Characterizing COX-2-associated gene network in triple negative breast cancer: its role in distant metastasis and COX-2 inhibitor resistance

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Abstract

Triple-negative breast cancers (TNBCs) have an aggressive biology and portend a poor prognosis as compared with other breast cancer (BC) subtypes. These tumors harbor an enriched population of breast cancer stem cells (BCSCs), which are intrinsically chemo-resistant and allow for self-renewal and differentiation into non-BCSCs to repropagate the tumor. Thus, targeting BCSCs is a critical step towards improving the durability in clinical response for patients with TNBCs. Our lab has previously shown that cyclooxygenase-2 (COX-2) is highly expressed in TNBCs and that its expression is required for TGF-beta-induced BCSC proliferation and expansion. In addition, neoadjuvant Phase I/II trials and preclinical studies also suggest that targeted inhibition of COX-2 by celecoxib, a selective COX-2 inhibitor, can effectively reduce breast tumor growth and metastasis, highlighting the value of COX-2 as a therapeutic target in breast cancer. However, it remains unclear why celecoxib failed to elicit clinical benefits in a phase III, multicenter, double-blind, randomized trial of celecoxib vs placebo in primary breast cancer patients (REACT trial). To elucidate the molecular basis underlying tumorigenesis and resistance to COX-2 inhibition in breast cancer, we employed a comprehensive *in silico* approach to identify 10 COX-2 associated genes (TPM4, RGS2, LAMC2, SERPINB5, KLK7, MFGE8, KLK5, ID4, RBP1, SLC2A1) that are highly altered and expressed in aggressive BCs and BC cell lines classified as “less-sensitive” to COX-2 inhibitor, and that also predict poor prognosis in BC patients. Using CRISPR/Cas9 gene editing technology, we generated individual knockouts (KOs) for each candidate gene and assessed their roles in regulating 1) TNBC metastasis and 2) resistance to celecoxib, using cell lines and preclinical models of TNBC. Importantly, we found that individual deletion of 6 genes (TMP4, RGS2, SERPINB5, MFGE8, KLK5, ID4) resulted in a ~90% reduction in lung metastatic

burden, and KOs of the remaining 4 genes (LAMC2, KLK7, RBP1, SLC2A1) suppressed lung colonization in TNBC by 60-80%. Further, we demonstrated that KLK5, KLK7, and MFGE8 KO can effectively restore TNBC sensitivity to celecoxib both *in vitro* and *in vivo*, as evidenced by reduced cell viability and attenuated primary tumor growth. Altogether, our results provide an important rationale for developing novel combination therapies with COX-2 inhibitors in the treatment of TNBC.

Résumé

Les cancers du sein triple négatifs (TNBC) ont une biologie agressive et laissent présager un mauvais pronostic par rapport aux autres sous-types de cancer du sein (CB). Ces tumeurs abritent une population enrichie de cellules souches du cancer du sein (BCSC), qui sont intrinsèquement chimiorésistantes et permettent l'auto-renouvellement et la différenciation en non-BCSC pour repopager la tumeur. Ainsi, le ciblage des BCSC est une étape critique vers l'amélioration de la durabilité de la réponse clinique pour les patients atteints de TNBC. Notre laboratoire a précédemment montré que la cyclooxygénase-2 (COX-2) est fortement exprimée dans les TNBC et que son expression est requise pour la prolifération et l'expansion des BCSC induites par le TGF-bêta. En outre, les essais de phase I / II néoadjuvants et les études précliniques suggèrent également que l'inhibition ciblée de la COX-2 par le célécoxib, un inhibiteur sélectif de la COX-2, peut réduire efficacement la croissance des tumeurs mammaires et les métastases, soulignant la valeur de la COX-2 en tant cible dans le cancer du sein. Cependant, on ne sait toujours pas pourquoi le célécoxib n'a pas obtenu de bénéfices cliniques dans un essai randomisé de phase III, multicentrique, en double aveugle, du célécoxib par rapport au placebo chez des patientes atteintes d'un cancer du sein primaire (essai REACT). Pour élucider la base moléculaire sous-jacente à la tumorigenèse et à la résistance à l'inhibition de la COX-2 dans le cancer du sein, nous avons utilisé une approche in silico complète pour identifier 10 gènes associés à la COX-2 (TPM4, RGS2, LAMC2, SERPINB5, KLK7, MFGE8, KLK5, ID4, RBP1, SLC2A1) qui sont fortement altérées et exprimées dans les BC agressifs et les lignées cellulaires BC classées comme «moins sensibles» à l'inhibiteur de la COX-2, et qui prédisent un mauvais pronostic chez les patients BC. En utilisant la technologie d'édition de gène CRISPR / Cas9, nous avons généré des knockouts (KO) individuels pour chaque gène candidat et évalué leurs rôles dans la

régulation 1) des métastases TNBC et 2) de la résistance au célécoxib, en utilisant des lignées cellulaires et des modèles précliniques de TNBC. Surtout, nous avons constaté que la suppression individuelle de 6 gènes (TMP4, RGS2, SERPINB5, MFGE8, KLK5, ID4) entraînait une réduction d'environ 90% de la charge métastatique pulmonaire et des KO des 4 gènes restants (LAMC2, KLK7, RBP1, SLC2A1) a supprimé la colonisation pulmonaire du TNBC de 60 à 80%. En outre, nous avons démontré que KLK5, KLK7 et MFGE8 KO peuvent efficacement restaurer la sensibilité de TNBC au célécoxib à la fois in vitro et in vivo, comme en témoigne la viabilité cellulaire réduite et la croissance de la tumeur primaire atténuée. Dans l'ensemble, nos résultats fournissent une justification importante pour le développement de nouvelles thérapies combinées avec des inhibiteurs de la COX-2 dans le traitement du TNBC.

Acknowledgements

First, I would like to extend my utmost gratitude to my supervisor, Prof. Jean-Jacques Lebrun, for giving me the opportunity to join his lab and for placing his faith in me to undertake a productive M.Sc. His approach to self-directed learning, guidance and constant encouragement have fostered my strong interest in cancer research and allowed me to become an autonomous learner when facing the unknown. I must also acknowledge the fact that I have been very fortunate to have the luxury of creative freedom under his guidance, as I was trained to lead my own project, conduct independent literature review and design experiments as a Master's student.

I would also like to thank my committee members: Dr. Suhad Ali, Dr. Bertrand Jean-Claude and Dr. Jun-Li Liu for their support, valuable suggestions and inputs for my research project throughout my Master's training.

I would like to thank my fellow lab members, both present and past, for their guidance and support throughout my graduate studies: Dr. Jun Tian, Dr. Ni Wang, Dr. Meiou Dai, Girija Daliah, Sophie Poulet, Julien Boudreault, Gang Yan, as well as lab members from Dr. Ali's lab. Special thanks to Dr. Jun Tian for training me before I took on the COX-2 project, and Dr. Ni Wang for her expertise in animal work.

Finally, I would like to thank my parents for their unconditional love, patience and guidance throughout this journey. It is because of their understanding and support that I get to pursue my dreams without any fears or regret.

Preface

Contribution of the Authors

The entire thesis is written by myself, and revised by Prof. Jean-Jacques Lebrun. The thesis is presented in a monograph format with five chapters, as follows:

Chapter 1 is an introduction to breast cancer and its classification by histological and molecular subtypes, TGF-β signaling pathway, celecoxib and its mechanisms of action to target cyclooxygenase-2 in cancer.

