# INDEX

<table>
<thead>
<tr>
<th>Acknowledgements</th>
<th>i</th>
</tr>
</thead>
<tbody>
<tr>
<td>Index</td>
<td>iii</td>
</tr>
<tr>
<td>1. ABBREVIATIONS</td>
<td>1-3</td>
</tr>
<tr>
<td>2. ABSTRACT OF THE THESIS</td>
<td>4-5</td>
</tr>
<tr>
<td>3. INTRODUCTION AND REVIEW OF LITERATURE</td>
<td>6-26</td>
</tr>
<tr>
<td>3.1 Cancer</td>
<td>6</td>
</tr>
<tr>
<td>3.2 Breast Cancer</td>
<td>7</td>
</tr>
<tr>
<td>3.2.1 Breast Cancer Symptoms and Signs</td>
<td>7</td>
</tr>
<tr>
<td>3.2.2 Causes of Breast Cancer</td>
<td>8</td>
</tr>
<tr>
<td>3.2.2.1 Genetic Causes</td>
<td>8</td>
</tr>
<tr>
<td>3.2.2.2 Hormonal Factor</td>
<td>8</td>
</tr>
<tr>
<td>3.2.2.3 Lifestyle and Dietary Agents</td>
<td>8</td>
</tr>
<tr>
<td>3.2.2.4 Environmental Factor</td>
<td>8</td>
</tr>
<tr>
<td>3.2.3 Stages of Breast Cancer</td>
<td>8</td>
</tr>
<tr>
<td>3.2.4 Medical Treatment</td>
<td>9</td>
</tr>
<tr>
<td>3.3 Melanoma</td>
<td>9</td>
</tr>
<tr>
<td>3.3.1 Causes of Melanoma</td>
<td>9</td>
</tr>
<tr>
<td>3.3.2 Symptoms of Melanoma</td>
<td>9</td>
</tr>
<tr>
<td>3.3.3 Treatment</td>
<td>10</td>
</tr>
<tr>
<td>3.4 Osteopontin</td>
<td>10</td>
</tr>
<tr>
<td>3.4.1 Location of OPN in Genome</td>
<td>10</td>
</tr>
<tr>
<td>3.4.2 Splice Variants and Structural Features of OPN</td>
<td>11</td>
</tr>
</tbody>
</table>
3.4.3 OPN in Tumor Biology

