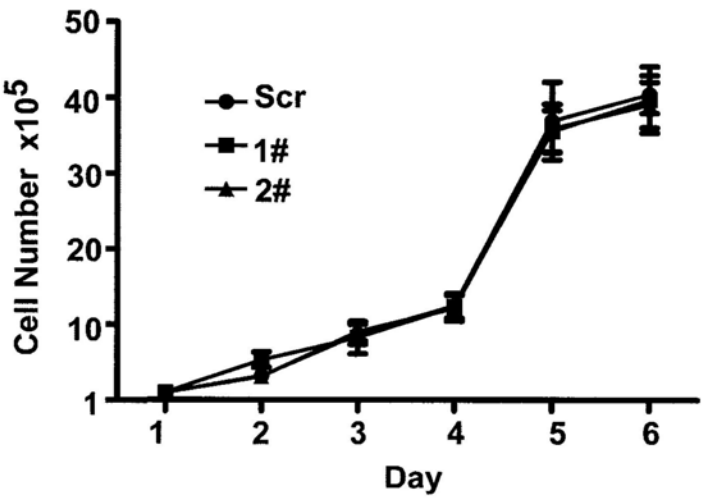

Figure 3.6.2 Bmi-1 knockdown did not affect the proliferation of MDA-MB-435S.

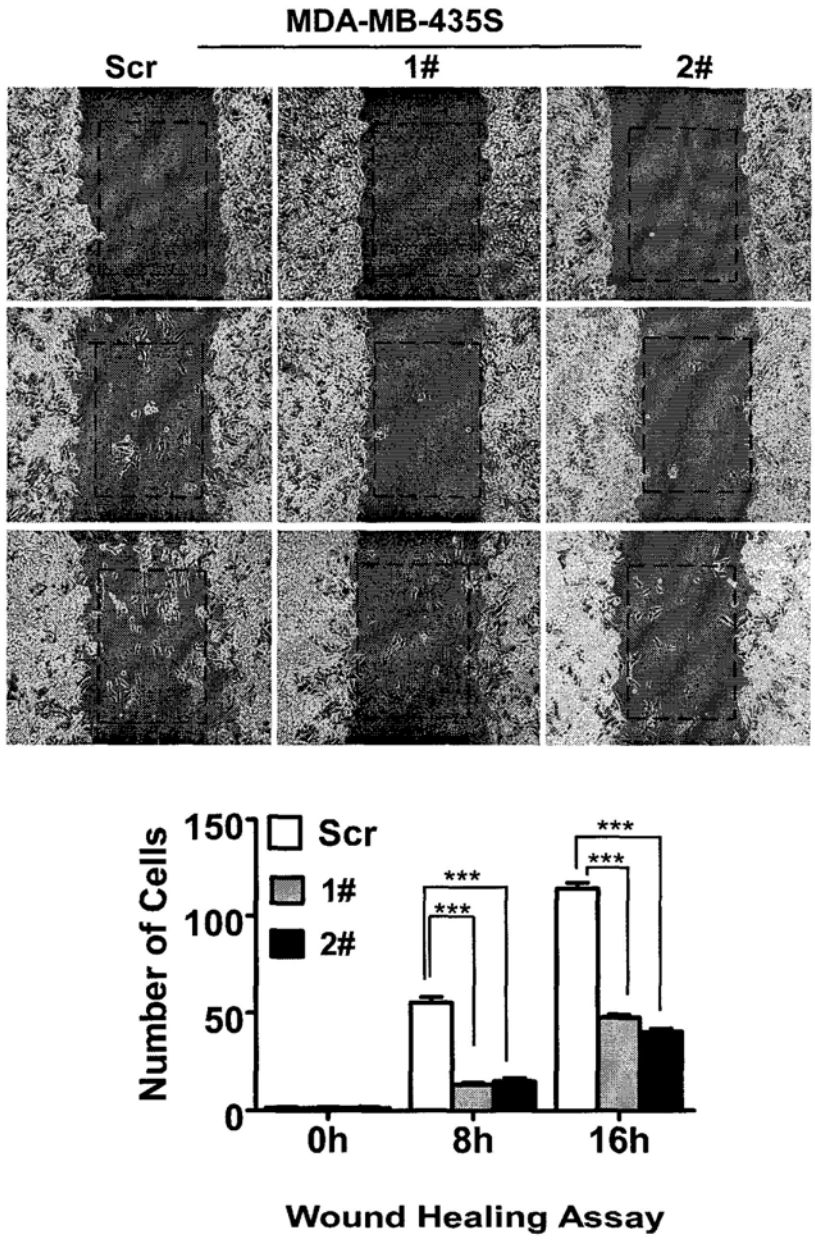

Figure 3.6.3 Mobility of MDA-MB-435S cells was dramatically hampered by the ablation of Bmi-1. The mobility was measured by examining the rate of wound closure at 0, 8, 16 hours (200 x).

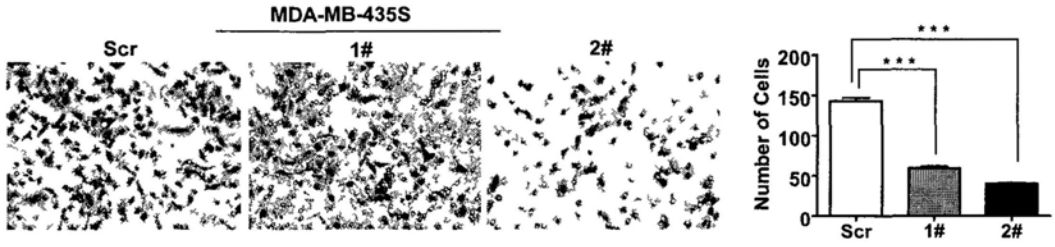

Figure 3.6.4 Bmi-1 knockdown significantly depressed the invasiveness of MDA-MB-435S cells. The invasive properties of cells were analyzed by using Matrigel-coated Boyden chamber assay (200 x, ***P<0.001).

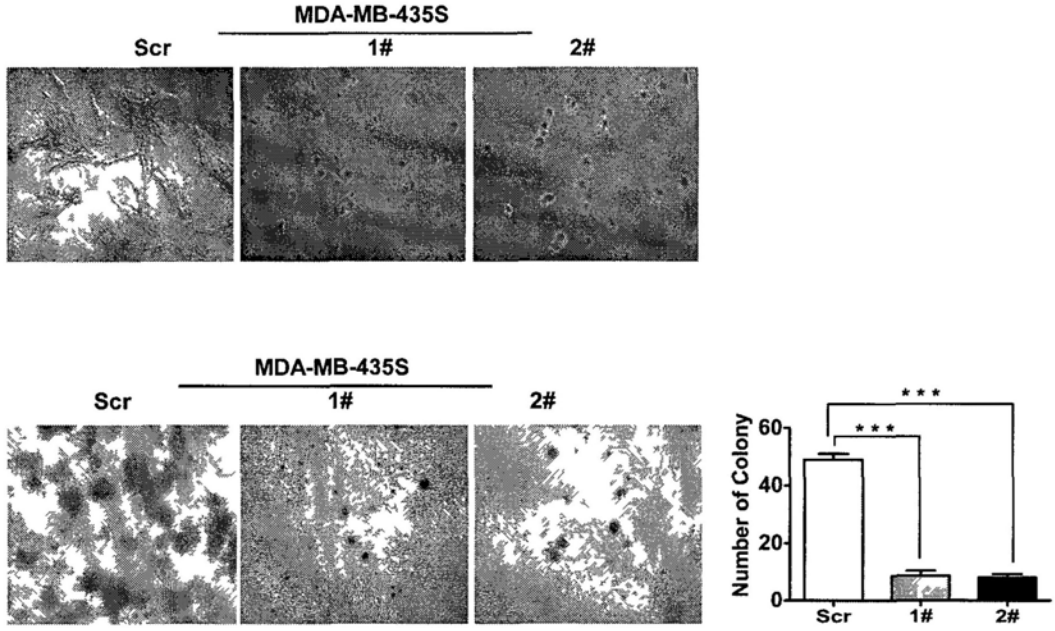

Figure 3.6.5 Invasiveness and transformation of MDA-MB-435S in 3-D Matrigel and soft agar were declined by the repression of Bmi-1. Bmi-1 repression caused the disappearance of the irregular branched structures in Matrigel culture (200 x, ***P<0.001).

3.7 The Repression of Bmi-1 Slowed Down the Tumor Progression and Reduced the Spontaneous Lung Metastasis in Nude Mice

In order to further evaluate the effects of Bmi-1 on the development of breast cancer, 2 x 10^6 MDA-MB-435S/shBmi-12# and MDA-MB-435S/shScr cells were injected into the fat pad of nude mice. Although the xenografts were observed at each mouse at the sixth week, the repression of Bmi-1 could not only descend the xenografts' volumes (Figure 3.7.1, *$P=0.038$*) and weights (Figure 3.7.2,*$P=0.041$*) but also delay the tumor occurrence (Figures 3.7.1). Macroscopic xenografts were observed in the fat pad of all nude mice after MDA-MB-435S/shScr cells injection in two weeks, whereas only 60% (6 of 10) nude mice were found to have tumor burden in the fat pad in MDA-MB-435S/shBmi-12# group. Among the other four nude mice after MDA-MB-435S/shBmi-12# cells injection, tumors were found on 25th day, 28th day, 30th day and 33th day, respectively. Accordingly, the persistent knockdown of Bmi-1 in xenograft tissues was confirmed by western blotting (Figure 3.7.3). The tumors arising from MDA-MB-435S/shBmi-12# cells were poorly differentiated adenocarcinoma, similar to those from controls [277], as assessed by hematoxylin and eosin staining and reviewed by a veterinary pathologist (Figure 3.7.4, 3.7.5). The xenografts from MDA-MB-435S/shScr cells invaded the adjacent muscles deeply, whereas the MDA-MB-435S/shBmi-12# cells showed declined invasiveness (Figure 3.7.4). In addition, Necropsy revealed fulminant large gross lung metastatic lesions involving large portions of all lung

lobes were found in 80% (8 in 10) mice injected with the MDA-MB-435S/shScr cells. On the contrary, only tiny and limited lung metastatic lesions were observed in 50% (5 in 10) mice injected with the MDA-MB-435S/shBmi-12# cells (Figure 3.7.5, *$P=0.036$). However, the injection of MCF-10A/Bmi-1 cells neither formed xenografts in fat pad nor caused metastatic lesions in nude mice, even if SCID mice were used (data not shown). These results indicated that overexpression of Bmi-1 was not sufficient for the fully malignant transformation of immortalized HMECs. The knockdown of Bmi-1 strongly slows down the tumor progression and represses spontaneous lung metastasis in nude mice.

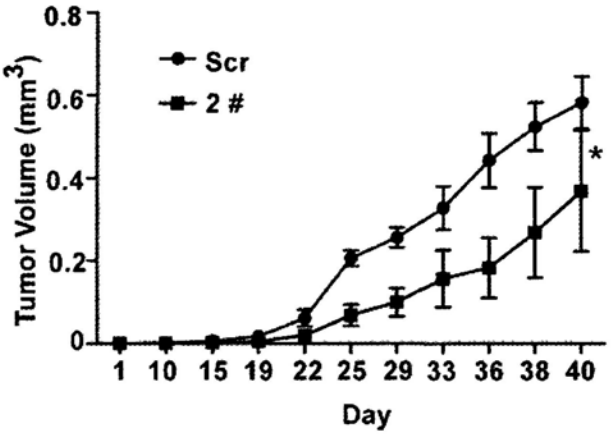

Figure 3.7.1 The repression of Bmi-1 reduced the xenografts' volumes and delayed the tumor occurrence (**P=0.038*).

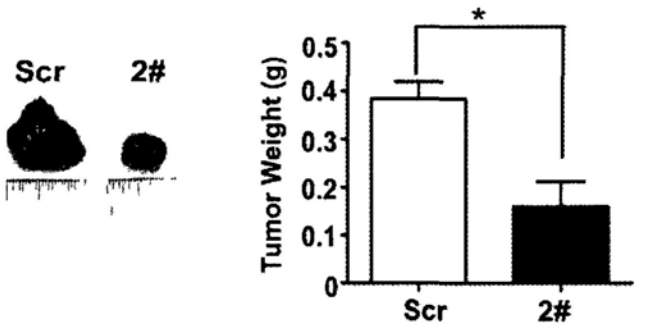

Figure 3.7.2 The repression of Bmi-1 depressed the xenografts' weight (**P=0.041*).

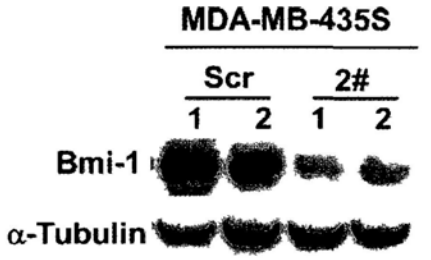

Figure 3.7.3 Bmi-1 expressions in the primary xenografts tissues were detected by immunoblotting (1 and 2 were abbreviated from sample 1 and sample 2).

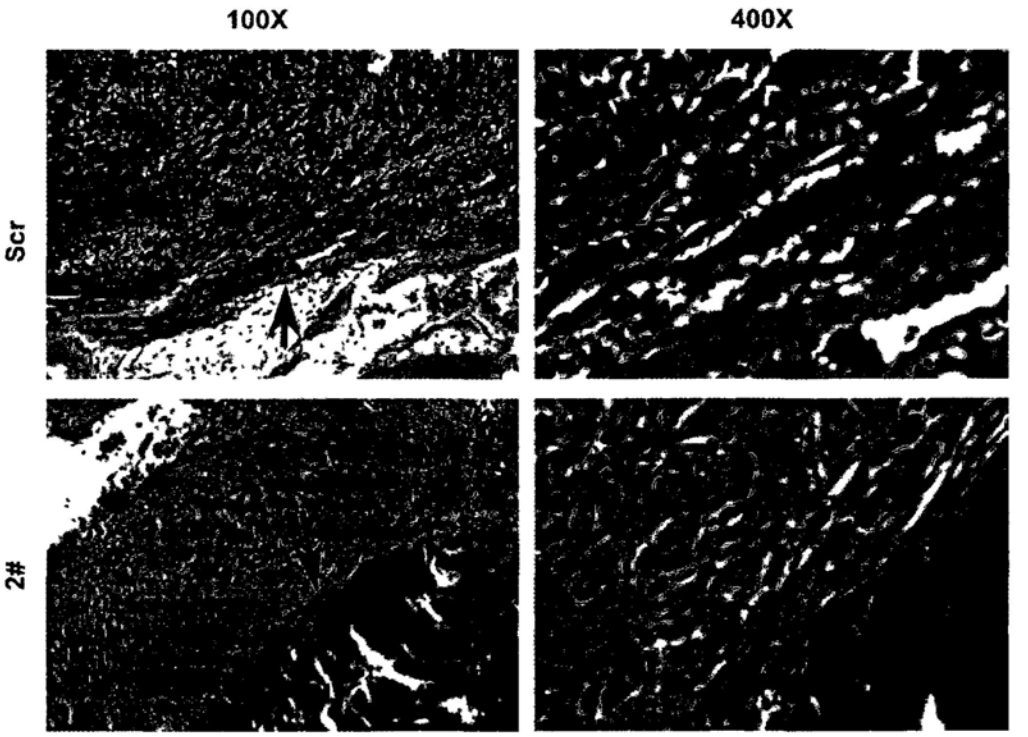

Figure 3.7.4 H&E staining confirmed the invasiveness of the primary xenografts by analyzing the encroachment of adjacent muscle. The xenografts from MDA-MB-435S/shScr cells invaded the adjacent muscles deeply, whereas the MDA-MB-435S/shBmi-1 2# cells showed reduced invasiveness (100 x and 400 x).

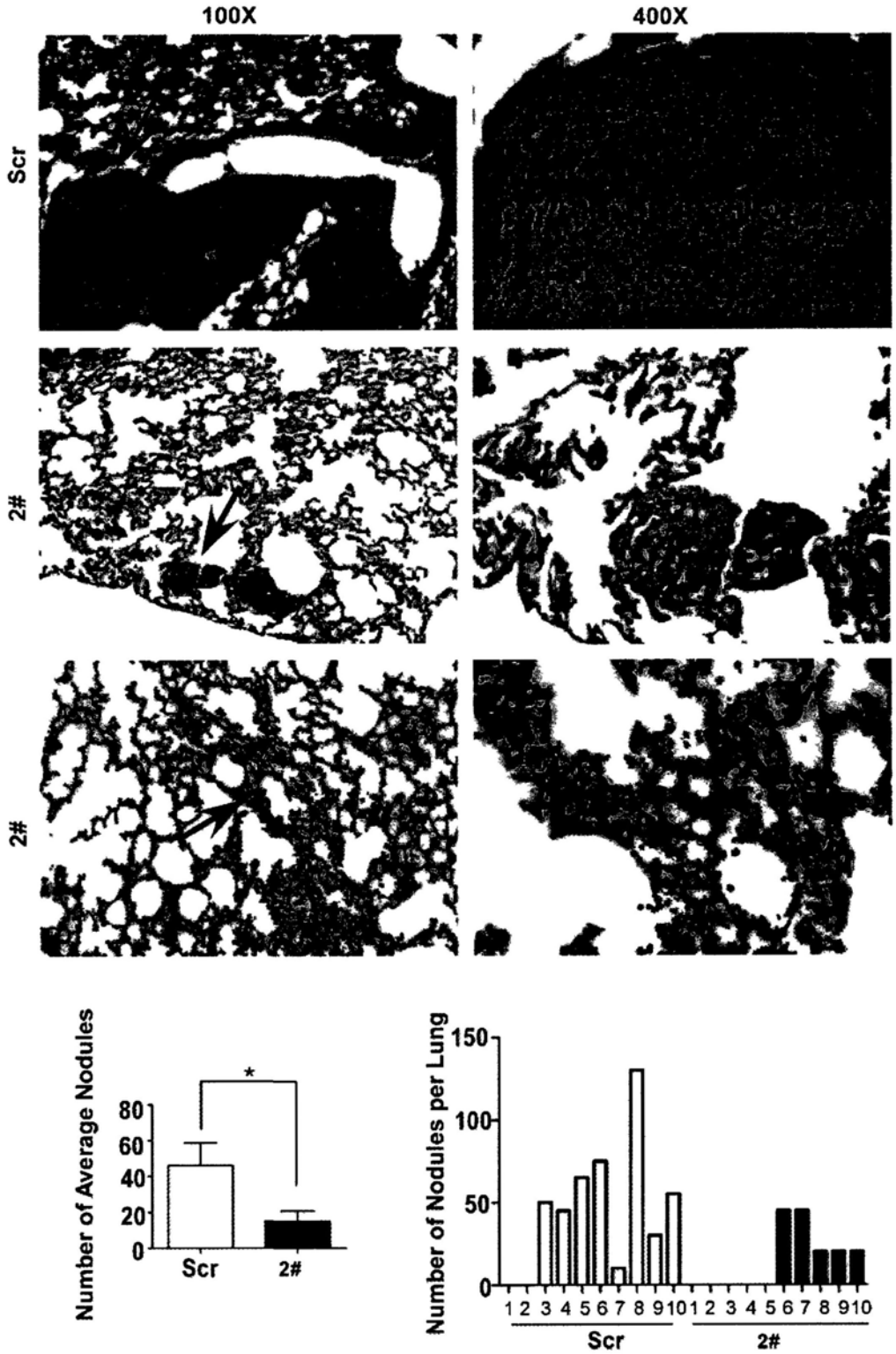

Figure 3.7.5 Repression of Bmi-1 decreased lung metastatic lesions. The number of spontaneous lung metastatic lesions in mice (N=10 per group) was analyzed by counting serial ten sections for each sample (**P=0.036*).