Chapter 2-5 was adapted from the COX-2 paper that I co-authored and published in *Breast Cancer Research*. As I am not the first author of the article, I cannot present it in a manuscript-based format. Therefore, I have quoted excerpts taken from the publication that were my own writing in this thesis. Chapter 2 presents the rationale, objective and background of the study, including previous *in silico* work conducted by Dr. Jun Tian that forms the basis of my M.Sc. thesis project. Chapter 3 details the methodology for the experiments I conducted and/or assisted in. Chapter 4 highlights the data I generated independently and in collaboration with Dr. Jun Tian, Dr. Ni Wang, and Julien Boudreault. Chapter 5 discusses the implications of all findings and summarizes the contributions of this study to the advancement of knowledge. As I am not the first author on the COX-2 paper, which this thesis cannot be presented in a manuscript-based format.

For this project, I assisted in the validation of CRISPR/Cas9 knockouts (KOs) of 10 candidate genes generated in two TNBC cell lines—MDA-MB-231 and SUM159—by surveyor assay and western blot alongside Dr. Jun Tian, who designed the study. In addition, I performed *in vitro* cell viability assays (i.e. SRB and Prestoblue) to assess MDA-MB-231- and SUM159-derived

individual gene KOs for their abilities to sensitize TNBC to celecoxib. To functionally validate top candidates for their contributions to TNBC metastasis and resistance to celecoxib *in vivo*, I amplified selected KO cells *in vitro* and prepared drug solutions for injections by Dr. Ni Wang, who is in charge of all animal work including tumor volume measurement. Further, I generated celecoxib-resistant variants of MDA-MB-231 and assessed COX-2 expression by western blot. Julien Boudreault designed primers for all 10 COX-2 associated genes and assessed mRNA expression of each candidate gene by qPCR. We also collaborated with two pathologists Dr. Baharak Khadang and Dr. Khldoun Bakdounes, who assessed the total lung involvement mediated by each candidate gene in H&E-stained lung tissues extracted from spontaneous metastasis models of TNBC. Finally, I was involved in data analysis and figure design for all *in vitro* and *in vivo* work, as well as the drafting of the manuscript alongside Dr. Jun Tian. Prof. Suhad Ali provided input for the design for the study and contributed to the editing of the manuscript, and Prof. Jean-Jacques Lebrun is involved in research design, data interpretation, and supervision of the project.

During my Master's studies, I have published the following article as a second author:

1 Tian J, **Wang V**, Wang N, Khadang B, Boudreault J, Bakdounes K, et al. Identification of MFGE8 and KLK5/7 as mediators of breast tumorigenesis and resistance to COX-2 inhibition. *Breast Cancer Research*, 2021 Feb 15; 23(1):23.

Chapter 1: Introduction – Literature Review

1.1 Breast cancer overview

In 2020, breast cancer is expected to be the most commonly diagnosed cancer in Canadian women (27 400) and the fourth leading cause of cancer death in Canada (6.1%), preceded by lung (25.5%), colorectal (11.6%), and pancreatic cancers (6.4%) [1]. Current state of knowledge on breast cancer indicates that it is no longer considered as a single entity—rather—as a heterogenous yet complex type of disease with diverse cellular compositions, mutational landscapes, histological patterns, and signaling profiles that differentially regulates tumor progression, metastasis and drug resistance, in concert with its surrounding microenvironment [2,3]. Thanks to early screening efforts and improvements in breast cancer diagnostics and follow-up care, female mortality rate has declined by 49% since 1986 [1] and 5-year survival rate has increased up to 88% [4]. However, due to a lack of understanding of the mechanisms driving cancer cell diversity at a molecular, phenotypic, and functional level, considerable therapeutic challenges still remain with the treatment and diagnosis of this disease. To address this unmet clinical need, recent advances in high-throughput, multi-omics profiling of breast tumors have enabled the characterization of intra- and inter-tumoral heterogeneity at single-cell resolution to track clonal evolution, measure cell states, predict treatment response, as well as to infer lineage relationship between metastatic founder clones and its subclones. These information should enable the robust development of diagnostic, prognostic and predictive biomarkers in the future to improve risk stratification in patients and to individualize their care through personalized therapy. This section provides an overview on the histopathological and molecular classifications of breast cancer subtypes as well as their current treatment modalities.

1.1.1 Histological classifications of breast cancer subtypes

Adenocarcinomas of the breast is the most common histologic type of breast malignancy, accounting for more than 95% of breast cancers [5]. Depending on whether or not the tumor has grown beyond the mammary epithelium, breast carcinomas can be generally categorized into *in situ* and invasive carcinomas. *In situ* carcinomas are non-invasive pre-malignant lesions that are confined to the basement membrane of the mammary tissue with the potential of developing into invasive cancers [6]. They arise from either the milk-producing lobules or ducts of the breast and can be ductal or lobular. Ductal carcinoma in situ (DCIS) is the most common type of non-invasive breast cancer, representing 20% of all newly diagnosed breast cancer cases [7]. It constitutes a heterogenous group of lesions that differ in morphologies and can be sub-divided into comedo, cribriform, solid, micropapillary and papillary. Lobular carcinoma in situ (LCIS), on the other hand, comprises only 1-2% [8] of all breast cancers and is less common, but patients with this benign proliferative disease exhibit a 5- to 6-fold higher risk of developing invasive breast cancers than those without it [9]. Invasive carcinomas are cancers that have broken through the duct wall, invaded the surrounding stroma and into nearby breast tissues. These malignancies are heterogeneous in nature and consist of infiltrating ductal, invasive lobular, ductal/lobular, mucinous (colloid), tubular, medullary, and papillary carcinomas [10] that demonstrate a wide variety of cytoarchitectural features. Of which, infiltrating ductal carcinoma (IDC) is the most common subtype and represents 70-80% of all invasive cases [11]. Based on the pattern of differentiation, IDC can be histologically sub-classified into grade 1 (well-differentiated) -3 (poorly differentiated) according to 3 main factors: tubule formation, nuclear pleomorphism, and mitotic rate [12]. However, more than three quarters of the IDC lack distinguishable morphological

characteristics to be classified as a special subtype, thus they are termed “no special type” (NST)[13].

1.1.2 Molecular classifications of breast cancer subtypes

For many years, clinicians have relied solely on conventional pathologic assessment to diagnose, classify and treat breast cancer without taking into account its molecular underpinnings [10]. The consequence is that patients often relapse after receiving cytotoxic therapies without demonstrating durable clinical response, suggesting that the characterization of morphological features and the use of clinical variables alone in predicting breast tumor behaviour and therapeutic response are insufficient [13]. This prompts the need for the research community to elucidate the molecular heterogeneity of breast cancer and search for additional parameters to guide treatment planning and risk stratification in this disease. In early 2000’s, Perou et al. pioneered the identification of five intrinsic molecular subtypes of breast cancer: luminal A, luminal B, normal breast-like, ErbB2(Her2/neu)+ , and basal-like [14], by subjecting patient tumor specimens to cDNA-based microarray gene expression profiling and hierarchical clustering analysis. This approach was later validated and standardized by another group [15,16], and has led to the discovery of a new subtype classified as “claudin-low” in 2007 [17,18].

Luminal A: It is the most common molecular subtype and accounts for 50% of all breast cancers [6]. Immunohistochemical profiling of this subtype reveals that these tumors express estrogen receptor (ER) (≥ 1%), high levels of progesterone receptor (PR) (≥ 20%), low levels of human epidermal growth factor receptor 2 (HER2) (≤ 10%), and low levels of the proliferation marker Ki-67 (< 14%) [19]. These cancer cells are believed to originate from the luminal epithelium of the

breast as they still retain strong expressions of cytokeratin (CK) 7/8/18/19 [20]. Luminal A contains low-grade variants including mucinous, cribriform, tubular, IDC-NST, and the classic type of invasive lobular carcinoma (ILC) [20,21], and shows the best prognosis of all subtypes [6].

