Pre-clinical analysis of fluvastatin as a metastasis-prevention agent in breast cancer

by

Rosemary Yu

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Medical Biophysics
University of Toronto

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Doctor of Philosophy

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2017

Abstract

Fluvastatin is a member of the statin family of drugs, widely prescribed to lower serum cholesterol. Accumulating evidence from epidemiological, pre-clinical, and clinical studies indicate that statins have pleiotropic anti-cancer activities. Previous work have suggested a mechanistic model where depletion of farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), and consequently inhibition of prenylation of RAS family members, underlies statin-induced cancer cell death. However, several lines of evidence suggest that this model is incomplete, yet alternative mechanisms have not been described. The purpose of this thesis is to (i) elucidate the molecular mechanism of fluvastatin sensitivity in breast cancer cells, and (ii) identify the point of therapeutic intervention in which fluvastatin treatment will provide clinical benefit in breast cancer patients. We first demonstrate that fluvastatin-induced cell death can be uncoupled from inhibition of protein prenylation, in breast cancer cells that have undergone epithelial-mesenchymal transition (EMT). In these cells, the mechanism of fluvastatin sensitivity is an increased dependency on the biosynthesis of dolichol, required for co-translational protein N-glycosylation. Fluvastatin treatment impairs the expression of tri- and tetra-antennary β1,6-branched complex type N-glycans associated with breast cancer cell EMT and metastasis. These results, along with supporting evidence from epidemiological studies, provided rationale to examine the efficacy of fluvastatin as a metastasis-prevention agent in
breast cancer. Using a mouse model of post-surgical metastatic breast cancer that fully recapitulates the intravasation and dissemination process, we show that fluvastatin treatment in the adjuvant setting significantly delays breast cancer metastasis, reduces metastatic burden, and improves overall survival. Overall, this research provides novel insight into the mechanism of the anti-cancer effects of fluvastatin, and demonstrates efficacy of fluvastatin use in the adjuvant therapeutic setting to prevent breast cancer metastasis.
Acknowledgments

To my supervisor, Dr. Linda Penn, thank you for your unwavering support throughout my time in your lab. I am sincerely grateful for the freedom and belief that you have given me to pursue my research ideas; your scientific guidance and intellectual support; and above all, the rich and engaging environment that you have built in which to learn, explore, and grow. For all this and a lot more, thank you.

To my supervisory committee members, Dr. Mark Minden and Dr. Brad Wouters, thank you both for your insight and advice. I have always felt more inspired about my research and its contribution to the field of cancer research after discussing my projects with you.

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To Broom-Broom, you have been a constant source of comfort and joy. To Mom and Dad, thank you for your never-ending love.
# Table of Contents

Acknowledgments ........................................................................................................ iv

Table of Contents ........................................................................................................... v

List of Figures ................................................................................................................ ix

List of Abbreviations ..................................................................................................... xi

Chapter 1 Introduction .................................................................................................... 1

1 Introduction .................................................................................................................. 2

1.1 Treatment of cancer .................................................................................................. 2

1.1.1 Treatment of breast cancer ................................................................................. 4

1.2 Drug repurposing .................................................................................................... 7

1.2.1 Opportunities ..................................................................................................... 8

1.2.2 Challenges ......................................................................................................... 11

1.3 Repurposing statins as anti-breast cancer agents ................................................... 15

1.3.1 Statins as cholesterol-lowering agents .............................................................. 15

1.3.2 Evidence of anti-breast cancer effects ............................................................... 18

1.4 The mevalonate pathway in cancer metabolism .................................................... 21

1.4.1 End products ..................................................................................................... 22

1.4.2 Deregulation ..................................................................................................... 26

1.5 Mechanism of statin-induced cancer cell apoptosis .............................................. 29

1.6 Objectives and thesis outline .................................................................................. 33

1.7 References .............................................................................................................. 35

Chapter 2 Statin-induced cancer cell death can be mechanistically uncoupled from
prenylation of RAS family proteins ................................................................................ 57

2 Statin-induced cancer cell death can be mechanistically uncoupled from prenylation of
RAS family proteins .................................................................................................... 58

2.1 Abstract .................................................................................................................. 58

2.2 Introduction .............................................................................................................. 58
2.3 Results .............................................................................................................................................. 60

2.3.1 HRAS\textsuperscript{G12V} and KRAS\textsuperscript{G12V}, but not other proteins in the RAS superfamily, sensitize MCF10A cells to fluvastatin ................................................................. 60

2.3.2 Inhibition of RAS prenylation is uncoupled from fluvastatin-induced cell death ........................................................................................................................................ 61

2.3.3 The RAS-ZEB1-EMT signaling axis underlies increased sensitivity to fluvastatin ......................... 65

2.3.4 Enrichment of EMT phenotype is associated with sensitivity to statins in a large panel of cancer cell lines ......................................................................................................................................... 75

2.4 Discussion ........................................................................................................................................ 78

2.5 Materials and Methods .......................................................................................................................... 81

2.5.1 Reagents ............................................................................................................................................ 81

2.5.2 Cell culture ...................................................................................................................................... 81

2.5.3 MTT assay ...................................................................................................................................... 81

2.5.4 Immunoblotting ............................................................................................................................... 82

2.5.5 Soft agar colony formation ............................................................................................................ 82

2.5.6 Membrane fractionation ................................................................................................................ 82

2.5.7 Cell death assay ............................................................................................................................. 82

2.5.8 qRT-PCR ....................................................................................................................................... 83

2.5.9 Small hairpin RNA-mediated gene silencing .................................................................................. 83

2.5.10 Pharmacogenomic analysis ......................................................................................................... 84

2.6 References ......................................................................................................................................... 85

Chapter 3 Fluvastatin inhibits EMT-associated protein N-glycosylation and delays breast cancer metastasis ........................................................................................................................................... 93

3 Fluvastatin inhibits EMT-associated protein N-glycosylation and delays breast cancer metastasis ........................................................................................................................................... 94

3.1 Abstract .............................................................................................................................................. 94

3.2 Introduction ....................................................................................................................................... 94

3.3 Results .............................................................................................................................................. 97
3.3.1 EMT sensitizes breast cancer cells to fluvastatin and tunicamycin

3.3.2 Fluvastatin inhibits dolichol-mediated protein N-glycosylation

3.3.3 Fluvastatin inhibits EMT-associated protein N-glycosylation

3.3.4 Fluvastatin impairs protein N-glycosylation in vivo

3.3.5 Post-surgical adjuvant fluvastatin treatment delays metastatic outgrowth and prolongs survival

3.4 Discussion

3.5 Materials and Methods

3.5.1 Reagents

3.5.2 Cell culture

3.5.3 MTT assay

3.5.4 Immunoblotting

3.5.5 Immunohistochemistry

3.5.6 Cell death assay

3.5.7 qRT-PCR

3.5.8 N-glycan extraction

3.5.9 Total glycan analysis by LC-MS/MS

3.5.10 Animal models

3.5.11 Fluvastatin quantification by HPLC-MS/MS

3.6 References

Chapter 4 Discussion

4 Discussion

4.1 Challenges in the treatment of cancer

4.2 Mechanism of statin-induced cancer cell death

4.3 Biomarkers of statin sensitivity

4.4 Point of therapeutic intervention for statin use in breast cancer
List of Figures

Figure 1-1. Pharmacology of statins. ........................................................................................................ 17

Figure 1-2. The mevalonate pathway........................................................................................................ 23

Figure 1-3. Possible mechanisms of deregulation of the MVA pathway in cancer cells. ............ 27

Figure 1-4. Prenylation substrate is determined by the amino acid sequence in the CaaX domain. ........................................................................................................................................... 32

Figure 2-1. HRAS$^{G12V}$ and KRAS$^{G12V}$, but not other prenylated proteins, sensitize MCF10A cells to fluvastatin................................................................................................................................. 62

Figure 2-2. Inhibition of RAS prenylation is uncoupled from fluvastatin-induced cell death. .... 63

Figure 2-3. Overexpression of HRAS$^{G12V}$ or myr-HRAS$^{G12V}$ does not alter HMGCR or HMGCS1 expression. ................................................................................................................................. 66

Figure 2-4. Fluvastatin cytotoxicity is not mediated by the BRAF, RALA, PI3K, or MYC downstream mediators of RAS signaling. .................................................................................................................. 67

Figure 2-5. Transforming oncogenes do not all sensitize MCF10As to fluvastatin. ............... 68

Figure 2-6. RAS induces EMT through ZEB1, and induction of EMT is sufficient for sensitizing cells to fluvastatin. ................................................................................................................................. 70

Figure 2-7. TGF-β induced EMT and fluvastatin sensitivity are both reversible....................... 72

Figure 2-8. shRNA knockdown of ZEB1 rescues the increased sensitivity in HRAS$^{G12V}$ and myr-HRAS$^{G12V}$ cells. ........................................................................................................................................... 73

Figure 2-9. Unimodal and bimodal gene expression in the CCLE database. ......................... 76

Figure 2-10. Statin sensitivity is associated with cancer cell EMT. ........................................ 77

Figure 3-1. Induction of EMT by SNAIL overexpression increases cell sensitivity to inhibition of dolichol-dependent protein N-glycosylation by fluvastatin and tunicamycin. ......................... 99
Figure 3-2. Induction of EMT increases cell sensitivity to fluvastatin and tunicamycin. 102

Figure 3-3. Fluvastatin effect is independent from induction of ER stress 105

Figure 3-4. Inhibition of dolichol synthesis underlies fluvastatin sensitivity in mesenchymal breast cancer cell lines. 107

Figure 3-5. Exogenous addition of dolichol (dolichyl[C95]-PP) did not rescue viability of cells with fluvastatin treatment in DMEM supplemented with 10% FBS. 109

Figure 3-6. Fluvastatin treatment decreases complex branched N-glycans associated with EMT. 113

Figure 3-7. Fluvastatin treatment decreases complex branched N-glycans in vivo. 117

Figure 3-8. Post-surgical adjuvant fluvastatin treatment delays metastasis and prolongs survival. 120

Figure 3-9. Proposed mechanism of statin-mediated attenuation of breast cancer metastasis by targeting EMT-associated protein N-glycosylation. 125

Figure 4-1. Pre-clinical models of the points of therapeutic intervention in breast cancer. 145

Figure 5-1. Two-component mechanisms of feedback regulation. 161

Figure 5-2. MVA and GGPP reverses fluvastatin-induced cell death but does not reverse upregulation of MVA pathway genes. 164

Figure 5-3. HMGCS1 and HMGCR upregulation is sustained in the absence of negative feedback. 166

Figure 5-4. HMGCS1 and HMGCR upregulation is dependent on SREBP2 in the absence of negative feedback. 167

Figure 5-5. The MVA pathway may be regulated by a positive feedback loop. 168
List of Abbreviations

μg  microgram
μL  microliter
μM  micromolar
μm  micrometer or micron
2-TTFA  2-thenoyltrifluoroacetone
5-FU  5-fluorouracil
ABI  Applied Biosystems
ABL  Abelson murine leukemia viral oncogene homolog
ACAT2  acetyl-CoA acetyltransferase
Acetyl-CoA  acetyl coenzyme A
ACN  acetonitrile
ADP  adenosine diphosphate
AFP  alpha-fetoprotein
AKT/PKB  Protein kinase B
ALL  acute lymphoblastic leukemia
AML  acute myeloid leukemia
AMPK  5' adenosine monophosphate-activated protein kinase
ANOVA  Analysis of variance
APL  acute promyelocytic leukemia
ASCO  American Society of Clinical Oncology
<table>
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<tr>
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<tr>
<td>ASM</td>
<td>aggressive systemic mastocytosis</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>ATRA</td>
<td>all-trans-retinoic acid</td>
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<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
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<td>B/R</td>
<td>benefit/risk assessments</td>
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<td>BCR</td>
<td>breakpoint cluster region</td>
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<td>BD</td>
<td>Becton Dickinson Biosciences</td>
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<tr>
<td>BiP</td>
<td>Binding immunoglobulin protein</td>
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<td>BRAF</td>
<td>v-Raf murine sarcoma viral oncogene homolog B</td>
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<td>Breast Cancer susceptibility gene 1/2</td>
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<tr>
<td>BSA</td>
<td>body surface area</td>
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<td>bovine serum albumin</td>
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<td>cancer antigen 125</td>
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<tr>
<td>DBCO</td>
<td>dibenzocyclooctyne group</td>
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</table>
DC   dendritic cell
DFS  disease-free survival
DFSP dermatofibrosarcoma protuberans
DNA deoxyribonucleic acid
DHDDS dehydrodolichyl diphosphate synthase
dNTP Deoxynucleotide triphosphate
DOLK dolichol kinase
DOLPP1 dolichyl diphosphate phosphatase 1
DP   dipyridamole
DTT  Dithiothreitol
EDTA Ethylenediaminetetraacetic acid
EGF  epidermal growth factor
EGFR epidermal growth factor receptor
EMT epithelial to mesenchymal transition
ER   endoplasmic reticulum
ERdj4 Endoplasmic reticulum-localized DnaJ 4
ERK extracellular signal-regulated kinase
ERα  estrogen receptor α
ESMO European Society for Medical Oncology
ESR1 estrogen receptor α
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<td>electron transport chain</td>
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<td>fatty acid synthesis</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>FDPS</td>
<td>farnesyl diphosphate synthase</td>
</tr>
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<td>FDR</td>
<td>false discovery rate</td>
</tr>
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<td>FFPE</td>
<td>formalin-fixed and paraffin-embedded</td>
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<td>fluvastatin</td>
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<td>fibronectin</td>
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<td>FPKM</td>
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<td>farnesyltransferase inhibitor</td>
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<td>GIST</td>
<td>gastrointestinal stromal tumours</td>
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<td>Glc</td>
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<td>GlcNAc, N</td>
<td>N-acetylglucosamine</td>
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<td>high density lipoprotein</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>HRAS</td>
<td>Harvey rat sarcoma viral oncogene homolog</td>
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<tr>
<td>HTC</td>
<td>high-throughput screening</td>
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<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
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<td>IHC</td>
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<td>INSIG</td>
<td>insulin-induced gene</td>
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<td>IPP</td>
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<td>MM</td>
<td>multiple myeloma</td>
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<td>mM</td>
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<td>mouse mammary tumour virus</td>
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<td>MPD</td>
<td>myeloproliferative diseases</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MTD</td>
<td>maxim tolerated dose</td>
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<td>mTORC1</td>
<td>mammalian target of rapamycin complex 1</td>
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<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium</td>
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<td>MVA</td>
<td>mevalonate</td>
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<td>MVAC</td>
<td>methotrexate, vincristine, doxorubicin, cisplatin</td>
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<td>NSCLC</td>
<td>non-small cell lung cancer</td>
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<td>NUS1</td>
<td>dehydrodolichyl diphosphate synthase regulatory subunit</td>
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<td>pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>PDE5</td>
<td>phosphodiesterase type 5</td>
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<tr>
<td>PDGF-R</td>
<td>platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PDO</td>
<td>patient-derived organoid</td>
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<tr>
<td>PDX</td>
<td>patient-derived xenograft</td>
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<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
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<tr>
<td>PGAM1</td>
<td>phosphoglycerate mutase</td>
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<td>graphitized carbon</td>
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<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
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<tr>
<td>Ph+</td>
<td>Philadelphia chromosome positive</td>
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<tr>
<td>PHA-E</td>
<td>Phytohaemagglutinin-erythroagglutinin</td>
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<tr>
<td>PHA-L</td>
<td>Phytohaemagglutinin-leucoagglutinin</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PI3K-p110a</td>
<td>phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha</td>
</tr>
<tr>
<td>PKM1/2</td>
<td>pyruvate kinase, isoform M1/M2</td>
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PMVK  phosphomevalonate kinase
POLR2A  RNA polymerase II, subunit A
PP  diphosphate
PR  progesterone receptor
PRO  patient-reported outcomes
PSA  prostate-specific antigen
PTGS2  prostaglandin-endoperoxide synthase 2
PyMT  polyoma middle T
qRT-PCR  real-time quantitative reverse transcription polymerase chain reaction
RAC1  Ras-related C3 botulinum toxin substrate 1
RALA  Ras-related protein Ral-A
RAP1A  Ras-related protein Rap-1A
RECIST  Response Evaluation Criteria in Solid Tumors
RCC  renal cell carcinoma
RDI  relative dose-intensity
RHOA/B  Ras homolog gene family member A/B
RNA  ribonucleic acid
RNA-seq  ribonucleic acid sequencing
rv  reverse
SCAP  SREBP cleavage-activating protein
<table>
<thead>
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<th>Term</th>
<th>Definition</th>
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<td>SCB</td>
<td>Santa Cruz Biotechnology</td>
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<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<td>SD</td>
<td>standard deviation</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>shRNA</td>
<td>small hairpin ribonucleic acid</td>
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<tr>
<td>SILAC</td>
<td>Stable isotope labeling with amino acids in cell culture</td>
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<td>sterol regulatory element-binding protein 1/2</td>
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<td>transcriptional co-activator with PDZ-binding motif</td>
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<td>TCA</td>
<td>tricarboxylic acid</td>
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<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
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<td>trifluoroacetic acid</td>
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<td>transforming growth factor beta</td>
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<td>T helper 1/2 cell</td>
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<td>thapsigargin</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
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</tr>
<tr>
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<td>tyrosine kinase inhibitor</td>
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<tr>
<td>TNBC</td>
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<tr>
<td>Treg</td>
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<td>transfer ribonucleic acid</td>
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<tr>
<td>TTP</td>
<td>time to progression</td>
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<tr>
<td>tuni</td>
<td>tunicamycin</td>
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<td>Twist Family BHLH Transcription Factor 1</td>
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<td>UGT1A3</td>
<td>UDP glucuronosyltransferase family 1 member A3</td>
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<td>very low density lipoprotein</td>
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<td>yes-associated protein 1</td>
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<td>ZEB1</td>
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Chapter 1
Introduction
1 Introduction

1.1 Treatment of cancer

*Surgery and radiotherapy.* Cancer deaths account for 14-15% of human deaths each year worldwide (1), approximately 20% in the US (2), and up to 30% in Canada (3). For most solid tumours presenting without metastases, the standard of care and often the cure, involves complete surgical resection of the tumour, surrounding tissue, and/or the axillary lymph node(s) (4). Surgery is often accompanied by radiotherapy, where ionizing radiation is administered to the target tissue to destroy cancer cells by inducing DNA damage (5). In rare cases, radiotherapy alone is curative (6). In general, however, additional treatment is needed, in the form of cytotoxic chemotherapies, targeted chemotherapies, and/or immunotherapies.

*Cytotoxic chemotherapy.* Cancer has traditionally been treated with general cytotoxic chemotherapy targeted to rapidly dividing cells, with little tumour specificity (7). Many of these drugs still constitute the standard of care for many cancers, often in combination, such as CAF for breast cancer (cyclophosphamide, Adriamycin (doxorubicin), 5-FU (5-fluorouracil)) and MVAC for bladder cancer (methotrexate, vincristine, doxorubicin, cisplatin) (7). Their typical mechanisms of action are by inducing DNA damage (alkylating agents), by inhibiting DNA synthesis (antimetabolites and topoisomerase inhibitors), or by inhibiting cell division (anti-microtubule agents, cytotoxic antibiotics) (7). However, they are associated with wide-ranging toxicities and reduced quality of life, leading to questions regarding their clinical benefit in many cancers (8).

*Targeted chemotherapy.* In the past two decades, growing understanding of the hallmarks of cancer (9) and the molecular alterations leading to their selection (10) has given rise to rationally designed inhibitors to target these drivers (11). These targeted therapeutics also represent the
growing effort to combat tumour heterogeneity, by stratifying patients based on predicted response (11). One strategy for targeted therapy is the use of monoclonal antibodies, such as the anti-CD20 antibody rituximab, approved by the US Food and Drug Administration (FDA) in 1997 for B-cell malignancies; and the anti-HER2 (human epidermal growth factor receptor 2) antibody trastuzumab, approved in 1998 for HER2+ breast cancer. Another class of targeted therapeutics is small molecule drugs, including a large family of tyrosine kinase inhibitors (TKI’s), such as imatinib, approved by the FDA in 2001 for chronic myelogenous leukemia (CML); and gefitinib, approved in 2003 for non-small cell lung cancer (NSCLC). Recent advances in targeted chemotherapies include the rapid development of drugs to target epigenetic regulators (eg. JQ-1 (12, 13)) and hypoxia (eg. TH-302 (14, 15)).

Targeted therapeutics have dramatically improved the survival rate of some cancers. For example, the 5-year survival rate for CML was 31% in the early 1990s, and was improved to 63% by 2011, due in part to the use of imatinib and related agents (16). Unfortunately, despite the best of intentions, it is now clear that targeted agents are generally more toxic than the older chemotherapeutics (17). Inhibition of EGFR (gefitinib and related agents) causes Grade 3/4 – severe to life-threatening – adverse events in 40% of NSCLC patients (18), and cardiotoxicity is a huge problem in patients treated with TKI’s (19). In addition to questions about their safety, the clinical utility of targeted agents is further challenged by their small margin of benefit and high cost. As many tumours quickly gain resistance against targeted therapeutics, many drugs are only able to extend survival by a few days to weeks, with significant declines in quality of life (QOL) (20). For example, the addition of bevacizumab to carboplatin and paclitaxel, now the standard of care for metastatic NSCLC, extends progression-free survival (PFS) by 12-18 days, depending on the bevacizumab dose. Bevacizumab costs US$90,000 for one cycle. In pancreatic cancer patients, addition of erlotinib to gemcitabine extends median overall survival (OS) by 10 days,
and costs US$16,000 (20). Clearly, development of safe, effective, and inexpensive drugs for the treatment of cancer remains a gap that urgently needs to be filled.

*Immunotherapy*. Immunotherapies manipulate the interaction between the body’s immune system and the tumour. Recently there has been unprecedented interest in this field, owing in part to remarkable successes seen with the use of an anti-CTLA-4 (cytotoxic T lymphocyte-associated protein 4) antibody (ipilimumab) against melanoma (21), and anti-PD-1 (programmed cell-death protein 1) antibodies (nivolumab, pembrolizumab) for melanoma (22, 23) and NSCLC (24). Anti-PD-1 and anti-CTLA-4 immunotherapies belong to a class of immunotherapy strategies called checkpoint blockade, where the mechanism(s) by which tumours suppress T cell activation – immune-checkpoint signaling – is inhibited (25). T cell function is re-activated as a result, and can eliminate malignant cells through immune surveillance (25). Although it is currently associated with severe immune-related adverse events (26) and staggering cost (27), immunotherapy holds the unique possibility of generating immunological memory that will be able to prevent the rise of therapy-resistant malignant clones, thereby preventing disease recurrence (28-31). Other modalities of immunotherapy, including adoptive cellular therapy, chimeric antigen receptor (CAR) T-cell therapy, and oncolytic viruses, are under intensive clinical investigation at present (25, 32).