3.8 Bmi-1 expression Induced the Expression of Mesenchymal Markers and Inhibited the Expression of Epithelial Markers

The expression of EMT markers was analyzed in order to address the mechanism of Bmi-1-facilitated aggressiveness and metastasis of breast cells. Bmi-1 overexpression repressed the expression of epithelium markers, such as E-cadherin and β-Catenin, accompanied by up-regulation of the expression of mesenchymal markers including Vimentin and Fibronectin (Figure 3.8.1). By contrast, the knockdown of Bmi-1 expression inhibited the expression of Vimentin and Fibronectin, but partially rescued the expression of β-Catenin (Figure 3.8.1). However, the expression of E-cadherin could not be detected in MDA-MB-435S cells because of its unique properties [39, 280]. To further validate the role of Bmi-1 in EMT, mRNA levels of Bmi-1 and E-cadherin were measured in 34 breast cancer tissues and paired non-cancerous tissues from the same patient by real-time quantitive PCR. As shown in Figure 3.8.2, Bmi-1 was strongly up-regulated in breast cancer tissues compared with paired non-cancerous tissues (A, $**P=0.001$), whereas E-cadherin was markedly down-regulated (A, $*P=0.042$). Additionally, there was a converse correlation between Bmi-1 and E-cadherin at the transcriptional level (B, Spearman's rho=-0.418, $*P=0.041$). To further decipher the role of Bmi-1 in the invasion and metastasis of breast cancer, EMT markers were detected in the primary xenografts and spontaneous metastatic lung lesions by immunohistochemistry. As shown in Figure 3.8.3, the Bmi-1 repression enhanced the expression of

β-Catenin and concomitantly reduced the expression of Fibronectin in primary xenografts and metastatic lung lesions. As demonstrated above, Bmi-1 expression is negatively correlated with the E-cadherin expression, which is important for the EMT of breast cancer cells.

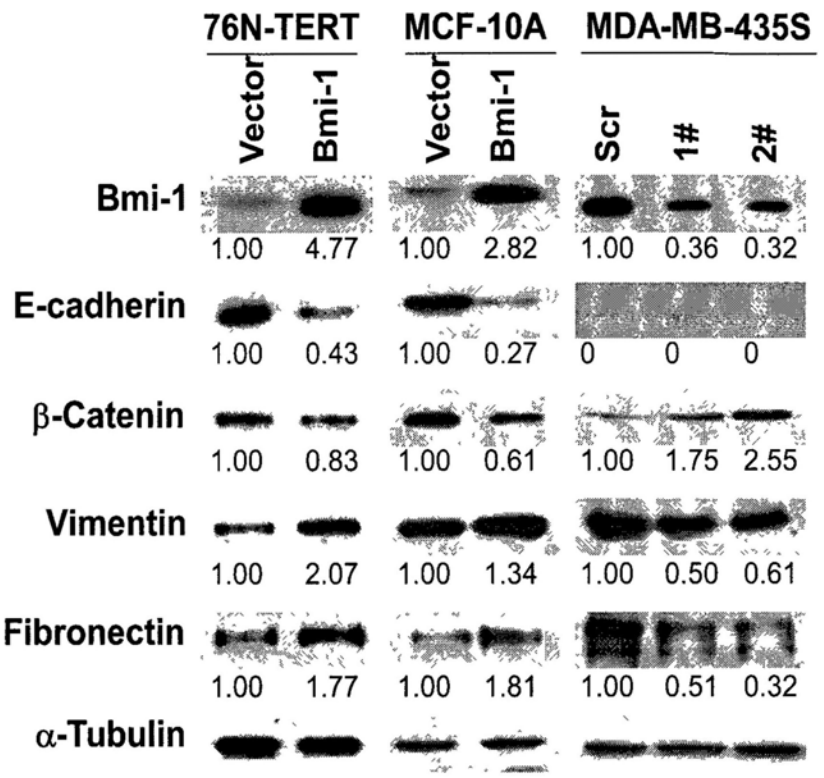

Figure 3.8.1 Bmi-1 expression induced the expression of mesenchymal markers and inhibited the expression of epithelial markers. The expressions of indicated proteins were analyzed by immunoblotting. Anti-α-Tubulin was used as the loading control.

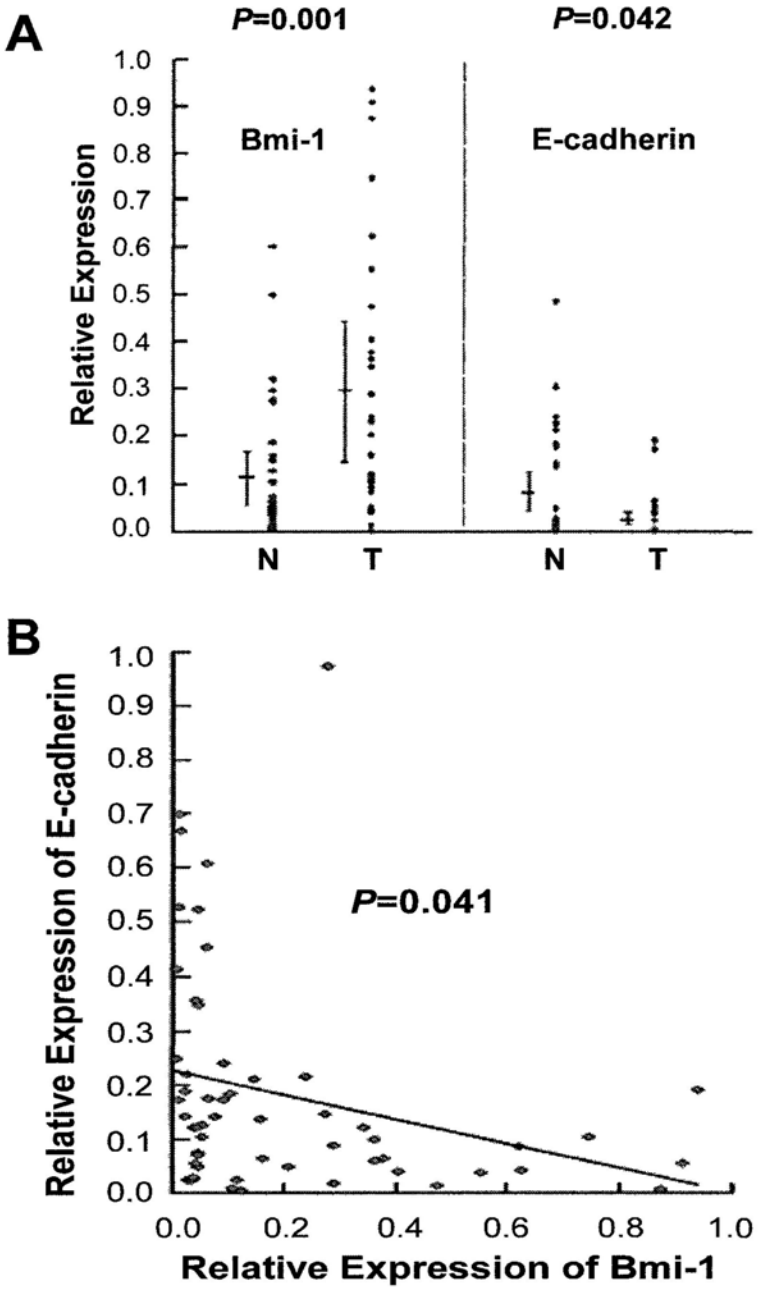

Figure 3.8.2 Converse correlation between Bmi-1 and E-cadherin expression at the transcriptional level. Top: The mRNA levels of Bmi-1 and E-cadherin were compared between the breast cancer tissues and the adjacent non-cancer tissues (**A**, ***P=0.001*; **B**, **P=0.042*). Bottom: The converse relationship between Bmi-1 and E-cadherin expression levels was plotted (**B**, Spearman's rho=-0.418, **P=0.041*).

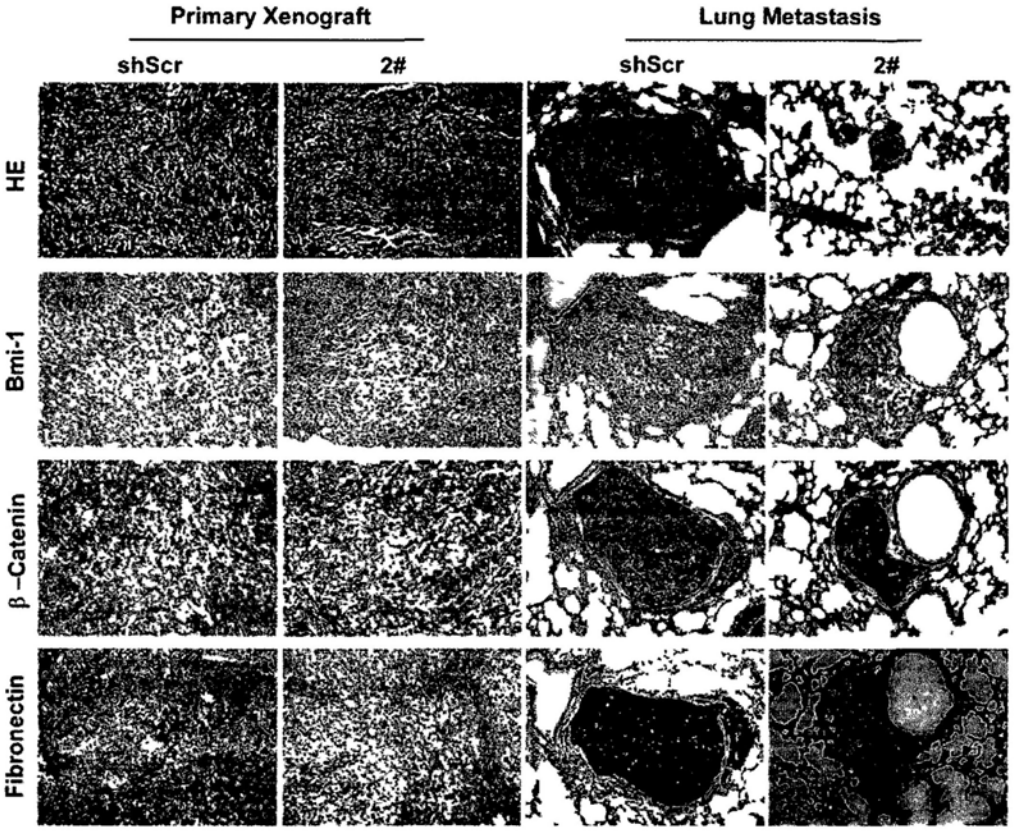

Figure 3.8.3 Suppression of the endogenous Bmi-1 expression reversed EMT markers in nude mice. H&E staining indicated the similarity of histopathology of primary xenografts and lung metastases in two groups of mice. The indicated proteins were analyzed by immunohistochemistry. Bmi-1 and Fibronectin expression detected in both primary xenografts and lung metastasis were down-regulated, whereas the expression of β-Catenin was up-regulated in the MDA-MB-435S/2# group, compared with the MDA-MB-435S/shScr group.

3.9 Bmi-1 activated the Akt /GSK-3β/Snail Pathway

Because Akt/GSK3β pathway was often involved in the EMT, we analyzed Akt, GSK-3β, and Snail expression to determine the mechanism of Bmi-1-induced alteration of EMT markers. Consistent with previous studies which indicated that Bmi-1 expression could regulate Akt activity in breast cancer cells [167] and the Akt /GSK-3β/Snail pathway in NPC cells [28], overexpression of Bmi-1 facilitated phosphorylated Akt expression and knockdown of Bmi-1 inhibited phosphorylated Akt expression, but total Akt remained unaffected (Figures 3.9.1). Accordingly, the expression of phosphorylated GSK-3β was up-regulated by Bmi-1 overexpression, and down-regulated by Bmi-1 knockdown, but no appreciable changes were observed in the total GSK-3β expression (Figures 3.9.1). Surprisingly, MCF-10A had vey little Snail expression, whereas MCF-10A/Bmi-1 expressed noticeable amount of Snail expression (Figures 3.9.1). In addition, Bmi-1 knockdown led to the distinct fall of Snail in MDA-MB-435S (Figures 3.9.1). Nevertheless, the transcriptional level of Snail was not affected by Bmi-1 overexpression examined by RT-PCR (Figures 3.9.2), suggesting that the modulation of Snail might be due to a post-transcriptional modification. It was reported that Bmi-1 could extend the half-life of Snail in NPEC cells by identifying the subcellular localization [28]. Therefore, we analyzed the localization of Snail in MCF-10A cells. As shown in Figure 3.9.3, Snail protein could be detected in the nucleus and cytoplasm of the controls, but it was primarily localized in the nucleus of the Bmi-1-mediated cells.

Collectively, Bmi-1 seems to activate Akt and GSK-3β by phosphorylation, release Snail, down-regulate E-cadherin expression, and finally result in the deregulation of EMT markers, whereby it promotes migration and invasion of the breast cells.

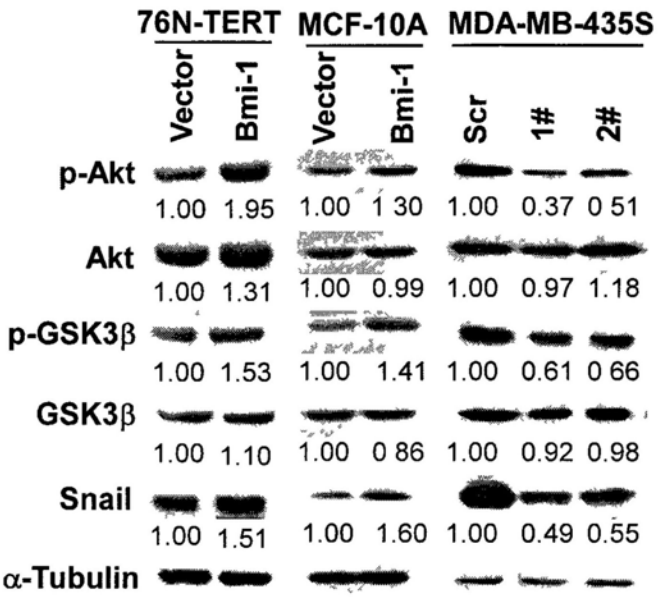

Figure 3.9.1 Bmi-1 modulated Akt/GSK3β/Snail pathway. Cell extracts were analyzed by immunoblotting with antibodies against the indicated proteins. Anti-α-Tubulin was used as the loading control.

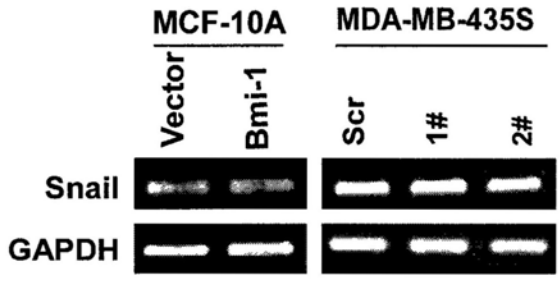

Figure 3.9.2 Transcriptional level of Snail was not affected by Bmi-1 overexpression. GAPDH was used as the loading control.

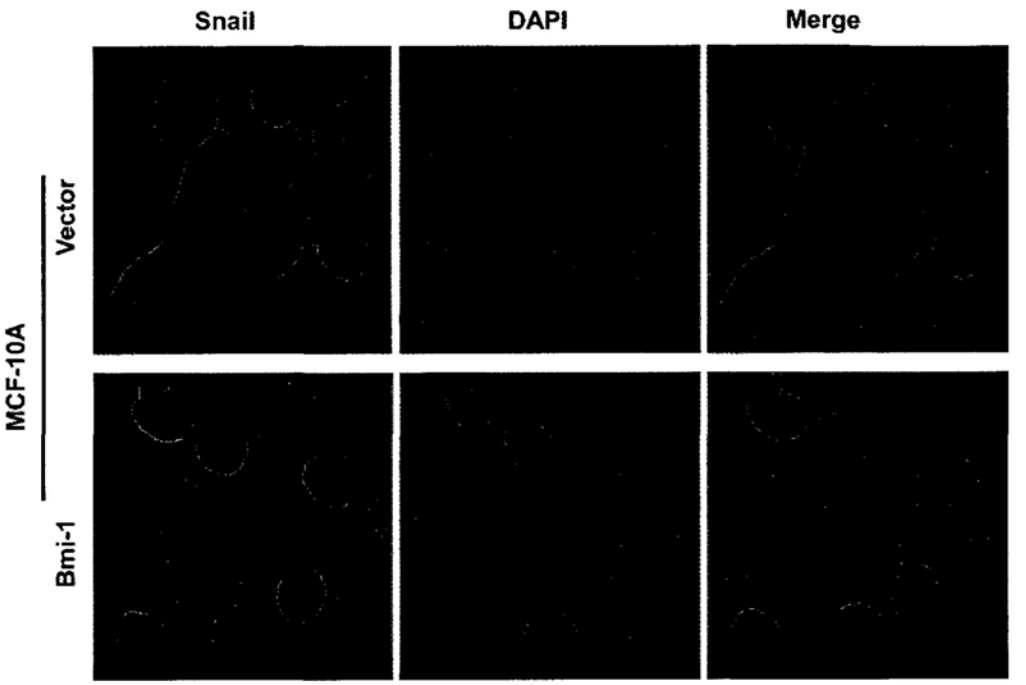

Figure 3.9.3 Immunofluorescence identified the effect of Bmi-1 on the subcellular localization of Snail proteins (Green) in MCF-10A cells.

Chapter 4 Discussion&Conclusion

4.1 The Impact of Bmi-1 in the Invasion and Metastasis of Breast Cancer

Breast cancer, a common malignant disease in women, is prone to invade the adjacent regions and inclined to metastasize to lymph nodes and distant organs. In order to develop novel treatments and cures, it is indispensable to address the nature underlying the tumorigenesis, invasion and metastasis. Although Bmi-1 expression in breast cancer was detected previously, the correlation of Bmi-1 and clinicopathologic parameters was not analyzed. In addition, the role of Bmi-1 expression in the prognosis and survival remained unknown [167]. Kim *et al* brought out the relationship between Bmi-1 expression and clinicopathologic parameters in only 37 samples, but the survival of patients depending on Bmi-1 expression was not mentioned [16]. Choi *et al* revealed the role of Bmi-1 in the prognosis of patients and the correlation with clinicopathologic parameters, but Bmi-1 expression in adjacent non-cancerous tissues was not investigated. The current study first illustrated Bmi-1 expression in primary breast cancer tissues, followed by stating the association between Bmi-1 expression and clinicopathologic parameters, and finally addressed the role of Bmi-1 in the prognosis and overall survival in a large series of 252 samples. Herein, the discordant expression of Bmi-1 was observed between primary cancer tissues and matched adjacent non-cancerous tissues in a significant number of patients. Only 35.9% of matched adjacent non-cancerous tissues displayed high Bmi-1 expression. However, as many as 72.2% of the cancer tissues were defined as high Bmi-1 expression. The rate of Bmi-1

expression was much higher in breast cancer than previous data (53.2%) [20]. The difference may reflect the Bmi-1 status in the samples used in different studies, which recruited tissues samples from patients with different disease stages, or samples from different populations. As shown in Table 4.1, 46.4 % of Chinese samples with early stage (Ⅰ , Ⅱ) were recruited in our study, while 75.8% of Korean samples with early stage (Ⅰ , Ⅱ) were used in Choi's study [20]. In addition, no samples with stage Ⅳ had been recruited in Choi's study [20] (Table 4.1). Furthermore, the difference may come from variations in antigen retrieval, antibody dilution, development time and the positive criteria adjusted (Table 4.3), especially the score of the positive number. For example, we used the EDTA buffer to retrieve antigen in our research compared with citrate buffer in Choi's study [20] (Table 4.2). In addition, the development time in our study was 10 min compared with 5 min in Choi's study [20] (Table 4.2). The criteria used in the immunohistochemistry varied in different studies. Choi *et al* tested Bmi-1 expression by tissue microarray, which might not be good representative for the whole paraffin-embedded tissue (Table 4.1). Choi *et al* quantified positive cells as a percentage of the total number of tumor cells with observation of 100 cells in five high power fields (400 x), and assigned to one of five categories: 0:<5%, 1: 5-25%, 2: 26-50%, 3: 51-75% and 4: >75% [20] (Table 4.3). However, in our study, the percentage of positive cells was defined as <5% of the cells (1 point), 6% to 35% of the cells (2 points), 36% to 70% of the cells (3 points), >71% of the cells (4 points) in 4 lower power field (40 x)

(Table 4.3). Furthermore, cells were considered positive for Bmi-1 only when nuclear staining was observed [20]. Whereas, nuclear and cytoplasm staining were both observed in our samples. However, to further confirm Bmi-1 expression in breast cancers, multi-central studies are required.