Luminal B: This subtype accounts for 20-30% of invasive breast cancers [22], exhibits robust expression of ER/PR but variable expression of HER2, and is sub-divided into HER2- and HER2+. The HER2- subcluster is marked by positive expression of ER (≥ 1%), negative or low expression of PR (< 20%), low expression of HER2 (≤ 10%), and high expression of Ki-67 (≥ 20%), whereas the HER2+ subcluster is marked by positive expression of ER (≥ 1%) and HER2 (> 10%), as well as a range of expression of PR and Ki-67 [19,23]. Similar to luminal A, luminal B expresses CKs of mammary epithelial origin [22]. But unlike luminal A, luminal B contains variants that have moderately higher grades such as micropapillary carcinoma and grade 2 IDC NST, and is associated with a higher risk of locoregional relapse and less favorable prognosis as compared to luminal A[24,25].

HER2+: Overexpression or amplification in receptor tyrosine-protein kinase erbB-2 (ERBB2), the proto-oncogene encoding HER2/neu, is found in 15-20% of all breast cancers [26]. These tumors frequently harbor TP53 mutations [27], express high levels of HER2 (>10%) and Ki-67 (>20%), but low levels of ER (< 1%) and PR (<20%) [19]. Generally speaking, HER2-overexpressing tumors contain high-grade variants such as pleomorphic ILC [28] and are associated with increased lymph-node metastasis, early and high relapse rate, as well as short disease-free survival (DFS) [22]. Overall, this subtype displays worse prognosis than its HER2- counterpart.

Basal-like: 10-20% of all breast cancers are basal-like (BL) [29], as evidenced by their lack of expression of ER (<1%), PR (<20%) and HER2 (≤ 10%) [6]. Thus, they are also known as “triple-

negative" (TN), although not all BL tumors are TN, and vice versa [30,31]. The basal subtype expresses high levels of basal epithelial/myoepithelial genes of the breast including CK5/14/17, vimentin, integrin α6, laminin, fatty-acid binding protein 7, and EGFR [14], but low levels of luminal genes, and is characterized by a high Ki-67 index (>30%) [19]. Similar to TN, BL breast cancers are recurrently mutated in BRCA1 and TP53 [32,33], present an extremely poor prognosis with high risks of systemic relapse, and are more prevalent in young Black/African American women (AA) [34].

Normal breast-like: This subtype comprises 5-10% of all breast cancers and is found to cluster with fibroadenomas and normal breast samples in microarray studies. Similar to TN, normal breast-like cancers are ER-, PR-, and HER2-negative. Yet, they are not classified as basal-like because these tumors do not express basal epithelial markers and cytokeratins such as CK5 and EGFR. Instead, they harbor a gene expression pattern related to adipogenesis, and present an intermediate prognosis between their basal-like and luminal counterparts [35]. Some researchers have expressed concerns regarding the true existence of this subtype, as they believe that normal breast-like tumors are an artifact originating from contamination with normal breast tissues [36].

Claudin-low: Similar to basal-like, claudin-low is a subtype of TNBC [18] and corresponds to 10% of all breast cancers [37]. The majority of these tumors consist of medullary and metaplastic IDCs and are enriched in the expression of epithelial-to-mesenchymal transition (EMT), immune response and stem cell-like markers, but depleted in the expression of luminal and proliferation-related genes. They are clinically manifested as ER-, PR- and HER2-negative, and are associated with poor overall survival (OS) and relapse-free survival (RFS) outcomes [38].

1.1.3 Systemic therapy for breast cancer

In luminal-like breast cancer (ER+ and/or PR+, HER2-) , endocrine therapy (ET) is the mainstay of treatment in both the adjuvant and metastatic setting [39]. It is a type of hormonal intervention that consists of ER blockers such as tamoxifen, a selective estrogen receptor modulator (SERM), and fulvestrant, a selective estrogen receptor down-regulator (SERD), and estrogen blockers such as aromatase inhibitors (AIs) and ovarian suppression (OvS) therapies [40]. The menopausal status of the patients dictate the types of treatment regimens they are subscribed to. Typically, for premenopausal women with early-stage luminal breast cancer, it is recommended that they receive at least 5 years of tamoxifen after surgery. For postmenopausal women, 5 years of tamoxifen or exemestane, an AI, as an initial or sequential monotherapy should be considered. In metastatic luminal-like breast cancer, premenopausal women are subjected to OvS in combination with tamoxifen or an AI, while postmenopausal women without visceral crisis should exhaust all ET options until they develop anti-estrogen resistance, before attempting chemotherapy [39]. In general, luminal B responds poorer than luminal A to ET and chemotherapy, and is associated with worse prognosis [41].

In HER2-positive breast cancer (luminal-like and non-luminal-like), the standard of care is chemotherapy plus anti-HER2 therapy [39]. For grade 1, node-negative, early-stage breast cancer patients, standard regimens include paclitaxel-based chemotherapy in the adjuvant setting in addition to trastuzumab, a HER2 targeted agent. For patients with tumours that are grade 2 or above regardless of nodal status, neoadjuvant trastuzumab, pertuzumab plus docetaxel have been shown to improve their rates of pathological complete response (pCR) as compared with adjuvant pertuzumab plus docetaxel [42]. However, real-world evidence argues the opposite—in favor of adjuvant dual HER2-blockade—due to the observation that patients with early HER2-

positive breast cancer, particularly those who were node-positive at diagnosis, are still highly susceptible to relapse, despite achieving pCR after receipt of neoadjuvant pertuzumab and trastuzumab [45]. In the metastatic setting, continued inhibition of the HER2 pathway remains a priority and multi-line treatment combining chemotherapy with anti-HER2 agents should be adopted to stabilize disease progression [39,43,44]. For the first-line therapy, patients without prior exposure to trastuzumab are eligible for dual HER2-blockade in addition to concurrent taxane- or vinorelbine-based chemotherapy, whereas patients with prior exposure to trastuzumab are subjected to a single HER2-targeted agent plus cytotoxic chemotherapy [39,43]. For the second-line therapy, patients can receive either ado-trastuzumab (T-DM1), an antibody-drug conjugate that selectively targets and kills HER-2 overexpressing cells by linking trastuzumab to a cytotoxic agent, or trastuzumab in combination with lapatinib, a dual tyrosine kinase inhibitor that inhibits both the HER2/neu and epidermal growth factor receptor (EGFR) pathways [39,43,44]. For later lines of therapy targeting advanced HER2+ breast cancer, trastuzumab in combination with sequential monochemotherapy is a possible strategy to slow tumor growth and prolong time to progression [39,43]. Overall, patients with HER2+ breast cancer have experienced substantial improvements in their long-term outcomes since the introduction of HER2-targeted drugs [46].

In ER-/PR-/HER2-negative TNBC, cytotoxic chemotherapy is the standard of care with few molecularly-guided therapies available [39]. Although basal-like, normal-like and claudin-low tumours are all defined as “triple-negative” (TN) by immunohistochemistry, TNBC and the basal-like breast cancer share the greatest overlap in their gene expression profiles [54]. For early-stage TNBC, neoadjuvant chemotherapy with an anthracycline or taxane backbone is preferred for patients to achieve pCR [47]. In cases of non-pCR, patients with minimal disease burden can still benefit from adjuvant capecitabine to improve survival outcomes [48]. For late-stage TNBC

without BRCA mutations, there are currently no recommended indications for chemotherapy [43,49]. Yet, for late-stage BRCA mutation carriers, treatment with a poly (ADP-ribose) polymerase (PARP) inhibitor such as olaparib and talazoparib can improve their quality of life and progression-free survival (PFS) compared with monochemotherapy alone [50,51]. Moreover, patients with advanced TNBC expressing programmed death-ligand 1(PD-L1) could also benefit from a first-line chemoimmunotherapy with nab-paclitaxel and an anti-PD-L1 antibody, atezolizumab, before receiving subsequent lines of chemotherapy [52]. Nonetheless, the lack of well-defined biomarkers predictive of clinical efficacy in the context of TNBC limits its available treatment options, as patients who were not cured with prior lines of chemotherapy tend to suffer from shortened therapeutic response, and rapid development of relapse and drug resistance. Indeed, metastatic TNBC patients have considerably worse prognosis than their non-TNBC counterparts, with a median overall survival of merely 13-18 months in the case of therapeutic intervention [55]. Thus, efforts are clearly warranted to identify novel therapeutic strategies capable of eliciting sustained clinical response in this aggressive and lethal disease.