1.1.1 Treatment of breast cancer

**By stage and grade.** Current prognosis and treatment of breast cancer depends on a variety of clinical and pathological factors, including patient age, tumour stage and grade, and receptor status (33). Tumour stage considers the size, axillary lymph node involvement, and metastatic status of breast cancer, combined to give a score between 0 and 4 (Stage 0 to IV) (16). Stage 0 breast cancers are non-invasive ductal carcinoma in situ (DCIS) or lobular carcinoma in situ
(LCIS), and are curable with surgery and/or radiation. Stage I-II are “early-stage”, and Stage III is “locally advanced”, breast cancers with increasing size and lymph node involvement. Stage I-III breast cancers are generally treatable with a combination of surgery, radiotherapy, and chemotherapy. Early-stage breast cancers have a 5-year survival rate of 85-99% (16). Stage IV breast cancer are metastatic breast cancer, with secondary sites often in the lung, liver, brain and bones. Metastatic breast cancer has a 5-year survival rate of 25% (16). While less than 10% of patients are diagnosed with metastatic breast cancer, 20-30% of early-stage breast cancer patients go on to develop metastatic breast cancer, despite aggressive treatment (34).

Breast cancer grade (Grade I-III, or low-intermediate-high) is determined histologically, by scoring tumour cell differentiation (33). Grading determines how fast the cancer cells are likely to grow and spread, and helps determine the aggressiveness of therapy (35).

**By subtype.** In clinical practice, three biomarkers are routinely used to assess breast cancers: ERα (estrogen receptor α), PR (progesterone receptor), and HER2 (16, 36). This gives rise to three major subtypes of breast cancers: ERα/PR positive (+) tumours have a more favourable prognosis, and respond well to hormone therapy (eg. tamoxifen, aromatase inhibitors); HER2+ tumours are more aggressive and more likely to metastasize and reoccur, but respond well to anti-HER2 therapies (eg. trastuzumab, lapatinib); and triple-negative breast cancers (TNBC) follow the most aggressive clinical course with early recurrence, and have limited therapeutic options other than surgery, radiotherapy, and cytotoxic chemotherapy (eg. CAF) (16, 36). Virtually all TNBC relapse within 5 years of diagnosis, after which the median survival is only 6 months (37).

Breast cancer can also be classified by molecular profiling, historically through gene expression profiling and more recently by exome sequencing, DNA copy number, DNA methylation, and
many others (33, 38). These methods can subtype breast cancers into 20 or more subgroups, largely falling into luminal A/B, HER2+, claudin-low, normal-like, and basal-like categories (33). A number of prognostic tests based on molecular profiling have been developed and incorporated in patient management, such as OncotypeDX (Genomic Health), PAM50 (Nanostring Technologies), and MammaPrint (Agendia) (38). Molecular profiling has also been successful in identifying novel treatment options for breast cancer, such as the use of poly(ADP-ribose) polymerase (PARP) inhibitors (e.g., iniparib, olaparib) to treat breast cancers with mutations in BRCA1/2 (39).

**Points of therapeutic intervention.** Surgery is the most common breast cancer treatment given with curative intent, which involves breast conservation surgery or mastectomy, and removal of axillary lymph nodes if needed (40). Even in patients with evidence of metastases, surgery of either the primary tumour or at the metastatic site is still performed for palliative purposes (40). Neo-adjuvant therapy (NAT), consisting of radiotherapy and/or chemotherapy, is given in the space between diagnosis and surgery, usually to locally advanced breast cancer patients, with the goal of reducing tumour size to facilitate surgery (41). NAT is also used in the attempt to (i) convert a previously inoperable tumour into an operable one; (ii) downstage a tumour to allow breast conservation surgery; and (iii) provide prognostic information and guide selection of appropriate follow-up therapies (41). As evidence accumulates that response to NAT can be correlated to long-term outcome (41, 42), there is increased interest to conduct clinical trials in the neoadjuvant setting (often called “window of opportunity” trials), as they require smaller sample sizes, are less expensive, and provide rapid assessment of efficacy (43). Importantly, most pre-clinical drug assessment techniques using 2D cell culture, as well as tumour xenografts, represent pre-clinical models of NAT.
Adjuvant therapies of either systemic or local-regional radiotherapy, chemotherapy (including targeted therapies against ERα or HER2), or both, is given after surgery. The duration of adjuvant therapies varies from a few weeks (chemo and/or radiotherapy) (44), to up to 1 year (anti-HER2 therapy), to 5-10 years (anti-ERα therapy) (36). The goal of adjuvant therapies is to decrease the risk of breast cancer recurrence and prevent metastasis, but unfortunately 20-30% of tumours still recur (34).

In the 20-30% of early-stage patients that experience recurrence, treatment options are limited. For local-regional recurrence, surgical removal of the recurrent tumour followed by radiotherapy, if radiotherapy has not previously been given, is recommended (34, 35). For metastatic recurrence, patients may be encouraged to enroll in clinical trials for novel therapeutics (34). As such, treatment of metastasis is the therapeutic setting that most oncology drugs are clinically tested in. As metastatic recurrences are highly aggressive and likely resistant to all current therapies, this may be contributing to the high failure rate of oncology drugs during clinical testing, discussed below.

1.2 Drug repurposing

As of 2011, the success rate of investigational drugs from clinical development to approval by the FDA is the lowest (6.7%) for oncology drugs, compared to drugs for all other indications (12.1%) (45). This discrepancy alone reduces the probability of FDA approval for all investigational drugs from nearly one in eight to over one in ten (45). This high failure rate of investigational cancer drugs is alarming, considering the high cost of clinical trials, the long timeline for drug development (46), as well as the unmet needs from cancer patients, clinicians, and policy makers. In light of these challenges, repurposing drugs that are already approved for non-oncology use is an alternative strategy to bring more treatment options to cancer patients.
Drug repurposing has the potential to reduce the time, cost, and risk investments in phase I/II clinical trials, since the safety, dosage range, formulation, and side effects are already documented and approved. Additionally, many older drugs are now off-patent and manufactured as inexpensive generics. Drug repurposing is therefore attractive and timely, as the national healthcare budgets world-wide are unlikely to continue to support the exponential increase in the cost of new oncology drugs (20, 47).

1.2.1 Opportunities

Clinical and epidemiological observations. Association between use of a drug and an unexpected effect or outcome is primarily reported as clinical or epidemiological observations. A well-known example of clinical observations leading to drug repurposing is the case of sildenafil, originally developed to treat hypertension and angina (48), now widely marketed as Viagra due to patient reports of erection as an unexpected effect (49). Another example is thalidomide, originally developed and marketed as a sedative (50), then failed as a remedy for nausea in pregnant women due to severe fetal toxicity leading to birth defects (51, 52). Observations by clinicians that thalidomide relieved leprosy symptoms prompted a repurposing investigation (53), leading to its approval for the treatment of leprosy complications in 1998 (50).

The stories of sildenafil and thalidomide do not end here. Sildenafil targets PDE5 (phosphodiesterase type 5) (48), and the observation that PDE5 is upregulated in pulmonary hypertensive lungs (54) prompted its current development for this indication. Thalidomide was found to have anti-angiogenic and anti-cancer activities (55, 56), leading to its approval for the treatment of multiple myeloma (MM) in 2006 (50, 57). These successes in drug repurposing were made possible by scientific progress in the understanding of disease biology and
vulnerabilities, highlighting the importance of basic and translational research to confirm and follow-up on opportunities of drug repurposing identified by clinical observations.

Epidemiological studies and post hoc analyses are additional avenues commonly used to examine the association between use of an already-approved drug and a specific outcome, usually from non-primary indications. Although rife with statistical bias and interpretation errors (58, 59), epidemiological studies are nevertheless useful as a first-pass tool to identify drugs for repurposing (47). The power of epidemiological studies lies in the size of the population examined, which is particularly important for cases where effect sizes are small, or when the rate and magnitude of response are highly variable. The disadvantage is that, due to the need for a large sample size, conclusions from epidemiological studies are only meaningful when examining widely-prescribed drugs and prevalent outcomes (eg. risk of common cancers, as opposed to rare diseases). Some examples of epidemiologically reported association between drug use and response in oncology include metformin (60), currently used to treat diabetes; aspirin (61, 62), used as a painkiller and for prevention of stroke and coronary events; and statins (63, 64), used to treat hypercholesterolemia and to prevent cardiovascular diseases (CVD). As indicated above, basic and translational research is needed prior to the successful repurposing of these drugs as oncology drugs. Pre-clinical studies of the mechanism and efficacy of fluvastatin, a member of the statin family of cholesterol-lowering drugs, for its application as an anti-breast cancer therapeutic, is the focus of this thesis.

**Drug screening and data mining.** Identification of anti-cancer activities in existing drugs can occur through high-throughput screening (HTS), which has been used for new drug discovery for decades (65), and are now being increasingly applied for drug repurposing (66-70). Libraries of approved small molecules ranging from ~100 drugs (69, 70) to >2,500 clinically approved and
bioactive compounds (66) have been assembled for this purpose. In light of growing evidence that multiple machineries are simultaneously derailed in most cancer cells (9), a particularly attractive application of the HTS method to identify anti-cancer drugs is the possibility to test for synergistic drug combinations. For economic reasons, development of novel oncology drugs focuses primarily on showing efficacy and/or superiority of the novel drugs when administered alone. In contrast, synergistic pairs of FDA-approved drugs have been successfully identified through chemical screening and validated in pre-clinical models, including chlorpromazine + pentamidine against lung cancer (71), and atorvastatin + dipyridamole against multiple myeloma (MM) and acute myeloid leukemia (AML) (72).

Another emerging method to identify oncological targets and drug candidates is through in silico data mining (73), made possible by the public establishment and maintenance of large databases of genomics (eg. EntrezGene), cancer genomics (eg. The Cancer Genome Atlas; TCGA), proteomics (eg. UniProt), pharmaceuticals (eg. PubChem), cancer cell lines (eg. Cancer Cell Line Encyclopedia; CCLE), and drug perturbations (eg. Connectivity Map; CMAP) (74). Major strategies for the selection of drugs for repurposing include searching for similarities based on gene expression profiles (75, 76), side-effect profiles (77, 78), and correlation between drug targets and patient survival (79). Although these computational approaches to assess the therapeutic potential of a known drug for repurposing is still highly experimental (80, 81), successes have been made such as the identification and pre-clinical validation of imipramine and clomipramine against small cell lung cancer (SCLC) (75), and pentamidine against renal cell carcinoma (RCC) (76).
1.2.2 Challenges

**Targeted vs. non-targeted repurposing.** The successful repurposing of sildenafil is partially attributable to its well-characterized mechanism of action as a PDE5 inhibitor, and the developing scientific understanding between PDE5 activity and penile erection. Similarly, all-*trans*-retinoic acid (ATRA) was originally marketed as a topical acne treatment (82). Later it was found that acute promyelocytic leukemia (APL) is driven by a fusion protein RARA-PML (RARA, retinoic acid receptor α; PML, promyelocytic leukemia gene) (83), rendering the leukemic promyelocytes sensitive to ATRA-induced differentiation and subsequent apoptosis. ATRA is currently the standard of care for APL patients, with concurrent use of arsenic trioxide (84) or anthracyclines (85). Such are cases of “targeted” repurposing, and is well-represented in oncology as repurposing between cancer types. The best example is imatinib, a specific inhibitor of tyrosine kinases ABL (Abelson murine leukemia viral oncogene homolog), c-Kit, and PDGF-R (platelet-derived growth factor receptor). Imatinib was first approved in 2001 for Philadelphia chromosome positive (Ph+) CML, harbouring the BCR-ABL fusion protein (BCR; breakpoint cluster region) (86). Since it is known to also inhibit c-Kit, imatinib was immediately tested for the treatment of gastrointestinal stromal tumours (GIST), which express c-Kit in up to 95% of cases examined, and was approved by 2002 (87). Today, imatinib is also FDA-approved to treat Ph+ ALL (acute lymphoblastic leukemia); MPD (myeloproliferative diseases); ASM (aggressive systemic mastocytosis); HES (hypereosinophilic syndrome); CEL (chronic eosinophilic leukemia); and DFSP (dermatofibrosarcoma protuberans) (86), all via its on-target effects as a selective tyrosine kinase inhibitor.

In contrast to the above examples, some drugs have been observed to exert potential anti-cancer effects, but the mechanism of action is either unclear, or independent from known target(s). For example, the anti-diabetic drug metformin has been reported to reduce cancer mortality by up to
34% (88, 89), with validation in pre-clinical models of breast cancer (90, 91) and response in clinical trials (92-94). However, the molecular target of metformin leading to cancer cell death remains elusive, and it is unclear whether metformin directly induces cancer cell death, or indirectly through its systemic effects on blood sugar levels and insulin sensitivity (95).

Similarly, ample evidence exists to support the use of aspirin in post-surgical colorectal cancer (CRC), including positive results from randomized trials (96-98), without fully elucidating the molecular target. Recently, however, several studies have implicated modulation of cyclooxygenase-2 (COX-2; officially known as prostaglandin-endoperoxide synthase 2, PTGS2) as the underlying mechanism of aspirin-induced CRC cell death (99-101). If validated, this would move aspirin into the “targeted” category, and aspirin treatment may be stratified based on COX-2 activity (47).

Patient selection and the search for biomarkers. Whereas the attrition rate of oncological drugs as a whole is estimated to be 82-95% (102, 103), for a subset of these drugs – kinase inhibitors – the attrition rate is reduced to an astounding 53% (102). This is largely attributable to a careful selection of patients with an appropriate molecular drug target (104). In other words, knowledge of the molecular target and mechanism of action for a given drug is critical for its successful development. In drug repurposing, this is exemplified by the quick acceptance of ATRA by the medical community to treat APL, once it became clear that APL is driven by the fusion protein RARA-PML, which can be targeted by ATRA (83).

In the case of thalidomide, its clinical development for MM between 1990s to 2000s (50, 57) is paralleled by intense pre-clinical research to identify its molecular target, cumulating in the identification of the Cereblon (CRBN) E3 ubiquitin ligase complex as its target for both
teratogenicity and immunomodulation in 2010-2012 (105, 106). CRBN was immediately developed as a predictive biomarker for thalidomide and thalidomide-analogues (107).

A drug is less likely to be accepted for repurposing without a definitive molecular target to aid patient selection (although it is not impossible, as seen with thalidomide). Aspirin has been reported to reduce CRC recurrence by 6.7-10% in multiple double-blinded, randomized, placebo-controlled phase II/III clinical trials (96-98), and yet is still not recommended by most clinical guidelines, including the most influential US National Comprehensive Cancer Network (NCCN) and European Society for Medical Oncology (ESMO). For the repurposing of statins, currently prescribed to patients with high cholesterol, the question of patient selection becomes even more challenging, with (i) conflicting data on the association between cholesterol levels and cancer risk (eg. positive association with breast cancer (108); negative association with breast cancer (109)), (ii) conflicting data on cholesterol levels after treatment with standard of care (eg. positive association with aromatase inhibitors (110, 111); negative association with tamoxifen (112)), and (iii) pre-clinical indication that the anti-cancer effects of statins are independent from its cholesterol-lowering effects altogether (113, 114). As a result, after two decades of pre-clinical research, statins have yet to reach the clinic as an oncology drug.

Understanding the target and mechanism of a drug for repurposing is also important for the search for molecular biomarkers of response. Currently, FDA-approval of oncology drugs relies on direct measurements of clinical benefit, such as survival (OS, overall survival; DFS, disease-free survival), quality of life (QOL; PRO, patient-reported outcomes), and safety (B/R, benefit/risk assessments) (115, 116). Endpoints often used in oncology trials, defined by Response Evaluation Criteria in Solid Tumors (RECIST), are also objective measures of tumour response (progressive disease, stable disease, partial response, and complete response) (115,
In contrast, molecular biomarkers of response are routinely used in the evaluation of drugs for other diseases, such as cholesterol and triglyceride levels for atherosclerotic disease, and CD4 (cluster of differentiation 4) T lymphocyte count for HIV (human immunodeficiency virus) infection (116). Drug trials and drug repurposing trials could be faster, easier, and cheaper to conduct if a molecular biomarker of response is incorporated as a surrogate endpoint (116).

Partially contributing to this challenge is the criticism that biomarkers of response in oncology have not been reliable to date. For example, the utility of PSA (prostate-specific antigen) to monitor response in prostate cancer (117), CEA (carcinoembryonic antigen) for colon cancer (118), and CA-125 (cancer antigen 125) for ovarian cancer (119), are all being debated. It is therefore recommended that clinical trials simultaneously assess both the direct and the surrogate endpoints (116). Thus the efficacy, the mechanism of action, and biomarker(s) of response all need to be pre-clinically determined, for the successful development or repurposing of a drug.

**Dosing.** Particularly relevant to the issue of drug repurposing is that it is often unknown whether the dose recommended for other indications is sufficient to achieve anti-cancer activity. For example, pre-clinical experiments with statins often require higher doses than the recommended cholesterol-lowering dose (72, 120), but lower than the maximum tolerated dose (MTD) in cancer patients (121, 122). The anti-cancer effects of metformin are often examined at millimolar (mM) concentrations in cell culture, which is >100 fold higher than the achievable serum concentration in patients taking diabetic doses of metformin (123). Additionally, high variability in intra-individual pharmacokinetics are commonly observed for orally-administered drugs (124), highlighting an additional aspect of personalized medicine that requires optimization.

Identification of the optimal dose is further challenged by the growing realization that (i) the optimal biological dose (OBD) of a non-cytotoxic drug is not always associated with the MTD
(124), (ii) the schedule of drug delivery may influence its cellular target (125-127), and (iii) novel dose-escalation strategies with optimized scheduling may be needed for effective execution of drug combinations (128, 129). Together, these factors mean that Phase I dose-finding/schedule-finding trials, and the associated safety assessments, are required in the (new) target population for repurposed drugs.

**Financial difficulties.** Without financial incentives, the task of repurposing a non-oncology drug depends on combined efforts from the scientific community and society. Mobilization of not-for-profit foundations, health insurance companies, and governments are needed to fund pre-clinical studies and clinical trials (91, 130). Additional responsibilities may need to be taken on by project initiators (often academics), from dedicated project management to realistic budgeting, efficient and transparent trial conduct, and targeted communication to interest groups and stakeholders. Influential clinical guideline authorities and regulating bodies should be consulted from the beginning.

1.3 Repurposing statins as anti-breast cancer agents

1.3.1 Statins as cholesterol-lowering agents

**Pharmacology of statins.** High levels of cholesterol in the blood, specifically cholesterol that is carried in low density lipoprotein (LDL) particles, is a major risk factor for cardiovascular diseases (CVD) (131). The statins family of drugs are profoundly successful in lowering cholesterol and reducing CVD (132-134), reducing all-cause mortality by 30% and coronary deaths by 42% in the landmark “4S study” (Scandinavian Simvastatin Survival Study) (132) while having no effect on non-cardiovascular deaths (133). Since the discovery of the first statin, mevastatin, in 1976 (135) and the first FDA-approval of a statin (lovastatin, also known as mevinolin) in 1987 (136), statins have become one of the most successful pharmaceuticals to
date. As of 2012, statins are prescribed to approximately 23% of US adults over 45 years of age (137), and approximately 18% of Canadians at high to intermediate risk of CVD (138).

There are seven approved statins in North America, with differing pharmacological characteristics (139, 140) (Fig 1-1). In general, the lipophilic statins are more likely to be detected in extra-hepatic tissues, while hydrophilic statins are only consistently detected in the liver (141). Although simvastatin is the most frequently used statin for cardiovascular indications, its peak plasma concentration is one of the lowest, being 13-fold lower than fluvastatin (Fig 1-1). While most statins are metabolized by the liver CYP3A4 system commonly used to process many other drugs, fluvastatin, pitavastatin, and rosuvastatin are metabolized by alternative enzymes (139, 140), decreasing the chance of adverse pharmacokinetic interactions between these statins and other co-administered therapeutics. Lovastatin and simvastatin are lactone pro-drugs that require acidic activation, and both have bioactivities independent from inhibition of HMGCR, interacting directly with P-glycoprotein (P-gp) (142) and the proteasome (143-145) (Fig 1-1).

**Mechanism of action.** Statins competitively inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), the rate-limiting enzyme of the MVA pathway, responsible for the biosynthesis of cholesterol as well as isoprenoid end products (135, 146) (reviewed below). The liver is the major site of cholesterol production in the human body (147). In liver cells, cellular cholesterol can either be synthesized via the MVA pathway, or be absorbed from the blood plasma through receptor-mediated uptake of LDL-cholesterol (148). Inhibition of HMGCR by statins lower the cholesterol biosynthetic activity in the cell, and as a response cells upregulate the LDL receptor (LDLR), leading to increased uptake of LDL-cholesterol from the blood plasma (147). As a result, plasma LDL levels are decreased, and risk of cardiovascular events is
Figure 1-1. Pharmacology of statins.

Pharmacodynamic/pharmacokinetic characteristics (top) and chemical structures (bottom) of seven members of the statin family of drugs that are available in North America.

<table>
<thead>
<tr>
<th>Statin</th>
<th>Trade Name</th>
<th>Solubility</th>
<th>Rate of use (%)</th>
<th>Peak plasma concentration (ng/ml)</th>
<th>Primary liver metabolism</th>
<th>HMGCR-independent effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluvastatin</td>
<td>Lescol</td>
<td>Lipophilic</td>
<td>&lt;20</td>
<td>448</td>
<td>CYP2C9</td>
<td>No</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>Lipitor</td>
<td>Lipophilic</td>
<td>20.2</td>
<td>66</td>
<td>CYP3A9</td>
<td>No</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>Livalo</td>
<td>Lipophilic</td>
<td>&lt;20</td>
<td>41</td>
<td>UGT1A3</td>
<td>No</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>Zocor</td>
<td>Lipophilic</td>
<td>42.0</td>
<td>34</td>
<td>CYP3A4</td>
<td>Yes</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>Mevacor</td>
<td>Lipophilic</td>
<td>7.4</td>
<td>20</td>
<td>CYP3A4</td>
<td>Yes</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>Pravachol</td>
<td>Lipophilic</td>
<td>11.2</td>
<td>55</td>
<td>CYP3A4</td>
<td>No</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>Crestor</td>
<td>Lipophilic</td>
<td>8.2</td>
<td>37</td>
<td>CYP2C9</td>
<td>No</td>
</tr>
</tbody>
</table>

Adapted from references (139, 140)
Molecularly, this mechanism of action exploits the robust negative feedback regulation of MVA pathway genes, mediated transcriptionally by the SREBP (sterol regulatory element-binding protein) family of transcription factors (148). Of the three SREBP family members (SREBP1a, 1c, and 2), their strength of MVA pathway regulation is ranked as follows: SREBP2 >> SREBP1a > SREBP1c (149–151). SREBP activity is controlled by the cholesterol sensor SCAP (SREBP cleavage-activating protein) (152). When cells have adequate supply of cholesterol, SCAP retains SREBP in the endoplasmic reticulum (ER) in an inactive state, through a three-way interaction with the ER-resident protein INSIG (insulin-induced gene). When cholesterol levels in the ER are low, such as when HMGCR is inhibited by statins, SCAP dissociates from INSIG and escorts SREBP into the Golgi, where SREBP is activated by sequential cleavage by Golgi-resident proteases S1P (site-1 protease) and S2P (site-2 protease) (151). Cleavage-activated SREBP then translocates into the nucleus to upregulate the transcription of MVA pathway genes, as well as LDLR, thus replenishing intracellular cholesterol stores (147, 148). Although the main site of statin action as a cholesterol-lowering agent is in the liver, this pathway and its extraordinary feedback mechanism can be observed in liver cells as well as all normal cells of the body (153, 154).