Interestingly, we found that Bmi-1 expression was incremental in the progression of cancer from normal ductal tissue, lobular glandules hyperplasia, carcinoma *in situ* to invasive breast cancer. The similar results were seen in ovarian cancer, colon cancer, liver cancer, bronchial cancer and gastric cancer, which reported the increased positive staining of Bmi-1 occurred from normal tissues to cancer tissues [176, 184, 186, 281]. However, the consistent expression of Bmi-1 appeared to be a feature of biliary epithelial cells and intrahepatic cholangiocarcinoma (ICC) [282]. The immunohistochemical expression level of Bmi-1 was almost the same or slightly lower in proliferative bile ductules (ductular reactions) frequently seen in various conditions such as chronic viral hepatitis and regeneration after submassive hepatic necrosis, when compared with intrahepatic bile ducts [283]. In addition, the immunostaining for Bmi-1 failed to detect a difference between nonneoplastic biliary epithelial cells and ICC [282], however, this study depended on the only 30 samples including ICC of hilar type (N=13) and peripheral type (N= 8) and bile ductular carcinoma (BDuC) (N= 9) [282]. And the statistic difference was not measured because the small number samples [282]. In our study, there was a significant difference of

Bmi-1 expression between adjacent non-cancerous tissues and cancer tissues ($P<0.001$). Although only 35.9% of adjacent non-cancerous tissues displayed high Bmi-1 expressions, Bmi-1 staining signals were detected in 69.2% adjacent non-cancerous tissues. Hyperplasia often occurred in adjacent non-cancerous tissues, so we speculated the staining of Bmi-1 was originated from the hyperplasia. In addition, the presence of free circulating nucleic acids (CNAs) had been observed in healthy controls and patients with metastasis had higher CNA levels than patients with localized disease [284, 285]. Bmi-1 expression had also been reported be at lower levels in plasma of healthy controls than breast cancer [18]. Therefore, it was not surprising to find Bmi-1 expression in non-cancerous tissues. In addition, Bmi-1 alone was not enough to transform immortalized HMECs. All these results implied that some other oncogenes or tumor suppress genes, together Bmi-1, contributed the transformation of Bmi-1. Therefore, it suggested that these genes probably up-regulated Bmi-1 expression.

We also studied Bmi-1 mRNA expression extracted from 30 breast cancer tissues with adjacent non-cancer tissues. There was a significant discrepancy between breast cancer tissues and adjacent non-cancer tissues, which was in accord with the protein levels in tissues. Besides its expression in breast cancer tissues [21], the higher expression of Bmi-1 was also detected in the plasma of breast cancer than the healthy control, although Bmi-1 mRNA in plasma was

identified in 43.2% breast cancer samples (48 in 111) and in 55% the healthy controls (11 in 20) [18]. These reports suggested that Bmi-1 plays an important role in the carcinogensis of breast cancer.

In this study, Bmi-1 was localized in the nucleus and cytoplasm. Interestingly, the higher rate of cytoplasm staining was observed in adjacent non-cancerous tissues, whereas breast cancer tissues exhibited the higher rate of nucleus staining. This indicated that Bmi-1 protein seemed to localize in the nucleus of the breast cancer cells and in the cytoplasm of the non-cancer cells. Previous data also addressed Bmi-1 proteins showed the cytoplasm or nucleus staining in the breast cancer [167], but it did not confirm the different staining in adjacent non-cancerous tissues and breast cancer tissues. However, Kim's and Choi's study only mentioned the nucleus staining of breast cancer [16, 20] and the cytoplasm staining was considered as non-specific staining [286]. Saeki *et al* revealed that high Bmi-1 expression resulted in strong nuclear staining in breast cancer, while low Bmi-1 expression was primarily observed in the cytoplasm [21]. Bmi-1 acted as a transcriptional repressor by regulating chromatin silencing, because Bmi-1 dots appeared in different parts of the nucleus, often very near to or partially coincident with heterochromatin. However, the nuclear staining of Bmi-1 was subjected the regulation of cell cycle [287]. The nucleus and cytoplasm staining was also observed in ovarian cancer [176] and gastric cancer [288], but it showed the nucleus staining in squamous cell carcinoma of the

tongue [175], hepatocellular carcinoma [184], colon cancer [23] and ICC [282]. Although all gastric samples tested positive for Bmi-1 localization in the cytoplasm [186, 288], the majority of gastric-cancer-adjacent samples expressed cytoplasmic Bmi-1 staining in more than 50% of the cell population, and less than 15% of the gastric carcinoma samples expressed cytoplasmic Bmi-1 staining in more than 50% of the cell population. The opposite results were observed in the nuclear localization of Bmi-1 in gastric-cancer-adjacent samples and gastric carcinoma samples [288]. These results are in line with our study.

In fact, a lot of polycomb families were the mainly nuclear localized proteins, including EZH2 [289], RING1 [290], Nervous System Polycomb 1(NSPc1) [291]. But the NSPc1 protein also existed at least partially in the cytoplasm. The phosphorylation could explain the different subcellular localization [291]. Early studies had also depicted that hyperphosphorylated M33, a mouse polycomb protein, was shown to be localized in the nucleus, whereas hypophosphorylated M33 was localized to the cytoplasm. The nuclear M33 isoforms treated with alkaline phosphatase had increased the mobilities corresponding to cytoplasmic M33 [292]. Accordingly, when cells ceased proliferating, the nuclear M33 isoforms were dephosphorylated and returned to the cytoplasm [292]. However, nuclear localization is a prerequisite for M33 to exert its function [293]. Fujisaki *et al* had found that Mel-18 dimerization might be regulated by its phosphorylation status, with dephosphorylated Mel-18 forming homodimers,

which dissociated when phosphorylated by PKC [294]. As reported before, there was a rich proline/serine region at the carboxyl terminus of Bmi-1 protein, where phosphorylation often occurred [142]. Additionally, there were growing evidences suggesting that Bmi-1 could activate Akt/ PKB pathway [167] and Bmi-1 was cell cycle-regulated and correlated with its phosphorylation status [287]. Whether phosphorylation of Bmi-1 was a direct cause or merely associated with the nucleus-cytoplasm shuttling events remained to be determined, however.

Herein, high Bmi-1 expression showed the obvious converse correlation with the larger tumor size, lymphonode involvement, organ metastasis and advanced clinical stages. This finding was converse to previous data [20], which indicated that the expression of Bmi-1 was significantly correlated with favorable prognostic index at diagnosis. Our result revealed that high Bmi-1 expression was related to the more aggressive behavior of breast cancer. In addition, the rate of high Bmi-1 level was low than 50% in adjacent non-cancerous tissues and with clinical stage Ⅰ / Ⅱ. However, the rate was over 90% in breast cancer tissues with clinical stage Ⅲ/Ⅳ. This result further revealed that Bmi-1 expression served as a possible marker to identify late stage cancer. In fact, more statistically significant effects of Bmi-1 expression in advanced stages were observed when OS was analyzed. Another study pointed out positive signals with a more intense Bmi-1 staining pattern was observed in invading

fronts stronger than in the central portions of primary invasive ductal breast cancers [16]. In addition, high level of Bmi-1 expression was significantly correlated with lymph node metastasis [16]. However, not in concordance with our studies, higher mRNA expression extracted from tissues was observed in the patients without lymph node metastasis. High Bmi-1 mRNA expression levels of tissues were observed in the relatively early stages (stage I and II) and Bmi-1 could serve as a diagnosis marker for breast cancer [21]. Choi *et al* demonstrated that the expression rates of Bmi-1 protein were higher in the early stages of breast cancer (20.2% in stage I, 55.6% in stage II and 24.2% in stage III) [20] (Table 4.1). But Bmi-1 mRNA in the plasma of breast cancer had no significant correlation with clinical stage [18]. It was unclear whether high expression of Bmi-1 mRNA causes carcinogenesis or whether carcinogenesis leads to the elevation of Bmi-1 mRNA expression. Furthermore, Bmi-1 overexpression showed converse correlations with clinicopathologic characteristics in many carcinomas, including NPC, bladder cancer, ovarian cancer and colon cancer [22, 23, 174, 176, 179]. These study supported high level of Bmi-1 protein might contribute to the invasion and progression of breast cancer. However, the study did not investigate the correlation between Bmi-1 expression and p53, Ki67, BRCA1/BRCA2 expression, which was the well-established poor prognosis marker in breast cancer, because these markers were not available in our records. In addition, pathologic grade were not considered in this study, because more than majority of patients showed ductal

carcinoma.

It is also important to note that high levels of Bmi-1 expression were a predictor of short overall survival for breast cancer, independent of TNM classification, clinical stage and hormone receptor. Although our study contradicts with others' reports [19, 20], it is in agreement with the results report by Silva *et al's* [18]. There was lack of consistency in the prognostic significance in their studies. Table 4.4 clarified the difference among the previous researches on Bmi-1 expression of breast cancer. Consisting with this study, the expression of Bmi-1 also predicted the poor prognosis in leukemia, lymphoma, colorectal cancer, squamous cell lung cancer and nasopharyngeal cancer [22, 25, 172, 182, 183, 295, 296].

In our study, Bmi-1 was not significantly correlated with ER and PR, which was similar to the previous report which indicated that Bmi-1 mRNA levels of tissue had no significant correlation with ER or PR [21]. However, higher Bmi-1 mRNA level in the plasma was related to negative progesterone receptors, which was a well-established marker of poor clinical outcome in breast cancer [18]. Nevertheless, a statistically significant association was observed between Bmi-1 expression and survival when ER or PR was included in the analysis in our study. ER^+ cancer cells depend on the estrogen presence for their growth, so they can be treated with drugs to reduce the expression of estrogen (Tamoxifen).

Patients with the ER presence were offered adjuvant hormone therapy in our study. The prognostic role of Bmi-1 expression in breast cancer seemed to be irrelevant to adjuvant hormonal therapy in this study. The discrepancy may come from subpopulation, area and therapy. Patients with metastatic breast cancer may take tamoxifen for varying lengths of time, depending on the cancer's response to this treatment and other factors (http://www.cancer.gov/cancertopics). When used as adjuvant therapy for early-stage breast cancer, tamoxifen is generally prescribed for 5 years. However, the ideal length of treatment with tamoxifen is not known. Then, different time of therapy depending on the response to it maybe resulted in different outcome in our study. In addition, the combined endocrine therapy and other therapy remained the research issue (http://www.cancer.gov/cancertopics). Generally, patients with positive HER-2 showed a worse prognosis [297]. However, these 252 patients did not receive the anti-HER-2 therapy because Herceptin (a humanized monoclonal antibody directed at the HER2 ectodomain) did not come to use at that time. Although there was no significant correlation between Bmi-1 expression and HER-2, patients with high Bmi-1 expression showed poor survival stratified by HER-2, just like ER and PR. Having demonstrated the significance of Bmi-1 expression in overall survival of breast cancer patients, it will be our next focus to investigate the prognostic value in disease-free survival and cancer-specific survival in terms of Bmi-1 expression. Additionally, a possible correlation between Bmi-1 expression and the outcome after hormonal

therapy and chemotherapy is a worthy investigation, which would require large samples.

The univariate Cox regression hazard analyses revealed that higher level of Bmi-1 expression predicted higher risk of death in breast cancer patients. The relative risk increased nearly 4 fold in patients with high Bmi-1 expression than the other. After the adjustment for potential confounding factors, Bmi-1 expression by multivariate Cox regression hazard analyses was found to predict poor survival. It was worth noting that large tumor size, lymph node involvement, distant metastasis and advanced clinical stage were significant unfavorable prognostic factors. In addition, PR and clinical stage still claimed the prognosis by multivariate Cox regression hazard analyses. Choi's study explained the tumor size was not a prognostic factor, but lymphonode metastasis could predict the prognosis of breast cancer. Unfortunately, they did not study the prognosis of clinical stage, which was the best indicator for the prognosis, although they analyzed the relationship between Bmi-1 expression and clinical stages [20]. Except clinical stages, they concluded that Bmi-1 expression was an independent prognosis factor [20]. However, the Bmi-1 impact on the prognosis of breast cancer was not explicated on other studies [16, 21]. Recently, the immunoreactivity of Bmi-1 emerged as an independent prognostic factor by multivariate Cox regression analysis in many carcinoma, including colon cancer [23], ovarian cancer [281], bladder cancer [174] and gastric cancer [24]. But the

absence of Bmi-1 protein in squamous cell carcinoma of the tongue was bound up with a higher risk of the recurrence [281]. The exact prognosis of Bmi-1 expression should be stated depending on large number of samples and commonly-used markers included. The tumor size and lymph node involvement were both valuable prognostic indicators [95-97, 99]. A larger tumor size had been related to more positive lymph nodes [99], thus their interaction further influenced the survival from breast cancer. Node positive patients had about a 4-8 times higher mortality than those without the nodal involvement [97, 100, 102]. In our study, the more nodes and larger size were involved in the worse prognosis. The survival of node-positive patients was improved due to better staging procedures and application of systemic treatment [95, 98, 298]. It was reported that patients with metastasis (stage: M1) at diagnosis exhibited very poor 10-year survival (3.4%) [299]. Table 4.1 –Table 4.4 showed the difference of Bmi-1 in breast cancer in previous reports.

Table 4.1 Patients' informations in different studies

Author	Number	Diagnosis	Population	Mean Age	Follow-up	Method	TNM (%, N)
Nalwoga H	192	1990-2002	African (Ugandan)	46.2Y (18-80)	9 M(0.5-108)	TMA and IHC	2003 (WHO) Stage 1-2: 9% (2) Stage 3: 36% (8) Stage 4: 54% (12)
Pietersen AM	295	1984-1995	Dutch	Unknown	10.2 Y	TMA and IHC	Stage 1-2: 295 samples
Choi YJ	960	1995-2002	Korean	47Y (20-80)	77.1 M(8-149)	TMA and IHC	6th AJCC Stage Ⅰ: 20.2% (94) Stage Ⅱ: 55.6% (534) Stage Ⅲ: 24.2% (232)
Kim JH	71	2000-2002	Korean	Unknown	Unknown	IHC	TNM residual tumor classfication
Arners JB	176	1996-2001	Norwegian	60Y (49-72)	69M (10-105)	TMA and IHC	Unknown
Guo BH	252	1999-2001	Chinese	47Y (26-78)	59M (4-78)	IHC	2002 (UICC) Stage Ⅰ: 9.1% (23) Stage Ⅱ: 37.3% (94) Stage Ⅲ: 35.7% (90) Stage Ⅳ: 17.9% (45)

Table 4.2 Antigen retrieval method, recourse of antibody and developmental time in different studies

Author	Antigen Retrieval Method	Antibody	Development
Nalwoga H	Tris–EDTApH9.0 microwaving (750W for 10min) (350W for 20min)	6C9 60min, RT	10min
Pietersen AM	citrate buffer, Unknown	Millipore, F6, Ati-mouse 1:400	Unknown
Choi YJ	citrate buffer, microwaving	Upstate Biotechnology Lake Placid, NY, USA	5min
Kim JH	citrate buffer (PH=6.0), microwaving	Upstate Biotechnology, Lake Placid, NY, USA Anti-mouse 1:100, 90min, RT	Unknown
Arners JB	Tris–EDTApH9.0, microwaving	Upstate Biotechnology, Billerica, MA, USA Clone F6, 1:800, overnight at 4°C	
Guo BH	EDTA PH8.0, microwaving	Upstate Biotechnology, Lake Placid, USA clone F6, 1:100, overnight at 4°C	10 min

Table 4.3 Positive criteria used in different studies

Author	Evaluation
Nalwoga H	a staining index (values0–9) was determined by multiplying the score for intensity of staining(none=0,weak=1, moderate=2 and strong=3) with the score for proportion of tumor cells stained (<10%=1, 10–50%=2, >50%=3). The majority of cases (75%) had staining index 0, and therefore the cut off was 0=negative and 1–9=positive.
Pietersen AM	38% patients having high levels of expression
Choi YJ	The staining intensity was subclassified as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Positive cells were quantified as a percentage of the total number of tumor cells with observation of 100 cells in 5 high power field (9400), and assigned to one of five categories: 0: <5%, 1: 5–25%, 2: 26–50%, 3: 51–75% and 4: >75%. The score of percentage of positive tumor cells, and the score of staining intensity were added to generate the immunoreactive score (IS) for each tumor specimen. The cases were grouped by IS value as 0, 1+ (IS 1, 2), 2+ (IS 3, 4), 3+ (IS 5, 6) and 4+ (IS 7). The slide of higher than 2+ was classified as a positive case.
Kim JH	Moderate to strong nuclear staining was regarded as a positive reaction. The distribution of Bmi-1 was scored on a semi-quantitative scale, as follows: negative (<10% of tumor positive), focally positive(10–50% of tumor positive), and diffusely positive (>50% of tumor positive).
Arners JB	The intensity of reactivity was graded 0, (no reactivity) to 3 (strong reactivity).Tumor cell area was graded 1(0–10%), 2 (11–50%) and 3 (>50%). A composite staining index (SI) was defined as the product of the intensity and area scores, giving values from 0 to 9. Expression categories were initially based on quartiles for SI in the present dataset,

	considering also the distribution plots as well as the number of cases and events when dichotomous variables were constructed. After evaluation, the SI median (SI=3) was chosen for analysis of associations and outcome.
Guo BH	positive cells were quantified as a percentage of the total number of tumor cells in 10 low power field (40X) and assigned to one of four categories: $\leq$ 5% of the cells (1 point), 6% to 35% of the cells (2 points), 36% to 70% of the cells (3 points), $\geq$ 71% of the cells (4 points). The staining intensity was subclassified as negative staining (1 point), weak staining (2 points), moderate staining (3 points) and strong staining (4 points). A final score was then calculated by multiplying the above two scores. If the final score was >4, Bmi-1 expression was considered high, otherwise, Bmi-1 expression was considered negative.