1.2 TGF-β signaling pathway

In humans, more than 33 members of the transforming growth factor-beta (TGF-β) superfamily have been discovered, including the TGF-β subfamilies, the bone morphogenic proteins (BMPs) and the growth differentiation factors (GDFs), the activin/inhibin subfamilies, the nodal and the left-right determination factors, the anti-Müllerian hormone, as well as the glial cell line-derived neurotrophic factor family of ligands (GFL) [56,57]. These multifunctional polypeptides play pivotal roles in the regulation of embryogenesis, immune homeostasis and inflammation, erythropoiesis,

bone remodelling and tissue repair [57-61], among other biological processes, and are evolutionarily conserved and structurally related to each other.

The TGF- β subfamily comprises 3 isoforms—TGF-β1, 2, 3—encoded by the TGFB1, TGFB2, TGFB3 gene, respectively. Although all 3 TGF-β ligands feed into the same receptor-mediated pathway [62,63], TGF-β1 is the most abundant and the best characterized isoform in mammals [64]. Prior to its activation, TGF-β1 is secreted as an inactive precursor in complex with its latency associated peptide (LAP), before being deposited in the extracellular matrix by the latent TGF-β-binding protein (LTBP). It is a homodimer that requires proteolytic cleavage from its pro-peptide, LAP, in order to become bioavailable. Once activated, the TGF-β ligand is capable of engaging with a complex of two transmembrane receptor serine/threonine kinases (RSKs)— the type I (TβRI) and type II receptor (TβRII) —to transduce its signals downstream via SMAD-dependent and SMAD-independent pathways [61].

Importantly, dysregulation of TGF-β expression and activity as well as its signaling pathway is implicated in the pathogenesis of many human diseases, including cancers [64]. In fact, TGF-β1 is known to serve a dual purpose in cancer: acting as a tumor suppressor in normal, premalignant cells as well as early carcinomas, and as an oncogene in locally advanced tumors that are ready to metastasize [61] (Figure 1.1). This section details the canonical and noncanonical TGF-β signaling through SMAD-dependent and -independent pathways, and elucidates the paradoxical roles played by TGF-β during the course of malignant progression.

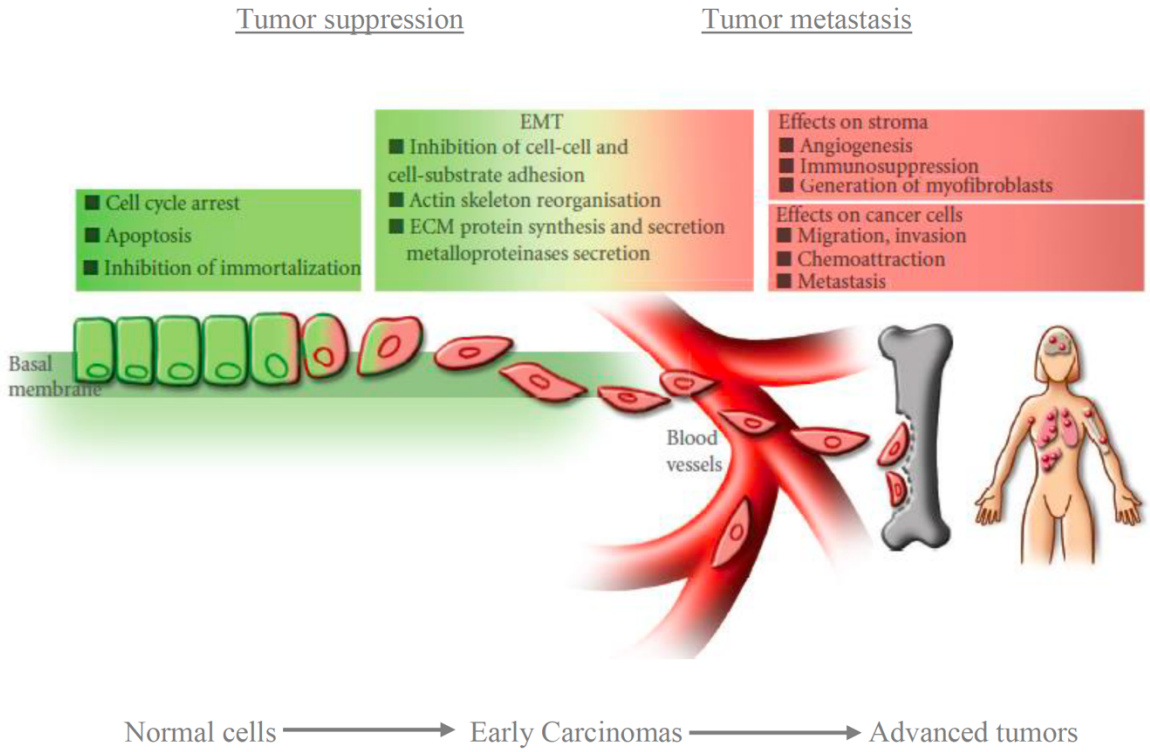


Figure 1.1: The paradoxical effects of TGF-β in human cancer

(Adapted from *Neel J-C, 2012*) [61]

1.2.1 SMAD-Dependent Pathway

Intracellular TGF-β signaling begins with the activation and phosphorylation of two cell surface proteins: TβRI and TβRII. In the absence of ligands, these transmembrane RSKs remain unphosphorylated and act as homodimers without coming into close contact with each other. In the presence of active TGF-β, however, TβRII becomes constitutively auto-phosphorylated at its cytoplasmic tail and is capable of dimerizing with TβRI to form a stable tetrameric ligand-receptor complex (Figure 1.2) [64-66]. There are three type I receptors with which TβRII interact:

Activin-Like Kinase 1(ALK1), ALK2 and ALK5 [67]. Of which, ALK5 is the "classical" TβRI that is most frequently expressed in epithelial cells [61].

Ligand binding to the extracellular domains of both receptors also induces conformational changes in their respective kinase domains. This enables auto-phosphorylated TβRII to trans-phosphorylate the glycine/serine-rich motif within the intracellular juxtamembrane region of TβRI, causing it to in turn activate two receptor-regulated SMADs (R-SMADs) —SMAD2 and SMAD3—via phosphorylation of serines at their C-termini [68-70]. The phospho R-SMADs composed of two moieties of SMAD2 and/or SMAD3 then form a heterotrimeric complex with cytoplasmic SMAD4, a common SMAD (Co-SMAD), before translocating into the nucleus to regulate TGF-β/SMAD-mediated signaling output [68].