3.4.3.1 Breast Cancer

3.4.3.2 OPN in Melanoma Cancer

3.4.4 OPN in Angiogenesis and Metastasis

3.4.5 Stromal OPN

3.4.6 OPN a potential Target for the Therapy of Cancer

3.5 Andrographolide

3.6 Apoptosis

3.7 Phosphatidylinositol 3-kinases

3.8 Nuclear Factor kappa B (NF-κB)

3.9 AP-1 transcription factor

3.9.1 c-Jun

3.9.2 c-Fos

3.9.3 Activation and Regulation of AP-1

3.10 ERK MAPKs

3.11 ABCG2 Transporter

3.12 Side Population

4. AIMS AND OBJECTIVE

5. MATERIALS AND METHODS

5.1 Sources of chemicals, antibodies and plasmid constructs

5.2 Maintenance of cell lines

5.3 Extraction, Isolation and Characterization of Andro

5.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

5.4.1 Reagents and Solutions for SDS-PAGE
5.4.2 Gel Composition
5.4.3 Coomassie Blue Staining Solutions
5.4.4 Reagents and Solutions for Western Blotting
5.5 Preparation of Nuclear and Cytoplasmic Extracts
5.6 Electrophoretic Mobility Shift Assay (EMSA)
  5.6.1 Labeling of Oligonucleotide
  5.6.2 DNA-protein Binding
5.7 Preparation of LB/Amp
5.8 Preparation of Competent Cells
5.9 Transformation
5.10 Plasmid Preparation
5.11 Agarose Gel Electrophoresis
5.12 Preparation of Glycerol Stocks
5.13 Transfection
5.14 Luciferase Reporter Assay
5.15 RNA Isolation and Reverse Transcription-PCR (RT-PCR)
5.16 MTT Cell Viability Assay
5.17 Wound Healing Assay
5.18 Cell Migration Assay
5.19 Tumor-Endothelial Cell Comigration/Coinvasion Assay
5.20 In vitro Tube Formation Assay
5.21 Matrigel Colony Formation Assay
5.22 Cell Cycle Analysis
5.23 Annexin V/Propidium Iodide Staining
5.24 Detection of Oxidative Stress
5.25 Hoechst 33342 Staining and SP analysis 46
5.26 Tumor Xenografts and Bioluminescence Analysis 46
5.27 Development and Establishment of Murine Primary Culture from the Tumors of OPN +/+ and OPN -/- mice 47
5.28 In Vivo Metastasis study by Intra-Cardiac Injection 47
5.29 Immunofluorescence 47
5.30 Immunohistochemistry 48
5.31 Statistical analysis 49

6. ANDRO INHIBITS BREAST TUMOR GROWTH BY DOWN REGULATING OPN EXPRESSION AND PI3-KINASE SIGNALING

6.1 Introduction 50
6.2 Results 51
  6.2.1 Purification and characterization of Andro 51
  6.2.2 Andro inhibits breast cancer cell proliferation 52
  6.2.3 Andro induces G2/M phase arrest and apoptosis in a dose dependent manner 53
  6.2.4 Andro induces cellular apoptosis through caspase independent pathway 54
  6.2.5 Breast cancer cells motility is inhibited by Andro 56
  6.2.6 Andro blocks endothelial cell motility, tumor-endothelial interactions and in vitro angiogenesis 57
  6.2.7 Andro inhibits OPN expression 60
  6.2.8 Andro downregulates PI3-kinase/Akt pathway 60
  6.2.9 Andro downregulates NF-κB and AP-1 activation in breast cancer cells 62
  6.2.10 Andro regulates NF-κB and AP-1-regulated Cox-2, cyclin D1, VEGF and Flk1 expression 62
6.2.11 Andro inhibits breast tumor growth in \textit{in vitro} and \textit{in vivo} models 66

6.3 Discussion 68

7. STUDY ON THE ROLE OF STROMAL OPN IN MELANOMA GROWTH, ANGIOGENESIS AND METASTASIS

7.1 Introduction 71

7.2 Results 73

7.2.1 Abrogation of melanoma growth and angiogenesis in OPN Knockout mice 73

7.2.2 Stromal OPN promotes lung and liver metastasis in experimental mice model 73

7.2.3 Functional characterization of primary melanoma cells isolated from OPN$^{+/+}$ and OPN$^{-/-}$ mice tumors 75

7.2.4 B16-WT cells exhibit vasculogenic mimicry and tumor endothelial interaction 76

7.2.5 Expression and activation of protein kinases in parental and primary melanoma cells 79

7.2.6 Reintroduction of B16-WT cells in OPN$^{-/-}$ mice exhibit enhanced tumor growth, angiogenesis and metastasis 79

7.2.7 Stromal OPN selectively enriches SP phenotype in murine melanoma cells 82

7.2.8 Functional characterization of sorted SP murine melanoma cells 84

7.2.8.1 Efflux of mitoxantrone from melanoma SP cells 84

7.2.8.2 SP regenerates SP and non-SP cells 84

7.2.8.3 SP cells are more tumorigenic in nature 84

7.2.8.4 SP cells display increased lung metastasis in mice model 86

7.2.9 Stromal OPN regulates SP phenotype by upregulating ABCG2 expression through ERK signaling 87
7.2.10 ERK2 but not ERK1 regulates SP phenotype in response to stromal-OPN in B16F10 cells 88

7.3 Discussion 91

8. SUMMARY AND CONCLUSION 93

9. BIBLIOGRAPHY 97

10. APPENDIX 118

10.1 List of Accomplishment 118

10.2 List of conference presentation 119