AJCC: the American Joint Committee on Cancer

UICC: International Union against Cancer

Table 4.4 Description of results in different studies

Author	Reuslts
Nalwoga H	46 tumors (25%) were positive for Bmi-1 staining. Bmi-1 positivity was mostly associated with low histological grade, low mitotic counts and ER positivity. Bmi-1 expression was inversely associated with the TNP. No survival Analysis
Pietersen AM	High expression of Bmi-1 predicted better OS. The 'protective' effect of Bmi-1 expression was limited to lymph node-positive patients. Bmi-1 was correlated low grade and positive ER. Bmi-1 was independent prognostic factor
Choi YJ	511 (53.2%) cases had positive staining. Bmi-1 expression was smaller tumor size, negative axillary lymph node, earlier stage, low nuclear grade, positive ER and positive PR. Bmi-1 was independent prognostic factor for OS.
Kim JH	58% (30 in 44) was observed more intensely in the invading fronts than in the central portions of the primary invasive breast cancers. Bmi-1 oncoprotein was negative, focally and diffusely positive in 27 (38%), 21 (30%), and 23 (32%) cases. There was a positive correlation between Bmi-1 expression and axillary LN metastases or ER.
Arners JB	75 (42.6%) cases had positive staining. There were positive associations between Bmi-1 with ER and PR positivity, Negative correlation between Bmi-1 and basallike profiles. Univariate survival analysis did not show a significant influence of Bmi-1 status on overall survival.
Guo BH	72.2% (182) cases were defined as high expression. 96.5% (241) cases showed positive staining. Bmi-1 expression was strongly correlated with large tumor size, lymph node involvement, distant metastasis, and advanced clinical stage. High Bmi-1 expression is associated with an unfavorable prognosis.

Understanding of the mechanisms of transformation and metastasis of human breast epithelial cells required a reliable source of normal cells which had not been affected by viral or chemical agents. To address the role of Bmi-1 in tumor progression, Bmi-1 expression was overexpressed in two HMEC lines, including 76N-TERT, a p53-positive and p16-negative spontaneously immortalized HMEC line by hTERT [300, 301], and MCF-10A, which did not express $p16^{INK4a}$, $p14^{ARF}$, $p15^{INK4b}$ and estrogen receptor-negative [302, 303]. MDA-MB-435S with Bmi-1 repression were established by RNA interference, which was an estrogen independent breast cancer cell lines derived from mammary ductal carcinoma [304]. Although MDA-MB-435 was reported to originate from melanoma based on the expression of certain melanocyte genes [305, 306]. On the other hand, there had been other reports that MDA-MB-435 cells produced milk lipid droplets on induction of differentiation [307]. Recently, MDA-MB-435 xenograft tissue sections stained entirely positive for epithelium-specific markers but only partially positive for melanocyte-specific markers [308]. MDA-MB-435S could form progressively growing tumors in the lungs and regional lymph nodes metastases in following injection into the mammary fat pads of 3-4 week old athymic nude mice [277]. In our study, Bmi-1 did not change the morphology of 76N-TERT and MCF-10A cells. MCF-10A/Bmi-1 and 76N-TERT/Bmi-1 cells still formed flat monolayers with individual cell varying in size and shape. The amount of cytoplasm varied from cell to cell, whereas the nuclei were homogeneous in size and round in shape.

These morphology of cells transfected with Bmi-1 was similar with 76N-TERT and MCF-10A cells [309]. In addition, The spindle-shaped phenotype and non-contact inhibited disorganized proliferation of MDA-MB-435S [310] was not altered by the inhibition of Bmi-1. In this study, Bmi-1 did not alter the proliferation of immortalized HMECs and MDA-MB-435S. Because 76N-TERT and MCF-10A both did not show P16 expression, so we tested the P16 level in MDA-MB-435S. Interestedly, P16 was up-regulated by Bmi-1 repression. Notably, P53 levels showed no obvious difference and P21 could not be detected in MDA-MB-535S with Bmi-1 repression or sramble control. Although Bmi-1 failed to affect the proliferation of cells in vitro, Bmi-1 did decrease the tumor volume and weight in vivo. The discrepancy might come from the culture time. The proliferation rate was tested in vitro for just six days contrary to two months in vivo.

Metastatic relapse remains a major challenge in breast cancer management. Many factors are involved in tumor progression, including changes in cell adhesion, cell communication, increased migration or motility and invasiveness [311]. In this study, Bmi-1 expression contributed to all of these events as observed in clinical samples and cell lines. In the present study, Bmi-1 overexpression could contribute to the malignancy of breast cancer involving in controlling cell adhesion migration and invasiveness, whereas Bmi-1 knockdown was demonstrated to be effective in inhibiting cell adhesion,

migration, invasiveness and colony formation of a highly metastatic human breast cancer cell line, MDA-MB-435S. In the 'wound healing assay', cells were inspected over time as they fill a damaged area in the cell monolayer. Similarly, the capacity to invasiveness was assessed by 'cell migration assays' performed in Boyden chambers in response to a chemotactic gradient. Majority of normal cells are adherence-dependent on solid inert support in contrast to transformed cells. The shRNAi-treated MDA-MB-435S cells formed significantly fewer colonies in soft agar than control cells, suggesting that Bmi-1 knockdown could effectively suppress anchorage independent growth ability of breast cancer cells. Unfortunately, Bmi-1 expression could not induce the transformation of MCF-10A and 76N-TERT, however, Bmi-1 expression can promote the invasiveness and mobility of immortalized HMECs, in agreement with immortalized NPECs and MDCKs [161]. These cells transfected with Bmi-1 could not grow in soft agar in our study. In addition, Bmi-1 expression did not alter the morphology of these cells in 3-D Matrigel culture (data not shown). These were different from MCF-10A/Ras, which could grow in soft agar and form tumor in nude mice [312]. Alternatively, Bmi-1 cooperated with Ras to induce the brain metastasis of breast cancer from the fat pad of mouse [29]. Furthermore, together with Ras, Bmi-1 induced fulminant metastatic disease in the lung using a tail vein model of haematogenous spread through accelerated cellular proliferation and inhibition of apoptosis [29]. Unlike MCF-10A/ Bmi-1 cells, the morphology of MCF-10A/HER-2 and

MCF-10A/TGF cells were transformed *in vitro*, they were not tumorigenic in normal or γ-irradiated nude mice [312]. The overexpression of Bmi-1 was not sufficient to elicit tumor formation *in vitro* and *in vivo,* suggesting that additional genetic changes were needed to complete these effects such as the activation of the other proto-oncogenes or loss of expression of tumor suppressor gene.

Use of athymic nude mice has allowed many and diverse studies of human tumor biology [313], including the analysis of metastatic properties in relatively recent years [314, 315]. The mouse fat pad was a more favorable site for the growth of mouse mammary tumors [316], compared with the subcutis and also for the development of metastasis, with a higher frequency of metastasis from the mammary fat pad tumors [317]. Therefore, the MDA-MB-435S with Bmi-1 knockdown and control were injected into the mammary fat pad to determine tumorigenicity and spontaneous lung metastasis. The knockdown by shRNAi suppressed tumorigenicity and spontaneous lung metastasis of human metastatic breast carcinoma MDA-MB-435S, suggesting that Bmi-1 knockdown might have the potential to be a new therapeutic agent for the treatment of human breast cancer. To our knowledge, this was the first study reporting the Bmi-1 knockdown effect of anti-tumor on breast cancer. In our study, lung metastasis occurred in 80% mice, in accordance with previous study [277]. Taken in conjunction with the current work, Hu1A12, a mouse anti-human Osteopontin

(OPN) antibody and OPN knockdown could inhibit MDA-MB-435S tumor growth and spontaneous lung metastasis in nude mice [279, 318], but the mechanism remained unclear. In order to further examine the potential oncogenic role of Bmi-1 in immortalized HMECs, $1x10^7$ MCF-10A/Bmi-1 cells were injected into the mouse fat pad. However, no tumor formation was found after two months in nude mice and SCID mice, in accordance with previous report, which indicated that athymic male mice implanted in the axillary fat pad and female athymic mice given i.v.(intravenous injection) injections of $1x10^7$ MCF-10A cells did not develop tumors at the site of inoculation or in distant sites six months after inoculation [303]. Recent study indicated that the knockdown of Bmi-1 increased the effectiveness of radiotherapy and chemotherapy. The tumor growth in nude mice was inhibited by transplanting head and neck squamous cell cancer-derived ALDH1-positive cells with Bmi-1 knockdown [319]. This implies a clue to further determine the radiotherapy and chemotherapy sensitivity on MDA-MB-435S with Bmi-1 knockdown in the future.

4.2 The Mechanism of Bmi-1 in the Invasion and Metastasis

Approximately 90% of cancer deaths resulted from the local invasion and distant metastasis of tumor cells; therefore, an understanding of metastasis was urgently needed. One important insight came from the discovery that the increased motility and invasiveness of cancer cells is reminiscent of the EMT.

EMT is a biologic process that allows a polarized epithelial cell, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components [266, 320]. The connection between the loss of E-cadherin expression in cancer cells and passage through an EMT had been established by many studies. For example, induction of the c-Fos oncogene in normal mouse mammary epithelial cell lines induced an EMT and was associated with the decrease in E-cadherin expression [321].

Because tumors are 3-D tissues with a complex architecture and a plethora of interconnected cell types and surrounding extracellular matrix, it is obvious that EMT represents only part of the processes of tumor cell invasiveness and metastasis. Starting with a primary carcinoma, EMT of the transformed carcinoma cells can produce migratory mesenchymal derivatives. Such migratory cells use extracellular matrix structures (e.g. collagen fibers) to reach the pericyte/endothelial wall of nearby blood vessels in order to start the process of intravasation [322]. *In vitro* the force of cell-matrix interaction can be studied by 'cell adhesion assays' carried out on cellular supports that mimic the extracellular matrix like Fibronectin, Collagen, Laminin or Fibrinogen [323]. Indeed, identification of anchorage independent growth with a 'soft agar colony

formation assay' which measures proliferation in a semisolid culture medium, is a characteristic of malignancy and/or EMT *in vitro* [202, 215].

In order to determine whether Bmi-1 affected EMT, we probed the cells with epithelial and mesechymal markers and examined the changes induced by Bmi-1 expression in soft agar and 3-D Matrigel. In this study, the overexpression of Bmi-1 alone could not fully transform 76N-TERT or MCF-10A. This result was in accordance with the previous observation, which demonstrated that Bmi-1 alone did not result in the EMT phenotype in MCF-10A cells, but the co-overexpression of Bmi-1 and Ras readily did [29]. Unlike NPECs and MDCKs [28], Bmi-1 expression alone was not sufficient to induce the EMT typical morphological changes in immortalized HMECs in the current study. However, Bmi-1 could alter the expression of EMT markers in cell lines. In addition, Datta's study addressed very little expression alteration in EMT markers between MCF-10A/ Bmi-1 and MCF-10A/vector [166]. The transition from epithelial- to mesenchymal-cell characteristics encompasses a spectrum of inter- and intracellular changes, not all of which are always seen during EMT. EMT does not therefore necessarily refer to a lineage switch [203]. The converse correlation between Bmi-1 expression and E-cadherin expression was also interpreted in human breast cancer tissues and adjacent non-cancerous tissues. In cancer, downregulation of E-cadherin is the key step towards the invasive phase of carcinoma, and dominant transcriptional repression is largely

responsible for the loss of E-cadherin expression [38]. The mechanism for the Bmi-1-mediated metastasis of NPC came from the EMT [28]. This cell-specific function was also observed in Ras-induced cells [166, 324] and ILEI-induced cells [325]. Conversely, when the cells expressing ILEI were treated with a Ras farnesylation inhibitor, EMT was reverted lending further support to the notion that ILEI-induced EMT requires activation of the Ras-signaling pathway [326]. These data suggest that additional oncogenic events, such as H-Ras, are probably involved in the EMT transformation of immortalized HMECs. Thus, Bmi-1-induced EMT depends on the specificity of cells.

Crosstalk between different pathways, recognized as the mechanism capable of expanding the cellular communication signaling network, is now receiving further attention. The activated PI3K/Akt pathway was well documented for various human malignancies and sometimes correlated with an aggressive phenotype [327]. The Akt signaling cascade initiates at the cell surface when growth factors or other extracellular stimuli activate phosphoinositide 3-kinase (PI3K). Activated PI3K generates a lipid second messenger, phosphatidylinositol-3, 4, 5-trisphosphate (PIP3), causing translocation of Akt to the plasma membrane where it becomes phosphorylated and activated [328]. Previous study addressed that the down-regulation of Bmi-1 by RNA interference (RNAi) approach was accompanied by the down-regulation of Akt/protein Kinase B (PKB) activity [167]. In addition, MCF-10A had vey little

phosphorated Akt expression, whereas MCF-10A/Bmi-1 expressed a significant amount of activated Akt expression [166]. There are two phosphorylation motifs within Snail to dually regulate the function of this protein, one for protein degradation and the other for subcellular localization [38]. Recently, a new report deciphered that the depletion of Bmi-1 was able to downregulate phospho-Akt in pancreatic cell lines [182]. In this study, we confirmed that Bmi-1 triggered invasion partially due to the Akt pathway activation in breast cancer cells.

Recently, intensive study demonstrated that Snail expression directly repressed E-cadherin expression and induced EMT in MDCK cells [38, 39], indicating that Snail expression had a fundamental role in EMT through its suppression of E-cadherin. The expression of Snail and E-cadherin was correlated with the prognosis of patients suffering from breast cancer [41]. Furthermore, the loss of E-cadherin expression promoted Wnt signaling and was associated with high levels of Snail in the nucleus [41]. The central role of
Snail in the regulation of EMT has been underscored in the complex regulation of Snail stability, subcellular localization and function through different phosphorylation events [203]. GSK3β phosphorylates two Ser residues on Snail, one of which targets Snail for ubiquitination and degradation, whereas the other promotes its nuclear export [40].

Despite these various observations, there was a high debate about the

involvement of EMT in human cancer metastasis, in part due to clinical observations showing that majority of human breast carcinoma metastases expressed E-cadherin expression and maintained their epithelial morphology, suggesting that they had disseminated without switching to a mesenchymal phenotype [329, 330]. No obvious EMT phenotype was achieved in tumor invasion and metastasis in several mouse tumor models. The occurrence of distant metastases was not inhibited by blocking the EMT program with a dominant-negative TGF-β type 1 receptor [331]. Indeed, cell migration during several developmental events does not require a complete EMT phenotype. Partial EMT and collective cell migration are not sufficient to explain how individual tumor cells can intravasate into the blood circulation and travel to distant organs. Cancer cells may, more often than not, pass through a partial EMT program rather than a complete one; such cells may concomitantly express epithelial and mesenchymal markers [197]. In ductal breast carcinomas there was no correlation between the immunoreactive levels of E-cadherin and lymph node status [332, 333]. Moreover, transfection of E-cadherin was not sufficient to revert the fibroblastic phenotype of spindle carcinoma cells (such as CarB cells) to the epithelial character, even in the presence of other epithelial molecules such as Plakoglobin [334, 335]. The highly malignant CarB cells were completely deficiently in E-cadherin expression and exhibited a fibroblastic-like phenotype [336]. Despite the acquisition of invasive and metastatic properties, a block in the E-cadherin expression by stable antisense

transfection, was not sufficient to induce a full EMT [337]. In the current study, E-cadherin expression was not detected in MDA-MB43S cells, suggesting that a battery of genes were involved in the invasiveness and metastasis. To answer this question, we tried to repressBmi-1 expression in MDA-MB43S. Interestingly, Bmi-1 knockdown did not influence the morphological behavior of MDA-MB43S, albeit it significantly inhibited carcinogensis and metastasis induced by MDA-MB43S cell. These implied that other genes participated in carcinogensis and metastasis of breast cancer induced by Bmi-1 expression, apart from E-cadherin expression.

Our data suggested that Bmi-1 expression showed critical effect in tumorigenesis and lung metastasis of breast cancer. We believed this was an extremely important observation for studying lung metastasis of breast cancer, as lung is the most common location of breast cancer metastasis. We suggested that Bmi-1 played a vital role in breast cancer metastasis. E-cadherin, which mediated adherence junctions and restrains carcinoma invasion, was a useful molecule to protect breast cancer from metastasis [338]. As metastasis could be acquired from the early stage of tumor development, some genes may constantly regulate tumor development. They may not only facilitate primary tumor initiation but also promote tumor transformation and metastasis [339]. The expression patterns of Bmi-1, together with function studies, indicate that Bmi-1 plays a prominent role in breast cancer progression and metastasis and opens a

door to the future studies for Bmi-1 target therapy in breast cancer.

Conclusions

In summary, breast cancer shows a high prevalence of Bmi-1 expression, which is significantly correlated with aggressive features and unfavorable prognosis.

Assessment of Bmi-1 expression might help to identify a high-risk subgroup of breast cancers.

Bmi-1 plays a crucial role in the invasion and metastasis by modulating the Akt /GSK-3β/Snail pathway and the expression of EMT markers in breast cancer.

References

1. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massague J: **Genes that mediate breast cancer metastasis to lung.** *Nature* 2005, **436:**518-524.
2. Parkin DM, Bray F, Ferlay J, Pisani P: **Global cancer statistics, 2002.** *CA Cancer J Clin* 2005, **55:**74-108.
3. Carter CL, Allen C, Henson DE: **Relation of tumor size, lymph node status, and survival in 24,740 breast cancer cases.** *Cancer* 1989, **63:**181-187.
4. Braun S, Pantel K, Muller P, Janni W, Hepp F, Kentenich CR, Gastroph S, Wischnik A, Dimpfl T, Kindermann G, et al: **Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer.** *N Engl J Med* 2000, **342:**525-533.
5. Zhang L, Sullivan P, Suyama J, Marchetti D: **Epidermal growth factor-induced heparanase nucleolar localization augments DNA topoisomerase I activity in brain metastatic breast cancer.** *Mol Cancer Res*, **8:**278-290.
6. Wolfer A, Wittner BS, Irimia D, Flavin RJ, Lupien M, Gunawardane RN, Meyer CA, Lightcap ES, Tamayo P, Mesirov JP, et al: **MYC regulation of a "poor-prognosis" metastatic cancer cell state.** *Proc Natl Acad Sci U S A.*
7. Hayashida T, Takahashi F, Chiba N, Brachtel E, Takahashi M, Godin-Heymann N, Gross KW, Vivanco MM, Wijendran V, Shioda T, et al: **HOXB9, a gene overexpressed in breast cancer, promotes tumorigenicity and lung metastasis.** *Proc Natl Acad Sci U S A*, **107:**1100-1105.
8. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, et al: **Molecular portraits of human breast tumours.** *Nature* 2000, **406:**747-752.
9. Wang Y, Klijn JG, Zhang Y, Sieuwerts AM, Look MP, Yang F, Talantov D, Timmermans M, Meijer-van Gelder ME, Yu J, et al: **Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer.** *Lancet* 2005, **365:**671-679.
10. Kallioniemi OP, Wagner U, Kononen J, Sauter G: **Tissue microarray technology for high-throughput molecular profiling of cancer.** *Hum Mol Genet* 2001, **10:**657-662.
11. Bracken AP, Helin K: **Polycomb group proteins: navigators of lineage pathways led astray in cancer.** *Nat Rev Cancer* 2009, **9:**773-784.
12. Haupt Y, Alexander WS, Barri G, Klinken SP, Adams JM: **Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in E mu-myc transgenic mice.** *Cell* 1991, **65:**753-763.
13. van Lohuizen M, Verbeek S, Scheijen B, Wientjens E, van der Gulden H, Berns A: **Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging.** *Cell* 1991, **65:**737-752.
14. Dimri GP, Martinez JL, Jacobs JJ, Keblusek P, Itahana K, Van Lohuizen M, Campisi J, Wazer DE, Band V: **The Bmi-1 oncogene induces telomerase activity and immortalizes human mammary epithelial cells.** *Cancer Res* 2002, **62:**4736-4745.
15. Lessard J, Sauvageau G: **Bmi-1 determines the proliferative capacity of normal and**

leukaemic stem cells. *Nature* 2003, **423:**255-260.