Upon nuclear entry via importin-dependent or -independent mechanisms [71,72], SMAD3/4 of the heterotrimer, but not SMAD2, bind via their MH1 domains to a site-specific DNA sequence of CAGAC [64]. This GC-rich sequence is known as the SMAD-binding element (SBE) and its physical interaction with the R-SMAD-SMAD4 complex is required for transcriptional activation of TGF-β/SMAD target genes. However, in the absence of essential co-activators such as CREB-binding protein (CBP) and p300, SMAD3/4 bind with low affinity to SBE and result in only limited TGF-β-induced transcriptional activation [68]. Thus, to enhance its binding affinity for the SBE and to upregulate TGF-β-dependent transcription, the R-SMAD-SMAD4 complex must functionally cooperate with CBP/p300 as well as other co-activators including SMAD4-interacting factor (SMIF), melanocyte-specific gene 1(MSG1) and p300/CBP-associated factor (P/CAF) [68, 73-75]. Similarly, SMADs may associate with its co-repressors to downregulate SMAD-activated gene expression. This is mediated by proto-oncogenes—c-Ski, SnoN, and cMyc [76,77]—that compete with p300/CBP for binding to SMAD2/3/4, followed by their

recruitment of histone deacetylases (HDACs) to suppress SMAD-associated histone acetyltransferase (HAT) activity [78]. In addition to its interaction with co-activators and co-repressors, the R-SMAD-SMAD4 complex also partners with sequence-specific transcription factors that bind to the promoters of its target genes to regulate their transcription. The SMAD heterotrimer can either associate with FoxO forkhead and Sp1 to activate expression of two cyclin-dependent kinase inhibitors—p15 and p21 [79], or with CCAAT/enhancer-binding protein (C/EBPβ) to inhibit TGF-β-mediated adipocyte differentiation [80]. To ensure the specificity of TGF-β-induced transcriptional activity, SMADs interact with a subset of DNA-binding cofactors that have distinct expression patterns [81-84].

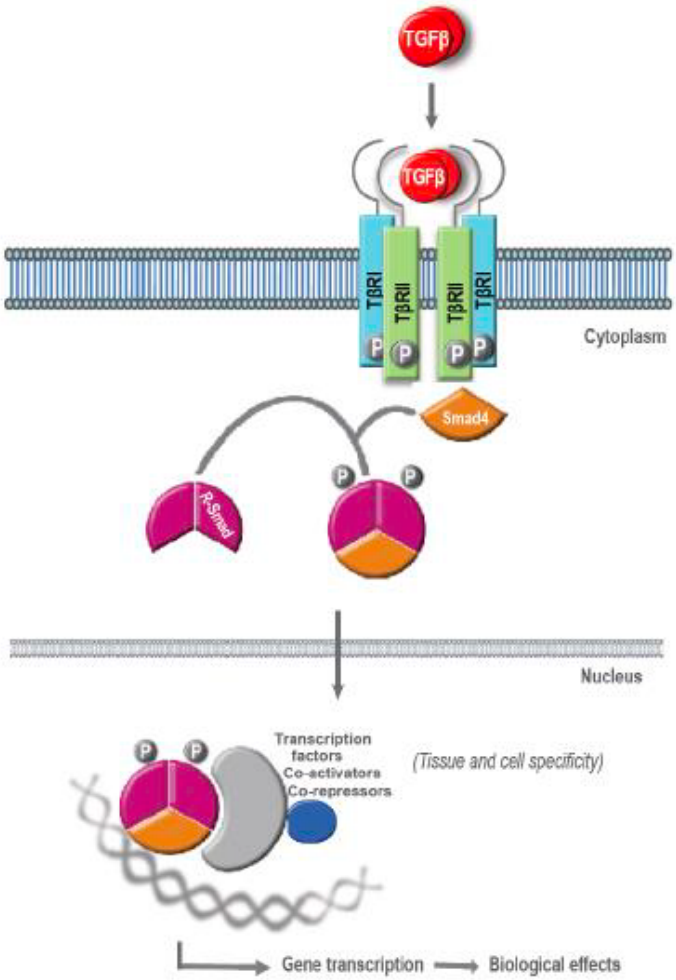


Figure 1.2: TGF-β signaling via canonical SMAD-dependent pathways

(Adapted from *Neel J-C, 2012*) [61]

1.2.2 SMAD-Independent Pathways

TGF-β is capable of signaling through other SMAD-independent pathways in addition to the canonical SMAD-dependent pathway to mediate its diverse cellular responses. These major intracellular effectors of TGF-β include phosphatidylinositol-3 kinase (PI3K) and Akt, the Rho

family of GTPases, as well as members of the mitogen-activated protein kinase (MAPK) family, as depicted in Figure 1.3 [61].

PI3K/Akt pathway: In the absence of TGF-β, TβRII constitutively associates with the regulatory subunit of PI3K, p85 [85]. In the presence of TGF-β, trans-phosphorylated TβRI interacts with p85 to induce direct activation of PI3K, which in turn phosphorylates and activates AKT. Activated AKT regulates protein synthesis in response to TGF-β-induced EMT, invasion and metastasis via two pathways: the direct and indirect way. The direct way is whereby AKT, upon TGF-β stimulation, phosphorylates heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1) to prevent its interaction with eukaryotic translation elongation factor 1 α1 (eEF1A1), thus allowing the latter to reverse translational silencing of target EMT transcripts [86]. The indirect way is via AKT-mediated activation of mammalian target of rapamycin complex 1 (mTORC1) and its downstream effectors to promote *de novo* protein synthesis. Briefly, AKT phosphorylates tuberous sclerosis complex 2 (TSC2), a core protein of the TSC complex, to induce its release of a GTP-binding protein known as RSD homolog enriched in brain (RHEB) that activates mammalian target of rapamycin complex 1 (mTORC1). Upon activation, mTORC1 in turn phosphorylates S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) to stimulate translation of proteins that propagate anti-apoptotic, pro-survival signaling during EMT [85,87,88]. Conversely, activated AKT also inhibits TGF-β-induced growth arrest and apoptosis by sequestering unphosphorylated SMAD3 in the cytoplasm. This prevents SMAD3 from entering the nucleus in complex with SMAD4 and results in attenuated SMAD-mediated transcription [89,90].

Rho-like GTPase pathway: The Rho family of small GTPases plays critical roles in TGF-β-induced EMT and its associated processes including cell migration, invasion, and metastasis [91,92]. On one hand, TGF-β can rapidly activate RhoA and Rho-associated protein kinase (ROCK) to induce formation of stress fibers and reorganization of actin cytoskeleton [93]. On the other hand, TGF-β-dependent phosphorylation of PAR6, a cell polarity regulator, can lead to dissolution of junctional complexes and subsequent loss of cell-cell adhesion via SMURF1-mediated localized RhoA degradation [94,95]. Further, TGF-β signaling through small GTPases CDC42/RAC1 has been shown to activate p21-activated kinases (PAKs) [93,96], a family of serine/threonine kinases that are involved in the regulation of cell motility, filopodia formation and cytoskeleton reorganization during TGF-β-induced EMT [97].

JNK/p38 pathway: TGF-β signaling through its receptors also activates the c-Jun amino terminal kinase (JNK) and p38 MAPK pathways. Upon ligand binding, the tetrameric TβRII/TβRI complex associates with and induces Lys63-linked polyubiquitination of a RING-domain E3 ligase known as tumor necrosis factor receptor-associated factor 6 (TRAF6) [98]. While ubiquitylation at Lys48 of TRAF6 targets the adaptor protein for degradation [98], polyubiquitylation at Lys63 of TRAF6 facilitates its engagement with TGF-β-activated kinase 1 (TAK1), a MAP kinase kinase kinase (MAPKKK) that signals through MAP kinase kinases (MKKs), MKK4 and MKK3/6, to activate p38 and JNK [99-101]. Upon MKK-induced dual phosphorylation on tyrosine and threonine residues, p38 and JNK MAPK activity is stimulated. This enables the two MAPKs to in turn phosphorylate their downstream effectors, which cooperate with canonical SMAD signaling to regulate TGF-β-induced apoptosis, invasion and metastasis [93].

ERK/MAPK pathway: Activation of the extracellular signal-regulated kinase (ERK) MAPK pathway begins with the phosphorylation of tyrosine residues on TβRII and TβRI. While these two transmembrane RSKs are serine- and threonine-phosphorylated upon ligand binding, they also contain numerous phospho-tyrosine residues that serve as docking sites for homology 2 domain-containing (Shc) proteins, such as ShcA and growth factor receptor-bound protein 2 (Grb2) [102-104]. Upon binding of ShcA to the activated receptor complex, TβRI phosphorylates it on serine and tyrosine residues to facilitate its binding with Grb2 and Sos [105], a Ras-activating guanine exchange factor (GEF) that converts membrane-bound Ras from the inactive GDP-bound state to the active GTP-bound state [106,107]. Activation of Ras results in a phosphorylation cascade through downstream protein kinases including Raf, MEK1/2 and ERK1/2, and culminates with the induction of ERK targets that are important for TGF-β-induced EMT [108].