1.3.2 Evidence of anti-breast cancer effects

*Epidemiological studies.* As a part of the Women’s Health Initiative, Cauley *et al.* (155) examined breast cancer incidence in 150,000 post-menopausal women, and reported that use of lipophilic statins is associated with an 18% reduction of breast cancer incidence. Nielson *et al.* (64) assessed the mortality of the entire Danish population from 1995 to 2007 (n=300,000), and reported statin use to be associated with a 15% reduction in death from all cancers, and 13%
reduction in death from breast cancer. Additional epidemiological studies with smaller population cohorts (n=2,000-3,000) similarly reported reduced cancer incidence or death in statin users, with effect sizes as high as 55% (63, 156-158). Particularly interesting is the observation by Kumar et al. (156) that use of lipophilic statins for >1 year (yr) have a 37% reduction in ERα/PR-negative tumour incidence, compared to never- or <1 yr-users. Consistently, breast cancer cases in the >1 yr-user cohort are significantly more likely to be low grade and stage (156).

Not all studies agree on the association of statin use and reduced breast cancer risk and/or death described above (159-163). In a meta-analysis of 42 studies, Kuoppala et al. (164) reported that statin use is associated with reduced risk of stomach cancer, liver cancer, and lymphoma, but not of breast cancer. It should be noted that many of these studies do not report covariates and/or confounding factors, such as physical activity and diet, duration of follow-up times, and duration of statin use.

In addition to the mixed results described above, several epidemiological studies have examined the effect of statin use on breast cancer recurrence, after front-line treatment. Ahern et al. (165) reported with a cohort of 18,000 stage I-III breast cancer patients that any-time statin use is associated with 10% reduction in risk of recurrence. Studies with smaller cohorts (n=700-4,000) also report reductions in recurrence, with effect sizes as high as 60% (166-168). Furthermore, increasing duration of statin use after diagnosis is associated with decreasing risk of recurrence (168). These epidemiology data suggest that statins may have different clinical utility depending on the breast cancer subtype, and the points of therapeutic intervention.

**Pre-clinical studies.** Pre-clinical evaluations of statins in breast cancer cell lines or tumour xenografts have demonstrated that some breast cancer cells are highly sensitive to statin-induced
apoptosis, while others are resistant (169-171). This indicates that repurposing statins into the breast cancer is dependent on identification of predictive markers of sensitivity, in order to inform clinical trial designs, targeted patient population, and co-development of diagnostics for assessment of therapeutic response. To this end, our group has previously assessed the statin sensitivity of a panel of 25 breast cancer cell lines, the largest to date, and reported an association of statin sensitivity with three factors (169): (i) relatively lower expression of HMGCR; (ii) and ERα-negative, basal-like tumour subtype; and (iii) a 10-gene candidate signature. Other studies, using much smaller panels of breast cancer cell lines, have also reported statin sensitivity to be associated with ERα negativity (170, 171), activation of NFκB (170), activation of RAS family members (170, 172-174), and specific p53 mutations (175, 176).

Additional pre-clinical support for the potential use of statins in breast cancer include evidence where ectopic introduction of the catalytic domain of HMGCR (177) or exogenous addition of MVA (178) is sufficient to promote breast tumour xenograft growth. These data, combined with the observation that high mRNA expression of HMGCR is correlated with poor prognosis of breast cancer patients (176, 177), suggest that HMGCR is a metabolic oncogene capable of driving breast cancer cell transformation. Therefore, inhibition of HMGCR by statins may have therapeutic activity.

**Clinical trials.** The clinical efficacy of various statins, at cholesterol-lowering doses or higher doses, administered with or without combination therapeutics, have been tested in a variety of solid and liquid cancer types (for examples see review (179)). In breast cancer, however, clinical evaluations of statins have thus far been restricted to small-cohort (n=40-50) window-of-opportunity trials (180-182). In the two trials published to date, both reported that some patients responded to statin treatment, while others did not, which mirrors results from pre-clinical
models of breast cancer (169). Efforts were therefore concentrated in identifying biomarkers of statin sensitivity.

Garwood et al. (180) treated 40 patients with a diagnosis of DCIS or stage I breast cancer with cholesterol-lowering doses of fluvastatin (20-80 mg/d) for 3-6 weeks, and reported that high grade tumours were more likely to respond. Bjarnadottir et al. (181) treated 50 patients with primary invasive breast cancer with a cholesterol-lowering dose of atorvastatin (80 mg/d) for 2 weeks, and reported that tumours with higher expression of HMGCR prior to treatment, assessed at the protein level by immunohistochemical (IHC) analysis of formalin-fixed and paraffin-embedded (FFPE) tissue samples, were more likely to respond. A companion study (182) performed mRNA analysis using materials harvested from Bjarnadottir et al. (181), and reported that tumours with lower mRNA expression of CCND1 (cyclin D1) prior to treatment were more likely to respond, although this result does not reach statistical significance. A later follow-up publication (183) then reported that tumours with higher mRNA expression of HMGCR prior to treatment were less likely to respond to statin treatment, which directly contradicts results from the original study published by Bjarnadottir et al. (181). Thus the search for biomarkers of statin sensitivity continues.

1.4 The mevalonate pathway in cancer metabolism

Cancer cells reprogram their metabolism to ensure survival and sustain abnormal growth (184). The mevalonate (MVA) pathway is an anabolic pathway fueled by acetyl-CoA to produce both steroid and isoprenoid end products critical for proliferation and cell signaling (151) (Fig 1-2). Inhibition of MVA production by the statin family of drugs potently inhibits cancer cell growth in many cancer types, both in tissue culture (113, 185-187) and in tumour xenografts (72, 120, 170, 188), indicating that consumption of MVA pathway end products from the blood plasma is
insufficient to sustain cancer cell growth, and MVA pathway activity is indispensable. In particular, cell cycle progression at G1/S is dependent on production of isoprenoids by the MVA pathway (153, 189), while G2/M progression requires adequate supplies of cholesterol (190, 191). Recently it has also been shown that, in a p53-null setting, the conversion of MVA to isopentenyl diphosphate (IPP) is essential for maintaining dNTP levels and S phase progression, although the exact mechanism of this is currently unknown (192).

Evidence indicates that increased flux through the MVA pathway, experimentally induced either by ectopic expression of the catalytic domain of HMGCR (177), or by exogenous supplementation of MVA (178), can directly promote tumourigenesis. In primary patient samples, high expression of MVA pathway genes are correlated with poor prognosis in breast cancer (177), corroborating these experimental findings. In a cancer cell, the MVA pathway may be deregulated at multiple levels and through multiple mechanisms (152), although it is currently unknown whether this deregulation can act as an independent oncogenic signal, or simply creates a dependency in a fast-growing cell (reviewed below). In either case, tumours become uniquely vulnerable to the depletion of MVA pathway end products, which can be targeted for therapy.

1.4.1 End products

Cholesterol. The best-known end product of the MVA pathway is cholesterol (Fig 1-2), which is required for all animal life to build and maintain membranes. In addition to its requirement for mitosis, cancer cells often have an increased demand for cholesterol to maintain elevate levels of lipid rafts and caveolae, important for protein membrane trafficking and signal transduction (193-195). These are sometimes exemplified in cancer patients that present with extremely low levels of serum cholesterol (196), likely due to high LDLR activity in the tumour (197).

Cholesterol also serves as a precursor for the biosynthesis of steroid hormones in the estrogen,
Enzymes of the mevalonate (MVA) pathway are shown in blue. Chemical structures of fluvastatin and biologically important end products of the MVA pathway are shown. Red box represents the chemical group of statins that inhibit HMGCR. ACAT2, acetyl-CoA acetyltransferase; HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; MVK, mevalonate kinase; PMVK, phosphomevalonate kinase; MVD, mevalonate diphosphate decarboxylase; FDPS, farnesyl diphosphate synthase; GGPS1, geranylgeranyl diphosphate synthase 1; DHDDS, dehydrodolichyl diphosphate synthase; NUS1, dehydrodolichyl diphosphate synthase regulatory subunit; DOLPP1, dolichyl diphosphate phosphatase 1; SRD5A3, steroid 5-alpha-reductase 3; DOLK, dolichol kinase; PP, diphosphate.

Figure 1-2. The mevalonate pathway.
progestogen, and androgen families, which are important for the transformation and therapeutic resistance in some subtypes of breast and prostate cancers (198, 199).

**Isopentenyl diphosphate.** The MVA pathway is the sole *de novo* synthetic pathway for isoprenoid end products in all mammalian cells (200) (Fig 1-2). Isopentenyl diphosphate (IPP) is the smallest isoprenoid metabolite, and is used to modify tRNAs on adenosine-37 (A37) located in the anti-codon loop (201). The function of tRNA isopentenylation in human cells, both in normal and in cancerous cells, is an understudied area. One study (202) has reported that overexpression of the enzyme TRIT1, which catalyzes the isopentenylation of tRNAs, has tumour-suppressing activities in lung cancer cell lines. However, the yeast homologue of TRIT1 has been reported to have distinct cytoplasmic (ie. tRNA-isopentenylating) and nuclear functions (203). It is currently unknown whether the apparent tumour suppressing activities of TRIT1 in lung cancer are due to its tRNA isopentenylation activity, or its nuclear function, if it is indeed located in the nucleus.

**Farnesyl diphosphate and geranylgeranyl diphosphate.** Sequential polymerization of IPP produces isoprenoid metabolites farnesyl diphosphate (FPP), composed of 3 isopentenyl moieties, and geranylgeranyl diphosphate (GGPP), composed of 4 isopentenyl moieties (151) (Fig 1-2). FPP and GGPP are essential substrates for protein geranylgeranylation and farnesylation, respectively, together referred to as protein prenylation (204-206). These processes add a hydrophobic farnesyl or geranylgeranyl moiety to proteins containing a C-terminal CaaX domain, thus localizing them to cellular membranes (207, 208). A large body of work has implicated GGPP, and to a lesser extent FPP, as the limiting metabolic end products of the MVA pathway in cancer cells (209-214). However, despite clear evidence of reduced protein
prenylation when the MVA pathway is inhibited, the functional role of these small GTPases in conferring statin sensitivity have been conflicting (reviewed below).

**Heme A and coenzyme Q.** Two components of the electron transport chain (ETC) require isoprenoid metabolites produced by the MVA pathway. Heme A is an iron-chelating compound that participates in the reduction of dioxygen to water by cytochrome c oxidase during aerobic respiration (215). Heme A contains a hydroethylfarnesyl group derived from FPP (216) (Fig 1-2), which is functionally important for the conservation of the energy of oxygen reduction (217). Larger isoprenoids containing 6-10 IPP subunits are also used in the production of coenzyme Q (CoQ) (Fig 1-2). The hydrophobic isoprenoid chain localizes CoQ to the inner membrane of the mitochondria, where the quinone group transfers electrons from complex I or II to complex III of the electron transport chain, thus enabling ATP (adenosine triphosphate) production (218). Synthesis of isoprenoid end products via the MVA pathway is therefore critical for ATP production during oxidative phosphorylation.

**Dolichol.** Dolichol is a large isoprenoid end product of the MVA pathway, derived from 19-21 IPP molecules (Fig 1-2). Dolichol resides in the endoplasmic reticulum (ER) as the carrier of N-glycans such as N-acetylglucosamine (GlcNAc), mannose (Man), glucose (Glc), as well as the precursor glycan (GlcNAc2-Man9-Glc3) prior to its *en bloc* transfer onto nascent polypeptides (219, 220). The cellular demand for dolichol synthesis is large and continuous, as the synthesis of each precursor glycan to be transferred to nascent peptides requires 8 dolichol molecules (221). Recycling of dolichol is poorly characterized (222) and thought to be inefficient, as it accumulates with aging (223). Furthermore, *N*-glycosylation occurs on an estimated 50-70% of all proteins (224), many of which contain multiple sites for *N*-glycosylation (although not all
may be occupied) (225). Protein N-glycosylation is frequently altered in cancer and plays central roles in tumour formation, proliferation and metastasis (226).

### 1.4.2 Deregulation

**LDL-cholesterol uptake.** Deregulation of the MVA pathway can occur at multiple levels.

Atypical plasma lipid profiles are commonly observed in all cancer types, usually with lower levels of serum cholesterol and elevated levels of triglycerides (227). The lowered cholesterol levels are thought to be mediated by a 2- to 11-fold increased expression and activity of LDLR in malignant cells (Fig 1-3A), leading to removal of LDL-cholesterol from the serum (228, 229). Cholesterol is then used for rapid proliferation (230) or to populate elevated levels of cholesterol-rich lipid rafts (231).

**Genomic alterations and protein expression.** Analysis of large cancer genomics datasets using cBioPortal (232, 233) shows that MVA pathway genes are frequently amplified (eg. HMGCS1, GGPS1) or mutated (eg. SREBP2) in a variety of cancer types. These genomic alterations (Fig 1-3B), combined with multiple lines of evidence indicating that cancer cells upregulate the MVA pathway enzymes (Fig 1-3F) for acquisition of MVA pathway end products (113, 178, 234, 235), indicate that deregulation of the MVA pathway can contribute to cell transformation.

Remarkably, overexpression of the catalytic domain of HMGCR could cooperate with activated RAS to transform normal diploid mouse embryonic fibroblasts (MEFs) (177).

The MVA pathway could also be deregulated through altered SREBP2 processing (Fig 1-3D) or aberrant sterol feedback mechanism (Fig 1-3E). Our lab has previously reported that a panel of 17 multiple myeloma (MM) cell lines can be split into two categories: those that can and do mount a sterol feedback response after treatment with statins, and those that cannot or do not do so (113). Why or how this arises in cancer cells is currently unknown, but clearly indicates that
Figure 1-3. Possible mechanisms of deregulation of the MVA pathway in cancer cells.

A simplified MVA pathway is shown on the left in the cytoplasm of the schematic of a cell. The MVA pathway is regulated by a robust negative feedback mechanism involving cholesterol-mediated inhibition of cleavage of the transcription factor sterol regulatory element binding transcription factor 2 (SREBP2). Representative SREBP2-regulated genes is shown in the nucleus on the right. HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDLR, low density lipoprotein receptor. A-G, possible mechanisms of MVA pathway deregulation in cancer cells. Deregulation of the MVA pathway can contribute to cancer initiation and/or progression.
deregulation of the sterol feedback response is selected for in certain cancer patients.

**Intersection with cancer cell signaling.** Multiple oncogenic and tumour-suppressive pathways regulate the MVA pathway (Fig 1-3C), and can contribute to deregulation of the MVA pathway in cancer cells. The PI3K-AKT/PKB-mTORC1 signaling axis (PI3K, phosphoinositide 3-kinase; AKT/PKB, protein kinase B; mTORC1, mammalian target of rapamycin complex 1) is the major regulator of cell proliferation in response to growth factor stimulation, and mediates its effects partly through increasing lipid and cholesterol production (236-238). This occurs through activation of SREBP by multiple mechanisms, resulting in upregulation of the FAS and MVA pathways (236-238). AMPK (5' adenosine monophosphate-activated protein kinase) is a negative regulator of cell growth in response to low intracellular ATP levels, and reduces the activity of both SREBP and HMGCR (239, 240). Several gain-of-function p53 mutations commonly found in cancer cells have been shown to functionally interact with nuclear SREBP2, and increase the transcription of MVA pathway genes (176). Additionally, metabolites produced by the MVA pathway play integral roles in multiple signaling pathways (Fig 1-3F). Signaling through the YAP/TAZ (yes-associated protein 1/transcriptional co-activator with PDZ-binding motif) pathway, a major pro-growth and anti-apoptosis signal in some cancer cells, requires MVA pathway activity to produce GGPP (175, 241). GGPP allows prenylation and membrane localization of RHOA (Ras homolog gene family member A), whose activity is necessary for YAP/TAZ signaling transduction (175, 241). Cholesterol-derived end products of the mevalonate pathway, particularly steroid hormones, are also important drivers of initiation and progression of hormone-dependent cancers such as breast and prostate cancers (151).

**Intersection with cancer cell metabolism.** Recent studies in cancer metabolism indicate that cancer cells preferentially uptake glucose and alternative carbon sources from the external
milieu, including acetate (242, 243) and glutamine (244, 245). This leads to abnormally elevated rate of acetyl-CoA production, which is used for many cellular processes, including metabolic processes such as the TCA (tricarboxylic acid) cycle, FAS pathway, and MVA pathway (242). The Appendix of this thesis presents preliminary data showing that the MVA pathway can be deregulated by metabolites upstream of MVA, such as acetyl-CoA produced from altered upstream metabolic pathways (Fig 1-3G).

Systemic contribution. MVA pathway activity in non-malignant tissue may have systemic activity to fuel cancer cell growth. For example, cholesterol synthesized from the liver may be preferentially used to fuel cancer cell growth due to upregulation of LDLR in cancer cells, as reviewed above. Steroid hormones required for hormone-dependent breast and prostate cancers may be produced by gonads and adrenal glands. On the other hand, MVA pathway products have been associated with activation of the immune system: cholesterol skews T cell programming to promote a T helper (Th)1-to-Th2 switch, and sustains an increase in Tregs in the spleen (246, 247). The isoprenoid metabolites of the MVA pathway, including IPP, FPP, and GGPP, can activate the innate-like γδ T cells, with the diphosphate moiety being recognized as a non-self mycobacterial antigen (248), thus promoting immune-surveillance of cancer cells with elevated flux through the MVA pathway.

1.5 Mechanism of statin-induced cancer cell apoptosis

Current model. We and others have shown that statin-induced cancer cell apoptosis is an on-target effect that can be rescued by co-administration with MVA (113, 187, 249), or can be rescued with FPP and GGPP (113, 214, 249-252). FPP and GGPP are essential substrates for protein geranylgeranylation and farnesylation, respectively, together referred to as protein prenylation (204-206). These processes add a hydrophobic farnesyl or geranylgeranyl moiety to
proteins containing a C-terminal CaaX domain (cysteine-aliphatic-aliphatic-any amino acid), thus localizing them to cellular membranes (207, 208). Prenylation-driven membrane localization is required for all proteins in the RAS GTPase superfamily (207, 208), prompting many groups to examine the effect of statin treatment on prenylation status of RAS family proteins. Statin treatment leads to a decrease in the prenylated and membrane-associated forms of RAS super family proteins, including those in the RAS, RHO, RAC, RAP, and RAB subfamilies (209-214). This inhibition of protein prenylation is reversed with FPP or GGPP co-treatment with statins (113, 174, 209-211, 213, 249, 250). On the other hand, cholesterol, CoQ, and dolichol cannot rescue cancer cell apoptosis when co-administered with statins (113, 114). Based on these data, the current model of the mechanism statin-induced cancer cell apoptosis is via inhibition of FPP and GGPP synthesis, which becomes limiting for prenylation of proteins in the RAS superfamily.

In cells that are dependent on RAS, such as many cancer cells that harbor mutations in RAS proteins or upregulated RAS signaling pathways, inhibition of RAS prenylation may be therapeutic (253-255). This was the scientific basis for the development of farnesyltransferase inhibitors (FTIs) and geranylgeranyltransferase inhibitors (GGTIs), both of which have shown efficacy in pre-clinical models but have been limited in clinical application due to high general toxicity (256). A number of studies have therefore tested the use of low-dose GGTIs/FTIs in combination with statins, and have shown that the safety profiles can be improved (257, 258).

Discrepancies. The current model of the mechanism of action of statins described above predicts that cancer cells that are dependent on RAS or RAS family members would be more sensitive to statins, as they cannot survive without the requisite protein prenylation. However, this has not been consistently observed. For example, cancer cells with upregulated or hyperactivated RAS or
RHO are associated with increased statin sensitivity in some studies (209-211), but not others (113, 174, 251). The majority of epidemiological studies and clinical trials report no association between response to statins and RAS mutations (259-264). In cell lines that were sensitive to statin-induced cell kill, rescuing RAS localization (113, 265) or RAF-MEK-ERK signaling (266) did not make cells resistant, and intrinsic sensitivity to statin kill was largely independent of RAS function (113, 169, 267). The literature on other prenylated proteins are also inconsistent with this model of statin kill.

The amino acid sequence in the CaaX domain dictates whether a given protein prefers to be prenylated using FPP, GGPP, or have no preference for either one over the other (Fig 1-4) (254, 255). Evidence from studies using GGTIs and/or FTIs indicate that many proteins can be alternatively prenylated when their preferred process is limited: for example, when farnesylation is inhibited, KRAS (CaaX sequence: CVIM) and NRAS (CaaX sequence: CVVM) can be alternatively prenylated by geranylgeranylation (Fig 1-4), and remain fully functional (268, 269). Curiously, however, many studies have reported that while GGPP consistently rescues statin-induced cancer cell death (113, 210, 211, 213, 249-252), FPP does so less consistently, sometimes rescuing completely (249), sometimes partially (113, 210, 250, 251), and sometimes not at all (174, 210, 252). In general, these results were interpreted in two ways. The first interpretation is that protein geranylgeranylation plays a more important role than protein farnesylation in cancer cell survival. However, this has not been supported by studies using GGTIs and FTIs to specifically block these processes (255, 268, 269). The second interpretation suggests that FPP is shunted towards cholesterol synthesis rather than being used for protein prenylation. Indeed, differential K_M suggest that decreasing FPP concentrations are 1,000-fold more likely to impact flux through squalene synthase, the first step of sterol synthesis, than the activity of farnesyltransferase (270). Accordingly, it has been suggested that all observations of
Figure 1-4. Prenylation substrate is determined by the amino acid sequence in the CaaX domain.

The CaaX amino acid sequence determines requirement or preference for farnesylation or geranylgeranylation. The chemical structures of the farnesyl group (left) and geranylgeranyl group (right) are shown. FTase, farnesyltransferase; FTIs, farnesyltransferase inhibitors; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GGTase, geranylgeranyltransferase; GGTIs, geranylgeranyltransferase inhibitors.
statins inhibiting protein prenylation are artefacts of supra-pharmacologic levels of statins used in tissue culture (271), and statins in fact have no effect on protein prenylation in vivo (254). These contradicting data raise the possibility that inhibition of protein prenylation is likely not the sole contributor to statin sensitivity (254). This implicates not only an alternative mechanism of statin-induced cell death, but also the potential to develop better biomarkers for the identification of patients that will benefit from statin treatment.

1.6 Objectives and thesis outline

The overarching hypothesis of this thesis is that inhibition of the MVA pathway by statins can induce cell death in a subset of breast cancer cells, and improve clinical outcome. My objectives were (i) to understand the rationale behind the discrepancies in the current model of statin mechanism of action; (ii) to identify molecular biomarkers that could predict sensitivity to statins in breast cancer cell lines; and (iii) to identify the point of therapeutic intervention in which statin treatment will impact breast cancer patient care.

Chapter 2 of this thesis presents direct evidence that inhibition of protein prenylation is independent from statin-induced apoptosis. We showed that increased cellular demand for GGPP and FPP did not always sensitize breast cancer cells to fluvastatin, and when it did, the effect was independent from inhibition of protein prenylation. We then identified breast cancer cell EMT to be the underlying cause of increased sensitivity to statin-induced cell death. Five bi-modally distributed EMT genes was sufficient to accurately predict statin sensitivity in ~700 cancer cell lines, suggesting cancer cell EMT to be a universal biomarker of statin sensitivity.