16. Kim JH, Yoon SY, Jeong SH, Kim SY, Moon SK, Joo JH, Lee Y, Choe IS, Kim JW: **Overexpression of Bmi-1 oncoprotein correlates with axillary lymph node metastases in invasive ductal breast cancer.** *Breast* 2004, **13:**383-388.
17. Glinsky GV, Berezovska O, Glinskii AB: **Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer.** *J Clin Invest* 2005, **115:**1503-1521.
18. Silva J, Garcia V, Garcia JM, Pena C, Dominguez G, Diaz R, Lorenzo Y, Hurtado A, Sanchez A, Bonilla F: **Circulating Bmi-1 mRNA as a possible prognostic factor for advanced breast cancer patients.** *Breast Cancer Res* 2007, **9:**R55.
19. Nalwoga H, Arnes JB, Wabinga H, Akslen LA: **Expression of aldehyde dehydrogenase 1 (ALDH1) is associated with basal-like markers and features of aggressive tumours in African breast cancer.** *Br J Cancer*, **102:**369-375.
20. Choi YJ, Choi YL, Cho EY, Shin YK, Sung KW, Hwang YK, Lee SJ, Kong G, Lee JE, Kim JS, et al: **Expression of Bmi-1 protein in tumor tissues is associated with favorable prognosis in breast cancer patients.** *Breast Cancer Res Treat* 2009, **113:**83-93.
21. Saeki M, Kobayashi D, Tsuji N, Kuribayashi K, Watanabe N: **Diagnostic importance of overexpression of Bmi-1 mRNA in early breast cancers.** *Int J Oncol* 2009, **35:**511-515.
22. Song LB, Zeng MS, Liao WT, Zhang L, Mo HY, Liu WL, Shao JY, Wu QL, Li MZ, Xia YF, et al: **Bmi-1 is a novel molecular marker of nasopharyngeal carcinoma progression and immortalizes primary human nasopharyngeal epithelial cells.** *Cancer Res* 2006, **66:**6225-6232.
23. Li DW, Tang HM, Fan JW, Yan DW, Zhou CZ, Li SX, Wang XL, Peng ZH: **Expression level of Bmi-1 oncoprotein is associated with progression and prognosis in colon cancer.** *J Cancer Res Clin Oncol* 2009.
24. Liu JH, Song LB, Zhang X, Guo BH, Feng Y, Li XX, Liao WT, Zeng MS, Huang KH: **Bmi-1 expression predicts prognosis for patients with gastric carcinoma.** *J Surg Oncol* 2008, **97:**267-272.
25. Zhang XW, Sheng YP, Li Q, Qin W, Lu YW, Cheng YF, Liu BY, Zhang FC, Li J, Dimri GP, Guo WJ: **BMI1 and Mel-18 oppositely regulate carcinogenesis and progression of gastric cancer.** *Mol Cancer*, **9:**40.
26. Mihic-Probst D, Kuster A, Kilgus S, Bode-Lesniewska B, Ingold-Heppner B, Leung C, Storz M, Seifert B, Marino S, Schraml P, et al: **Consistent expression of the stem cell renewal factor BMI-1 in primary and metastatic melanoma.** *Int J Cancer* 2007, **121:**1764-1770.
27. Jiang Y, Su B, Meng X, Liu C, Liu B, Liu D, Fan Y, Yang H: **Effect of siRNA-mediated silencing of Bmi-1 gene expression on HeLa cells.** *Cancer Sci* 2010, **101:**379-386.
28. Song LB, Li J, Liao WT, Feng Y, Yu CP, Hu LJ, Kong QL, Xu LH, Zhang X, Liu WL, et al: **The polycomb group protein Bmi-1 represses the tumor suppressor PTEN and induces epithelial-mesenchymal transition in human nasopharyngeal epithelial cells.** *J Clin Invest* 2009.

29. Hoenerhoff MJ, Chu I, Barkan D, Liu ZY, Datta S, Dimri GP, Green JE: **BMI1 cooperates with H-RAS to induce an aggressive breast cancer phenotype with brain metastases.** *Oncogene* 2009, **28:**3022-3032.
30. Langley RR, Fidler IJ: **Tumor cell-organ microenvironment interactions in the pathogenesis of cancer metastasis.** *Endocr Rev* 2007, **28:**297-321.
31. Sleeman JP: **The lymph node as a bridgehead in the metastatic dissemination of tumors.** *Recent Results Cancer Res* 2000, **157:**55-81.
32. Pantel K, Brakenhoff RH: **Dissecting the metastatic cascade.** *Nat Rev Cancer* 2004, **4:**448-456.
33. Thiery JP: **Epithelial-mesenchymal transitions in tumour progression.** *Nat Rev Cancer* 2002, **2:**442-454.
34. Nieto MA: **The snail superfamily of zinc-finger transcription factors.** *Nat Rev Mol Cell Biol* 2002, **3:**155-166.
35. Behrens J, Lowrick O, Klein-Hitpass L, Birchmeier W: **The E-cadherin promoter: functional analysis of a G.C-rich region and an epithelial cell-specific palindromic regulatory element.** *Proc Natl Acad Sci U S A* 1991, **88:**11495-11499.
36. Hajra KM, Ji X, Fearon ER: **Extinction of E-cadherin expression in breast cancer via a dominant repression pathway acting on proximal promoter elements.** *Oncogene* 1999, **18:**7274-7279.
37. Giroldi LA, Bringuier PP, de Weijert M, Jansen C, van Bokhoven A, Schalken JA: **Role of E boxes in the repression of E-cadherin expression.** *Biochem Biophys Res Commun* 1997, **241:**453-458.
38. Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, Garcia De Herreros A: **The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells.** *Nat Cell Biol* 2000, **2:**84-89.
39. Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA: **The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression.** *Nat Cell Biol* 2000, **2:**76-83.
40. Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, Hung MC: **Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition.** *Nat Cell Biol* 2004, **6:**931-940.
41. Blanco MJ, Moreno-Bueno G, Sarrio D, Locascio A, Cano A, Palacios J, Nieto MA: **Correlation of Snail expression with histological grade and lymph node status in breast carcinomas.** *Oncogene* 2002, **21:**3241-3246.
42. Cheng CW, Wu PE, Yu JC, Huang CS, Yue CT, Wu CW, Shen CY: **Mechanisms of inactivation of E-cadherin in breast carcinoma: modification of the two-hit hypothesis of tumor suppressor gene.** *Oncogene* 2001, **20:**3814-3823.
43. Come C, Magnino F, Bibeau F, De Santa Barbara P, Becker KF, Theillet C, Savagner P: **Snail and slug play distinct roles during breast carcinoma progression.** *Clin Cancer Res* 2006, **12:**5395-5402.
44. Takkunen M, Grenman R, Hukkanen M, Korhonen M, Garcia de Herreros A, Virtanen I: **Snail-dependent and -independent epithelial-mesenchymal transition in oral squamous carcinoma cells.** *J Histochem Cytochem* 2006, **54:**1263-1275.
45. Micalizzi DS, Christensen KL, Jedlicka P, Coletta RD, Baron AE, Harrell JC, Horwitz

KB, Billheimer D, Heichman KA, Welm AL, et al: **The Six1 homeoprotein induces human mammary carcinoma cells to undergo epithelial-mesenchymal transition and metastasis in mice through increasing TGF-beta signaling.** *J Clin Invest* 2009, **119:**2678-2690.

46. Evdokimova V, Tognon C, Ng T, Ruzanov P, Melnyk N, Fink D, Sorokin A, Ovchinnikov LP, Davicioni E, Triche TJ, Sorensen PH: **Translational activation of snail1 and other developmentally regulated transcription factors by YB-1 promotes an epithelial-mesenchymal transition.** *Cancer Cell* 2009, **15:**402-415.
47. Tryndyak VP, Beland FA, Pogribny IP: **E-cadherin transcriptional down-regulation by epigenetic and microRNA-200 family alterations is related to mesenchymal and drug-resistant phenotypes in human breast cancer cells.** *Int J Cancer* 2009.
48. Sant M, Aareleid T, Berrino F, Bielska Lasota M, Carli PM, Faivre J, Grosclaude P, Hedelin G, Matsuda T, Moller H, et al: **EUROCARE-3: survival of cancer patients diagnosed 1990-94--results and commentary.** *Ann Oncol* 2003, **14 Suppl 5:**v61-118.
49. Botha JL, Bray F, Sankila R, Parkin DM: **Breast cancer incidence and mortality trends in 16 European countries.** *Eur J Cancer* 2003, **39:**1718-1729.
50. Leung GM, Thach TQ, Lam TH, Hedley AJ, Foo W, Fielding R, Yip PS, Lau EM, Wong CM: **Trends in breast cancer incidence in Hong Kong between 1973 and 1999: an age-period-cohort analysis.** *Br J Cancer* 2002, **87:**982-988.
51. Tam CY, Martin LJ, Hislop G, Hanley AJ, Minkin S, Boyd NF: **Risk factors for breast cancer in postmenopausal Caucasian and Chinese-Canadian women.** *Breast Cancer Res*, **12:**R2.
52. Hesketh T, Lu L, Xing ZW: **The effect of China's one-child family policy after 25 years.** *N Engl J Med* 2005, **353:**1171-1176.
53. Parkin DM, Fernandez LM: **Use of statistics to assess the global burden of breast cancer.** *Breast J* 2006, **12 Suppl 1:**S70-80.
54. Giordano SH, Cohen DS, Buzdar AU, Perkins G, Hortobagyi GN: **Breast carcinoma in men: a population-based study.** *Cancer* 2004, **101:**51-57.
55. Peppercorn J: **Breast cancer in women under 40.** *Oncology (Williston Park)* 2009, **23:**465-474.
56. Correa P: **The epidemiology of cancer of the breast.** *Am J Clin Pathol* 1975, **64:**720-727.
57. **Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without the disease.** *Lancet* 2002, **360:**187-195.
58. Apter D, Vihko R: **Early menarche, a risk factor for breast cancer, indicates early onset of ovulatory cycles.** *J Clin Endocrinol Metab* 1983, **57:**82-86.
59. Yager JD, Davidson NE: **Estrogen carcinogenesis in breast cancer.** *N Engl J Med* 2006, **354:**270-282.
60. Duffy SW, Nagtegaal ID, Astley SM, Gillan MG, McGee MA, Boggis CR, Wilson M, Beetles UM, Griffiths MA, Jain AK, et al: **Visually assessed breast density, breast cancer risk and the importance of the craniocaudal view.** *Breast Cancer Res* 2008, **10:**R64.
61. Morimoto LM, White E, Chen Z, Chlebowski RT, Hays J, Kuller L, Lopez AM,

Manson J, Margolis KL, Muti PC, et al: **Obesity, body size, and risk of postmenopausal breast cancer: the Women's Health Initiative (United States).** *Cancer Causes Control* 2002, **13:**741-751.

62. Chlebowski RT, Hendrix SL, Langer RD, Stefanick ML, Gass M, Lane D, Rodabough RJ, Gilligan MA, Cyr MG, Thomson CA, et al: **Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial.** *Jama* 2003, **289:**3243-3253.
63. Boffetta P, Hashibe M, La Vecchia C, Zatonski W, Rehm J: **The burden of cancer attributable to alcohol drinking.** *Int J Cancer* 2006, **119:**884-887.
64. Thune I, Brenn T, Lund E, Gaard M: **Physical activity and the risk of breast cancer.** *N Engl J Med* 1997, **336:**1269-1275.
65. Marcus JN, Watson P, Page DL, Lynch HT: **Pathology and heredity of breast cancer in younger women.** *J Natl Cancer Inst Monogr* 1994:23-34.
66. **Familial breast cancer: collaborative reanalysis of individual data from 52 epidemiological studies including 58,209 women with breast cancer and 101,986 women without the disease.** *Lancet* 2001, **358:**1389-1399.
67. Pharoah PD, Day NE, Duffy S, Easton DF, Ponder BA: **Family history and the risk of breast cancer: a systematic review and meta-analysis.** *Int J Cancer* 1997, **71:**800-809.
68. Claus EB, Risch N, Thompson WD: **Genetic analysis of breast cancer in the cancer and steroid hormone study.** *Am J Hum Genet* 1991, **48:**232-242.
69. Iniesta MD, Chien J, Wicha M, Merajver SD: **One-hit effects and cancer.** *Cancer Prev Res (Phila Pa)*, **3:**12-15.
70. Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, Collins N, Gregory S, Gumbs C, Micklem G: **Identification of the breast cancer susceptibility gene BRCA2.** *Nature* 1995, **378:**789-792.
71. Eerola H, Vahteristo P, Sarantaus L, Kyyronen P, Pyrhonen S, Blomqvist C, Pukkala E, Nevanlinna H, Sankila R: **Survival of breast cancer patients in BRCA1, BRCA2, and non-BRCA1/2 breast cancer families: a relative survival analysis from Finland.** *Int J Cancer* 2001, **93:**368-372.
72. Lichtenstein P, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, Koskenvuo M, Pukkala E, Skytthe A, Hemminki K: **Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland.** *N Engl J Med* 2000, **343:**78-85.
73. Potter JD, Cerhan JR, Sellers TA, McGovern PG, Drinkard C, Kushi LR, Folsom AR: **Progesterone and estrogen receptors and mammary neoplasia in the Iowa Women's Health Study: how many kinds of breast cancer are there?** *Cancer Epidemiol Biomarkers Prev* 1995, **4:**319-326.
74. Huang WY, Newman B, Millikan RC, Schell MJ, Hulka BS, Moorman PG: **Hormone-related factors and risk of breast cancer in relation to estrogen receptor and progesterone receptor status.** *Am J Epidemiol* 2000, **151:**703-714.
75. Enger SM, Ross RK, Paganini-Hill A, Carpenter CL, Bernstein L: **Body size, physical activity, and breast cancer hormone receptor status: results from two case-control studies.** *Cancer Epidemiol Biomarkers Prev* 2000, **9:**681-687.

76. Yoo KY, Tajima K, Miura S, Takeuchi T, Hirose K, Risch H, Dubrow R: **Breast cancer risk factors according to combined estrogen and progesterone receptor status: a case-control analysis.** *Am J Epidemiol* 1997, **146:**307-314.
77. Britton JA, Gammon MD, Schoenberg JB, Stanford JL, Coates RJ, Swanson CA, Potischman N, Malone KE, Brogan DJ, Daling JR, Brinton LA: **Risk of breast cancer classified by joint estrogen receptor and progesterone receptor status among women 20-44 years of age.** *Am J Epidemiol* 2002, **156:**507-516.
78. Vacek PM, Geller BM: **A prospective study of breast cancer risk using routine mammographic breast density measurements.** *Cancer Epidemiol Biomarkers Prev* 2004, **13:**715-722.
79. Barlow WE, White E, Ballard-Barbash R, Vacek PM, Titus-Ernstoff L, Carney PA, Tice JA, Buist DS, Geller BM, Rosenberg R, et al: **Prospective breast cancer risk prediction model for women undergoing screening mammography.** *J Natl Cancer Inst* 2006, **98:**1204-1214.
80. Boyd NF, Guo H, Martin LJ, Sun L, Stone J, Fishell E, Jong RA, Hislop G, Chiarelli A, Minkin S, Yaffe MJ: **Mammographic density and the risk and detection of breast cancer.** *N Engl J Med* 2007, **356:**227-236.
81. London SJ, Connolly JL, Schnitt SJ, Colditz GA: **A prospective study of benign breast disease and the risk of breast cancer.** *Jama* 1992, **267:**941-944.
82. Hartmann LC, Sellers TA, Frost MH, Lingle WL, Degnim AC, Ghosh K, Vierkant RA, Maloney SD, Pankratz VS, Hillman DW, et al: **Benign breast disease and the risk of breast cancer.** *N Engl J Med* 2005, **353:**229-237.
83. Collins LC, Baer HJ, Tamimi RM, Connolly JL, Colditz GA, Schnitt SJ: **Magnitude and laterality of breast cancer risk according to histologic type of atypical hyperplasia: results from the Nurses' Health Study.** *Cancer* 2007, **109:**180-187.
84. Connolly JL, Schnitt SJ: **Benign breast disease. Resolved and unresolved issues.** *Cancer* 1993, **71:**1187-1189.
85. Gail MH, Brinton LA, Byar DP, Corle DK, Green SB, Schairer C, Mulvihill JJ: **Projecting individualized probabilities of developing breast cancer for white females who are being examined annually.** *J Natl Cancer Inst* 1989, **81:**1879-1886.
86. Schoenmakers HP: **[Dental impression materials--discriminating characteristics of elastomers and alginates].** *Rev Belge Med Dent* 1979, **34:**275-286.
87. Fitzgibbons PL, Henson DE, Hutter RV: **Benign breast changes and the risk for subsequent breast cancer: an update of the 1985 consensus statement. Cancer Committee of the College of American Pathologists.** *Arch Pathol Lab Med* 1998, **122:**1053-1055.
88. Hildreth NG, Shore RE, Dvoretsky PM: **The risk of breast cancer after irradiation of the thymus in infancy.** *N Engl J Med* 1989, **321:**1281-1284.
89. Ng AK, Travis LB: **Radiation therapy and breast cancer risk.** *J Natl Compr Canc Netw* 2009, **7:**1121-1128.
90. Ron E: **Cancer risks from medical radiation.** *Health Phys* 2003, **85:**47-59.
91. Houssami N, Lord SJ, Ciatto S: **Breast cancer screening: emerging role of new imaging techniques as adjuncts to mammography.** *Med J Aust* 2009, **190:**493-497.
92. Michael M, Garzoli E, Reiner CS: **Mammography, Sonography and MRI for**

Detection and Characterization of Invasive Lobular Carcinoma of the Breast. *Breast Dis* 2009, **30:**21-30.