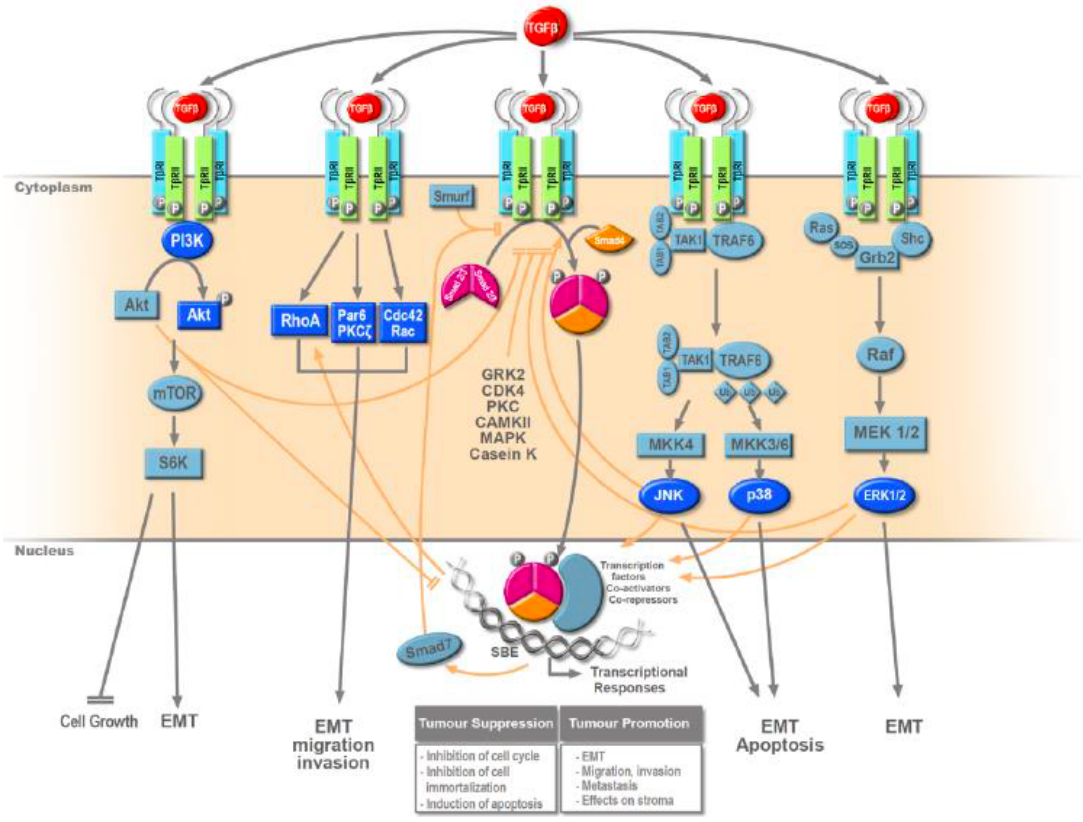


Figure 1.3: TGF-β signaling via SMAD-independent pathways

(Adapted from *Neel J-C, 2012*) [61]

1.2.3 TGF-β signaling in tumor suppression

TGF-β mediates cytostatic signaling in myeloid, lymphoid, epithelial and endothelial cell types to induce growth inhibition and apoptosis, as well as to prevent cellular immortalization [61]. Constitutive expression of TGF-β1 and its associated receptors has been shown to attenuate formation of mammary tumors induced by expression of mouse mammary tumor virus (MMTV) and/or oncogene HER2/neu in transgenic mouse models [109,110]. This affirms the role of TGF-β as a tumor suppressor in normal cells and early carcinomas of its target tissues.

Inhibition of Cell Cycle: TGF-β induces expression of cyclin-dependent kinase inhibitors (CDKIs) $p15^{INK4B}$, $p21^{CIP1}$ and $p27^{KIP1}$ to inactivate G1 phase CDKs that are required for S phase progression [111]. Briefly, $p15^{INK4B}$ binds to CDKs 4 and 6 to prevent their association with and activation by regulatory cyclin D. This catalyzes the displacement of $p21^{CIP1}$ and $p27^{KIP1}$ from the CDK4/6-cyclin D complex to bind and inactivate the CDK2-cyclin A/E complexes at the G1/S checkpoint [112,113]. In addition, TGF-β also downregulates the expression of oncogene c-MYC [114] and inhibitors of DNA binding 1 and 2 (ID1, ID2) [115] to inhibit cellular growth and proliferation. Repression of c-MYC is mediated by SMADs in association with G1/S transcriptional repressors E2F4/5 and co-repressors p107 [116], whereas inhibition of ID1 and ID2 is mediated by SMADs in association with target gene activating transcription factor-3 (ATF3), or c-MYC antagonists MAD2/4 [115,116]. Further, TGF-β-mediated repression of CDC25A results in prolonged G1 arrest due to the inability of the tyrosine phosphatase to de-phosphorylate the inhibitory sites on CDK4/6, thereby inactivating the G1/S checkpoint [117].

Induction of apoptosis: TGF-β integrates diverse signaling responses to elicit a cell- or tissue-specific apoptotic response. In hepatocarcinomas, for instance, TGF-β activates SMAD-dependent transcription of death-associated protein kinase (DAPK) to sensitize tumor cells to mitochondria-mediated apoptosis [118], and stimulates crosstalk with the JNK-mediated apoptosis pathways via TβRII-bound adaptor protein Daxx [119]. In epithelial cells of the pancreas, TGF-β induces expression of TGF-β-inducible early-response gene (TIEG1) to promote apoptosis and growth inhibition [120]. The core mechanism by which TGF-β induces cell death is mediated by the binding of an E2F1-pRB-P/CAF transcriptional complex to the promoters of its pro-apoptotic targets [121], however we have yet to clearly define all the players involved in this process.

Prevention of cellular immortalization: Unlike immortalized and cancer cells, non-immortalized cells experience progressive shortening of telomeres with each replication cycle to avoid the acquisition of limitless replicative potential [122]. Telomerase, encoded by hTERT, is an enzyme that regulates the length of telomeres, and its expression is elevated in cancer cells [61]. To prevent non-immortalized cells from undergoing immortalization, TGF-β induces SMAD3-dependent transcriptional repression of hTERT by recruiting p38, JNK and HDAC to its promoter [123].

1.2.4 TGF-β signaling in tumor promotion

When tumors have grown past its early stages, they start to lose TGF-β-induced cytostatic responses. This is primarily driven by two mechanisms: 1) deletions or mutations in the TGF-β signaling components that deprives the cytokine of its antitumoral functions [124], and 2) activation of oncogenic MAPK, PI3K, Ras and c-MYC pathways that renders tumor cells resistant to TGF-β/SMAD-mediated growth inhibition [125-128]. By disabling the tumor-suppressive arm of the TGF-β pathway, advanced-stage tumors can exploit its pro-metastatic arm instead to facilitate their growth, migration, invasion and metastasis by co-opting the surrounding microenvironment [61]. In many human malignancies including those of the breast, increased TGF-β production correlates with increased aggressiveness and higher tumor grade [129-132]. In fact, previous studies have shown that TGF-β can exert both autocrine actions on tumors themselves to induce EMT and metastatic dissemination [61,111,133,134] , and paracrine actions on nearby stromal tissues to stimulate angiogenesis, immunosuppression, and myofibroblast differentiation [61].