In Chapter 3, we further delineated the mechanism of statin-induced breast cancer cell death, and identified the inhibition of dolichol biosynthesis to be the mechanism of statin sensitivity in breast cancer cells that have undergone EMT. Dolichol is a large isoprenoid metabolite produced
by the MVA pathway, and is important for N-linked glycosylation of nascent peptides.

Fluvastatin treatment impairs the presentation of the EMT-associated β1,6-branched, tri- and tetra-antennary, unfucosylated and singly fucosylated complex glycans, both in cell culture and \textit{in vivo}. We then tested the efficacy of fluvastatin in a mouse model of post-surgical metastatic breast cancer, that closely mimics the treatments experienced by early-stage breast cancer patients. We showed that fluvastatin treatment in the adjuvant setting effectively delayed breast cancer metastasis and improved survival.

The Appendix of this thesis document observations suggesting a novel mechanism of MVA pathway (de)regulation, that may contribute to oncogenic transformation through intersection with cancer cell metabolism.

Overall, this thesis significantly advances our understanding of how statins induce breast cancer cell death. Two biomarkers of statin sensitivity are identified, one based on gene expression and one based on glycan presentation. We further show in a pre-clinical mouse model of breast cancer that fluvastatin treatment in the adjuvant therapeutic setting can effectively delay metastatic recurrence. By demonstrating efficacy, elucidating mechanism, and identifying biomarkers of fluvastatin sensitivity, this thesis provides evidence to support the immediate repurposing of this effective, safe, and inexpensive drug to target breast cancer metastasis.
1.7 References


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Chapter 2
Statin-induced cancer cell death can be mechanistically uncoupled from prenylation of RAS family proteins

Contributions: The work presented in this chapter was performed by the author except for Fig 2-9 and Fig 2-10, where Dr. Wail Ba-Alawi and Dr. Benjamin Haibe-Kains performed datamining, bimodal index calculations, and pharmacogenomic analyses.

This project was completed with supervisory support from Dr. Linda Z. Penn.
Statin-induced cancer cell death can be mechanistically uncoupled from prenylation of RAS family proteins

2.1 Abstract

Statins inhibit the rate-limiting enzyme of the mevalonate pathway, responsible for the *de novo* production of farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP). FPP and GGPP are essential substrates for post-translational prenylation of RAS family proteins, and a long-standing open question has been whether inhibition of protein prenylation of RAS family members underlies statin-induced cancer cell death. Here, we demonstrate that statin-induced cancer cell death can be uncoupled from protein prenylation of RAS family members. Increased cellular demand for GGPP and FPP for prenylation does not uniformly sensitize cells to inhibition of GGPP and FPP synthesis, as only HRAS and KRAS, but not other family members, sensitized MCF10A cells to fluvastatin-mediated cell death. However, this sensitivity was independent of RAS prenylation, but dependent on the RAS-ZEB1-EMT signaling axis. Induction of EMT was sufficient to phenocopy the increase in fluvastatin sensitivity in RAS-transformed cells, and knocking down ZEB1 rescued RAS-transformed cells from fluvastatin-induced cell death. By mining publicly available gene expression and statin sensitivity databases, we showed that enrichment of EMT features is associated with increased sensitivity to statins in a large panel of cancer cell lines, suggesting that this association can be generalized across multiple cancer types. Taken together, these results indicate that the anti-cancer effect of statins is independent from prenylation of RAS family proteins, even in RAS-transformed cancer cells.

2.2 Introduction

Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) (Fig 2-1A), and have been widely prescribed to lower cholesterol levels (1). Epidemiological evidence
and clinical trials (6-10) indicate that statins have anti-cancer activities, particularly in breast, prostate, lung, and colorectal cancers. Pre-clinical data demonstrate that statin treatment induces cancer cells to undergo apoptosis (11-14), and lowers disease burden in tumour xenografts (15-18). Despite the promising potential to repurpose statins as anti-cancer agents, the molecular mechanism of how inhibition of HMGCR can specifically kill cancer cells remains unclear.

HMGCR catalyzes the conversion of HMG-CoA to mevalonate (MVA), the sole precursor for the de novo synthesis of sterols, geranylgeranyl diphosphate (GGPP), farnesyl diphosphate (FPP), and several other metabolic end products (19, 20) (Fig 2-1A). Statin-induced apoptosis can be rescued by co-administration with MVA (13, 14, 21), demonstrating that this is an on-target effect, or with GGPP or FPP (14, 21-25). Sterols cannot rescue cancer cell apoptosis induced by statins (14, 26). These results have led to a model where statins induce apoptosis by inhibiting GGPP and FPP synthesis.

GGPP and FPP are essential substrates for protein geranylgeranylation and farnesylation, respectively, together referred to as protein prenylation (27-29). Prenylation with the hydrophobic geranylgeranyl or farnesyl moiety localizes proteins to the cell membrane (30, 31). Prenylation-driven membrane localization is required for all proteins in the RAS GTPase superfamily, and several groups have shown that statin treatment decreases the prenylated and membrane-associated forms of RAS, RHO, RAC, RAP, and RAB subfamily proteins (22, 32-36). However, evidence for the functional importance of these small GTPases in conferring statin sensitivity have been conflicting. For example, cancer cells with upregulated or hyperactivated RAS or RHO are associated with increased statin sensitivity in some studies (32-34), but not
others (14, 24, 37). Understanding the mechanism driving these discrepancies has remained a challenge and has been an area of debate for many years (19, 38).

In this Chapter, we directly address these discrepancies and show that inhibition of RAS family protein prenylation can be uncoupled from statin-induced apoptosis. We systematically introduced several members of the RAS superfamily in the MCF10A basal breast cell line (39-41), producing a panel of sublines with increased demand for GGPP and/or FPP. This did not uniformly sensitize cells to inhibition of GGPP and FPP synthesis, as only HRAS\textsuperscript{G12V} and KRAS\textsuperscript{G12V} exhibited an increased sensitivity to fluvastatin. HRAS\textsuperscript{G12V} and myristoylated-HRAS\textsuperscript{G12V} sensitized cells to statins to a similar extent, indicating that statin-induced cell death is independent of RAS prenylation, even in RAS-transformed cells. We then showed that overexpression of RAS induced epithelial-to-mesenchyme transition (EMT) in these cells, in part by upregulating the EMT driver ZEB1. Exogenous expression or knockdown of ZEB1 conferred or rescued statin sensitivity, respectively, suggesting that EMT was the critical feature that was functionally important for statin-induced cell death. Taking a computational pharmacogenomics approach, we demonstrated that EMT was associated with statin sensitivity across a large panel of cancer cell lines. Taken together, our results provide a rationale for why RAS-related oncogenes have been poor biomarkers of statin sensitivity, and suggest that a set of EMT-associated genes should be further evaluated in the pre-clinical and clinical setting.

2.3 Results

2.3.1 HRAS\textsuperscript{G12V} and KRAS\textsuperscript{G12V}, but not other proteins in the RAS superfamily, sensitize MCF10A cells to fluvastatin

Using the MCF10A breast epithelial cell line as a non-transformed, genomically stable cell background (39-41), we ectopically expressed representative proteins from the RAS, RHO,
RAC, and RAP subfamilies in their dominantly active forms (42) (Fig 2-1B). These mutants remain dependent on prenylation for activity, allowing us to simulate the increase in demand for FPP and/or GGPP as a result of aberrant activation of these GTPases. The increase in demand for FPP and/or GGPP did not universally sensitize cells to fluvastatin (Fig 2-1C), as only cells overexpressing HRAS$^{G12V}$ or KRAS$^{G12V}$ had lowered fluvastatin IC$_{50}$ values (13.6 μM and 13.2 μM, respectively) compared to the vector control (22.2 μM), which indicated an increased sensitivity to fluvastatin (Fig 2-1C). A colony formation assay in soft agar was used to test the effect of fluvastatin treatment on the transformation potential of cells overexpressing HRAS$^{G12V}$ or KRAS$^{G12V}$ (Fig 2-1D). In this and all subsequent colony formation assays, 20 μM fluvastatin was used to allow for adequate penetration into the soft agar. Both colony count (Fig 2-1E) and colony size (Fig 2-1F) were significantly reduced compared to the untreated control.

2.3.2 Inhibition of RAS prenylation is uncoupled from fluvastatin-induced cell death

If inhibition of RAS localization was the mechanism of fluvastatin-induced cell death, cells overexpressing myristoylated HRAS$^{G12V}$ (myr-HRAS), which localizes to the cell membrane independently of prenylation with FPP or GGPP, should remain insensitive to fluvastatin. Fig 2-2A shows that overexpression of HRAS$^{G12V}$ and myristoylated HRAS$^{G12V}$ both activated downstream signaling to a similar extent, as seen by the increase in Erk and Akt phosphorylation. While membrane-bound HRAS$^{G12V}$ began being mislocalized into the cytoplasmic compartment after treatment with 10 μM of fluvastatin for 24 h, myr-HRAS$^{G12V}$ remained in the membrane fraction, confirming that myristoylation occurs independent of FPP and GGPP (Fig 2-2B). EGFR, HMGCS1, and actin were used as controls for membrane-localized, cytosol-localized, and total proteins, respectively (Fig 2-2B). Unexpectedly, the fluvastatin IC$_{50}$ value of cells overexpressing myr-HRAS$^{G12V}$ was lowered similarly to
**Figure 2-1. HRAS\textsuperscript{G12V} and KRAS\textsuperscript{G12V}, but not other prenylated proteins, sensitize MCF10A cells to fluvastatin.**

A, a simplified schematic of the MVA pathway. B, representative RAS family proteins selected for ectopic expression in MCF10A cell lines. C, ectopic expression of HRAS\textsuperscript{G12V} and KRAS\textsuperscript{G12V} sensitized MCF10A cells to fluvastatin, as assessed by MTT assay following 72 h of treatment. Bars are mean + SD, n=3. **, p<0.01 (one-way ANOVA with a Dunnett post-test, comparing all columns vs. vector control column). D-F, treatment with fluvastatin decreased colony count and colony size of RAS-driven anchorage-independent growth in soft agar. Colonies were treated with 20 μM fluvastatin 2x weekly for 18 days. Bars are mean + SD, n=4. *, p<0.05; **, p<0.01 (unpaired, two-tailed t test, comparing fluvastatin-treated vs. no treatment control).
Figure 2-2. Inhibition of RAS prenylation is uncoupled from fluvastatin-induced cell death.

A, overexpression of HRAS<sup>G12V</sup> and myr-HRAS<sup>G12V</sup> activated Erk phosphorylation and Akt phosphorylation to a similar extent. B, the proportion of HRAS<sup>G12V</sup> in the cytoplasmic (c) fraction was increased, and the proportion in the membrane (m) proportion was decreased, after 24 h of treatment with 10 μM fluvastatin. In contrast, the localization of myr-HRAS<sup>G12V</sup> was unaffected by fluvastatin treatment. C, both HRAS<sup>G12V</sup> and myr-HRAS<sup>G12V</sup> sensitized MCF10As
to fluvastatin as assessed by MTT assay following 72 h of treatment. Bars are mean ± SD, n=3. *, p<0.05; **, p<0.01 (one-way ANOVA with a Dunnett post-test, comparing all columns vs. vector control column). D-F, treatment with fluvastatin decreased colony count and colony size of myr-HRAS<sup>G12V</sup>-driven anchorage-independent growth in soft agar. Colonies were treated with 20 μM fluvastatin 2x weekly for 18 days. Bars are mean ± SD, n=4. *, p<0.05 (unpaired, two-tailed t test, comparing fluvastatin-treated vs. no treatment control). G-I, 10 μM fluvastatin induced cell death in MCF10A cells overexpressing HRAS<sup>G12V</sup> and myr-HRAS<sup>G12V</sup>, fully reversed by co-administration with MVA, GGPP, or FPP. Bars are mean ± SD, n=3. **, p<0.01; ***, p<0.001 (one-way ANOVA with a Dunnett post-test, comparing all columns vs. no treatment control column).
HRAS$^{G12V}$ (Fig 2-2C). Colony formation was also inhibited by fluvastatin treatment in cells overexpressing myr-HRAS$^{G12V}$ (Fig 2-2D-F), to the same extent as cells overexpressing HRAS$^{G12V}$ (Fig 2-1D-F). Addition of MVA, GGPP, or FPP rescued the fluvastatin-induced cell death in both HRAS$^{G12V}$ and myr-HRAS$^{G12V}$ cells (Fig 2-2G-I). Together, these data uncouple statin-induced cell death from GGPP and FPP demand for prenylation of RAS family proteins, and implicate that events downstream of RAS signaling are responsible for the increase in statin sensitivity in this cell system.

Overexpression of HRAS$^{G12V}$ or myr-HRAS$^{G12V}$ in MCF10A cells did not affect the expression of MVA pathway genes HMGCR and HMGCS1, either basally or in response to fluvastatin exposure (Fig 2-3A-B). This rules out deregulation of the MVA pathway, or its sterol feedback response, as the mechanism of increased statin sensitivity in this model.

2.3.3 The RAS-ZEB1-EMT signaling axis underlies increased sensitivity to fluvastatin

Since RAS signaling, rather than RAS localization, was implicated as the driver of fluvastatin sensitivity, we overexpressed constitutively active forms of several classic effectors of RAS signaling, namely RALA$^{G23V}$, BRAF$^{V600E}$, PI3K-p110$^{α}$E545K, PI3K-p110$^{α}$H1047R, and MYC$^{T58A}$, in MCF10As (Fig 2-4A-B). None of these sublines exhibited a lowered fluvastatin IC$_{50}$; overexpression of PIK3CA-p110$^{α}$ led to significant increases in IC$_{50}$ values (Fig 2-4C). Similar results were found in colony formation assays in soft agar (Fig 2-5A-C). Therefore, the observed increase in fluvastatin sensitivity in RAS-transformed cells was not mediated through these downstream mediators of RAS signaling.

The RAS sublines were phenotypically distinct from the MCF10A parental cells. Instead of an epithelial phenotype with a cobblestone-like appearance, the RAS sublines appeared more
Figure 2-3. Overexpression of HRAS$^{G12V}$ or myr-HRAS$^{G12V}$ does not alter HMGCR or HMGCS1 expression.

A-B, Cells were treated with 10 μM fluvastatin for 16 h and analyzed for HMGCR and HMGCS1, mRNA and protein expression. Bars are mean + SD, n=3. *, p<0.05; **, p<0.01 (unpaired, two-tailed t test, comparing fluvastatin-treated vs. no treatment control).
### Figure 2-4. Fluvastatin cytotoxicity is not mediated by the BRAF, RALA, PI3K, or MYC downstream mediators of RAS signaling.

A-B, ectopic expression of downstream mediators of RAS signaling. C, none of the oncoproteins indicated in A-B sensitized MCF10A cells to fluvastatin; p110α overexpression desensitized MCF10As to fluvastatin. Bars are mean ± SD, n=3. *** p<0.001 (one-way ANOVA with a Dunnett post-test, comparing all columns vs. vector control column).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Activating mutation</th>
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<tbody>
<tr>
<td>RALA</td>
<td>G23V</td>
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<td>BRAF</td>
<td>V600E</td>
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<tr>
<td>p110α</td>
<td>E545K</td>
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<td>p110α</td>
<td>H1047R</td>
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<td>MYC</td>
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![Figure 2-4: Fluvastatin cytotoxicity is not mediated by the BRAF, RALA, PI3K, or MYC downstream mediators of RAS signaling.](image)

**Protein Activating mutation**

- **RALA G23V**
- **BRAF V600E**
- **p110α E545K**
- **H1047R**
- **MYC T58A**
Figure 2-5. Transforming oncogenes do not all sensitize MCF10As to fluvastatin.

A-C, Cells with ectopic expression of PI3K-\(p_110\alpha^{E545K}\), PI3K-\(p_110\alpha^{H1047R}\), and MYC\(^{T58A}\) were assayed for colony formation in soft agar. Without fluvastatin treatment, all three oncogenes transformed MCF10As as assayed by soft agar colony formation assay. Bars are mean \(\pm\) SD, \(n=3\). *, \(p<0.05\) (one-way ANOVA with a Dunnett post-test, comparing all columns vs. vector control column). Fluvastatin treatment had no significant effect on the colony size or count. Colonies were treated with 20 \(\mu\)M fluvastatin 2x weekly for 18 days. Bars are mean \(\pm\) SD, \(n=3\). ns, not significant (unpaired, two-tailed \(t\) test, comparing fluvastatin-treated vs. no treatment control).
mesenchymal, with an elongated and spindle-shaped morphology (Fig 2-6A). These cells had undergone epithelial-to-mesenchymal transition (EMT), with a dramatic loss of E-cadherin expression and a gain of vimentin expression (Fig 2-6B). To test whether induction of EMT confers sensitivity to fluvastatin, we treated MCF10A cells with transforming growth factor beta (TGF-β) for 3 days, which induces EMT independently of RAS status (Fig 2-6C). This led to an increased sensitivity to fluvastatin, as indicated by a decrease in fluvastatin IC$_{50}$ in TGF-β-treated cells (Fig 2-6D). After TGF-β treatment, removal of TGF-β from the culture media gradually reverses EMT (Fig 2-7). MCF10A cells fully reverted back to an epithelial phenotype after 7 days of TGF-β removal, and sensitivity to fluvastatin was restored to control levels (Fig 2-7). These data indicate that a mesenchymal state is sufficient to confer sensitivity to fluvastatin.

RAS induces EMT by upregulating the EMT-driving transcription factor ZEB1 through several potential mechanisms (43, 44), and not by other EMT regulators such as SNAIL or TWIST (45, 46) in our MCF10A system (Fig 2-6E). ZEB1-overexpression in MCF10As led to a loss of E-cadherin and gain of vimentin expression independent of RAS status (Fig 2-6F), and decreased the fluvastatin IC$_{50}$ value similarly to the RAS sublines (Fig 2-6G). ZEB1 overexpression had no effect on the IC$_{50}$ value of GGTI-298 (Fig 2-6H), a specific inhibitor to geranylgeranyltransferase I, reinforcing the model that prenylation of RAS family proteins is uncoupled from fluvastatin-induced tumour cell death. We then knocked down ZEB1 in cells overexpressing HRAS$^{G12V}$ and myr-HRAS$^{G12V}$ using two independent shRNAs (Fig 2-8A). ZEB1 knockdown could partially rescue the RAS-driven fluvastatin sensitivity, both by IC$_{50}$ measurements (Fig 2-8B) and by colony formation in soft agar (Fig 2-8C-E).
Figure 2-6. RAS induces EMT through ZEB1, and induction of EMT is sufficient for sensitizing cells to fluvastatin.

A. RAS-overexpressing cells appear more mesenchymal than the vector control cells. B, RAS overexpression reduced expression of E-cadherin, an epithelial cell marker, and increased...
expression of vimentin, a mesenchymal cell marker. C, treating MCF10A cells with 5 ng/mL TGF-β for 3 days induced EMT. D, induction of EMT by 5 ng/mL TGF-β treatment sensitized MCF10A cells to fluvastatin, as assessed by MTT assay following 72 h of treatment. Dashed line represents IC\textsubscript{50} of HRAS\textsuperscript{G12V}-overexpressing cells. Bars are mean ± SD, n=3. *, p<0.05 (unpaired, two-tailed t test, comparing TGF-β-treated vs. no treatment control). E, HRAS\textsuperscript{G12V} and myr-HRAS\textsuperscript{G12V} cells upregulate the EMT transcription factor ZEB1. Bars are mean ± SD, n=3. **, p<0.01 (one-way ANOVA with a Dunnett post-test, comparing all columns vs. vector control column). F, ectopic expression of ZEB1 induced EMT. G, ZEB1 overexpression sensitized MCF10A cells to fluvastatin, as assessed by MTT assay following 72 h of treatment. Dashed line represents IC\textsubscript{50} of HRAS\textsuperscript{G12V}-overexpressing cells. Bars are mean ± SD, n=3. **, p<0.01 (unpaired, two-tailed t test, comparing TGF-β-treated vs. no treatment control). H, ZEB1 overexpression did not sensitize cells to GGTI-298. Bars are mean ± SD, n=3.
Figure 2-7. TGF-β induced EMT and fluvastatin sensitivity are both reversible.

After 3 days of treatment with 5 ng/mL TGF-β, MCF10A cells undergo EMT and become more sensitive to fluvastatin, as assessed by MTT assays for 72 h. Dashed line represents IC$_{50}$ of HRAS$^{G12V}$-overexpressing cells. Cells gradually reverted to epithelial with continued culturing after TGF-β removal; after 7 days, sensitivity to fluvastatin was restored to control levels. Bars are mean + SD, n=3. **, p<0.01; ***, p<0.001 (one-way ANOVA with a Dunnett post-test, comparing all columns vs. no treatment control column).
Figure 2.8. shRNA knockdown of ZEB1 rescues the increased sensitivity in HRAS<sup>G12V</sup> and myr-HRAS<sup>G12V</sup> cells.

A, two independent shRNAs were used to knockdown ZEB1 in cells overexpressing HRAS<sup>G12V</sup> and myr-HRAS<sup>G12V</sup>. B, knockdown of ZEB1 rescued the decreased fluvastatin IC<sub>50</sub> observed in
HRAS$^{G12V}$ and myr-HRAS$^{G12V}$ cells. Bars are mean $\pm$ SD, n=3. *, p<0.05; **, p<0.01 (one-way ANOVA with a Dunnett post-test, comparing all columns vs. shRNA control column). C-E, knockdown of ZEB1 in HRAS$^{G12V}$ and myr-HRAS$^{G12V}$ cells led to increased colony formation in soft agar under fluvastatin treatment. Colonies were treated with 20 μM fluvastatin 2x weekly for 18 days. Bars are mean $\pm$ SD, n=4. *, p<0.05 (one-way ANOVA with a Dunnett post-test, comparing all columns vs. shRNA control column).
2.3.4 Enrichment of EMT phenotype is associated with sensitivity to statins in a large panel of cancer cell lines

The Cancer Cell Line Encyclopedia (CCLE) database (47) contains RNA-seq data on 927 cancer cell lines across >20 cancer types (Fig 2-9A). Mining this large database, we observed that while the expression pattern for most genes follow a unimodal (Gaussian) distribution, as exemplified by POLR2A (RNA polymerase II, subunit A; Fig 2-9B), some genes are bimodally expressed, such as ESR1 (estrogen receptor α; Fig 2-9C-D). ESR1 is known for a strong bimodal expression in the breast tissue (48-50), representing ERα-low (left peak) and ERα-high (right peak) cell lines (Fig 2-9D). We then examined the expression profile of 11 well-characterized EMT-associated genes, and found that their expression were strongly bimodal, with bimodality indices (50, 51) higher than that of ESR1, our positive control (Fig 2-10A). The distribution of the top five bimodally expressed genes (VIM, CDH1, ZEB1, FN1, and CDH2) is shown in Fig 2-10B. For each gene, we computed the cutoff optimally discriminating between the two modes of the expression distribution, and classified the cell lines with either low or high expression of the gene of interest (Fig 2-10B). Each cell line was therefore characterized by a binary vector representing the activation of the top five bimodally expressed EMT-associated genes.