93. Brezina K: **[Results of mammography combined with selected needle biopsy (author's transl)].** *Wien Klin Wochenschr* 1975, **87:**666-671.
94. Wilhelm MC, de Paredes ES, Pope T, Wanebo HJ: **The changing mammogram. A primary indication for needle localization biopsy.** *Arch Surg* 1986, **121:**1311-1314.
95. Aebi S, Gelber S, Castiglione-Gertsch M, Gelber RD, Collins J, Thurlimann B, Rudenstam CM, Lindtner J, Crivellari D, Cortes-Funes H, et al: **Is chemotherapy alone adequate for young women with oestrogen-receptor-positive breast cancer?** *Lancet* 2000, **355:**1869-1874.
96. Vorgias G, Koukouras D, Paleogianni V, Tzoracoeleftherakis E: **Prognostic significance of factors affecting disease free interval and overall survival for Stage II breast cancer in Greece. A multivariate cohort study.** *Eur J Obstet Gynecol Reprod Biol* 2001, **95:**100-104.
97. Arriagada R, Le MG, Dunant A, Tubiana M, Contesso G: **Twenty-five years of follow-up in patients with operable breast carcinoma: correlation between clinicopathologic factors and the risk of death in each 5-year period.** *Cancer* 2006, **106:**743-750.
98. Warwick J, Tabar L, Vitak B, Duffy SW: **Time-dependent effects on survival in breast carcinoma: results of 20 years of follow-up from the Swedish Two-County Study.** *Cancer* 2004, **100:**1331-1336.
99. Weiss RB, Woolf SH, Demakos E, Holland JF, Berry DA, Falkson G, Cirrincione CT, Robbins A, Bothun S, Henderson IC, Norton L: **Natural history of more than 20 years of node-positive primary breast carcinoma treated with cyclophosphamide, methotrexate, and fluorouracil-based adjuvant chemotherapy: a study by the Cancer and Leukemia Group B.** *J Clin Oncol* 2003, **21:**1825-1835.
100. D'Eredita G, Giardina C, Martellotta M, Natale T, Ferrarese F: **Prognostic factors in breast cancer: the predictive value of the Nottingham Prognostic Index in patients with a long-term follow-up that were treated in a single institution.** *Eur J Cancer* 2001, **37:**591-596.
101. Chia SK, Speers CH, Bryce CJ, Hayes MM, Olivotto IA: **Ten-year outcomes in a population-based cohort of node-negative, lymphatic, and vascular invasion-negative early breast cancers without adjuvant systemic therapies.** *J Clin Oncol* 2004, **22:**1630-1637.
102. Fisher ER, Anderson S, Tan-Chiu E, Fisher B, Eaton L, Wolmark N: **Fifteen-year prognostic discriminants for invasive breast carcinoma: National Surgical Adjuvant Breast and Bowel Project Protocol-06.** *Cancer* 2001, **91:**1679-1687.
103. Hatteville L, Mahe C, Hill C: **Prediction of the long-term survival in breast cancer patients according to the present oncological status.** *Stat Med* 2002, **21:**2345-2354.
104. Soerjomataram I, Louwman MW, Ribot JG, Roukema JA, Coebergh JW: **An overview of prognostic factors for long-term survivors of breast cancer.** *Breast Cancer Res Treat* 2008, **107:**309-330.
105. Jordan VC: **Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer.** *Br J Pharmacol* 2006, **147 Suppl 1:**S269-276.

106. Charlier C, Colin C, Merville MP, Gielen J, Lambotte R, Plomteux G, Castronovo V: **[Tamoxifen in the treatment of breast cancer].** *J Gynecol Obstet Biol Reprod (Paris)* 1994, **23:**751-756.

107. Zhukov NV, Tjulandin SA: **Targeted therapy in the treatment of solid tumors: practice contradicts theory.** *Biochemistry (Mosc)* 2008, **73:**605-618.

108. Classe JM, Houvenaeghel G, Sagan C, Leveque J, Ferron G, Dravet F, Pioud R, Catala L, Rousseau C, Curtet C, Descamps P: **[Sentinel node detection applied to breast cancer: 2007 update].** *J Gynecol Obstet Biol Reprod (Paris)* 2007, **36:**329-337.

109. Pagani O, Senkus E, Wood W, Colleoni M, Cufer T, Kyriakides S, Costa A, Winer EP, Cardoso F: **International guidelines for management of metastatic breast cancer: can metastatic breast cancer be cured?** *J Natl Cancer Inst*, **102:**456-463.

110. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, Rinn JL, et al: **Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis.** *Nature*, **464:**1071-1076.

111. Mumprecht V, Detmar M: **Lymphangiogenesis and cancer metastasis.** *J Cell Mol Med* 2009, **13:**1405-1416.

112. Freudenberg JA, Wang Q, Katsumata M, Drebin J, Nagatomo I, Greene MI: **The role of HER2 in early breast cancer metastasis and the origins of resistance to HER2-targeted therapies.** *Exp Mol Pathol* 2009, **87:**1-11.

113. Lu X, Kang Y: **Epidermal growth factor signalling and bone metastasis.** *Br J Cancer*, **102:**457-461.

114. Hinton CV, Avraham S, Avraham HK: **Contributions of integrin-linked kinase to breast cancer metastasis and tumourigenesis.** *J Cell Mol Med* 2008, **12:**1517-1526.

115. Ali S, Lazennec G: **Chemokines: novel targets for breast cancer metastasis.** *Cancer Metastasis Rev* 2007, **26:**401-420.

116. Li YM, Pan Y, Wei Y, Cheng X, Zhou BP, Tan M, Zhou X, Xia W, Hortobagyi GN, Yu D, Hung MC: **Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis.** *Cancer Cell* 2004, **6:**459-469.

117. Benovic JL, Marchese A: **A new key in breast cancer metastasis.** *Cancer Cell* 2004, **6:**429-430.

118. Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY: **MicroRNA-21 targets tumor suppressor genes in invasion and metastasis.** *Cell Res* 2008, **18:**350-359.

119. Zhang B, Cao W, Zhang F, Zhang L, Niu R, Niu Y, Fu L, Hao X, Cao X: **Protein interacting with C alpha kinase 1 (PICK1) is involved in promoting tumor growth and correlates with poor prognosis of human breast cancer.** *Cancer Sci.*

120. Albasri A, Aleskandarany M, Benhasouna A, Powe DG, Ellis IO, Ilyas M, Green AR: **CTEN (C-terminal tensin-like), a novel oncogene overexpressed in invasive breast carcinoma of poor prognosis.** *Breast Cancer Res Treat.*

121. Chan CH, Lee SW, Li CF, Wang J, Yang WL, Wu CY, Wu J, Nakayama KI, Kang HY, Huang HY, et al: **Deciphering the transcriptional complex critical for RhoA gene expression and cancer metastasis.** *Nat Cell Biol.*

122. Demers M, Rose AA, Grosset AA, Biron-Pain K, Gaboury L, Siegel PM, St-Pierre Y: **Overexpression of Galectin-7, A Myoepithelial Cell Marker, Enhances Spontaneous Metastasis of Breast Cancer Cells.** *Am J Pathol.*

123. Das S, Ladell DS, Podgrabinska S, Ponomarev V, Nagi C, Fallon JT, Skobe M: **Vascular endothelial growth factor-C induces lymphangitic carcinomatosis, an extremely aggressive form of lung metastases.** *Cancer Res*, **70:**1814-1824.

124. Gilbert PM, Mouw JK, Unger MA, Lakins JN, Gbegnon MK, Clemmer VB, Benezra M, Licht JD, Boudreau NJ, Tsai KK, et al: **HOXA9 regulates BRCA1 expression to modulate human breast tumor phenotype.** *J Clin Invest.*

125. Yan W, Cao QJ, Arenas RB, Bentley B, Shao R: **GATA3 inhibits breast cancer metastasis through the reversal of epithelial-mesenchymal transition.** *J Biol Chem.*

126. Du J, Yang S, An D, Hu F, Yuan W, Zhai C, Zhu T: **BMP-6 inhibits microRNA-21 expression in breast cancer through repressing deltaEF1 and AP-1.** *Cell Res* 2009, **19:**487-496.

127. Hurst DR, Edmonds MD, Scott GK, Benz CC, Vaidya KS, Welch DR: **Breast cancer metastasis suppressor 1 up-regulates miR-146, which suppresses breast cancer metastasis.** *Cancer Res* 2009, **69:**1279-1283.

128. Mizuma M, Katayose Y, Yamamoto K, Shiraso S, Sasaki T, Yabuuchi S, Oda A, Masuda K, Rikiyama T, Onogawa T, et al: **Up-regulated p27Kip1 reduces matrix metalloproteinase-9 and inhibits invasion of human breast cancer cells.** *Anticancer Res* 2008, **28:**2669-2677.

129. Nobumoto A, Nagahara K, Oomizu S, Katoh S, Nishi N, Takeshita K, Niki T, Tominaga A, Yamauchi A, Hirashima M: **Galectin-9 suppresses tumor metastasis by blocking adhesion to endothelium and extracellular matrices.** *Glycobiology* 2008, **18:**735-744.

130. Cavalli LR: **Molecular markers of breast axillary lymph node metastasis.** *Expert Rev Mol Diagn* 2009, **9:**441-454.

131. Bao T, Davidson NE: **Gene expression profiling of breast cancer.** *Adv Surg* 2008, **42:**249-260.

132. Tait CR, Waterworth A, Loncaster J, Horgan K, Dodwell D: **The oligometastatic state in breast cancer: hypothesis or reality.** *Breast* 2005, **14:**87-93.

133. Chang HY, Sneddon JB, Alizadeh AA, Sood R, West RB, Montgomery K, Chi JT, van de Rijn M, Botstein D, Brown PO: **Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds.** *PLoS Biol* 2004, **2:**E7.

134. Ding L, Ellis MJ, Li S, Larson DE, Chen K, Wallis JW, Harris CC, McLellan MD, Fulton RS, Fulton LL, et al: **Genome remodelling in a basal-like breast cancer metastasis and xenograft.** *Nature*, **464:**999-1005.

135. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, et al: **Gene expression profiling predicts clinical outcome of breast cancer.** *Nature* 2002, **415:**530-536.

136. Papp B, Muller J: **Histone trimethylation and the maintenance of transcriptional ON and OFF states by trxG and PcG proteins.** *Genes Dev* 2006, **20:**2041-2054.

137. Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL, Morrison SJ, Clarke MF: **Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells.** *Nature* 2003, **423:**302-305.

138. Jiang L, Li J, Song L: **Bmi-1, stem cells and cancer.** *Acta Biochim Biophys Sin*

(Shanghai) 2009, **41:**527-534.

139. Alkema MJ, Wiegant J, Raap AK, Berns A, van Lohuizen M: **Characterization and chromosomal localization of the human proto-oncogene BMI-1.** *Hum Mol Genet* 1993, **2:**1597-1603.

140. Alkema MJ, Jacobs H, van Lohuizen M, Berns A: **Pertubation of B and T cell development and predisposition to lymphomagenesis in Emu Bmi1 transgenic mice require the Bmi1 RING finger.** *Oncogene* 1997, **15:**899-910.

141. Itahana K, Zou Y, Itahana Y, Martinez JL, Beausejour C, Jacobs JJ, Van Lohuizen M, Band V, Campisi J, Dimri GP: **Control of the replicative life span of human fibroblasts by p16 and the polycomb protein Bmi-1.** *Mol Cell Biol* 2003, **23:**389-401.

142. Alkema MJ, Bronk M, Verhoeven E, Otte A, van 't Veer LJ, Berns A, van Lohuizen M: **Identification of Bmi1-interacting proteins as constituents of a multimeric mammalian polycomb complex.** *Genes Dev* 1997, **11:**226-240.

143. Borden KL, Boddy MN, Lally J, O'Reilly NJ, Martin S, Howe K, Solomon E, Freemont PS: **The solution structure of the RING finger domain from the acute promyelocytic leukaemia proto-oncoprotein PML.** *Embo J* 1995, **14:**1532-1541.

144. Cohen KJ, Hanna JS, Prescott JE, Dang CV: **Transformation by the Bmi-1 oncoprotein correlates with its subnuclear localization but not its transcriptional suppression activity.** *Mol Cell Biol* 1996, **16:**5527-5535.

145. Yadav AK, Sahasrabuddhe AA, Dimri M, Bommi PV, Sainger R, Dimri GP: **Deletion analysis of BMI1 oncoprotein identifies its negative regulatory domain.** *Mol Cancer*, **9:**158.

146. Belgiovine C, Chiodi I, Mondello C: **Telomerase: cellular immortalization and neoplastic transformation. Multiple functions of a multifaceted complex.** *Cytogenet Genome Res* 2008, **122:**255-262.

147. Wang J, Hannon GJ, Beach DH: **Risky immortalization by telomerase.** *Nature* 2000, **405:**755-756.

148. Milyavsky M, Shats I, Erez N, Tang X, Senderovich S, Meerson A, Tabach Y, Goldfinger N, Ginsberg D, Harris CC, Rotter V: **Prolonged culture of telomerase-immortalized human fibroblasts leads to a premalignant phenotype.** *Cancer Res* 2003, **63:**7147-7157.

149. Stampfer MR, Garbe J, Levine G, Lichtsteiner S, Vasserot AP, Yaswen P: **Expression of the telomerase catalytic subunit, hTERT, induces resistance to transforming growth factor beta growth inhibition in p16INK4A(-) human mammary epithelial cells.** *Proc Natl Acad Sci U S A* 2001, **98:**4498-4503.

150. Noble JR, Zhong ZH, Neumann AA, Melki JR, Clark SJ, Reddel RR: **Alterations in the p16(INK4a) and p53 tumor suppressor genes of hTERT-immortalized human fibroblasts.** *Oncogene* 2004, **23:**3116-3121.

151. Mori T, Kiyono T, Imabayashi H, Takeda Y, Tsuchiya K, Miyoshi S, Makino H, Matsumoto K, Saito H, Ogawa S, et al: **Combination of hTERT and bmi-1, E6, or E7 induces prolongation of the life span of bone marrow stromal cells from an elderly donor without affecting their neurogenic potential.** *Mol Cell Biol* 2005, **25:**5183-5195.

152. Haga K, Ohno S, Yugawa T, Narisawa-Saito M, Fujita M, Sakamoto M, Galloway DA, Kiyono T: **Efficient immortalization of primary human cells by p16INK4a-specific short hairpin RNA or Bmi-1, combined with introduction of hTERT.** *Cancer Sci* 2007, **98:**147-154.

153. Zhang X, Soda Y, Takahashi K, Bai Y, Mitsuru A, Igura K, Satoh H, Yamaguchi S, Tani K, Tojo A, Takahashi TA: **Successful immortalization of mesenchymal progenitor cells derived from human placenta and the differentiation abilities of immortalized cells.** *Biochem Biophys Res Commun* 2006, **351:**853-859.

154. Saito M, Handa K, Kiyono T, Hattori S, Yokoi T, Tsubakimoto T, Harada H, Noguchi T, Toyoda M, Sato S, Teranaka T: **Immortalization of cementoblast progenitor cells with Bmi-1 and TERT.** *J Bone Miner Res* 2005, **20:**50-57.

155. Kim RH, Kang MK, Shin KH, Oo ZM, Han T, Baluda MA, Park NH: **Bmi-1 cooperates with human papillomavirus type 16 E6 to immortalize normal human oral keratinocytes.** *Exp Cell Res* 2007, **313:**462-472.

156. Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M: **The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus.** *Nature* 1999, **397:**164-168.

157. Seger YR, Garcia-Cao M, Piccinin S, Cunsolo CL, Doglioni C, Blasco MA, Hannon GJ, Maestro R: **Transformation of normal human cells in the absence of telomerase activation.** *Cancer Cell* 2002, **2:**401-413.

158. Drayton S, Rowe J, Jones R, Vatcheva R, Cuthbert-Heavens D, Marshall J, Fried M, Peters G: **Tumor suppressor p16INK4a determines sensitivity of human cells to transformation by cooperating cellular oncogenes.** *Cancer Cell* 2003, **4:**301-310.

159. Delle Fave G, Corleto VD: **Oncogenes, growth factors, receptor expression and proliferation markers in digestive neuroendocrine tumours. A critical reappraisal.** *Ann Oncol* 2001, **12 Suppl 2:**S13-17.

160. Haupt Y, Bath ML, Harris AW, Adams JM: **bmi-1 transgene induces lymphomas and collaborates with myc in tumorigenesis.** *Oncogene* 1993, **8:**3161-3164.

161. Song LB, Li J, Liao WT, Feng Y, Yu CP, Hu LJ, Kong QL, Xu LH, Zhang X, Liu WL, et al: **The polycomb group protein Bmi-1 represses the tumor suppressor PTEN and induces epithelial-mesenchymal transition in human nasopharyngeal epithelial cells.** *J Clin Invest* 2009, **119:**3626-3636.

162. Wang Q, Li WL, You P, Su J, Zhu MH, Xie DF, Zhu HY, He ZY, Li JX, Ding XY, et al: **Oncoprotein BMI-1 induces the malignant transformation of HaCaT cells.** *J Cell Biochem* 2009, **106:**16-24.

163. Douglas D, Hsu JH, Hung L, Cooper A, Abdueva D, van Doorninck J, Peng G, Shimada H, Triche TJ, Lawlor ER: **BMI-1 promotes ewing sarcoma tumorigenicity independent of CDKN2A repression.** *Cancer Res* 2008, **68:**6507-6515.

164. He S, Iwashita T, Buchstaller J, Molofsky AV, Thomas D, Morrison SJ: **Bmi-1 over-expression in neural stem/progenitor cells increases proliferation and neurogenesis in culture but has little effect on these functions in vivo.** *Dev Biol* 2009, **328:**257-272.

165. Cui H, Hu B, Li T, Ma J, Alam G, Gunning WT, Ding HF: **Bmi-1 is essential for the tumorigenicity of neuroblastoma cells.** *Am J Pathol* 2007, **170:**1370-1378.

166. Datta S, Hoenerhoff MJ, Bommi P, Sainger R, Guo WJ, Dimri M, Band H, Band V, Green JE, Dimri GP: **Bmi-1 cooperates with H-Ras to transform human mammary epithelial cells via dysregulation of multiple growth-regulatory pathways.** *Cancer Res* 2007, **67:**10286-10295.

167. Guo WJ, Zeng MS, Yadav A, Song LB, Guo BH, Band V, Dimri GP: **Mel-18 acts as a tumor suppressor by repressing Bmi-1 expression and down-regulating Akt activity in breast cancer cells.** *Cancer Res* 2007, **67:**5083-5089.

168. Park IK, Morrison SJ, Clarke MF: **Bmi1, stem cells, and senescence regulation.** *J Clin Invest* 2004, **113:**175-179.

169. Bea S, Tort F, Pinyol M, Puig X, Hernandez L, Hernandez S, Fernandez PL, van Lohuizen M, Colomer D, Campo E: **BMI-1 gene amplification and overexpression in hematological malignancies occur mainly in mantle cell lymphomas.** *Cancer Res* 2001, **61:**2409-2412.

170. Vonlanthen S, Heighway J, Altermatt HJ, Gugger M, Kappeler A, Borner MM, van Lohuizen M, Betticher DC: **The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression.** *Br J Cancer* 2001, **84:**1372-1376.

171. van Kemenade FJ, Raaphorst FM, Blokzijl T, Fieret E, Hamer KM, Satijn DP, Otte AP, Meijer CJ: **Coexpression of BMI-1 and EZH2 polycomb-group proteins is associated with cycling cells and degree of malignancy in B-cell non-Hodgkin lymphoma.** *Blood* 2001, **97:**3896-3901.

172. Kim JH, Yoon SY, Kim CN, Joo JH, Moon SK, Choe IS, Choe YK, Kim JW: **The Bmi-1 oncoprotein is overexpressed in human colorectal cancer and correlates with the reduced p16INK4a/p14ARF proteins.** *Cancer Lett* 2004, **203:**217-224.

173. Wang H, Pan K, Zhang HK, Weng DS, Zhou J, Li JJ, Huang W, Song HF, Chen MS, Xia JC: **Increased polycomb-group oncogene Bmi-1 expression correlates with poor prognosis in hepatocellular carcinoma.** *J Cancer Res Clin Oncol* 2008, **134:**535-541.

174. Qin ZK, Yang JA, Ye YL, Zhang X, Xu LH, Zhou FJ, Han H, Liu ZW, Song LB, Zeng MS: **Expression of Bmi-1 is a prognostic marker in bladder cancer.** *BMC Cancer* 2009, **9:**61.