Autocrine effects: TGF-β elicits a SMAD-dependent EMT program through induction of key EMT regulatory factors such as high-mobility group A2 (HMGA2), zinc-finger proteins Snail and Slug, and basic helix-loop-helix factor Twist, [135] to upregulate expression of mesenchymal proteins (tenascin-C, fibronectin, vimentin, N-Cadherin), and downregulate expression of epithelial proteins (E-cadherin, ZO-1). In addition, TGF-β can also signal downstream via SMAD-independent mTOR [87,136], Erk MAPK [137-139], Ras/PI3K [140-142], and p38 stress-activated kinase pathways [143] to regulate the EMT process. Further, there are multiple mechanisms by which TGF-β contributes to increased migratory and invasive capacity of cancer cells, including through upregulation of p21, downregulation of TIMP3, microRNA regulation, increased production of matrix metalloproteinases (MMPs) 2 and 9, and activation of RhoGTPases, among other processes [144-147]. Before the onset of the metastatic cascade, TGF-β potentiates chemoattraction of primary tumors to their target tissues (i.e. bone) to favor the growth of secondary tumors. When circulating cancer cells have reached their sites of colonization, TGF-β activates expression of COX-2 and epidermal growth factor receptor (EGFR) to promote their extravasation into the lung parenchyma, as in the case of breast cancer metastasis to lung [110].

Paracrine effects: TGFβ-induced angiogenesis enables delivery of oxygen and nutrients to tumor cells via pre-existing or nascent blood vessels. In epithelial cells and fibroblasts, TGFβ mediates the vascularization process by upregulating expression of connective tissue growth factors (CTGF) and vascular endothelial growth factor (VEGF) [148,149]. When present in large quantity, TGFβ can assist tumor cells to evade host immune surveillance by exerting immunosuppressive effects on the diverse players involved in the immune landscape of the tumor microenvironment. For example, TGF-β has been shown to inhibit the production of cytolytic factors (i.e. interferon γ and Fas-ligand) to disable cytotoxic T-cells [150], and it also interferes with the function of

interleukin-2 and its receptors to prevent stimulation of T-lymphocytes by dendritic cells [151]. Further, TGFβ stimulates the generation and maturation of myofibroblasts, or cancer-associated fibroblasts (CAFs), to secrete cytokines that enhance the proliferative, invasive and angiogenic capacities of cancer cells prior to its dissemination [152].

1.3 Celecoxib

Celecoxib is a nonsteroidal anti-inflammatory drug (NSAID) that selectively targets the COX-2 enzyme. Marketed by Pfizer and initially discovered by a team at the Searle division of Monsanto led by John Talley [153,154], the drug first entered the US market in December 1998 and is indicated for the treatment of osteoarthritis, rheumatoid arthritis, acute pain, ankylosing spondylitis, and most recently, familial adenomatous polyposis (FAP) [155]. For the last 20 years, celecoxib remains one of the most widely prescribed medications for relief of pain and inflammation. It exerts its analgesic effects by blocking COX-2, a key enzyme involved in the biotransformation of arachidonic acid (AA) to inflammatory prostanoids (PGs). Celecoxib differs from traditional NSAIDs in that it is selective for COX-2 inhibition, thus minimizing unwanted gastrointestinal side effects including ulcers and bleeding mediated by inhibition of COX-1. Despite a more favorable gastrointestinal toxicity profile, the use of celecoxib is still largely restricted due to its questionable cardiovascular risk profile, as two selective COX-2 inhibitors of the same class as celecoxib—valdecoxib and rofecoxib—were subsequently withdrawn from the market for the same reason. It is not until the completion of a decade-long PRECISION trial in 2016 that concludes celecoxib may not pose more heart risks than other NSAIDs, though its absolute safety remains to be investigated. It is important to note that celecoxib is currently the only available selective COX-2 inhibitor in the market. Celecoxib has

also shown promise for its chemoprevention role as it is indicated as an adjunct for the treatment of FAP. Though the drug has not yet received approval for other types of malignancies, its antineoplastic actions and the pathways involved remain an area of intense research.

1.3.1 Mechanism of action: anti-inflammatory

Celecoxib is an NSAID and a selective inhibitor of prostaglandin-endoperoxide synthase 2 (PTGS2). There are two isoforms of PTGS—PTGS1 and PTGS2—both of which possess dual cyclooxygenase and hydroperoxidase activities. Thus, they are also known as COX-1 and COX-2. The COX enzymes catalyze a two-step reaction to convert arachidonic acid (AA) to prostaglandin endoperoxide H2 (PGH2), an important precursor of prostaglandins and thromboxanes. Importantly, synthesis of end products (prostaglandins and thromboxanes) depends on the availability of substrates (arachidonic acid). Thus, arachidonic acid (AA) must first be cleaved from phospholipids by the cytosolic or secretory enzyme phospholipase A2 (PLA_2) to allow both COX isoenzymes to act on it. First, COX-1 mediates an oxygenation reaction by inserting two molecules of O_2 into the C-H bonds of AA to produce Prostaglandin G2 (PGG2). Then, COX-2 reduces the peroxide functional group of PGG2 to form a secondary alcohol, PGH2. Via the action of tissue-specific prostaglandin (PG) synthases, PGH2 is then converted to active metabolites such as prostaglandins (PGE2, PGD2, PGF2a), prostacyclin (PGI2), and thromboxane A2 (TxA2) [156-158], which bind specific prostanoid G-protein-coupled receptors to mediate signaling responses in inflammation, blood pressure regulation, clotting, and gastrointestinal (GI) protection [159]. Of which, prostacyclin and thromboxane A2 exert antagonistic effects on platelet activity, with the former being a vasodilator that inhibits platelet

aggregation, and the latter being a vasoconstrictor that facilitates platelet aggregation. While COX-1 is a "housekeeping" gene present in many cell types that is involved in the regulation of gastric mucosal integrity, kidney hemodynamics and platelet thrombogenesis [160,161], COX-2 expression is mostly induced in response to inflammatory stimuli such as growth factors, cytokines, hormones and tumor promoters [160,162], although it is also constitutively expressed in the heart, the kidney, the reproductive tract, the brain and gastric mucosa [163]. Thus, selective inhibition of COX-2 by celecoxib is thought to reduce pain and inflammation while minimizing gastrointestinal, renal and hepatic toxicities that are typical of nonselective NSAIDs targeting both COX isoforms (Fig. 1.4). Structurally, celecoxib contains a polar sulfonamide moiety that binds to a hydrophilic side pocket near the active COX-2 binding site [156] and thus achieves a 10-20 fold higher inhibition of COX-2 over COX-1 [161,164] (Fig. 1.5).

However, since NSAIDs selective for COX-2 inhibit synthesis of COX-2-dependent prostacyclin without interfering with the production of COX-1-dependent thromboxane A2, their use is implicated in the pathogenesis of cardiovascular diseases due to reduced cardioprotection and hyper-platelet activity. Indeed, two selective COX-2 inhibitors (coxibs)—rofecoxib (Vioxx ®) and valdecoxib (Bextra®) were withdrawn from the market in 2004 and 2005, respectively, following clinical studies suggesting a cardiotoxic profile associated with their use [165,166]. Although celecoxib remains the only commercially available selective COX-2 inhibitor, it has long been viewed to exhibit an unfavorable cardiovascular profile similar to the other two coxibs that were discontinued. It is not until the completion of the PRECISION trial in 2016 that the use of celecoxib (at a dose of 100mg twice daily) was found to pose no greater risk of clot-related cardiovascular events than that of ibuprofen (dosed in the range of 600-800mg three times daily) and naproxen (dosed in the range of 375-500mg twice daily)—two nonselective NSAIDs [167].

These results have prompted FDA to consider changing its advice to physicians regarding celecoxib's safety [168].