Using the top five bimodally expressed EMT-associated genes as features, we built a binary classification rule that classified each solid tumour cell line as enriched with an EMT phenotype if at least one of the EMT-associated genes were activated (Fig 2-10B, right peak for VIM, ZEB1, FN1, and CDH2; left peak for CDH1). We then mined the Cancer Therapeutics Response Portal version 2 (CTRPv2) database (52-54) using the PharmacoGx R/Bioconductor package (55), and found that the EMT-enriched cell lines were associated with significantly higher AUC (more sensitive) to all three statin family members that have been assessed in CTRPv2: fluvastatin (Fig 2-10C), lovastatin (Fig 2-10D), and simvastatin (Fig 2-10E). Thus, cancer cell EMT is associated
Figure 2-9. Unimodal and bimodal gene expression in the CCLE database.

A, tissue type distribution across the Cancer Cell Line Encyclopedia (CCLE) database. B, unimodal (Gaussian) distribution of POLR2A. C-D, bimodal distribution of ESR1. Gene expression values are log2(FPKM+1) with FPKM = Fragments Per Kilobase of transcript per Million mapped reads. The script and data used for the generation of these figures can be downloaded at https://github.com/bhklab/StatinEMT.
Figure 2-10. Statin sensitivity is associated with cancer cell EMT.

A, bimodality index of 11 known EMT-associated genes. B, bimodal expression of the five EMT-associated genes used to classify cell lines based on EMT activity. Gene expression values are log2(FPKM+1) with FPKM = Fragments Per Kilobase of transcript per Million mapped reads. C-E, sensitivity to three statin family members are significantly associated with cell lines enriched for EMT features. AUC, area under the curve (higher values represent higher drug sensitivity); CI, Concordance Index; P-value, Wilcoxon rank sum test, comparing statin response on EMT ‘enriched’ vs. ‘not-enriched’ cell lines; n, number of cell lines. The script and data used for the generation of these figures can be downloaded at https://github.com/bhklab/StatinEMT.
with sensitivity to multiple statin family members in a large panel of cancer cell lines, across multiple cancer types. It should be noted that most, but not all, cell lines in the CTRPv2 database were evaluated for sensitivity to all three statin members, which is why the number of cell lines (n) is not identical between Fig 2-10C, D, and E.

2.4 Discussion

Previously, the increased cancer cell sensitivity to statins was thought to be mediated by inhibiting prenylation of proteins in the RAS superfamily. This model was built on three observations: i) statins inhibit prenylation of RAS family proteins (14, 21, 22, 32-37); ii) co-administration of GGPP or FPP with statins reverses the effect on protein prenylation (14, 21, 23, 32-34, 36, 37); and iii) co-administration of GGPP or FPP can rescue statin kill (14, 23-25, 33, 34, 36). However, most epidemiological studies and clinical trials do not support an association between response to statins and RAS mutations (56-61). Additionally, in cell lines that were sensitive to statins, rescuing RAS localization (14, 62) or RAF-MEK-ERK signaling (63) did not decrease statin sensitivity, and intrinsic sensitivity to statin kill was largely independent of RAS function (14, 64, 65). These contradicting data raise the possibility that inhibition of RAS family protein prenylation is not the sole contributor to statin sensitivity, implicating not only an alternative mechanism of statin-induced apoptosis, but also the potential to develop better biomarkers for the identification of patients that will benefit from statin treatment.

We show here that statin-induced cell death can indeed be uncoupled from inhibition of RAS family protein prenylation. First, increased cellular demand for GGPP and FPP for ectopic expression of RAS family proteins requiring prenylation for activity did not always sensitize MCF10A cells to fluvastatin (Fig 2-1). Although RAS overexpression led to an increased sensitivity to fluvastatin, this effect was independent from RAS prenylation and localization to
the cell membrane (Fig 2-2). Instead, RAS induced EMT by upregulating ZEB1, which was the underlying cause of increased sensitivity to fluvastatin-induced cell death (Fig 2-6, Fig 2-8). This can, in part, explain why statins have been reported to be more effective in more aggressive (invasive/metastatic) subtypes of breast (64, 66) and prostate cancers (67, 68), while mutations in RAS family proteins are poorly associated with statin response (56-61).

Interestingly, when we assayed for the expression profile of a list of 11 EMT-associated genes known for a strong association with EMT (45, 69), we found that they followed a bimodal distribution (Fig 2-10). Bimodal distribution of VIM, CDH1, ZEB1, FN1, or CDH2 were used as features to classify all solid tumour cell lines in the Cancer Cell Line Encyclopedia (47) into high-expression and low-expression populations (Fig 5B). As a comparison, ESR1 (estrogen receptor α) is known to be bimodally expressed in breast cancer (48-50), which could be used as a classifier of estrogen receptor-positive and -negative breast tumours in large bioinformatics databases (50) (Fig 2-9D). Mining the Cancer Therapeutics Response Portal (52-54), we interrogated sensitivity to three statin family members in >500 solid tumour cell lines for any association with an EMT phenotype. Cell lines enriched with EMT features were associated with significantly higher AUC in response to statin treatment (Fig 2-10C-E), indicating that they were more sensitive to statin kill. We have thus expanded our original observation in the MCF10A model system to include >20 cancer types and three statins, suggesting that the association between EMT and sensitivity to statins can be generalized across all solid tumour cell lines.

Activation of EMT is proposed to be the critical initiating step in metastatic dissemination of late-stage cancers (70, 71). Although it is still debated whether this process is required for metastasis, as opposed to being a phenotype of aggressive/metastatic disease (72, 73), it is nevertheless known to be associated with cell de-differentiation, stem-like properties, and anti-
apoptotic signaling (74, 75). Importantly, activation of EMT is typically associated with therapeutic resistance (72-75). We show here that activation of EMT increased cell sensitivity to fluvastatin kill (Fig 2-6, Fig 2-7), consistent with previous reports (76-78). This suggests the intriguing possibility that statins may be used to target disseminated and/or dormant cancer cells (that is, those that presumably have undergone EMT) that are responsible for therapeutic failure and refractory disease. Several epidemiological studies have reported supporting evidence, showing that statin use following front-line treatment was associated with better disease-free survival and overall survival in breast (4, 18, 79-82), prostate (83, 84), and renal cell carcinomas (85). Further testing of statins as adjuvant therapeutics in the pre-clinical and clinical setting is warranted.

It is perhaps tempting to ask which prenylated protein(s), other than the ones selected for testing in this study, is/are responsible for the anti-cancer effects of statins in the context of EMT. Indeed, we show that co-administration of GGPP or FPP could rescue fluvastatin kill in both HRAS\textsuperscript{G12V}- and myr-HRAS\textsuperscript{G12V}-overexpressing cells (Fig 2-2G-I). However, the two isoprenoids did not rescue to the same extent: GGPP and MVA completely rescued cell death to control levels, but FPP was less effective (Fig 2-2G-I). This is reminiscent of previous studies, where GGPP consistently rescued statin effects (14, 21, 23-25, 33, 34, 36), while FPP did so less consistently, rescuing completely (21), partially (14, 23, 24, 33), or not at all (25, 33, 37). Nonetheless, the observation that FPP did not rescue as well as GGPP in an HRAS\textsuperscript{G12V}-overexpressing system was unexpected, since HRAS prefers FPP over GGPP for prenylation (42, 86). Two explanations are possible for this observation. First, since FPP also acts as the precursor to sterols (19, 20), a portion of the supplemented FPP could be shunted towards cholesterol production, which does not play a role in statin-induced apoptosis (14, 26). However, the observation that MVA consistently rescues statin-induced cell death (13, 14, 21) (Fig 2-2G-I)
argues against this interpretation. Alternatively, GGPP also functions as the precursor for other isoprenoids such as dolichols and isoprenoid moieties on coenzyme Q (19, 20), and depletion of these larger isoprenoids could be contributing to statin sensitivity. Consistent with this is the observation that cells overexpressing ZEB1 were more sensitive to fluvastatin, but not to inhibition of geranylgeranylation itself (Fig 2-6G-H). Taken together, our data reinforce the new model presented here that, in the context of cancer cell EMT, fluvastatin-induced cell death is uncoupled from inhibition of RAS family protein prenylation. Why cells become dependent on the MVA pathway when undergoing EMT, and are therefore sensitive to fluvastatin inhibition, remains to be elucidated and will be an interesting area for future investigation that could lead to the identification of biomarkers of fluvastatin-responsive cancers.

2.5 Materials and Methods

2.5.1 Reagents

Fluvastatin was purchased from US Biologicals. TGF-β was purchased from PeproTech. All other chemicals were purchased from Sigma unless otherwise specified.

2.5.2 Cell culture

MCF10A cells were a kind gift of Dr. Senthil Muthuswamy and were cultured as previously described (87). Transgene expression was stably introduced into MCF10A cells using retroviral insertion with pBabePuro (88). Cells were imaged on the Leica MZ FLIII Stereomicroscope.

2.5.3 MTT assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed as previously described (64). Briefly, MCF10A cells were seeded at 750 cells/well in 96-well plates overnight, then treated in triplicate with 0-200 μM fluvastatin for 72 h. IC₅₀ values were computed using GraphPad Prism with a bottom constraint equal to 0.
2.5.4 Immunoblotting

Cell lysates were prepared by lysing directly in boiling SDS lysis buffer (1% SDS, 11% glycerol, 10% β-mercaptoethanol, 0.1 M Tris pH 6.8). The following antibodies were used: E-Cadherin (CST 3195), vimentin (CST 5741), actin (Sigma A2066), tubulin (Millipore CP06), FLAG (Sigma F1804), EGFR (CST 2232), ERK (CST 4695), p-ERK (CST 4370), AKT (CST 9272), p-AKT (CST 9271), HMGCS1 (SCB sc-32423), RALA (BD 610221), BRAF (Sigma HPA001328), MYC (9E10 in-house), ZEB1 (Sigma HPA027524), HMGCR (A9 in-house).

2.5.5 Soft agar colony formation

Anchorage-independent colony growth of MCF10A sublines in soft agar was evaluated using standard protocols (87). Colonies were imaged at 1.2x magnification on the Leica MZ FLIII Stereomicroscope after 18 days. Colony number and size were quantified using ImageJ.

2.5.6 Membrane fractionation

Cell were seeded at 2x10⁶/plate overnight and treated as indicated. Harvested cells were re-suspended in 1 mL HEPES buffer (0.25 M sucrose, 50 mM HEPES pH 7.5, 5 mM NaF, 5 mM EDTA, 2 mM DTT) and lysed by sonication. Homogenate was cleared at 2,000 xg for 20 min at 4°C, then ultracentrifuged at 115,000 xg for 70 min at 4°C for membrane fractionation. Membrane protein pellet was re-suspended in Triton buffer (1% Triton X-114, 50 mM Tris pH 7.5, 0.1 mM NaCl, 5 mM EDTA, 5 mM NaF, 2 mM DTT).

2.5.7 Cell death assay

Cells were seeded at 2.5x10⁵/plate overnight and treated as indicated. After 72 h, cells were fixed in 70% ethanol for >24 h, stained with propidium iodide, and analyzed by flow cytometry for the % sub-diploid DNA population as % cell death, as previously described (64).
2.5.8 qRT-PCR

Total RNA was harvested from subconfluent cells using TRIzol Reagent (Invitrogen). cDNA was synthesized from 500 ng of RNA using SuperScript III (Invitrogen). Real-time quantitative RT-PCR was performed using TaqMan probes for HMGCR (ABI Hs00168352), HMGCS1 (ABI Hs00266810), and GAPDH (ABI Hs99999905), and using SYBR Green for:

TWIST_Fw 5'-CCGGAGACCTAGATGTCATTG-3'

TWIST_Rv 5'-CCACGCCTGTTCCTTTT-3'

SNAIL_Fw 5'-CACTATGCCCGCTCTTTC-3'

SNAIL_Rv 3'-GGTCGAGGCTGCTGGAA-3'

ZEB1_Fw 5'-GCCAATAAGCAACGATTCTG-3'

ZEB1_Rv 5'-TTTGGCTGGATCACTTTCAAG-3'

18S_RNA_Fw 5'-GTAACCCGTTGAACCCCATT-3'

18S_RNA_Rv 3'-CCATCCAATCGGTAGTAGCG-3'

2.5.9 Small hairpin RNA-mediated gene silencing

Small hairpin RNA were cloned into single-vector inducible shRNA construct pLKO-TetON, and lentiviruses generated as previously described (89). Expression of shRNAs were induced where indicated using 100 ng/mL doxycycline. Sense strand of shRNAs cloned were:

shZeb1 A 5’-

CCGTTGTCTCCCCATAAGTATCAATTCTCGAGAATTGTACTTATGGGAGACATTTTTG-3’
shZeb1 B 5’-

CCGGCCTACCACTGGATGTAGTAAACTCGAGTTTACTACATCCAGTGGTAGGTTTTT
G-3’

2.5.10 Pharmacogenomic analysis

RNA-seq and drug sensitivity data were retrieved and curated from the Cancer Cell Line Encyclopedia (CCLE) (47) and the Cancer Therapeutics Portal version 2 (CTRPv2) (52-54) databases, and were mined using the R/Bioconductor PharmacoGx package (55). To calculate the bimodality index (50, 51), a mixture of two Gaussian models was used to fit the RNA-seq expression values of each gene across all cell lines in CCLE, as implemented in the bimod function in the R/Bioconductor genefu package (version 2.6.0) (50). The cutoff was calculated by taking the average of the end-points where the two Gaussian models meet and was used to classify cell lines into either showing low- or high-expression of a gene. A binary classification rule was developed to determine whether a cell line is ‘enriched’ with EMT phenotype or not. Cell lines from tumours of hematopoietic and lymphoid tissues, and those with unknown origin, were excluded from this analysis. If expression of any of VIM, ZEB1, FN1, or CDH2 was high, or if expression of CDH1 was low, in a cell line according to the bimodality cutoff, then it was classified as ‘enriched’ with EMT phenotype. Concordance index (CI) and p-value were calculated to measure the association between statin sensitivity, obtained from CTRPv2 database, and cell lines that were classified as either ‘enriched’ with EMT phenotype or ‘not enriched’. p-value was calculated using the non-parametric Wilcoxon rank sum test, comparing statins response on cell lines ‘enriched’ vs. ‘not enriched’ with EMT phenotype. The script and data used for the generation of these figures can be downloaded at

https://github.com/bhklab/StatinEMT.
2.6 References


60. Hong JY, Nam EM, Lee J, Park JO, Lee SC, Song SY, Choi SH, Heo JS, Park SH, Lim HY et al: Randomized double-blinded, placebo-controlled phase II trial of


Chapter 3
Fluvastatin inhibits EMT-associated protein N-glycosylation and delays breast cancer metastasis

**Contributions:** The work presented in this chapter was performed by the author except for the following:

Table 3-I and Fig 3-6A-E, Dr. Cunjie Zhang and Dr. Jim Dennis performed N-glycan profiling by LC-MS/MS.

Fig 3-7B-C, Dr. Wenjiang Zhang and Dr. Eric Chen performed HPLC-MS/MS analysis of fluvastatin in the mouse serum and tumour xenograft.

This project was completed with supervisory support from Dr. Linda Z. Penn.
Fluvastatin inhibits EMT-associated protein N-glycosylation and delays breast cancer metastasis

3.1 Abstract

Metastatic recurrence accounts for the majority of breast cancer-related deaths. Epidemiological studies indicate that the risk of metastatic recurrence in breast cancer patients is lowered by adjuvant statin use. Statins are routinely prescribed to lower serum cholesterol, but the efficacy and molecular mechanism of their anti-breast cancer activity as an adjuvant therapeutic are unclear. We show that fluvastatin induces cell death in breast cancer cells that have undergone epithelial-to-mesenchymal transition (EMT) by inhibiting the biosynthesis of dolichol, required for protein N-glycosylation. Total glycome analysis indicates that fluvastatin treatment impairs the expression of complex type N-glycans that are upregulated during EMT and associated with breast cancer metastasis, both in cultured cells and in vivo. In a mouse model of post-surgical metastatic breast cancer, adjuvant fluvastatin treatment delays metastasis, reduces metastatic burden, and improves overall survival. Our data support the immediate repurposing of fluvastatin as an adjuvant therapeutic to target metastatic recurrence in breast cancer.

3.2 Introduction

The front-line therapy for early-stage breast cancer entails surgical removal of the tumour followed by adjuvant therapies (1, 2). Despite aggressive treatment, 20-30% of these patients experience recurrence, often as distant metastases (2, 3). Novel and effective therapeutics to prevent metastatic recurrence will greatly impact breast cancer patient survival. Several retrospective epidemiological studies have indicated that the risk of post-surgical breast cancer recurrence is reduced by 30-60% in patients who are taking statins (4-7), a class of FDA-approved medications that are commonly prescribed to lower serum cholesterol levels.
Importantly, increasing duration of adjuvant statin use is associated with decreasing risk of recurrence (7), suggesting that long-term intake of statins in the adjuvant setting can prolong patient survival. This potential therapeutic option is attractive, as statins are inexpensive, their safety profiles are well understood, and they can be taken for many years without serious adverse events. These properties make statins well-suited to be repurposed for use in the adjuvant setting, which can extend for >10 years in breast cancer patients (1, 2).

Pre-clinical studies have shown that statins can induce apoptosis in breast cancer cells in vitro (8-10), inhibit growth of the primary breast tumour (11, 12), and prolong survival in mouse models of frank metastases, involving intravenous or intracardiac injections of a large bolus of cancer cells (13-15). These pre-clinical models have been well-established for studying neo-adjuvant and salvage therapies, but are poor models for evaluating the efficacy of adjuvant therapeutics (16). As such, the utility of statins in the adjuvant setting remains unclear. The molecular mechanism(s) through which statins may prevent metastatic recurrence of breast cancer also remains to be elucidated. Statins inhibit the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate (MVA), the rate-limiting step of the MVA pathway (Fig 3-1A) (17-19). The MVA pathway synthesizes cholesterol and isoprenoid metabolites including farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), required for post-translational prenylation of proteins such as RAS (20-22). Previous work has suggested that depletion of FPP and/or GGPP, and consequently inhibition of protein prenylation of RAS family members, underlies statin-induced breast cancer cell death (23-26). However, mutations in RAS family proteins could not predict response to statins in several prospective clinical trials (27-29), suggesting that alternative or additional mechanisms are at play. Understanding these mechanisms will be crucial to guide the design of clinical trials, identify biomarkers of statin
response, and identify novel anti-cancer pathways for the development of next-generation therapeutics to prevent metastatic breast cancer.

Here we sought to interrogate the mechanism of statin sensitivity in cell line and \textit{in vivo} models that are reflective of the adjuvant therapeutic space. The endpoint of adjuvant therapies is metastatic outgrowth, which arises from disseminated primary tumour cells (1-3). Activation of epithelial-to-mesenchymal transition (EMT) in cancer cells has been proposed to be the critical initiating step for dissemination, which needs to be reversed at the secondary site to give rise to metastatic outgrowth (30). Although it is still debated whether these processes are required for metastasis, the observation that disseminated cancer cells often gain mesenchymal characteristics while losing epithelial ones (30) suggests that therapeutic targeting of breast cancer cells that have undergone EMT will reflect utility in the adjuvant setting, after cells have disseminated from the primary tumour but prior to metastatic reactivation. Thus, in this Chapter, we first report the use of an EMT model system in breast cancer cell lines to evaluate the mechanism of statin sensitivity \textit{in vitro}. We show that breast cancer cells that have undergone EMT are sensitized to fluvastatin-induced cell death, and identify the molecular mechanism of statin sensitivity to be an increased dependency on the biosynthesis of dolichol via the MVA pathway (Fig 3-1A). Dolichol is a group of long-chain isoprenoids that comprises the lipid component of lipid-linked oligosaccharides (LLO), essential for \textit{N}-linked glycosylation of nascent peptides (17-19). Previous studies have shown that statin treatment can block \textit{N}-glycosylation on individual proteins such as P-gp (31), IGFR (32), EpoR (33), and FLT3 (34). Building on these studies, we show at a global level that fluvastatin treatment impairs the expression of complex type \textit{N}-glycans associated with breast cancer EMT and metastasis (35-38), both in cell lines and \textit{in vivo}. Finally, using an \textit{in vivo} model of post-surgical metastasis that closely follows the course of human breast cancer progression and treatment (16, 39), we show that post-surgical adjuvant
fluvastatin treatment delays breast cancer metastasis, reduces metastatic load, and improves survival. Taken together, our results warrant the immediate clinical testing of fluvastatin as a safe and effective adjuvant therapeutic to prevent breast cancer metastasis, and suggest the development of novel therapeutics to combat metastatic recurrence in breast cancer by inhibiting aberrant protein N-glycosylation.

### 3.3 Results

#### 3.3.1 EMT sensitizes breast cancer cells to fluvastatin and tunicamycin

We used MCF10A breast epithelial cells as our model system as they possess a highly stable genome, which allows for the evaluation of EMT in the absence of gross genetic instability (40). Fluvastatin was chosen for our studies based on its favorable pharmacokinetic properties and promising anti-breast cancer activities in pre-clinical and clinical settings (8). Ectopic expression of the EMT-inducing transcription factor SNAIL triggered EMT in MCF10A cells, as shown by downregulation of E-cadherin and upregulation of fibronectin (Fig 3-1B). Treatment with fluvastatin readily induced cell death in SNAIL-overexpressing cells, but not vector control cells, as assessed by quantification of DNA content by fixed propidium iodide staining followed by flow cytometry (Fig 3-1C). Fluvastatin-induced cell death in SNAIL-overexpressing cells was fully rescued by co-administration with MVA or GGPP, but interestingly not FPP (Fig 3-1C), a metabolic intermediate between MVA and GGPP (Fig 3-1A) (17-19). This has also been reported in several other cell lines (9, 41-45), together suggesting that exogenous FPP is shunted towards cholesterol synthesis and away from GGPP synthesis, while disruption of biological processes downstream of GGPP is critical for statin-induced cell death.

GGPP is used for three biological processes: protein prenylation; synthesis of coenzyme Q (CoQ) used in the electron transport chain (ETC); and synthesis of dolichol used for protein N-
glycosylation (Fig 3-1A) (17-19). We tested whether inhibiting any of these pathways individually using specific inhibitors (Fig 3-1A) could phenocopy statins and preferentially kill cells that have undergone EMT. EMT sensitized cells to fluvastatin, as indicated by a lowered IC₅₀ value in SNAIL-overexpressing cells (Fig 3-1D). To our surprise, EMT did not sensitize cells to GGTI-298 or GGTI-2133 (Fig 3-1D), suggesting that fluvastatin-induced cell death in the context of EMT is independent from inhibition of protein prenylation. Instead, inhibition of LLO assembly downstream of dolichol synthesis by tunicamycin (46, 47) phenocopied fluvastatin by decreasing the IC₅₀ in SNAIL-overexpressing cells (Fig 3-1D). The IC₅₀ for 2-thienoyltrifluoroacetone (2-TTFA) and rotenone, two inhibitors of the ETC (48), were similar between both cell lines (Fig 3-1D), showing that EMT does not sensitize cells to inhibition of the ETC.