175. Hayry V, Makinen LK, Atula T, Sariola H, Makitie A, Leivo I, Keski-Santti H, Lundin J, Haglund C, Hagstrom J: **Bmi-1 expression predicts prognosis in squamous cell carcinoma of the tongue.** *Br J Cancer*, **102:**892-897.

176. Yang GF, He WP, Cai MY, He LR, Luo JH, Deng HX, Guan XY, Zeng MS, Zeng YX, Xie D: **Intensive expression of Bmi-1 is a new independent predictor of poor outcome in patients with ovarian carcinoma.** *BMC Cancer*, **10:**133.

177. Bhattacharyya J, Mihara K, Yasunaga S, Tanaka H, Hoshi M, Takihara Y, Kimura A: **BMI-1 expression is enhanced through transcriptional and posttranscriptional regulation during the progression of chronic myeloid leukemia.** *Ann Hematol* 2009, **88:**333-340.

178. Li J, Gong LY, Song LB, Jiang LL, Liu LP, Wu J, Yuan J, Cai JC, He M, Wang L, et al: **Oncoprotein Bmi-1 renders apoptotic resistance to glioma cells through activation of the IKK-nuclear factor-kappaB Pathway.** *Am J Pathol*, **176:**699-709.

179. Vrzalikova K, Skarda J, Ehrmann J, Murray PG, Fridman E, Kopolovic J, Knizetova P, Hajduch M, Klein J, Kolek V, et al: **Prognostic value of Bmi-1 oncoprotein expression in NSCLC patients: a tissue microarray study.** *J Cancer Res Clin Oncol* 2008, **134:**1037-1042.

180. Hayry V, Tynninen O, Haapasalo HK, Wolfer J, Paulus W, Hasselblatt M, Sariola H, Paetau A, Sarna S, Niemela M, et al: **Stem cell protein BMI-1 is an independent marker for poor prognosis in oligodendroglial tumours.** *Neuropathol Appl Neurobiol* 2008, **34:**555-563.

181. Chowdhury M, Mihara K, Yasunaga S, Ohtaki M, Takihara Y, Kimura A: **Expression of Polycomb-group (PcG) protein BMI-1 predicts prognosis in patients with acute myeloid leukemia.** *Leukemia* 2007, **21:**1116-1122.

182. Song W, Tao K, Li H, Jin C, Song Z, Li J, Shi H, Li X, Dang Z, Dou K: **Bmi-1 is related to proliferation, survival and poor prognosis in pancreatic cancer.** *Cancer Sci.*

183. Mihara K, Chowdhury M, Nakaju N, Hidani S, Ihara A, Hyodo H, Yasunaga S, Takihara Y, Kimura A: **Bmi-1 is useful as a novel molecular marker for predicting progression of myelodysplastic syndrome and patient prognosis.** *Blood* 2006, **107:**305-308.

184. Effendi K, Mori T, Komuta M, Masugi Y, Du W, Sakamoto M: **Bmi-1 gene is upregulated in early-stage hepatocellular carcinoma and correlates with ATP-binding cassette transporter B1 expression.** *Cancer Sci*, **101:**666-672.

185. Kang MK, Kim RH, Kim SJ, Yip FK, Shin KH, Dimri GP, Christensen R, Han T, Park NH: **Elevated Bmi-1 expression is associated with dysplastic cell transformation during oral carcinogenesis and is required for cancer cell replication and survival.** *Br J Cancer* 2007, **96:**126-133.

186. Zhao J, Luo XD, Da CL, Xin Y: **Clinicopathological significance of B-cell-specific Moloney murine leukemia virus insertion site 1 expression in gastric carcinoma and its precancerous lesion.** *World J Gastroenterol* 2009, **15:**2145-2150.

187. Xiao J, Deng C: **Knockdown of Bmi-1 impairs growth and invasiveness of human gastric carcinoma cells.** *Oncol Res* 2009, **17:**613-620.

188. Wiederschain D, Chen L, Johnson B, Bettano K, Jackson D, Taraszka J, Wang YK, Jones MD, Morrissey M, Deeds J, et al: **Contribution of polycomb homologues Bmi-1 and Mel-18 to medulloblastoma pathogenesis.** *Mol Cell Biol* 2007, **27:**4968-4979.

189. Kozakowski N, Soleiman A, Pammer J: **BMI-1 expression is inversely correlated with the grading of renal clear cell carcinoma.** *Pathol Oncol Res* 2008, **14:**9-13.

190. Engelsen IB, Mannelqvist M, Stefansson IM, Carter SL, Beroukhim R, Oyan AM, Otte AP, Kalland KH, Akslen LA, Salvesen HB: **Low BMI-1 expression is associated with an activated BMI-1-driven signature, vascular invasion, and hormone receptor loss in endometrial carcinoma.** *Br J Cancer* 2008, **98:**1662-1669.

191. Bachmann IM, Puntervoll HE, Otte AP, Akslen LA: **Loss of BMI-1 expression is associated with clinical progress of malignant melanoma.** *Mod Pathol* 2008, **21:**583-590.

192. Iwama A, Oguro H, Negishi M, Kato Y, Morita Y, Tsukui H, Ema H, Kamijo T,

Katoh-Fukui Y, Koseki H, et al: **Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1.** *Immunity* 2004, **21:**843-851.

193. Molofsky AV, He S, Bydon M, Morrison SJ, Pardal R: **Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways.** *Genes Dev* 2005, **19:**1432-1437.
194. Molofsky AV, Pardal R, Iwashita T, Park IK, Clarke MF, Morrison SJ: **Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation.** *Nature* 2003, **425:**962-967.
195. Liu S, Dontu G, Mantle ID, Patel S, Ahn NS, Jackson KW, Suri P, Wicha MS: **Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells.** *Cancer Res* 2006, **66:**6063-6071.
196. Liu S, Dontu G, Wicha MS: **Mammary stem cells, self-renewal pathways, and carcinogenesis.** *Breast Cancer Res* 2005, **7:**86-95.
197. Yang J, Weinberg RA: **Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis.** *Dev Cell* 2008, **14:**818-829.
198. Hay ED: **The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it.** *Dev Dyn* 2005, **233:**706-720.
199. Guarino M: **Epithelial-to-mesenchymal change of differentiation. From embryogenetic mechanism to pathological patterns.** *Histol Histopathol* 1995, **10:**171-184.
200. Guarino M, Micheli P, Pallotti F, Giordano F: **Pathological relevance of epithelial and mesenchymal phenotype plasticity.** *Pathol Res Pract* 1999, **195:**379-389.
201. Peinado H, Olmeda D, Cano A: **Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype?** *Nat Rev Cancer* 2007, **7:**415-428.
202. Gupta GP, Massague J: **Cancer metastasis: building a framework.** *Cell* 2006, **127:**679-695.
203. Thiery JP, Sleeman JP: **Complex networks orchestrate epithelial-mesenchymal transitions.** *Nat Rev Mol Cell Biol* 2006, **7:**131-142.
204. Yilmaz M, Christofori G: **EMT, the cytoskeleton, and cancer cell invasion.** *Cancer Metastasis Rev* 2009, **28:**15-33.
205. Peinado H, Quintanilla M, Cano A: **Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions.** *J Biol Chem* 2003, **278:**21113-21123.
206. Guarino M, Rubino B, Ballabio G: **The role of epithelial-mesenchymal transition in cancer pathology.** *Pathology* 2007, **39:**305-318.
207. Zavadil J, Cermak L, Soto-Nieves N, Bottinger EP: **Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition.** *Embo J* 2004, **23:**1155-1165.
208. Savagner P, Yamada KM, Thiery JP: **The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition.** *J Cell Biol* 1997, **137:**1403-1419.
209. Lo HW, Hsu SC, Xia W, Cao X, Shih JY, Wei Y, Abbruzzese JL, Hortobagyi GN, Hung MC: **Epidermal growth factor receptor cooperates with signal transducer and**

activator of transcription 3 to induce epithelial-mesenchymal transition in cancer cells via up-regulation of TWIST gene expression. *Cancer Res* 2007, **67:**9066-9076.

210. Graham TR, Zhau HE, Odero-Marah VA, Osunkoya AO, Kimbro KS, Tighiouart M, Liu T, Simons JW, O'Regan RM: **Insulin-like growth factor-I-dependent up-regulation of ZEB1 drives epithelial-to-mesenchymal transition in human prostate cancer cells.** *Cancer Res* 2008, **68:**2479-2488.

211. Acevedo VD, Gangula RD, Freeman KW, Li R, Zhang Y, Wang F, Ayala GE, Peterson LE, Ittmann M, Spencer DM: **Inducible FGFR-1 activation leads to irreversible prostate adenocarcinoma and an epithelial-to-mesenchymal transition.** *Cancer Cell* 2007, **12:**559-571.

212. Leong KG, Niessen K, Kulic I, Raouf A, Eaves C, Pollet I, Karsan A: **Jagged1-mediated Notch activation induces epithelial-to-mesenchymal transition through Slug-induced repression of E-cadherin.** *J Exp Med* 2007, **204:**2935-2948.

213. Shintani Y, Maeda M, Chaika N, Johnson KR, Wheelock MJ: **Collagen I promotes epithelial-to-mesenchymal transition in lung cancer cells via transforming growth factor-beta signaling.** *Am J Respir Cell Mol Biol* 2008, **38:**95-104.

214. Kalluri R, Weinberg RA: **The basics of epithelial-mesenchymal transition.** *J Clin Invest* 2009, **119:**1420-1428.

215. Voulgari A, Pintzas A: **Epithelial-mesenchymal transition in cancer metastasis: mechanisms, markers and strategies to overcome drug resistance in the clinic.** *Biochim Biophys Acta* 2009, **1796:**75-90.

216. Andreolas C, Kalogeropoulou M, Voulgari A, Pintzas A: **Fra-1 regulates vimentin during Ha-RAS-induced epithelial mesenchymal transition in human colon carcinoma cells.** *Int J Cancer* 2008, **122:**1745-1756.

217. Birchmeier W, Behrens J: **Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness.** *Biochim Biophys Acta* 1994, **1198:**11-26.

218. Perl AK, Wilgenbus P, Dahl U, Semb H, Christofori G: **A causal role for E-cadherin in the transition from adenoma to carcinoma.** *Nature* 1998, **392:**190-193.

219. Geiger B, Ayalon O· **Cadherins.** *Annu Rev Cell Biol* 1992, **8:**307-332.

220. Kowalski PJ, Rubin MA, Kleer CG: **E-cadherin expression in primary carcinomas of the breast and its distant metastases.** *Breast Cancer Res* 2003, **5:**R217-222.

221. Dorudi S, Sheffield JP, Poulsom R, Northover JM, Hart IR: **E-cadherin expression in colorectal cancer. An immunocytochemical and in situ hybridization study.** *Am J Pathol* 1993, **142:**981-986.

222. Peinado H, Portillo F, Cano A: **Transcriptional regulation of cadherins during development and carcinogenesis.** *Int J Dev Biol* 2004, **48:**365-375.

223. Hajra KM, Chen DY, Fearon ER: **The SLUG zinc-finger protein represses E-cadherin in breast cancer.** *Cancer Res* 2002, **62:**1613-1618.

224. Eger A, Aigner K, Sonderegger S, Dampier B, Oehler S, Schreiber M, Berx G, Cano A, Beug H, Foisner R: **DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells.** *Oncogene* 2005, **24:**2375-2385.

225. Comijn J, Berx G, Vermassen P, Verschueren K, van Grunsven L, Bruyneel E, Mareel M, Huylebroeck D, van Roy F: **The two-handed E box binding zinc finger protein**

SIP1 downregulates E-cadherin and induces invasion. *Mol Cell* 2001, **7:**1267-1278.

226. Aigner K, Dampier B, Descovich L, Mikula M, Sultan A, Schreiber M, Mikulits W, Brabletz T, Strand D, Obrist P, et al: **The transcription factor ZEB1 (deltaEF1) promotes tumour cell dedifferentiation by repressing master regulators of epithelial polarity.** *Oncogene* 2007, **26:**6979-6988.

227. Spaderna S, Schmalhofer O, Wahlbuhl M, Dimmler A, Bauer K, Sultan A, Hlubek F, Jung A, Strand D, Eger A, et al: **The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer.** *Cancer Res* 2008, **68:**537-544.

228. Perez-Moreno MA, Locascio A, Rodrigo I, Dhondt G, Portillo F, Nieto MA, Cano A: **A new role for E12/E47 in the repression of E-cadherin expression and epithelial-mesenchymal transitions.** *J Biol Chem* 2001, **276:**27424-27431.

229. Fujita Y, Krause G, Scheffner M, Zechner D, Leddy HE, Behrens J, Sommer T, Birchmeier W: **Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex.** *Nat Cell Biol* 2002, **4:**222-231.

230. Pece S, Gutkind JS: **E-cadherin and Hakai: signalling, remodeling or destruction?** *Nat Cell Biol* 2002, **4:**E72-74.

231. Berx G, Cleton-Jansen AM, Strumane K, de Leeuw WJ, Nollet F, van Roy F, Cornelisse C: **E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain.** *Oncogene* 1996, **13:**1919-1925.

232. Risinger JI, Berchuck A, Kohler MF, Boyd J: **Mutations of the E-cadherin gene in human gynecologic cancers.** *Nat Genet* 1994, **7:**98-102.

233. Guilford P, Hopkins J, Harraway J, McLeod M, McLeod N, Harawira P, Taite H, Scoular R, Miller A, Reeve AE: **E-cadherin germline mutations in familial gastric cancer.** *Nature* 1998, **392:**402-405.

234. Guilford PJ, Hopkins JB, Grady WM, Markowitz SD, Willis J, Lynch H, Rajput A, Wiesner GL, Lindor NM, Burgart LJ, et al: **E-cadherin germline mutations define an inherited cancer syndrome dominated by diffuse gastric cancer.** *Hum Mutat* 1999, **14:**249-255.

235. Oda T, Kanai Y, Oyama T, Yoshiura K, Shimoyama Y, Birchmeier W, Sugimura T, Hirohashi S: **E-cadherin gene mutations in human gastric carcinoma cell lines.** *Proc Natl Acad Sci U S A* 1994, **91:**1858-1862.

236. Graff JR, Gabrielson E, Fujii H, Baylin SB, Herman JG: **Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression.** *J Biol Chem* 2000, **275:**2727-2732.

237. Lynch HT, Grady W, Lynch JF, Tsuchiya KD, Wiesner G, Markowitz SD: **E-cadherin mutation-based genetic counseling and hereditary diffuse gastric carcinoma.** *Cancer Genet Cytogenet* 2000, **122:**1-6.

238. Caldeira JR, Prando EC, Quevedo FC, Neto FA, Rainho CA, Rogatto SR: **CDH1 promoter hypermethylation and E-cadherin protein expression in infiltrating breast cancer.** *BMC Cancer* 2006, **6:**48.

239. Nass SJ, Herman JG, Gabrielson E, Iversen PW, Parl FF, Davidson NE, Graff JR: **Aberrant methylation of the estrogen receptor and E-cadherin 5' CpG islands**

increases with malignant progression in human breast cancer. *Cancer Res* 2000, **60:**4346-4348.

240. Azarschab P, Stembalska A, Loncar MB, Pfister M, Sasiadek MM, Blin N: **Epigenetic control of E-cadherin (CDH1) by CpG methylation in metastasising laryngeal cancer.** *Oncol Rep* 2003, **10:**501-503.

241. Wheeler JM, Kim HC, Efstathiou JA, Ilyas M, Mortensen NJ, Bodmer WF: **Hypermethylation of the promoter region of the E-cadherin gene (CDH1) in sporadic and ulcerative colitis associated colorectal cancer.** *Gut* 2001, **48:**367-371.

242. Hulit J, Suyama K, Chung S, Keren R, Agiostratidou G, Shan W, Dong X, Williams TM, Lisanti MP, Knudsen K, Hazan RB: **N-cadherin signaling potentiates mammary tumor metastasis via enhanced extracellular signal-regulated kinase activation.** *Cancer Res* 2007, **67:**3106-3116.

243. Hajra KM, Fearon ER: **Cadherin and catenin alterations in human cancer.** *Genes Chromosomes Cancer* 2002, **34:**255-268.

244. Andersen H, Mejlvang J, Mahmood S, Gromova I, Gromov P, Lukanidin E, Kriajevska M, Mellon JK, Tulchinsky E: **Immediate and delayed effects of E-cadherin inhibition on gene regulation and cell motility in human epidermoid carcinoma cells.** *Mol Cell Biol* 2005, **25:**9138-9150.

245. Kim K, Lu Z, Hay ED: **Direct evidence for a role of beta-catenin/LEF-1 signaling pathway in induction of EMT.** *Cell Biol Int* 2002, **26:**463-476.

246. Ueno H, Mochizuki H, Hatsuse K, Hase K, Yamamoto T: **Indicators for treatment strategies of colorectal liver metastases.** *Ann Surg* 2000, **231:**59-66.

247. Fodde R, Brabletz T: **Wnt/beta-catenin signaling in cancer stemness and malignant behavior.** *Curr Opin Cell Biol* 2007, **19:**150-158.

248. Peifer M, Polakis P: **Wnt signaling in oncogenesis and embryogenesis--a look outside the nucleus.** *Science* 2000, **287:**1606-1609.

249. Huber O, Korn R, McLaughlin J, Ohsugi M, Herrmann BG, Kemler R: **Nuclear localization of beta-catenin by interaction with transcription factor LEF-1.** *Mech Dev* 1996, **59:**3-10.

250. Brabletz T, Jung A, Reu S, Porzner M, Hlubek F, Kunz-Schughart LA, Knuechel R, Kirchner T: **Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment.** *Proc Natl Acad Sci U S A* 2001, **98:**10356-10361.

251. Ruoslahti E: **Fibronectin in cell adhesion and invasion.** *Cancer Metastasis Rev* 1984, **3:**43-51.

252. Korsching E, Packeisen J, Liedtke C, Hungermann D, Wulfing P, van Diest PJ, Brandt B, Boecker W, Buerger H: **The origin of vimentin expression in invasive breast cancer: epithelial-mesenchymal transition, myoepithelial histogenesis or histogenesis from progenitor cells with bilinear differentiation potential?** *J Pathol* 2005, **206:**451-457.

253. Gilles C, Polette M, Mestdagt M, Nawrocki-Raby B, Ruggeri P, Birembaut P, Foidart JM: **Transactivation of vimentin by beta-catenin in human breast cancer cells.** *Cancer Res* 2003, **63:**2658-2664.

254. Kokkinos MI, Wafai R, Wong MK, Newgreen DF, Thompson EW, Waltham M:

Vimentin and epithelial-mesenchymal transition in human breast cancer--observations in vitro and in vivo. *Cells Tissues Organs* 2007, **185:**191-203.

255. Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL: **Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism.** *Mol Biol Cell* 2001, **12:**27-36.

256. Bakin AV, Rinehart C, Tomlinson AK, Arteaga CL: **p38 mitogen-activated protein kinase is required for TGFbeta-mediated fibroblastic transdifferentiation and cell migration.** *J Cell Sci* 2002, **115:**3193-3206.

257. Janda E, Lehmann K, Killisch I, Jechlinger M, Herzig M, Downward J, Beug H, Grunert S: **Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways.** *J Cell Biol* 2002, **156:**299-313.

258. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL: **Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration.** *J Biol Chem* 2000, **275:**36803-36810.

259. Lee YI, Kwon YJ, Joo CK: **Integrin-linked kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition.** *Biochem Biophys Res Commun* 2004, **316:**997-1001.

260. Oft M, Heider KH, Beug H: **TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis.** *Curr Biol* 1998, **8:**1243-1252.

261. Oloumi A, McPhee T, Dedhar S: **Regulation of E-cadherin expression and beta-catenin/Tcf transcriptional activity by the integrin-linked kinase.** *Biochim Biophys Acta* 2004, **1691:**1-15.

262. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ: **The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1.** *Nat Cell Biol* 2008, **10:**593-601.

263. Thiery JP, Acloque H, Huang RY, Nieto MA: **Epithelial-mesenchymal transitions in development and disease.** *Cell* 2009, **139:**871-890.

264. Wu Y, Evers BM, Zhou BP: **Small C-terminal domain phosphatase enhances snail activity through dephosphorylation.** *J Biol Chem* 2009, **284:**640-648.

265. Husemann Y, Geigl JB, Schubert F, Musiani P, Meyer M, Burghart E, Forni G, Eils R, Fehm T, Riethmuller G, Klein CA: **Systemic spread is an early step in breast cancer.** *Cancer Cell* 2008, **13:**58-68.

266. Polyak K, Weinberg RA: **Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits.** *Nat Rev Cancer* 2009, **9:**265-273.

267. Borgquist S, Holm C, Stendahl M, Anagnostaki L, Landberg G, Jirstrom K: **Oestrogen receptors alpha and beta show different associations to clinicopathological parameters and their co-expression might predict a better response to endocrine treatment in breast cancer.** *J Clin Pathol* 2008, **61:**197-203.

268. Ryden L, Jirstrom K, Bendahl PO, Ferno M, Nordenskjold B, Stal O, Thorstenson S, Jonsson PE, Landberg G: **Tumor-specific expression of vascular endothelial growth factor receptor 2 but not vascular endothelial growth factor or human epidermal**

growth factor receptor 2 is associated with impaired response to adjuvant tamoxifen in premenopausal breast cancer. *J Clin Oncol* 2005, **23:**4695-4704.

269. Gong Y, Booser DJ, Sneige N: **Comparison of HER-2 status determined by fluorescence in situ hybridization in primary and metastatic breast carcinoma.** *Cancer* 2005, **103:**1763-1769.
270. Guo WJ, Datta S, Band V, Dimri GP: **Mel-18, a polycomb group protein, regulates cell proliferation and senescence via transcriptional repression of Bmi-1 and c-Myc oncoproteins.** *Mol Biol Cell* 2007, **18:**536-546.
271. Pear WS, Nolan GP, Scott ML, Baltimore D: **Production of high-titer helper-free retroviruses by transient transfection.** *Proc Natl Acad Sci U S A* 1993, **90:**8392-8396.
272. Cailleau R, Olive M, Cruciger QV: **Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization.** *In Vitro* 1978, **14:**911-915.
273. Koshida S, Kobayashi D, Moriai R, Tsuji N, Watanabe N: **Specific overexpression of OLFM4(GW112/HGC-1) mRNA in colon, breast and lung cancer tissues detected using quantitative analysis.** *Cancer Sci* 2007, **98:**315-320.
274. Sodunke TR, Turner KK, Caldwell SA, McBride KW, Reginato MJ, Noh HM: **Micropatterns of Matrigel for three-dimensional epithelial cultures.** *Biomaterials* 2007, **28:**4006-4016.
275. Lee GY, Kenny PA, Lee EH, Bissell MJ: **Three-dimensional culture models of normal and malignant breast epithelial cells.** *Nat Methods* 2007, **4:**359-365.
276. Chung H, Jung JY, Cho SD, Hong KA, Kim HJ, Shin DH, Kim H, Kim HO, Shin DH, Lee HW, et al: **The antitumor effect of LJ-529, a novel agonist to A3 adenosine receptor, in both estrogen receptor-positive and estrogen receptor-negative human breast cancers.** *Mol Cancer Ther* 2006, **5:**685-692.
277. Price JE, Polyzos A, Zhang RD, Daniels LM: **Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice.** *Cancer Res* 1990, **50:**717-721.
278. Shao R, Bao S, Bai X, Blanchette C, Anderson RM, Dang T, Gishizky ML, Marks JR, Wang XF: **Acquired expression of periostin by human breast cancers promotes tumor angiogenesis through up-regulation of vascular endothelial growth factor receptor 2 expression.** *Mol Cell Biol* 2004, **24:**3992-4003.
279. Dai J, Li B, Shi J, Peng L, Zhang D, Qian W, Hou S, Zhao L, Gao J, Cao Z, et al: **A humanized anti-osteopontin antibody inhibits breast cancer growth and metastasis in vivo.** *Cancer Immunol Immunother*, **59:**355-366.
280. Kremer M, Quintanilla-Martinez L, Fuchs M, Gamboa-Dominguez A, Haye S, Kalthoff H, Rosivatz E, Hermannstadter C, Busch R, Hofler H, Luber B: **Influence of tumor-associated E-cadherin mutations on tumorigenicity and metastasis.** *Carcinogenesis* 2003, **24:**1879-1886.
281. Yang GF, He WP, Cai MY, He LR, Luo JH, Deng HX, Guan XY, Zeng MS, Zeng YX, Xie D: **Intensive expression of Bmi-1 is a new independent predictor of poor outcome in patients with ovarian carcinoma.** *BMC Cancer*, **10:**133.
282. Sasaki M, Yamaguchi J, Ikeda H, Itatsu K, Nakanuma Y: **Polycomb group protein Bmi1 is overexpressed and essential in anchorage-independent colony formation, cell proliferation and repression of cellular senescence in cholangiocarcinoma: tissue and culture studies.** *Hum Pathol* 2009, **40:**1723-1730.

283. Sasaki M, Yamaguchi J, Itatsu K, Ikeda H, Nakanuma Y: **Over-expression of polycomb group protein EZH2 relates to decreased expression of p16 INK4a in cholangiocarcinogenesis in hepatolithiasis.** *J Pathol* 2008, **215:**175-183.

284. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ: **Free DNA in the serum of cancer patients and the effect of therapy.** *Cancer Res* 1977, **37:**646-650.

285. Shapiro B, Chakrabarty M, Cohn EM, Leon SA: **Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease.** *Cancer* 1983, **51:**2116-2120.

286. Silva J, Garcia JM, Pena C, Garcia V, Dominguez G, Suarez D, Camacho FI, Espinosa R, Provencio M, Espana P, Bonilla F: **Implication of polycomb members Bmi-1, Mel-18, and Hpc-2 in the regulation of p16INK4a, p14ARF, h-TERT, and c-Myc expression in primary breast carcinomas.** *Clin Cancer Res* 2006, **12:**6929-6936.

287. Voncken JW, Schweizer D, Aagaard L, Sattler L, Jantsch MF, van Lohuizen M: **Chromatin-association of the Polycomb group protein BMI1 is cell cycle-regulated and correlates with its phosphorylation status.** *J Cell Sci* 1999, **112 (Pt 24):**4627-4639.

288. Li W, Li Y, Tan Y, Ma K, Cui J: **Bmi-1 is critical for the proliferation and invasiveness of gastric carcinoma cells.** *J Gastroenterol Hepatol*, **25:**568-575.

289. He LR, Liu MZ, Li BK, Jia WH, Zhang Y, Liao YJ, Chen YC, Zhang LJ, Guan XY, Zeng YX, et al: **High expression of EZH2 is associated with tumor aggressiveness and poor prognosis in patients with esophageal squamous cell carcinoma treated with definitive chemoradiotherapy.** *Int J Cancer* 2009.

290. van Leenders GJ, Dukers D, Hessels D, van den Kieboom SW, Hulsbergen CA, Witjes JA, Otte AP, Meijer CJ, Raaphorst FM: **Polycomb-group oncogenes EZH2, BMI1, and RING1 are overexpressed in prostate cancer with adverse pathologic and clinical features.** *Eur Urol* 2007, **52:**455-463.

291. Gong Y, Wang X, Liu J, Shi L, Yin B, Peng X, Qiang B, Yuan J: **NSPc1, a mainly nuclear localized protein of novel PcG family members, has a transcription repression activity related to its PKC phosphorylation site at S183.** *FEBS Lett* 2005, **579:**115-121.

292. Noguchi K, Shiurba R, Higashinakagawa T: **Nuclear translocation of mouse polycomb m33 protein in regenerating liver.** *Biochem Biophys Res Commun* 2002, **291:**508-515.

293. Hirose S, Komoike Y, Higashinakagawa T: **Identification of a nuclear localization signal in mouse polycomb protein, M33.** *Zoolog Sci* 2006, **23:**785-791.

294. Fujisaki S, Ninomiya Y, Ishihara H, Miyazaki M, Kanno R, Asahara T, Kanno M: **Dimerization of the Polycomb-group protein Mel-18 is regulated by PKC phosphorylation.** *Biochem Biophys Res Commun* 2003, **300:**135-140.

295. Sawa M, Yamamoto K, Yokozawa T, Kiyoi H, Hishida A, Kajiguchi T, Seto M, Kohno A, Kitamura K, Itoh Y, et al: **BMI-1 is highly expressed in M0-subtype acute myeloid leukemia.** *Int J Hematol* 2005, **82:**42-47.

296. Breuer RH, Snijders PJ, Sutedja GT, Sewalt RG, Otte AP, Postmus PE, Meijer CJ, Raaphorst FM, Smit EF: **Expression of the p16(INK4a) gene product, methylation of the p16(INK4a) promoter region and expression of the polycomb-group gene**

BMI-1 in squamous cell lung carcinoma and premalignant endobronchial lesions. *Lung Cancer* 2005, **48:**299-306.

297. Sotiriou C, Pusztai L: **Gene-expression signatures in breast cancer.** *N Engl J Med* 2009, **360:**790-800.

298. Kato T, Kameoka S, Kimura T, Tanaka S, Nishikawa T, Kobayashi M: **p53, mitosis, apoptosis and necrosis as prognostic indicators of long-term survival in breast cancer.** *Anticancer Res* 2002, **22:**1105-1112.

299. Olivotto IA, Chua B, Allan SJ, Speers CH, Chia S, Ragaz J: **Long-term survival of patients with supraclavicular metastases at diagnosis of breast cancer.** *J Clin Oncol* 2003, **21:**851-854.

300. Herbert BS, Wright WE, Shay JW: **p16(INK4a) inactivation is not required to immortalize human mammary epithelial cells.** *Oncogene* 2002, **21:**7897-7900.

301. Shamanin VA, Androphy EJ: **Immortalization of human mammary epithelial cells is associated with inactivation of the p14ARF-p53 pathway.** *Mol Cell Biol* 2004, **24:**2144-2152.

302. Cowell JK, LaDuca J, Rossi MR, Burkhardt T, Nowak NJ, Matsui S: **Molecular characterization of the t(3;9) associated with immortalization in the MCF10A cell line.** *Cancer Genet Cytogenet* 2005, **163:**23-29.

303. Soule HD, Maloney TM, Wolman SR, Peterson WD, Jr., Brenz R, McGrath CM, Russo J, Pauley RJ, Jones RF, Brooks SC: **Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10.** *Cancer Res* 1990, **50:**6075-6086.

304. Leuschner C, Enright FM, Gawronska B, Hansel W: **Membrane disrupting lytic peptide conjugates destroy hormone dependent and independent breast cancer cells in vitro and in vivo.** *Breast Cancer Res Treat* 2003, **78:**17-27.

305. Ross DT, Scherf U, Eisen MB, Perou CM, Rees C, Spellman P, Iyer V, Jeffrey SS, Van de Rijn M, Waltham M, et al: **Systematic variation in gene expression patterns in human cancer cell lines.** *Nat Genet* 2000, **24:**227-235.

306. Ellison G, Klinowska T, Westwood RF, Docter E, French T, Fox JC: **Further evidence to support the melanocytic origin of MDA-MB-435.** *Mol Pathol* 2002, **55:**294-299.

307. You H, Yu W, Sanders BG, Kline K: **RRR-alpha-tocopheryl succinate induces MDA-MB-435 and MCF-7 human breast cancer cells to undergo differentiation.** *Cell Growth Differ* 2001, **12:**471-480.

308. Sellappan S, Grijalva R, Zhou X, Yang W, Eli MB, Mills GB, Yu D: **Lineage infidelity of MDA-MB-435 cells: expression of melanocyte proteins in a breast cancer cell line.** *Cancer Res* 2004, **64:**3479-3485.

309. Tait L, Soule HD, Russo J: **Ultrastructural and immunocytochemical characterization of an immortalized human breast epithelial cell line, MCF-10.** *Cancer Res* 1990, **50:**6087-6094.

310. Goldson TM, Vielhauer G, Staub E, Miller S, Shim H, Hagedorn CH: **Eukaryotic initiation factor 4E variants alter the morphology, proliferation, and colony-formation properties of MDA-MB-435 cancer cells.** *Mol Carcinog* 2007, **46:**71-84.

311. Yoshida BA, Sokoloff MM, Welch DR, Rinker-Schaeffer CW: **Metastasis-suppressor**

genes: a review and perspective on an emerging field. *J Natl Cancer Inst* 2000, **92:**1717-1730.

312. Ciardiello F, Kim N, McGeady ML, Liscia DS, Saeki T, Bianco C, Salomon DS: **Expression of transforming growth factor alpha (TGF alpha) in breast cancer.** *Ann Oncol* 1991, **2:**169-182.
313. Ovejera AA, Houchens DP: **Human tumor xenografts in athymic nude mice as a preclinical screen for anticancer agents.** *Semin Oncol* 1981, **8:**386-393.
314. Fidler IJ: **Rationale and methods for the use of nude mice to study the biology and therapy of human cancer metastasis.** *Cancer Metastasis Rev* 1986, **5:**29-49.
315. Kozlowski JM, Fidler IJ, Campbell D, Xu ZL, Kaighn ME, Hart IR: **Metastatic behavior of human tumor cell lines grown in the nude mouse.** *Cancer Res* 1984, **44:**3522-3529.
316. Miller FR, Medina D, Heppner GH: **Preferential growth of mammary tumors in intact mammary fatpads.** *Cancer Res* 1981, **41:**3863-3867.
317. Miller FR: **Comparison of metastasis of mammary tumors growing in the mammary fatpad versus the subcutis.** *Invasion Metastasis* 1981, **1:**220-226.
318. Suzuki M, Mose E, Galloy C, Tarin D: **Osteopontin gene expression determines spontaneous metastatic performance of orthotopic human breast cancer xenografts.** *Am J Pathol* 2007, **171:**682-692.
319. Chen YC, Chang CJ, Hsu HS, Chen YW, Tai LK, Tseng LM, Chiou GY, Chang SC, Kao SY, Chiou SH, Lo WL: **Inhibition of tumorigenicity and enhancement of radiochemosensitivity in head and neck squamous cell cancer-derived ALDH1-positive cells by knockdown of Bmi-1.** *Oral Oncol*, **46:**158-165.
320. Kalluri R, Neilson EG: **Epithelial-mesenchymal transition and its implications for fibrosis.** *J Clin Invest* 2003, **112:**1776-1784.
321. Eger A, Stockinger A, Schaffhauser B, Beug H, Foisner R: **Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear translocation of beta-catenin and upregulation of beta-catenin/lymphoid enhancer binding factor-1 transcriptional activity.** *J Cell Biol* 2000, **148:**173-188.
322. Romoser WS, Faran ME, Bailey CL, Lerdthusnee K: **An immunocytochemical study of the distribution of Rift Valley fever virus in the mosquito Culex pipiens.** *Am J Trop Med Hyg* 1992, **46:**489-501.
323. Eccles SA, Box C, Court W: **Cell migration/invasion assays and their application in cancer drug discovery.** *Biotechnol Annu Rev* 2005, **11:**391-421.
324. Grunert S, Jechlinger M, Beug H: **Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis.** *Nat Rev Mol Cell Biol* 2003, **4:**657-665.
325. Waerner T, Alacakaptan M, Tamir I, Oberauer R, Gal A, Brabletz T, Schreiber M, Jechlinger M, Beug H: **ILEI: a cytokine essential for EMT, tumor formation, and late events in metastasis in epithelial cells.** *Cancer Cell* 2006, **10:**227-239.
326. Mackenzie NC, Raz E: **Found in translation: A new player in EMT.** *Dev Cell* 2006, **11:**434-436.
327. Altomare DA, Testa JR: **Perturbations of the AKT signaling pathway in human cancer.** *Oncogene* 2005, **24:**7455-7464.

328. Robertson GP: **Functional and therapeutic significance of Akt deregulation in malignant melanoma.** *Cancer Metastasis Rev* 2005, **24:**273-285.
329. Tarin D, Thompson EW, Newgreen DF: **The fallacy of epithelial mesenchymal transition in neoplasia.** *Cancer Res* 2005, **65:**5996-6000; discussion 6000-5991.
330. Thompson EW, Newgreen DF, Tarin D: **Carcinoma invasion and metastasis: a role for epithelial-mesenchymal transition?** *Cancer Res* 2005, **65:**5991-5995; discussion 5995.
331. Han G, Lu SL, Li AG, He W, Corless CL, Kulesz-Martin M, Wang XJ: **Distinct mechanisms of TGF-beta1-mediated epithelial-to-mesenchymal transition and metastasis during skin carcinogenesis.** *J Clin Invest* 2005, **115:**1714-1723.
332. Shiozaki H, Tahara H, Oka H, Miyata M, Kobayashi K, Tamura S, Iihara K, Doki Y, Hirano S, Takeichi M, et al.: **Expression of immunoreactive E-cadherin adhesion molecules in human cancers.** *Am J Pathol* 1991, **139:**17-23.
333. Gamallo C, Palacios J, Suarez A, Pizarro A, Navarro P, Quintanilla M, Cano A: **Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma.** *Am J Pathol* 1993, **142:**987-993.
334. Lozano E, Cano A: **Induction of mutual stabilization and retardation of tumor growth by coexpression of plakoglobin and E-cadherin in mouse skin spindle carcinoma cells.** *Mol Carcinog* 1998, **21:**273-287.
335. Navarro P, Lozano E, Cano A: **Expression of E- or P-cadherin is not sufficient to modify the morphology and the tumorigenic behavior of murine spindle carcinoma cells. Possible involvement of plakoglobin.** *J Cell Sci* 1993, **105 (Pt 4):**923-934.
336. Navarro P, Gomez M, Pizarro A, Gamallo C, Quintanilla M, Cano A: **A role for the E-cadherin cell-cell adhesion molecule during tumor progression of mouse epidermal carcinogenesis.** *J Cell Biol* 1991, **115:**517-533.
337. Llorens A, Rodrigo I, Lopez-Barcons L, Gonzalez-Garrigues M, Lozano E, Vinyals A, Quintanilla M, Cano A, Fabra A: **Down-regulation of E-cadherin in mouse skin carcinoma cells enhances a migratory and invasive phenotype linked to matrix metalloproteinase-9 gelatinase expression.** *Lab Invest* 1998, **78:**1131-1142.
338. Ribeiro AS, Albergaria A, Sousa B, Correia AL, Bracke M, Seruca R, Schmitt FC, Paredes J: **Extracellular cleavage and shedding of P-cadherin: a mechanism underlying the invasive behaviour of breast cancer cells.** *Oncogene* 2009.
339. Bernards R, Weinberg RA: **A progression puzzle.** *Nature* 2002, **418:**823.