These observations were validated in MCF10A cells overexpressing additional EMT-inducing transcription factors SLUG, TWIST or ZEB1 (49-51), as well as two independent breast cancer cell lines, MDA-MB-231 and MCF-7 (Fig 3-2A-D). Overexpressing of TWIST or ZEB1 induced EMT in MCF10A cells, as indicated by downregulation of E-cadherin and upregulation of fibronectin, while overexpression of SLUG in the MCF10A cell system was unable to induce EMT (Fig 3-2A). Consistently, the mesenchymal TWIST- and ZEB1-overexpressing cells became more sensitive to fluvastatin and tunicamycin compared to the vector control and the SLUG-overexpressing cells, which remained epithelial (Fig 3-2B). The IC₅₀ for GGTIs and ETC inhibitors were unaffected by EMT (Fig 3-2B). Similarly, immunoblotting for E-cadherin and vimentin, another marker of mesenchymal cells, indicated that MCF-7 cells were epithelial and MDA-MB-231 cells were mesenchymal (Fig 3-2C). These two cell lines exhibited a 50-fold difference in sensitivity to both fluvastatin and tunicamycin, which could not be phenocopied by GGTI-298, GGTI-2133, 2-TTFA, or rotenone (Fig 3-2D). Together, these data indicate that
Figure 3-1. Induction of EMT by SNAIL overexpression increases cell sensitivity to inhibition of dolichol-dependent protein N-glycosylation by fluvastatin and tunicamycin.
A, a simplified schematic of the mevalonate (MVA) pathway. Inhibitors of specific components of the pathway are represented in red. CoA, coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; LLO, lipid-linked oligosaccharides; GGTI, geranylgeranyltransferase inhibitor; CoQ, coenzyme Q; ETC, electron transport chain; 2-TTFA, 2-thenoyltrifluoroacetone. B, immunoblot (IB) of E-cadherin, an epithelial cell marker, and fibronectin, a mesenchymal cell marker, revealed that overexpression of SNAIL induced EMT in MCF10A cells. Tubulin is used as loading control. C, Flow cytometric quantification of % dead cells (% population in the pre-G1 cell cycle) with propidium iodide DNA staining after fixation. Fluvastatin treatment for 72 h induced cell death in MCF10A cells overexpressing SNAIL, but not in vector control cells. Fluvastatin-induced cell death was fully rescued by co-administration with MVA or GGPP, but not FPP, at the indicated doses. Bars are mean + SD, n=3. *, p<0.05; **, p<0.01 (one-way ANOVA with a Dunnett post-test, comparing all columns vs. fluvastatin column). D, SNAIL overexpression sensitized cells to fluvastatin and tunicamycin, but not inhibitors of other components of the MVA pathway. IC_{50} values as calculated based on MTT assays after cells were treated with 8 doses of each drug for 72 h. Bars are mean + SD, n=3-4. *, p<0.05; **, p<0.01; (unpaired, two-tailed t test, comparing SNAIL vs. vector columns). E, IB for EGFR, GP130, and SLC3A2 for glycosylation status indicates that fluvastatin treatment for 48-72 h led to under-glycosylation of EGFR, GP130, and SLC3A2 in both vector control and SNAIL-overexpressing cells as indicated by the appearance of lower-molecular weight bands. Tunicamycin treatment for 24 h was a positive control for protein under-glycosylation. Ku80 is used as a loading control. Representative images are shown, n=3-4.
**Figure 3-2. Induction of EMT increases cell sensitivity to fluvastatin and tunicamycin.**

A, IB of E-cadherin, an epithelial cell marker, and fibronectin, a mesenchymal cell marker, revealed that MCF10A vector control and SLUG-overexpressing cells remained epithelial, while TWIST- and ZEB1-overexpressing cells underwent EMT. Tubulin is used as a loading control. B, Induction of EMT by TWIST or ZEB1 overexpression sensitized cells to fluvastatin and tunicamycin, but not inhibitors of other components of the MVA pathway. IC\textsubscript{50} values as calculated based on MTT assays after cells were treated with 8 doses of each drug for 72 h. Bars are mean + SD, n=3-4. *, p<0.05; **, p<0.01 (one-way ANOVA with a Dunnett post-test, comparing all columns vs. vector column). C, IB of E-cadherin, an epithelial cell marker, and vimentin, a mesenchymal cell marker, revealed that MCF-7 cells are more epithelial, while MBA-MB-231 cells are more mesenchymal. Tubulin is used as a loading control. D, Fluvastatin and tunicamycin preferentially killed the more mesenchymal MDA-MB-231 cells, compared to the more epithelial MCF-7 cells, by approximately 50-fold. This difference could not be phenocopied by GGTI-298, GGTI-2133, 2-TTFA, or rotenone. IC\textsubscript{50} values are calculated based on MTT assays after cells were treated with 8 doses of each drug for 72 h. Bars are mean + SD, n=3. *, p<0.05; **, p<0.01 (unpaired, two-tailed t test, comparing MDA-MB-231 and MCF-7 columns).
breast cancer cells with mesenchymal phenotypes are more sensitive to inhibition of dolichol synthesis and function by fluvastatin and tunicamycin.

3.3.2 Fluvastatin inhibits dolichol-mediated protein N-glycosylation

Dolichol is a group of large hydrophobic molecules that constitutes the lipid component of LLOs, playing an essential role in the assembly of precursor N-glycans used for co-translational protein N-glycosylation (Fig 3-1A) (52, 53). We therefore tested whether fluvastatin treatment can inhibit protein N-glycosylation. EGFR, GP130, and SLC3A2 are three membrane proteins with multiple glycosylation sites. Under-glycosylated proteins migrate faster on polyacrylamide gels and can be visualized as lower molecular weight bands by immunoblotting (54, 55). All three membrane proteins became under-glycosylated after 48-72 h of fluvastatin treatment, in both vector and SNAIL-overexpressing cells (Fig 3-1E). As a positive control, tunicamycin-mediated inhibition of protein glycosylation was observed after 24 h (Fig 3-1E).

As tunicamycin treatment is known to elicit ER stress, we tested whether the observed effect on protein glycosylation is specific to the inhibition of dolichol biosynthesis and/or function, or a general feature of ER stress. To this end, we treated cells with thapsigargin, another known inducer of ER stress that acts through a dolichol-independent mechanism (56, 57). First we confirmed that treatment with tunicamycin or thapsigargin both induced ER stress, as indicated by upregulation of ER stress markers ERdj4 and BiP (58) in both vector and SNAIL-overexpressing cells, within 24 h of treatment (Fig 3-3A). In contrast, treatment with fluvastatin had little effect on the expression of ERdj4 or BiP for up to 72 h (Fig 3-3A), suggesting that defects in protein glycosylation caused by fluvastatin treatment (Fig 3-1E) can be substantially uncoupled from induction of ER stress. Consistently, treatment with thapsigargin for up to 72 h did not result in under-glycosylation of EGFR, GP130, or SLC3A2 in either vector or SNAIL-
Figure 3-3. Fluvastatin effect is independent from induction of ER stress

A, qRT-PCR of ER stress markers ERdj4 and BiP revealed that fluvastatin treatment for up to 72 h did not induce ER stress, while tunicamycin and thapsigargin treatment both induced ER stress after 24 h of treatment. qRT-PCR of 18S rRNA was used as a housekeeping control. Bars are mean + SD, n=3. *, p<0.05; **, p<0.01; ***, p<0.001 (one-way ANOVA with a Dunnett post-test, comparing all columns vs. solvent control). B, IB for EGFR, GP130, and SLC3A2 for glycosylation status indicates that thapsigargin treatment for up to 72 h did not affect glycosylation status of these proteins in both vector control and SNAIL-overexpressing cells. Tunicamycin treatment for 24 h was a positive control for protein under-glycosylation. Ku80 is used as a loading control. Representative images are shown, n=3-4. C, IC_{50} values as calculated based on MTT assays after cells were treated with 8 doses of thapsigargin for 72 h, showed that sensitivity to thapsigargin was not modulated by EMT. Bars are mean + SD, n=3. **, p<0.01 (unpaired, two-tailed t test, comparing MDA-MB-231 and MCF-7 columns).
overexpressing cells (Fig 3-3B). Additionally, whereas overexpression of SNAIL sensitized cells to fluvastatin and tunicamycin (Fig 3-1D), their sensitivity to thapsigargin remained the same as vector control cells (Fig 3-3C). Comparing between MDA-MB-231 and MCF-7 cell lines, the mesenchymal MDA-MB-231 cells were in fact more resistant to thapsigargin than the epithelial MCF-7 cells (Fig 3-3C), further supporting that mesenchymal breast cancer cells are sensitized to fluvastatin and tunicamycin treatment through a dolichol-dependent mechanism.

As the presence of serum prohibits the uptake of exogenous dolichol (59), we used a panel of 5 breast cancer cell lines that can proliferate in serum-reduced media (Opti-MEM, Thermo Fisher) to test whether exogenous dolichol can functionally rescue statin-induced cell death. Immunoblot of E-cadherin and vimentin showed that MCF-7 and HCC1937 were epithelial, while MDA-MB-231, HCC1806, and BT549s acquired mesenchymal characteristics (Fig 3-4A). The mesenchymal cell lines were more sensitive to fluvastatin treatment, while the epithelial cell lines were more resistant, as indicated by the fluvastatin IC$_{50}$ in each cell line (Fig 3-4B). Consistently, fluvastatin treatment readily reduced cell viability in the mesenchymal breast cancer cell lines, but had no effect on the epithelial cell lines (Fig 3-4C). Exogenous addition of MVA, GGPP, or dolichol (dolichyl[C95]-PP) fully rescued the cell viability in the presence of fluvastatin (Fig 3-4C). The presence of serum abolished the ability of dolichol to rescue fluvastatin-induced reduction in cell viability (Fig 3-5A-B), which likely explains previous contradicting results (60-65). Taken together, these data suggest that breast cancer cells with mesenchymal characteristics are more sensitive to fluvastatin-induced cell death due to an increased dependency on dolichol-dependent protein N-glycosylation.
Figure 3-4. Inhibition of dolichol synthesis underlies fluvastatin sensitivity in mesenchymal breast cancer cell lines.

A, IB of E-cadherin, an epithelial cell marker, and vimentin, a mesenchymal cell marker, indicates that MCF-7 and HCC1937 cells are more epithelial, while MBA-MB-231, HCC1806, and BT549 cells are more mesenchymal. Ku80 is used as a loading control. B, fluvastatin
preferentially killed the more mesenchymal MDA-MB-231, HCC1806, and BT549 cells, compared to the more epithelial MCF-7 and HCC1937 cells. IC$_{50}$ values are calculated based on MTT assays after cells were treated with 8 doses of each drug for 72 h in Opti-MEM serum reduced media. Bars are mean + SD, n=3. C, MTT assays reveal that fluvastatin treatment for 72 h in Opti-MEM serum reduced media reduced cell viability in mesenchymal cells, but not in epithelial cell lines. Fluvastatin-induced reduction in cell viability was fully rescued by co-administration with MVA, GGPP, or dolichol (dolichyl[C95]-PP), at the indicated doses. Bars are mean + SD, n=3-4. *, p<0.05; **, p<0.01 (one-way ANOVA with a Dunnett post-test, comparing all columns vs. fluvastatin column).
Figure 3-5. Exogenous addition of dolichol (dolichyl[C95]-PP) did not rescue viability of cells with fluvastatin treatment in DMEM supplemented with 10% FBS.

Cell viability of MDA-MB-231 cells (A) or MCF-7 cells (B) were calculated based on MTT assays after cells were treated with 7-8 doses of fluvastatin for 72 h. Data points are mean ± SD, n=3.
3.3.3 Fluvastatin inhibits EMT-associated protein N-glycosylation

Using EGFR, GP130, and SLC3A2 as reporters of glycosylation status, we showed that proteins became under-glycosylated with fluvastatin or tunicamycin treatment, in both the vector control cells and the SNAIL-overexpressing EMT cells (Fig 3-1E). Why, then, are SNAIL-overexpressing cells more sensitive to fluvastatin- and tunicamycin-induced cell death? Since several studies have previously reported an aberrant expression of N-glycans during cancer cell EMT (66-70), we hypothesized that EMT leads to the upregulation of a subset of N-glycans, and are dependent on their expression for survival. To test this hypothesis, we quantified the total glycome of membrane proteins (71) in control and SNAIL-overexpressing cells by LC-MS/MS, both basally and after treatment with fluvastatin for 48 h (Table 3-I).

N-glycans share a common core region (Fig 3-6A, highlighted in box) and are classified based on the oligosaccharide composition in the antennae. The MCF10A membrane protein glycome consisted of 32% high mannose type N-glycans, containing exclusively mannose (M, Man) residues in the antennae (Fig 3-6A). Complex type N-glycans, characterized by alternating units of galactose (H, Gal) and N-acetylglucosamine (N, GlcNAc), represented a total of 59% of the MCF10A membrane protein glycome, which can be further subdivided by the degree of modification by the monosaccharide fucose (F, Fuc) (Fig 3-6A). In MCF10A cells, complex type N-glycans were commonly expressed in the unfucosylated and singly fucosylated (core) forms, with a small amount of doubly fucosylated (core and antennae) structures (Fig 3-6A). With induction of EMT, the expression of 12 N-glycans were significantly upregulated, all of which belonged to the complex type subgroup; and 15 N-glycans were downregulated, which included all 9 of the doubly fucosylated complex structures detected (Fig 3-6B, Table 3-I). This specific loss of doubly fucosylated complex type N-glycan structures in EMT has not been previously reported, and its underlying mechanism is unknown.
Table 3-I. Total membrane protein N-glycans in vector and SNAIL-overexpressing MCF10A cells with fluvastatin treatment.

<table>
<thead>
<tr>
<th>Glycan</th>
<th>Charge / ‘m/z’ (retention time)</th>
<th>Vector EtOH</th>
<th>Vector Fluva</th>
<th>SNAIL EtOH</th>
<th>SNAIL Fluva</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2M3+N2H2</td>
<td>2+/821.3034 (10-13)</td>
<td>7.945</td>
<td>8.570</td>
<td>8.357</td>
<td>7.860</td>
</tr>
<tr>
<td>N2FM3+N2H2</td>
<td>2+/894.3335 (11.2-15)</td>
<td>16.122</td>
<td>18.598</td>
<td>18.002</td>
<td>16.848</td>
</tr>
<tr>
<td>N2FM3+N2H2F</td>
<td>2+/967.36245 (10.5-14)</td>
<td>1.232</td>
<td>1.303</td>
<td>0.151</td>
<td>0.209</td>
</tr>
<tr>
<td>N2M3+N3H3</td>
<td>2+/1003.872 (10.5-14)</td>
<td>3.058</td>
<td>2.732</td>
<td>4.385</td>
<td>3.589</td>
</tr>
<tr>
<td>N2FM3+N3H3</td>
<td>2+/1076.8981 (12-15.5)</td>
<td>7.993</td>
<td>7.871</td>
<td>10.469</td>
<td>8.711</td>
</tr>
<tr>
<td>N2FM3+N3H3F</td>
<td>2+/1149.9352 (12-15.9)</td>
<td>0.991</td>
<td>1.065</td>
<td>0.147</td>
<td>0.225</td>
</tr>
<tr>
<td>N2FM3+N3H3F</td>
<td>3+/766.9568 (12-15.9)</td>
<td>0.289</td>
<td>0.326</td>
<td>0.023</td>
<td>0.047</td>
</tr>
<tr>
<td>N2M3+N4H4</td>
<td>2+/1186.4468 (12.3-15.5)</td>
<td>1.689</td>
<td>1.292</td>
<td>2.531</td>
<td>1.793</td>
</tr>
<tr>
<td>N2M3+N4H4</td>
<td>3+/791.6279 (12.3-15.5)</td>
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<td>N2FM3+N4H4</td>
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<td>N2FM3+N4H4</td>
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<td>5.107</td>
<td>4.306</td>
<td>5.848</td>
<td>4.193</td>
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<td>N2FM3+N4H4F</td>
<td>2+/1332.5011 (13.5-16.5)</td>
<td>0.511</td>
<td>0.518</td>
<td>0.046</td>
<td>0.071</td>
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<td>N2FM3+N4H4F</td>
<td>3+/888.9998 (13.5-16.5)</td>
<td>0.961</td>
<td>0.927</td>
<td>0.096</td>
<td>0.147</td>
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<td>2+/1369.0154 (13.5-16.5)</td>
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<td>N2M3+N5H5</td>
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<td>3+/1205.1132 (16-17.5)</td>
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Figure 3-6. Fluvastatin treatment decreases complex branched N-glycans associated with EMT.

A, schematic representation of high mannose type and complex type N-glycans, the two major classes of N-glycans (top), and distribution of major classes of N-glycans in the total cell surface glycome in MCF10A cells quantified by LC-MS/MS (bottom). B, heatmap of the expression of complex N-glycans following SNAIL-induced EMT. Data presented are the mean of 3 biological
replicates with 1-2 technical replicates each. *, q<0.0301 (unpaired, 2-tailed t test with Benjamini-Hochberg FDR correction). C, heatmap of the expression of complex N-glycans following treatment with 20 µM fluvastatin in vector cells (left column) and SNAIL-overexpressing cells (right column). Black arrowheads indicate glycan species that are significantly upregulated in EMT (B) and downregulated by fluvastatin treatment in SNAIL-overexpressing cells (C, right column), but not affected by fluvastatin treatment in control cells (C, left column). Data presented are the mean of 3 biological replicates with 1-2 technical replicates each. *, q<0.0108 (unpaired, 2-tailed t test with Benjamini-Hochberg FDR correction).

D-E, LC-MS/MS quantification of the β1,6-branched, tri-antennary (D) and tetra-antennary (E) N-glycans indicate that these N-glycan species are upregulated in EMT, which is inhibited by 20 µM fluvastatin treatment for 48 h. Bars are mean + SD, n=3. ns, not significant; *, p<0.05 (one-way ANOVA with a Bonferroni post-test, comparing selected pairs of columns). F, schematic representation of two mutually exclusive N-glycan branching pathways, catalyzed by different enzymes, recognized by different lectins, and with opposing effects on metastasis. G, IB of GnT-III (MGAT3) and GnT-V (MGAT5) showed that SNAIL-overexpressing cells upregulated GnT-V while GnT-III expression was unchanged, compared to vector control cells. Actin was used as a loading control. Representative images are shown, n=3. H, lectin blotting revealed that PHA-L ligand was upregulated in SNAIL-overexpressing cells, and was decreased by fluvastatin after 48 h. PHA-E ligand was expressed at comparable levels between the two cell lines and was not affected by fluvastatin treatment. Treatment with tunicamycin for 48 h was used as a positive control that decreased expression of both PHA-L and PHA-E ligands in both cell lines. Treatment with thapsigargin for 48 h was used as a negative control. Actin was used as a loading control. Representative images are shown, n=4.
We then examined the effect of fluvastatin treatment on N-glycan profiles. Our hypothesis predicts that fluvastatin treatment will inhibit the expression of N-glycans that are upregulated by EMT. Indeed, 6 of the 12 N-glycans upregulated following induction of EMT were specifically inhibited by fluvastatin treatment in SNAIL-overexpressing cells, but not control cells (Fig 3-6C, black arrowheads), suggesting that these N-glycans were important mediators of fluvastatin-induced cell death. Of these, the singly fucosylated tri-antennary (N2FM3+N3H3) and singly fucosylated tetra-antennary (N2FM3+N4H4) structures, each representing ~10% of the total surface glycome, remained unchanged with fluvastatin treatment in the vector control cells (Fig 3-6D-E). SNAIL-overexpressing cells upregulated the expression of these N-glycans, and this upregulation is blocked when cells were exposed to fluvastatin (Fig 3-6D-E).

The tri- and tetra-antennary complex type N-glycans contain a β1,6 glycosidic linkage (β1,6GlcNAc-branching) produced by the enzyme β1,6-N-acetylglucosaminyltransferase V (GnT-V; encoded by MGAT5) and recognized by the lectin phytohaemagglutinin-L (PHA-L; Fig 3-6F) (67, 72). β1,6GlcNAc-branched tri- and tetra-antennary N-glycans (PHA-L ligands) are known to promote metastasis (67, 72). As a control, we also examined the expression of β1,4-N-acetylglucosaminyltransferase III (GnT-III; encoded by MGAT3), which adds a bisecting GlcNAc to the trimannosyl core (PHA-E ligands), and has been suggested to have metastasis-suppressing activities (Fig 3-6F) (73). Consistent with the increase in the tri- and tetra-antennary complex type N-glycans with induction of EMT (Fig 3-6D-E), we showed that induction of EMT by SNAIL overexpression led to upregulation of GnT-V, while GnT-III expression remained unchanged (Fig 3-6G). SNAIL-overexpressing cells showed an upregulation of PHA-L ligands (74, 75) compared to the vector control (Fig 3-6H, compare lane 5 to lane 1). Following fluvastatin treatment, PHA-L binding is unaffected in vector cells (compare lane 2 to lane 1), but is decreased in SNAIL-overexpressing cells (compare lane 6 to lane 5). In contrast, PHA-E
binding (74, 75) was similar between SNAIL-overexpressing cells and vector control cells, and there was minimal effect of fluvastatin on PHA-E substrates (Fig 3-6H). Tunicamycin and thapsigargin were used as positive and negative controls, respectively, for inhibition of protein glycosylation (Fig 3-6H).

### 3.3.4 Fluvastatin impairs protein N-glycosylation in vivo

Our in vitro data indicate that fluvastatin treatment impairs the biosynthesis of dolichol, which is needed to support the expression of N-glycans associated with EMT and metastasis. To test this mechanism of fluvastatin action in vivo, we treated a xenograft model of the aggressive LM2-4 breast cancer cell line, a derivative of MDA-MB-231 cells that preferentially metastasizes to the mouse lung (16, 39), with PBS or 50 mg/kg/d fluvastatin. Since fluvastatin treatment delayed growth of the primary tumour (Fig 3-7A), we extended treatment time in the fluvastatin group so that the tumours harvested for further analyses were size-matched (Fig 3-7A). Fluvastatin was readily detected by HPLC-MS/MS in both the mouse serum (4.9 ± 0.3 µg/ml or 11.9 ± 0.9 µM) and the xenograft tissue (0.59 ± 0.08 µg/g tissue) (Fig 3-7B-C). To the best of our knowledge, this is the first report of fluvastatin detection in extrahepatic tumour tissue to support the model that statins can directly induce cancer cell death in vivo, independent from their systemic cholesterol-lowering and/or immunomodulatory activities.

To test whether fluvastatin can inhibit EMT-associated protein N-glycosylation in vivo, we performed lectin histochemistry (74, 76) using PHA-L, and showed that fluvastatin decreased the number of cells staining positive for PHA-L in Ki67-positive regions of tumour sections (Fig 3-7D-E). We confirmed that LM2-4 xenografts will readily metastasize to the mouse lung by identifying lung lesions that stained positive for human EGFR (hEGFR) (Fig 3-7F). In the PBS treated group, 3 out of 6 mice (50%) had one or more lung lesions (Fig 3-7G). In the fluvastatin
Figure 3-7. Fluvalstatin treatment decreases complex branched N-glycans *in vivo*.

A, fluvastatin delayed growth of the primary tumour. One million LM2-4 cells were subcutaneously implanted in SCID mice and treated with PBS or 50 mg/kg/d fluvastatin by oral gavage. Mice were sacrificed 6 days (PBS) or 12 days after treatment (fluvastatin) to ensure tumours from the two groups were size-matched for downstream analyses. Data points are mean ± SD, n=5-6. B-C, at time of sacrifice, mouse serum and a piece of tumour xenograft were flash-frozen in liquid N₂, and fluvastatin was extracted and quantified by HPLC-MS/MS. Data points are mean ± SD, n=4-6. D-E, lectin histochemistry of tumour xenografts indicated that PHA-L ligand in Ki67-positive tissue areas was decreased with fluvastatin treatment. Representative
images are shown, n=5. Scale bars = 50 μm. Data points are mean ± SD, n=5. **, p<0.01 (unpaired, two-tailed t test, comparing fluvastatin treatment vs. PBS control). F-G, fluvastatin treatment decreased proportion of mice with metastatic lesions in the lungs at the time of sacrifice. At time of sacrifice, mouse lungs were resected and FFPE. Two sequential slices were obtained every 200 μm for three depths containing all five lobes, and stained for H&E or hEGFR (F) Scale bars = 100 μm. Metastatic colonies were identified by hEGFR and confirmed by H&E. Each lung slice was independently reviewed by two personnel. Representative images are shown, n=5-6.
treated group, 1 out of 5 mice (20%) had hEGFR-positive metastatic lesions, providing the first indication that fluvastatin treatment can prevent breast cancer metastasis in this model (Fig 3-7G). Thus, fluvastatin treatment inhibits the expression of N-glycans associated with EMT and metastasis in vivo.

3.3.5 Post-surgical adjuvant fluvastatin treatment delays metastatic outgrowth and prolongs survival

To test the efficacy of fluvastatin treatment in the post-surgical adjuvant setting, we allowed LM2-4 xenografts to reach ~500 mm³, then surgically removed the primary tumours to mimic front-line treatment in breast cancer patients (16, 39). This experimental design also serves to uncouple the effect of fluvastatin on metastatic outgrowth from delay of metastasis due to inhibition of primary tumour growth. After surgery, mice were randomly assigned to receive PBS or 50 mg/kg/d fluvastatin orally (Fig 3-8A), mimicking a p.o./q.d. (per os/quaque die, by mouth/once a day) prescription. Adjuvant fluvastatin treatment significantly prolonged overall survival in this mouse model (Fig 3-8B).

To evaluate the potential anti-metastatic activity of fluvastatin, we analyzed lung samples at three time points during the course of the experiment: (i) at time of surgery; (ii) at 8-9 days post-surgery; and (iii) at endpoint (Fig 3-8A). At time of surgery, we confirmed that this model accurately represented early-stage breast cancer, as the majority of mice did not have any observable metastases, and 2/8 mice had very small lung lesions (Fig. 3-8C). At 8-9 days post-surgery, analysis of a small cohort of animals showed that adjuvant fluvastatin treatment effectively inhibited metastatic outgrowth from disseminated breast cancer cells after the primary tumour was removed (Fig 3-8D). Finally, at endpoint, we showed that fluvastatin treatment decreased the proportion of mice with heavy (>50 colonies per slice) or medium metastatic load (5-50 colonies per slice), while increasing the proportion of mice with light metastatic load
**Figure 3-8. Post-surgical adjuvant fluvastatin treatment delays metastasis and prolongs survival.**

A, schematic of the mouse model and the time points where mice were sacrificed. B, fluvastatin treatment at 50 mg/kg/d orally significantly prolonged survival of mice with post-surgical metastatic breast cancer. *, p<0.05 (Log-rank test, n=12). C-E, At the indicated time point, mice were sacrificed and lungs were resected for FFPE. Two sequential slices were obtained every 200 μm for three depths containing all five lobes, and stained for H&E or hEGFR. Metastatic foci were identified by hEGFR staining and confirmed by H&E. At time of surgery, mouse lungs
were clear of metastatic foci or had very small lesions (C). Scale bar = 20 μm. At 8-9 days post-surgery, mice receiving fluvastatin treatment had less metastatic tumour load than mice receiving PBS control (D). At endpoint, fluvastatin treatment decreased the proportion of mice with heavy (>50 colonies per slice) or intermediate metastatic load (5-50 colonies per slice). The proportion of mice with light metastatic load (<5 colonies per slice) were increased (E). Each lung slice was independently reviewed by two personnel. Representative images are shown. F-G, quantification of metastatic load by colony count (F) or by hEGFR positivity (G) both showed lowered metastatic load in fluvastatin-treated mice.
(<5 colonies per slice; Fig 3-8E-G). We have thus demonstrated using a post-surgical metastatic breast cancer model that closely follows the course of human disease, that adjuvant fluvastatin use can delay metastatic spread and prolong survival.

3.4 Discussion

Metastatic recurrence is the main cause of breast cancer deaths (1-3). Accumulating evidence supports a role for statins in reducing the risk of post-surgical breast cancer recurrence. A major obstacle to the repurposing of statins as anti-cancer drugs has been an incomplete understanding of their molecular mechanism of action. Statins were previously thought to induce apoptosis in cancer cells by inhibiting FPP and GGPP synthesis, thereby disrupting the membrane localization of RAS family members (23-26). However, mutations in RAS family members have been poor biomarkers of statin response (27-29), suggesting not only that an alternative mechanism is at play, but also that better biomarkers need to be developed to identify patients that will benefit from statin treatment. Here, we address this important gap by demonstrating that sensitivity to fluvastatin in the context of breast cancer cell EMT is mediated by depletion of dolichol, a class of metabolites downstream of FPP and GGPP, which functions as the essential lipid carrier of glycans prior to protein N-glycosylation (52, 53). Fluvastatin treatment impaired the upregulation of β1,6GlcNAc-branched tri- and tetra-antennary N-glycans associated with breast cancer EMT and metastasis (66-70), both in cell culture and in vivo. Finally, using a clinically relevant model of metastatic recurrence, we demonstrate that adjuvant use of fluvastatin delayed breast cancer metastasis and prolonged survival. Our results support that the statin family of inexpensive and well-tolerated drugs could be repurposed as adjuvant therapeutics, and suggest the development of novel therapeutics to prevent metastatic recurrence in breast cancer by targeting aberrant protein N-glycosylation.
Activation of EMT is proposed to be a key component in cancer cell dissemination and metastatic dormancy (30). Altered protein N-glycosylation, and notably the upregulation of GnT-V (MGAT5), is pivotal to EMT (70, 77, 78). The tri- and tetra-antennary β1,6GlcNAc-branched N-glycans produced by GnT-V are potent modulators of metastatic potential (35, 36).

Consistently, high levels of tri- and tetra-antennary complex N-glycans are associated with disease progression and poor prognosis in breast and colon cancer patients (37, 38). Importantly, reducing the glycan structures produced by GnT-V is known to suppress cancer metastasis and EMT. In polyomavirus middle T transgenic mice (MMTV-PyMT), an Mgat5-deficient background markedly suppressed breast cancer growth and metastasis (36). Mgat5-deficient PyMT tumour cells displayed a block to EMT and reduced sensitivity to growth factors that were both rescued by transfection with Mgat5 (70). Here we demonstrated that the aberrant upregulation of N-glycan structures caused by increased expression of GnT-V during EMT is dependent on dolichol availability to support protein N-glycosylation in the ER. The assembly of dolichol-GlcNAc2Man9Glc3, the donor substrate for N-glycosylation, occurs in two phases (79): phase I takes place in the cytoplasmic face of the ER, producing dolichol-GlcNAc2Man5 using dolichol, UDP-GlcNAc, and GDP-Man; and phase II occurs in the ER lumen and requires dolichol-Man (4 molecules) and dolichol-Glc (3 molecules) as substrates. Thus, the assembly of each molecule of dolichol-GlcNAc2Man9Glc3 requires 8 dolichol molecules, and the observation that dolichol cannot be efficiently recycled (80, 81) indicates that cells must continuously synthesize dolichol through the MVA pathway to meet this demand. Indeed, dolichol-GlcNAc2Man9Glc3 levels can become limiting during nutrient deprivation (82), or conversely in rapidly dividing cancer cells. Additionally, approximately 30% of the transcriptome in vertebrates are N-glycosylated at one or more sites in the ER, before transiting into the Golgi where the N-glycans are remodelled by the branching pathway (79). The N-glycan branching
enzymes Gnt-I to Gnt-V (MGAT1 to MGAT5) all compete for available substrates, UDP-GlcNAc (69) and N-glycan structures that are to be remodeled (79), with a ~300-fold stepwise decline in substrate affinity (69) moving from Gnt-I to Gnt-V. Consistently, we observed a proportional reduction in bi-, tri-, and tetra-antennary glycan structures (7.3%, 16.8%, and 30.0%; Fig 3-6C, Table 3-I) with fluvastatin treatment in SNAIL-overexpressing cells. Thus, our results here suggest that breast cancer cells that have undergone EMT display increased tri- and tetra-antennary glycan structures mediated by Gnt-V, that must be supported by synthesis of dolichol and assembly of dolichol-GlcNAc2Man9Glc3 in the ER. This vulnerability is exploited at the level of MVA pathway inhibition by fluvastatin treatment, in turn impacting the Golgi N-glycan branching pathway (Fig 3-9).

Two prospective window-of-opportunity clinical trials have been completed to evaluate the impact of statin use as a neo-adjuvant therapeutic in breast cancer patients (83, 84). Comparing tumour samples at biopsy and at surgery, both studies reported a decrease in Ki67 staining after atorvastatin (83) or fluvastatin (84) treatment, indicating that statins decreased tumour cell proliferation, and supporting that statins should be repurposed to treat breast cancer. Here, we have carefully chosen a mouse model of post-surgical metastatic breast cancer that closely mimics front-line treatment and disease progression (16, 39), to test the efficacy of fluvastatin when used in the adjuvant setting to prevent metastasis, where long-term use of this safe and inexpensive drug will likely have clinical benefit. Fluvastatin treatment in this therapeutic space effectively delayed metastasis and prolonged survival at a daily dose of 50 mg/kg in the mouse, equivalent to a well-tolerated daily dose of 4 mg/kg in human patients for the management of primary hypercholesterolemia (85). This study was also the first to measure the concentration of fluvastatin in the mouse serum and xenograft tissue, with a treatment regimen and route closely mimicking what would be prescribed to human patients (p.o./q.d.). A serum concentration
Figure 3-9. Proposed mechanism of statin-mediated attenuation of breast cancer metastasis by targeting EMT-associated protein N-glycosylation.

Breast cancer cells disseminate from the primary tumour by undergoing EMT, whereby they lose cell-cell interactions, degrade and invade through the basement membrane, enter the circulatory system, and ultimately give rise to metastatic outgrowth in a secondary site. Breast cancer cells that have undergone EMT are characterized by an upregulation of tri- and tetra-antennary complex type N-glycans, whose expression is dependent on dolichol synthesis via the MVA pathway, making them more sensitive to fluvastatin-induced cell death. Thus fluvastatin may have clinical impact if prescribed in the adjuvant therapeutic space to prevent metastatic recurrence of breast cancer.
of 4.9 μg/ml (11.9 μM) fluvastatin was achieved in our mouse experiments (Fig 3-7B). In comparison, in human volunteers, up to 7 μM fluvastatin was achieved when treated at 1 mg/kg/d (86) and up to 12.3 μM lovastatin, another statin family member, was achievable when prescribed at 10 mg/kg/d (87). Additionally, a fluvastatin concentration of 586.2 ng/g tissue was measured in the tumour xenograft (Fig 3-7C). Assuming a tissue density of 1 g/ml, this is equivalent to a fluvastatin concentration of approximately 1.4 μM, proving that fluvastatin can reach extrahepatic tumour tissues to exert direct anti-cancer effects. Together these results warrant the immediate clinical evaluation of fluvastatin at this well-tolerated dose in the adjuvant setting in breast cancer patients.

3.5 Materials and Methods

3.5.1 Reagents

Fluvastatin was purchased from US Biologicals (F5277-76). TGF-β was purchased from PeproTech (100-21). PNGase F was purchased from NEB (P0704). cOmplete protease inhibitor was purchased from Roche (11697498001). RapiGest SF was purchased from Waters (186001861). Sialidase was purchased from Glyko (GK80040). All other chemicals were purchased from Sigma unless otherwise specified.

3.5.2 Cell culture

MCF10A cells were a kind gift of Dr. Senthil Muthuswamy. MDA-MB-231 and LM 2-4 cells were a kind gift of Dr. Robert Kerbel. BT549, HCC1806, HCC1937, MCF-7, MCF10A, MDA-MB-231, LM2-4, and 293Tv cells were cultured as previously described (8, 10, 16, 39, 88). Transgene expression was stably introduced into MCF10A cells using retroviral insertion with pLPC, a kind gift of Dr. Roberta Maestro, or pBabePuro (89). Cells were imaged on a Leica Stereomicroscope (Leica MZ FLIII).
3.5.3 MTT assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed as previously described [6]. Cells were seeded at 750-5,000 cells/well in 96-well plates and treated in triplicate with 8 doses of drugs or the solvent control for 72 h, either in growth media or in Opti-MEM serum reduced media as indicated. IC$_{50}$ values were computed using GraphPad Prism with a bottom constraint equal to 0.

3.5.4 Immunoblotting

Lysates were prepared by lysing directly in boiling SDS lysis buffer (1% SDS, 11% glycerol, 10% β-mercaptoethanol, 0.1 M Tris pH 6.8). The following antibodies were used for detection: c-MYC (MAb 9E10, in-house), E-Cadherin (CST 3195), vimentin (CST 5741), fibronectin (Abcam ab32419), actin (Sigma A2066), tubulin (Millipore CP06), GP130 (SCB sc-655), EGFR (CST 2232), SLC3A2 (SCB sc-7095), Ku80 (CST 2180), GnT-III (Thermo PA5-12156), GnT-V (Thermo MA5-24325), PHA-L (EY Labs H-1801-1), PHA-E (EY labs H-1802-1).

3.5.5 Immunohistochemistry

Tissue samples were formalin-fixed and paraffin-embedded by standard protocols. For tumours, three sequential slices were stained for H&E, Ki67 (Novus NB110-90592), or biotin-PHA-L (EY Labs BA-1801-2). The number of PHA-L positive cells were counted at 20x magnification in Ki67-positive regions with a minimum of four views per slice. For lungs, two sequential slices were obtained every 200 μm for three depths containing all five lobes, and stained for H&E or hEGFR (Zymed 28005). Metastatic colonies were identified by hEGFR staining and confirmed by H&E. Each lobe was individually outlined and total hEGFR positivity was computed using ImageScope.
3.5.6 Cell death assay

Cells were seeded at 250,000/plate overnight, then treated with as indicated for 72 h. Cells were fixed in 70% ethanol overnight, stained with propidium iodide (Sigma), and analyzed for the sub-diploid DNA (“pre-G1”) population as previously described (8).

3.5.7 qRT-PCR

Total RNA was harvested from subconfluent cells using TIRzol Reagent (Invitrogen). cDNA was synthesized from 500 ng of RNA using SuperScript III (Invitrogen). Real-time quantitative RT-PCR was performed using SYBR Green (Applied Biosystems) with the following primers:

BiP fw 3’- TGACATTGAAGACTTCAAAGCT-5’

BiP rv 3’- CTGCTGTATCCTCTTCACCAGT-5’

ERdj4 fw 3’- AAAATAAGAGCCCGGATGCT-5’

ERdj4 rv 3’- CGCTTCTTGGATCCAGTGTT-5’

18S_rRNA fw 5’-GTAACCCGTTGAACCCCATT-3’

18S_rRNA rv 3’-CCATCCAATCGGTAGTAGCG-3’

3.5.8 N-glycan extraction

A total of 15 million cells were seeded overnight and treated as indicated for 48 h. Cells were harvested, suspended 1 mL of HEPES homogenization buffer (0.25 M sucrose, 50 mM HEPES pH 7.5, 5 mM NaF, 5 mM EDTA, 2 mM DTT, cOmplete protease inhibitor), and lysed using a probe sonicator. Homogenate was cleared at 2,000 xg for 20 min at 4°C, then ultracentrifuged at 115,000 xg for 70 min at 4°C. The pellet was vigorously suspended in 650 μL Tris buffer (0.8% Triton X-114, 50 mM Tris pH 7.5, 0.1 mM NaCl, 5 mM EDTA, 5 mM NaF, 2 mM DTT,
cOmplete protease inhibitor). The homogenate was chilled on ice for 10 min, incubated at 37℃ for 20 min, then phase partitioned at 1,950 xg for 2 min at room temperature. The upper phase was discarded. Membrane proteins in the lower phase was precipitated with 1 mL acetone at -20℃ overnight.

Precipitated proteins were suspended in 60 μL of suspension buffer (0.25% RapiGest SF, 50 mM ammonium bicarbonate, 5 mM DTT). The completely dissolved solution was heated for 3 min at 85℃. Approximately 30 μg proteins was mixed with 0.5 μL of PNGase F, 0.7 μL of sialidase, and 20 μL of 50 mM ammonium bicarbonate, and incubated at 42℃ for 2 h followed by 37℃ overnight. Released N-glycans were extracted with 4-5 volumes of 100% ethanol at -80℃ for 2 hours. The supernatant containing released N-glycans was speed vacuumed to dry.

Home-made porous graphitized carbon (PGC) microtips containing 10 mg PGC in a bed volume of 50 μL was washed with 500 μL of ddH₂O, 500 μL of 80% acetonitrile (ACN), and equilibrated with 500 μL 0.1% trifluoroacetic acid (TFA). N-glycan pellets were dissolved in 50 μL of 0.1% TFA and slowly loaded into microtips at a flow rate of ~100 μL/min. Microtips were washed with 500 μL 0.1% TFA. N-glycans were eluted several times with 500 μL of elution buffer (0.05% TFA, 40% ACN). The eluted N-glycans were speed vacuumed to dry.

3.5.9 Total glycan analysis by LC-MS/MS

Analysis of the eluted N-glycans was modified from a previous method [34]. Total glycan samples were applied to a nano-HPLC Chip using a Agilent 1200 series microwell-plate autosampler, and interface with a Agilent 6550 Q-TOF MS (Agilent Technologies, Inc., Santa Clara, CA). The HPLC Chip (glycan Chip) had a 40 nL enrichment column and a 75 μm x 43mm separation column packed with 5 μm graphitized carbon as stationary phase. The mobile phase was 0.1% formic acid in water (v/v) as solvent A, and 0.1% formic acid in ACN (v/v) as solvent
B. The flow rate at 0.3 μL/min with gradient schedule; 5% B (0–1 min); 5–20% B (1–15 min); 20–70% B (15–16 min); 70% B (16–19 min) and 70–5% B (19–20 min). PNGase F released free glycan was identified by Agilent Masshunter Quanlititive Analysis software by the presence of hexose and N-acetylhexosamine. Glycan structures were predict by online GlycoMod (http://web.expasy.org/glycomod/). Agilent Masshunter Quantitative Analysis software was used to quantify the extracted glycan peaks.

3.5.10 Animal models

Animal work was carried out with the approval of the Princess Margaret Hospital ethics review board in accordance to the regulations of the Canadian Council on Animal Care. LM2-4 cells (1 million cells in 50 μL) were implanted subcutaneously in female SCID mice (6-8 wks), obtained in-house from the University Health Network animal colony. Primary tumours were measured every two days and calculated by (width x width x length)/2. After surgical removal of the primary tumours, animals were monitored daily for endpoint, including signs of metastatic load in the lung (laboured breathing). Treatment was given daily orally with PBS or 50 mg/kg/d fluvastatin. Necropsy was performed at endpoint where any tissues with evidence of metastatic disease is rapidly excised and fixed in formalin for histopathology.

3.5.11 Fluvastatin quantification by HPLC-MS/MS

Fluvastatin concentrations were determined by a modified HPLC-MS/MS method with atorvastatin as the internal standard (IS). Mouse serum or xenograft tissue were incubated with methyl tert-butyl ether for 30 min followed by centrifugation at 3000 rpm for 30 min. The supernatant was separated, dried at room temperature, and reconstituted in methanol/water (1:1). The HPLC system consisted of a Shimadzu LC-20AD pump and a Shimadzu LC-20 AC autosampler (Shimadzu Corporation, Columbia, MD). The column used was a Phenomex
hyperclone BDS C18 column (50 × 2.0mm, 5 µm, Torrance, CA). The binary mobile phase consisted of mobile phase A: 5 mM ammonium acetate in water and mobile phase B: 5 mM ammonium acetate in acetonitrile. The gradient conditions for the mobile phase were as follows: 0.0-1.0 min, 20-100% B; 1.0-3.0 min, 100% B; 3.0-3.2 min, 100-10% B; 3.2-6.0 min, 20% B. The flow rate was 0.5 ml/min. The HPLC system was interfaced to an Applied Biosystem MDS Sciex triple quadrupole mass spectrometer (API 3200) (Applied Biosystems, Foster City, CA) operating in the negative electrospray ionization mode. For multiple-reaction monitoring, the transitions monitored were \textit{m/z} 410.3 to 209.9 for fluvastatin, and \textit{m/z} 557.0 to 278.1 for the IS (atorvastatin). Data collection, peak integration, and calculation were performed using Applied Biosystem MDS Analyst 1.4.2 software.
3.6 References


Chapter 4
Discussion
4 Discussion

4.1 Challenges in the treatment of cancer

The ultimate goal of non-palliative cancer treatment strategies is to completely eradicate tumour cells. Several challenges exist that limit the chemotherapy treatment modality from achieving this goal, with or without concomitant surgery and/or radiotherapy. Major clinical challenges include intrinsic and acquired resistance, excessive toxicities, and issues with dosing. Resistance to targeted chemotherapies arises from complex intra-tumoural heterogeneity (1, 2), and an unanticipated large number of mutations capable of participating in oncogenic transformation (3, 4). As the majority of cancer-initiating mutations occur in regulators of core cellular processes (cell fate, cell survival, and genome maintenance), the use of targeted chemotherapeutics is often associated with unexpected tissue-specific toxicities (5, 6). Additionally, effectiveness of chemotherapies can be greatly compromised by underdosing, an issue that is often overlooked. Despite consistent evidence demonstrating that most elderly patients can both tolerate and benefit from standard chemotherapy regimens (7-10), fear of excessive toxicity in elderly patients remains a major contributor to dose reduction by >15% relative dose-intensity (RDI), in up to 40% of cancer patients (11, 12). The second population that is consistently undertreated is obese patients. This is because the dose of chemotherapeutics is calculated based on body surface area (BSA), but many oncologists either use “ideal”, rather than the actual body weight of the patient, to calculate BSA, or cap BSA at 2.0cm² (13, 14). This leads to systematic underdosing of chemotherapy among overweight and obese patients (13), which could be contributing to the poorer outcomes often observed in this patient population (15, 16).

In addition to clinical challenges discussed above, several administrative difficulties could also undermine the effectiveness of chemotherapy, including access to care (eg. for intravenously
administered therapeutics), nonadherence (eg. due to poor patient education), and prohibitive cost (17). These clinical and administrative challenges are further compounded by aggressive screening and treatment practices (18), leading to an estimated 25% of mammographically detected breast cancers to be over-diagnosis (19), and an estimated 60% breast cancer cases to be over-treated with chemotherapy (20-22).

To address these challenges, significant effort has been put toward advancement of precision medicine, to account for individual variability in genomics, lifestyle, and environment of each person (23, 24). From the therapeutics perspective, precision medicine requires in-depth understanding of the efficacy, safety, mechanism of action, and biomarkers of response, for each drug used alone or in combination with others. In other words, to maximize therapeutic efficacy and minimize overtreatment, it is imperative to understand what tumours to treat with what drug(s), at what time, and why. In line with these overarching questions, this thesis provides novel insights to further our understanding of the anti-breast cancer effects of the statin family of drugs, described below.

4.2 Mechanism of statin-induced cancer cell death

The previous model of statin-induced cancer cell death is that statins inhibit the biosynthesis of FPP and GGPP, thus limiting protein prenylation of the RAS superfamily (25, 26). In Chapter 2 of this thesis, we addressed the on-going discrepancies in this prenylation model of statin sensitivity, by building an experimental system that directly tests the association between demand for GGPP and FPP for protein prenylation, and sensitivity to fluvastatin. We showed that fluvastatin induces cancer cell death through inhibition of GGPP synthesis, but independently from inhibition of protein prenylation, thus suggesting that an alternative process downstream of GGPP underlines the mechanism of statin-induced cancer cell death.
In Chapter 3, we further delineated this GGPP-involving alternative process to be the biosynthesis of dolichol, a large isoprenoid end product of the MVA pathway that is functionally important for the co-translational $N$-glycosylation of nascent peptides. In particular, breast cancer cells that have undergone EMT become dependent on the dolichol-mediated expression of a class of EMT-associated $N$-glycans, leading to a vulnerability that can be exploited by statin treatment. Together, these data provide an explanation to why RAS superfamily proteins have been poor biomarkers of statin sensitivity in epidemiological, pre-clinical, and clinical studies, and suggest that glycan and glycoproteins biomarkers may better predict statin response, discussed below.

### 4.3 Biomarkers of statin sensitivity

Increased sensitivity to statin-induced cancer cell death has previously been associated with ER$\alpha$ status (27-29), activation of RAS family members (27, 30-32), p53 mutations (33, 34), MVA pathway gene expression (28, 35), and a 10-gene candidate signature derived from a panel of breast cancer cell lines with a heterogeneous response to statin treatment (28). In Chapter 2 of this thesis, we identified a gene-expression based biomarker of statin sensitivity, which exploits the bi-modal distribution of five core EMT-associated genes, namely $CDH1$, $CDH2$, $FNI$, $VIM$, and $ZEB1$. The selective use of bi-modally distributed genes allowed us to calculate biologically meaningful cut-off values for the binning of disease subsets (in this case, statin-sensitive and -insensitive cancer cell lines), which is superior to the traditional use of expression median as an arbitrary cut-off in genes with Gaussian expression (36, 37). However, the rationale behind EMT markers as predictors of statin sensitivity remains poorly understood, and will be an intriguing area of research to be further explored.
In Chapter 3, sensitivity to fluvastatin is shown to be associated with the EMT-associated β1,6-branched, tri- and tetra-antennary, unfucosylated and singly fucosylated complex glycans. These biomarkers can be collectively recognized by the lectin PHA-L, which we have shown to be readily adaptable for histochemical assays. This is particularly important, as histochemistry is within the capability of most cancer diagnostic labs, as opposed to assays based on gene expression and/or sequencing. Thus, once validated, histochemical PHA-L staining of a biopsy specimen may be rapidly advanced into the clinic. Identification of the glycoprotein biomarker may further improve the sensitivity and specificity of these assays.

4.4 Point of therapeutic intervention for statin use in breast cancer

Current pre-clinical models of breast cancer fall into four basic categories: (i) cell lines and traditional xenografts; (ii) patient-derived xenograft and organoids (PDXs and PDOs); (iii) transgenic animal models; and (iv) infusion models of metastatic breast cancer (Fig 4-1). Pre-clinical testing of drug sensitivities, including statins, are therefore limited to the points of therapeutic intervention represented by these models: primarily neo-adjuvant, preventative, and metastatic settings (Fig 4-1). However, our data on the association of breast cancer cell EMT and statin sensitivity indicate that statin use will be effective as an adjuvant therapeutic. We have thus carefully chosen a post-surgical model of breast cancer metastasis that mimics the patient treatment experience, and showed that fluvastatin treatment effectively delayed breast cancer metastasis and improved survival. Statins are particularly suitable for use in the adjuvant setting, due to their superior safety profiles and cost-effectiveness, thus providing the greatest clinical benefit for the treatment of breast cancer.

Additional considerations for the pre-clinical to clinical translation of statins lie in the question
Figure 4-1. Pre-clinical models of the points of therapeutic intervention in breast cancer.

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<th>Pre-clinical model</th>
<th>Point of therapeutic intervention</th>
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<td>Cell lines and traditional xenografts</td>
<td>Neo-adjuvant</td>
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<tr>
<td>Patient-derived xenografts and organoids (PDXs and PDOs)</td>
<td>Neo-adjuvant</td>
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<td>Transgenic animals</td>
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<td>Infusion models of metastasis</td>
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<td>Post-surgical model of metastasis</td>
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of which statin to prescribe to patients, and at what dose. Studies consistently show that lipophilic statins outperform hydrophilic statins in their anti-cancer activities, due to increased systemic circulation and decreased hepatoselectiveness (40). We and others have shown that some statins have additional bioactivities independent from inhibition of HMGCR (41, 42, 79). This thesis has used exclusively fluvastatin for all biological studies, which was chosen based on superior pharmacological characteristics including high plasma concentration, and metabolism through a CYP3A4-independent mechanism (43, 44). In Chapter 2, our analysis of >700 cell lines for their sensitivity to three statin family members indicate that our observations with fluvastatin sensitivity can be extended to at least two other lipophilic statins, lovastatin and simvastatin. In Chapter 3, we further characterized the association between fluvastatin dose, serum concentration, and concentration in the xenografts tissue. We demonstrated that an equivalent dose of 4 mg/kg/d in humans is correlated to a plasma concentration of 4.9 μg/ml (11.9 μM) fluvastatin, and a concentration of 586.2 ng/g reaching the tumour xenograft. Pending clinical validation, these data suggest that fluvastatin should be used at the maximally tolerated dose of 4 mg/kg/d (45), rather than the cholesterol-lowering dose (approximately 1 mg/kg/d), to effectively delay breast cancer metastasis and impact patient care. Alternatively, in a truly personalized treatment model, fluvastatin could be dose-escalated on an individual-by-individual basis until a stable plasma concentration of 11.9 μM is reached without major safety concerns.

4.5 Perspectives

4.5.1 Limitations of this work and future directions

The role of EMT in cancer progression. The bulk of the cell culture experiments performed in Chapters 2 and 3 of this thesis use EMT as a surrogate indicator of the metastatic capability of breast cancer cells. However, the role of EMT in cancer progression, and particularly in cancer
metastasis, is hotly debated. EMT was proposed to be a prerequisite for cancer cell metastasis in order to explain why, in carcinomas bound by epithelial characteristics such as tight junctions and desmosomes, increasingly invasive cells could dissolve underlying basement membranes, invade adjacent stromal compartments, and eventually intravasate into the draining lymph nodes and/or systemic circulation (46). However, to date accumulating data disagree on whether the acquisition of these malignant traits is a required series of events for tumour progression, or an accidental consequence thereof (46-51). Moreover, metastatic outgrowths tend to exhibit epithelial phenotypes (46, 51). Proponents of the EMT model of cancer metastasis suggest that cells that have escaped the primary tumour site must have a mechanism to regain epithelial characteristics, a process termed mesenchymal-to-epithelial transition (MET). However, experimental evidence of MET following EMT has been limited (46, 51).

Cancer cell EMT has also been shown to share characteristics with cancer cell hypoxia, therapeutic resistance, dormancy, and various definitions of stemness (46, 51-53). Again, it is unknown if EMT is a prerequisite or a byproduct of these aggressive phenotypes, or if all of these phenotypes (including EMT) are functionally equivalent, and if so, to what degree (52-55).

In Chapter 3, we complemented our cell culture approach with a clinically relevant in vivo model of post-surgical breast cancer metastasis, and showed that fluvastatin can delay breast cancer metastasis when used in the adjuvant setting. The use of additional in vivo models of breast cancer metastasis independent of EMT will be necessary to address whether the anti-breast cancer effects of statins are specific to cancer cell EMT, or to cancer metastasis in general. Examples of such a model include the transgenic breast cancer lineage-tracing models developed by Fischer et al. (49), which crosses the highly metastatic MMTV-PyMT or MMTV-Neu with Vim-Cre or Fsp1-Cre mesenchymal-specific reporters, or the model developed by Zheng et al. (50), where EMT-inducers Twist1 or Snail1 were deleted in the KPC transgenic mouse model of
PDAC. In both models, cancer cell EMT was not required for metastasis, but mediated resistance to common chemotherapeutics (49, 50). Characterizing the fluvastatin sensitivity of these cells will significantly impact the translation of statins into the cancer clinic.

**Glycans and glycoproteins as cancer biomarkers.** Glycosylation of proteins occur on an estimated 50% of total proteins, and up to 80% in some glycoprotein-rich fluids (56). As most of the FDA-approved cancer biomarkers have been proteins derived from the serum (57), it is not surprising that the majority of these biomarkers are glycosylated. Importantly, for many of these protein biomarkers, different glycoforms are expressed in normal and in tumour cells, which could provide a valuable biochemical tool for diagnosis. For example, the AFP-L3% assay exploits the specific expression of the L3 glycoform of AFP in HCC, in order to differentiate between patients with HCC or chronic liver disease, which specifically express the L1 glycoform (38, 39). Similarly, glycosylation of PSA secreted by the prostate tumour cell line LNCaP is significantly different from PSA present in the normal seminal plasma (58), and glycosylation of human pancreatic ribonuclease 1 (RNase 1) isolated from healthy pancreas and pancreatic cancer cell lines are distinct (59).

In Chapter 3 of this thesis, we assessed global glycosylation changes in breast cancer cells after fluvastatin treatment using MS-based assays and PHA-L binding assays, both in tissue culture and in tumour xenografts. Although this was immensely useful and allowed the identification of β1,6-branched, tri- and tetra-antennary, unfucosylated and singly fucosylated complex N-glycans as biomarkers of fluvastatin response, the drawback of these approaches is that the glycan cannot be traced back to the glycoprotein and the glycosylation site of origin. Unfortunately, identification of glycopeptides is challenged by several technical difficulties, such as identification of monosaccharides with the same mass, low efficiencies in glycopeptide
fragmentation, and poor algorithms to identify glycopeptide spectra (56, 60). Nevertheless, progress is being made to address all of these limitations, and several work-arounds exist to obtain glycosylation information from intact proteins (top-down approaches), and to identify peptides after enrichment for a glycan moiety (bottom-up approaches) (56, 61-63).

An immediate next step to follow up on the identification of glycoproteins affected by fluvastatin treatment is to identify the site(s) of glycosylation where occupancy is reduced by fluvastatin treatment. This analysis involves releasing N-glycans using PNGase F, which deaminates the glycosylated asparagine residues (N) to aspartic acid (D), leading to a difference in mass by 0.984Da that can be distinguished by MS (64). More sophisticated methods, if needed, include glycan-tagging through click chemistry reactions between azide-labeled monosaccharides and dibenzocyclooctyne group (DBCO)-linked tags (usually biotin), which allows for both identification of glycosylated peptides, and their quantification if combined with SILAC (stable isotope labeling with amino acids in cell culture) (65, 66). Validation of the identified glycoprotein biomarkers of fluvastatin response will be prioritized by cross-referencing with known breast cancer-associated antigens that are reactive to PHA-L (67), and performed by immunoblotting and IHC on cell lysates and FFPE samples of tumour xenografts. Knockdown and overexpression of validated biomarkers may further provide an indication of their functional relevance to statin-induced cancer cell death.

**Combination strategies.** Prescribing cancer chemotherapeutics in rationally identified combination partners could have multiple benefits, from improving therapeutic efficacy to potentially preventing drug resistance. Statins has been combined with several currently available chemotherapies and targeted agents, demonstrating both synergism (41, 68-71) and improved safety profiles *in vivo* (70, 72-75). As statins are unlikely to replace the current
standard of care, a major limitation in the studies described in Chapter 3 of this thesis is that the anti-breast cancer effect of fluvastatin treatment was not tested in combination with chemotherapeutics currently prescribed in the adjuvant setting. We are immediately equipped to address this question using the same model of post-surgical metastatic breast cancer, or using the transgenic breast cancer models described above. Importantly, it should be noted that in both the post-surgical model and the transgenic models, classic chemotherapeutics treatment strategies were ineffective at reducing tumour burden or prolonging survival (49, 76-78), although metronomic delivery of these chemotherapeutics, alone or in combination with each other, were effective in the post-surgical model (76-78). This is in line with the epidemiology of breast cancers, where approximately 20% of patients receiving surgery followed by adjuvant chemotherapy and/or radiotherapy have experienced recurrence, despite aggressive treatment. Demonstrating the efficacy and safety of adjuvant statin use in combination with current standard-of-care chemotherapeutics will have immediate impact on patient care.

4.5.2 Anticipated impact

In this thesis, we have provided strong pre-clinical evidence for the repurposing of the statin family of drugs as anti-metastatic breast cancer chemotherapeutics. We have resolved a key discrepancy in the mechanism of action for the anti-cancer effects of statins, which is critical for the interpretation of epidemiological and pre-clinical studies where efficacy of statins was poorly associated with previously proposed biomarker(s). We have identified two new biomarkers of statin sensitivity, one based on gene expression and one based on glycan presentation, and demonstrated that statin use as an adjuvant therapeutic effectively delayed breast cancer metastasis. Statins are readily available, well-tolerated, and affordable drugs that can be rapidly repurposed into the breast cancer clinic. This body of work provides new insights on the efficacy, mechanism of action, and biomarkers of statin sensitivity for this purpose. In the era of
personalized medicine, these insights will facilitate the design of clinical trials to evaluate statins as anti-breast cancer agents, and ultimately to impact patient care.
4.6 References


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157


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Appendix
Positive feedback regulation of the mevalonate pathway

Contributions: The work presented in this chapter was performed by the author.
This project was completed with supervisory support from Dr. Linda Z. Penn.
5 Appendix – Positive feedback regulation of the mevalonate pathway

5.1 Introduction

The mevalonate (MVA) pathway is regulated by a robust negative feedback system (Fig 5-1A) that was first described in 1933 (1). In this landmark study, mice placed in sealed bottles did not synthesize cholesterol when their diet contained cholesterol, but began synthesizing it \textit{de novo} when their diet was cholesterol-free (1). Later it was elucidated that, when cholesterol levels are low, cells not only increase the expression of MVA pathway enzymes (eg. HMGCR and HMGCS1) to increase cholesterol synthesis (Fig 5-1A), but also upregulate LDLR to increase cholesterol uptake from the extracellular milieu (2-4). This negative feedback loop is exploited by the statins family of drugs, which blocks \textit{de novo} cholesterol synthesis by inhibiting HMGCR (5, 6). In response to a falling rate of cholesterol synthesis, cells increase the expression of LDLR, leading to a decrease in LDL-cholesterol in the plasma (2, 7). This prevents build-up of atherosclerotic plaques in the arteries, a major risk factor for cardiovascular diseases (8).

Negative feedback loops are widely used by biological systems to maintain homeostasis (9, 10). For example, each non-essential amino acid can allosterically inhibit an enzyme in its biosynthetic pathway, often the rate-limiting one, at high concentration (11). When the concentration of an amino acid is low, this inhibition is lifted, and cells begin synthesizing more of the amino acid (11). Negative feedback can also occur at the level of transcription, such as increased expression of System A transporters during amino acid starvation (12, 13), and of GLUT1 when glucose concentrations are low (14). Similarly, the negative feedback loop of the MVA pathway is also regulated at the level of gene transcription, mediated by the transcription factor SREBP2 (7) (Fig 5-1A). When cholesterol levels are high, SREBP2 is held in a latent form in the ER. When cholesterol levels are low, SREBP2 is activated by protein cleavage,
Figure 5-1. Two-component mechanisms of feedback regulation.

A, schematic of a negative feedback loop. The MVA pathway is regulated by a negative feedback loop. B, schematic of a positive feedback loop consisting of double-negative regulation. The bacterial lac operon is regulated by this mechanism. C, schematic of a true positive feedback loop. Cancer cells are known to regulate glycolysis through positive feedback.
translocates into the nucleus, and induces transcription of MVA pathway genes as well as LDLR (2, 7).

In contrast to negative feedback, positive feedback occurs when a deviation from homeostasis is further amplified by the control system (Fig 5-1B, C). Positive feedback commonly functions as a cascading mechanism such as in cell signaling (15, 16), and as a transcription network motif where a transcription factor upregulates its own expression (17). Positive feedback loops are uncommon in metabolism under normal conditions, but an example can be found in the bacterial lac operon, where availability of lactose induces the expression of the lactose transporter lacY, as well as enzymes to metabolize it (lacZ and lacA) (18). This positive feedback is mediated through a double-negative feedback loop (Fig 5-1B): the lac repressor lacI blocks the expression of lacY under normal circumstances, but activity of lacY (that is, transport of lactose) blocks the activity of lacI, thus leading to amplification of lacY expression (18).

In cancer cells, however, several examples of positive feedback with mutual activators (Fig 5-1C) has been reported in glycolysis. Cancer cells have been reported to exclusively express the M2 isoform of pyruvate kinase (PKM2), whose enzymatic activity is only 25-50% that of the M1 isoform (PKM1) expressed in normal differentiated tissues (19). This decreased PK activity leads to an accumulation of its substrate, phosphoenolpyruvate (PEP). PEP directly activates phosphoglycerate mutase (PGAM1) by donating a phosphate group for phosphorylation of this enzyme, leading to increased flux through PGAM1 and increased rate of glycolysis (19) (Fig 5-1C). Additionally, by donating the phosphate group to PGAM1, PEP is still converted to pyruvate, but without generating ATP as it would have been in the typical pathway, where the phosphate group is donated to ADP (19). Failure to generate ATP may further stimulate glycolysis through allosteric regulation of phosphofructokinase (20). A third positive feedback
loop involving PKM2 in cancer cells is in hypoxic conditions, where PKM2 translocates into the nucleus, interacts directly with HIF-1α, and promotes HIF1-dependent transcription, including transcription of PKM2 itself (21). These positive feedback loops act together to contribute to the Warburg effect and contribute to tumour development (22, 23).

Here, we describe preliminary data suggesting that, in addition to the well-characterized negative feedback loop, the MVA pathway also participates in a positive feedback loop that has not been previously described. Implications of this positive feedback in the context of metabolic deregulation in cancer is discussed.

5.2 Results and discussion

We and others have previously shown that statin-induced cancer cell death can be reversed by co-administration with MVA, GGPP, or FPP (Fig 5-2A), indicating that depletion of these metabolites underlies cell death. Due to the negative feedback loop, statin treatment leads to upregulation of MVA pathway genes including HMGCR and HMGCS1. We thus hypothesized that co-administration of MVA, GGPP, or FPP will also reverse this change in gene expression. Surprisingly, this was not the case. As shown in Fig 5-2B-C, co-administration of MVA, GGPP, and FPP with fluvastatin did not reverse the increase in expression of HMGCR and HMGCS1. This was observed in all three MCF10A sublines tested, namely those with ectopic expression of TWIST, SNAIL, SLUG, and the vector control. Although they have differing sensitivities to fluvastatin-induced cell death after 72 h of treatment (Fig 5-2A), this experiment was performed at 16 h after treatment, which should precede cell death. Nevertheless, we lowered the dose of fluvastatin to 1 μM in the second experiment, which does not lead to cell death in any of the three sublines, even after 72 h treatment. We’ve also extended our analysis to include samples at 24 h, 48 h, and 72 h after treatment. As shown in Fig 5-3, changing these parameters did not
Figure 5-2. MVA and GGPP reverses fluvastatin-induced cell death but does not reverse upregulation of MVA pathway genes.

A, 10 µM fluvastatin induced cell death in MCF10A cells overexpressing TWIST, SNAIL, and SLUG, fully reversed by co-administration with MVA, GGPP, but not FPP. Cells were treated as indicated for 72 h. B-C, 10 µM fluvastatin induced upregulation of HMGCS1 and HMGCR mRNA, which was not reversed by co-administration with MVA, GGPP, or FPP. Cells were treated as indicated for 16 h. Bars are mean ± SD, n=2.
affect our previous finding, and we observed a sustained HMGCR and HMGCS1 upregulation with MVA co-treatment with fluvastatin, a condition where negative feedback control should be absent. This is a first indication that the MVA pathway may be regulated by a positive feedback loop, where excess accumulation of HMG-CoA leads to further activation of HMGCS1 and HMGCR.

The negative feedback loop that regulates MVA pathway genes is mediated through the transcription factor SREBP2, which can be inhibited by the drug dipyridamole (DP) (24). Specifically, DP blocks the cleavage activation of SREBP2 in response to sterol depletion, retains SREBP2 in the inactive form in the ER, and blocks the upregulation of HMGCR and HMGCS1 in response to statin treatment (24). We thus tested whether positive feedback loop is also dependent on SREBP2 activation. As shown in Fig 5-4, DP treatment inhibited the upregulation of HMGCR and HMGCS1 in both the negative feedback scenario (fluva vs. fluva+DP), and the positive feedback scenario (fluva+MVA vs. fluva+MVA+DP). This suggests that SREBP2 activity mediates both the negative feedback loop (Fig 5-1A) and the positive feedback loop proposed by these experiments (Fig 5-5).

Our immediate plans to validate this model of MVA pathway regulation is to (i) assess SREBP2 activation in the context of HMGCS1 overexpression and (ii) knockdown. Since HMG-CoA cannot cross the cell membrane, future experiments will be focused on genetically manipulating cells so that they will accumulate HMG-CoA, including (i) knocking down HMGCR with independent shRNAs; (ii) knocking down HMGCL, which catabolizes HMG-CoA independently of HMGCR; and (iii) feeding cells with acetate as a carbon source, in conjunction with overexpression of HMGCS1 and/or upstream enzymes. If validated, this model could represent
Figure 5-3. HMGCS1 and HMGCR upregulation is sustained in the absence of negative feedback.

A-B, HMGCR and HMGCS1 mRNA is consistently upregulated for 72h with high-dose MVA co-treatment with low-dose fluvastatin. Bars are mean + SD, n=2.
Figure 5-4. HMGCS1 and HMGCR upregulation is dependent on SREBP2 in the absence of negative feedback.

A-B, upregulation of HMGCR and HMGCS1 mRNA with high-dose MVA co-treatment with low-dose fluvastatin is inhibited by DP, an inhibitor of SREBP2 processing. Cells were treated as indicated for 24 h. Bars are mean + SD, n=2.
Figure 5-5. The MVA pathway may be regulated by a positive feedback loop.
another mode of MVA pathway deregulation (Fig 1-3) that may be exploited by cancer cells to promote transformation (25).

5.3 Methods

5.3.1 Cell culture

MCF10A cells were a kind gift of Dr. Senthil Muthuswamy and were cultured as previously described. Transgene expression was stably introduced into MCF10A cells using retroviral insertion with pLPC, a kind gift of Dr. Roberta Maestro.

5.3.2 Cell death assay

Cells were seeded at 2.5x10^5/plate overnight, then treated as indicated. After 72 h of treatment, cells were fixed in 70% ethanol for >24 h, stained with propidium iodide, and analyzed by flow cytometry for the % sub-diploid DNA population as % cell death, as previously described.

5.3.3 qRT-PCR

Total RNA was harvested from subconfluent cells using TRIzol Reagent (Invitrogen). cDNA was synthesized from 500 ng of RNA using SuperScript II (Invitrogen). Real-time quantitative RT-PCR was performed using TaqMan probes for HMGCR (ABI Hs00168352), HMGCS1 (ABI Hs00266810), and GAPDH (ABI Hs99999905).
5.4 References