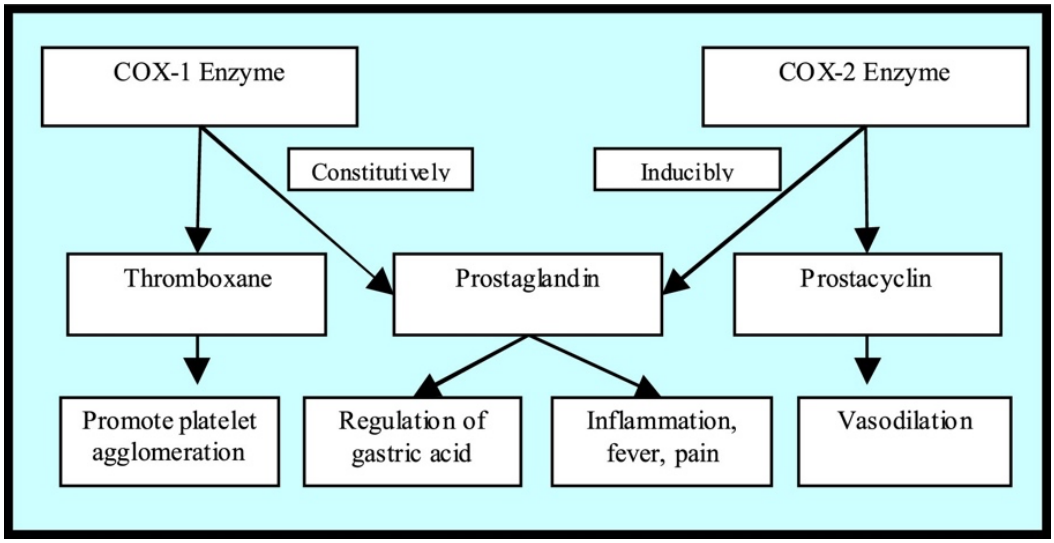


Figure 1.4: Key role of COX isoenzymes and their mediators

(Adapted from Pratiksha Saxena, 2019) [169]

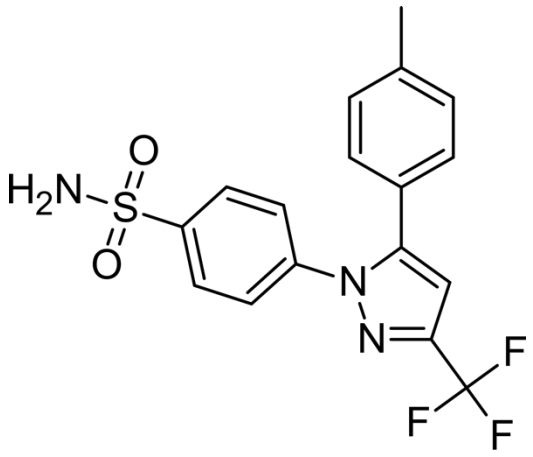


Figure 1.5: Structure of celecoxib

(Adapted from Neal M. Davies, 2000) [251]

1.3.2 Mechanism of action: anti-neoplastic

Since inflammation is highly linked to cancer progression, celecoxib as an NSAID has also been investigated in clinical studies for its anti-neoplastic effects in various cancers, including those of the lung, breast, colorectal, head and neck, bladder, cervix, esophageal, non-melanomatous and oral Mucositis [169]. Among which, celecoxib given at 400mg daily is shown to reduce the risk of colorectal adenoma recurrence by 41% in a phase III clinical trial [170], leading to its FDA approval as a chemo-preventive agent to reduce the number of adenomatous colorectal polyps in patients with familial adenomatous polyposis (FAP) [171-173]. Although the exact mechanisms by which celecoxib elicits an antitumor response is still unclear, evidence from *in vitro* and *in vivo* studies suggest that it targets both COX-dependent and COX-independent pathways to induce apoptosis, endoplasmic reticulum(ER) stress, cell cycle arrest, as well as to inhibit angiogenesis and tumor invasion [159]. Celecoxib in breast cancer appears to target COX-2, which is overexpressed in 40% of invasive breast carcinoma cases and results in poor prognosis and tumor progression [174,175]. Several preclinical studies in breast cancer also provide strong evidence for the use of selective COX-2 inhibitors (celecoxib, rofecoxib, etodolac) in the suppression of breast tumor growth and metastasis [176-182]. On the other hand, increased degradation of the oncoprotein β-catenin (encoded by gene *CTNNB1*) and downregulation of its target genes involved in angiogenesis, stemness and metastasis (FGF,

VEGF, MMPs) are observed in celecoxib-treated human colorectal cancer cell lines, leading to the hypothesis that celecoxib may target cancer stem cells (CSCs) via Wnt signaling pathway [183]. Similar observations are also made in gastric, pancreatic and lung cancer tissues or cell lines, wherein celecoxib treatment downregulates VEGF and MMP9 expression to inhibit angiogenesis and tumor invasion [184-186]. Further, celecoxib can induce apoptosis by either activating proapoptotic molecules such as caspases and CHOP (encoded by gene DDIT3) [187], or inhibiting antiapoptotic PDK/1/Akt signaling as seen in prostate and colon tumor cell lines [188-191]. Unlike other selective COX-2 inhibitors, celecoxib can also directly bind and inhibit sarcoplasmic/ER calcium ATPase, causing an influx of calcium into the cytosol, leading to ER stress and cell death [192,193].

Chapter 2: Background

This chapter is presented in a monograph format and adapted from the following published article:

Tian J, **Wang V**, Wang N, Khadang B, Boudreault J, Bakdounes K, et al. Identification of MFGE8 and KLK5/7 as mediators of breast tumorigenesis and resistance to COX-2 inhibition. *Breast Cancer Research*, 2021 Feb 15; 23(1):23 [194].

2.1 Rationale

Triple negative breast cancers (TNBCs) represent 10-20% of all breast cancers and are clinically manifested as negative for expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [195]. Due to its aggressive biology and "lack of [actionable] molecular targets [194]", TNBCs have a worse prognosis than other subtypes of breast cancer [196]. Given that these tumors are enriched in breast cancer stem cells (BCSCs), which are intrinsically chemo-resistant and allow for self-renewal and differentiation into non-BCSCs to repropagate the tumor, patients with TNBC are more likely to develop rapid loco-regional and distant recurrences after treatment with radiotherapy or cytotoxic chemotherapy [197]. Thus, devising strategies to induce durable clinical response in TNBC remains a priority for the development of anti-TNBC therapies.

COX-2 is a key enzyme involved in the biotransformation of arachidonic acid to inflammatory prostanoids with altered expression "in various types of cancers, including those of the breast" [194,198-200]. In fact, elevated levels of COX-2 occur in "40% of invasive breast carcinoma[s] [194]" and are an indicator of "poor prognosis and tumor progression [194, 201,202]". Preclinical studies in breast cancer suggest that contributions of COX-2/Prostaglandin E2 (PGE2) signaling to tumorigenesis are multi-faceted, ranging from regulation of primary tumor growth [203], to metastasis [204,205], angiogenesis [206] and immune evasion [207]. Consistent with these reports, our lab has also shown that cyclooxygenase-2 (COX-2) expression is upregulated in TNBC and corresponds to poor survival outcomes in patients with basal-like TNBC [208]. We further demonstrated that knocking down COX-2 expression or blocking its activity can effectively prevent the ability of breast cancer stem cells to self-renew and expand, thus highlighting its value as a potential therapeutic target in TNBC [208].

Celecoxib, marketed under the brand name CELEBREX, is a selective COX-2 inhibitor indicated for the treatment of osteoarthritis and rheumatoid arthritis. Unlike nonsteroidal anti-inflammatory drugs (NSAIDs) that target both COX-1 and COX-2, celecoxib can minimize inhibition of COX-1 induced gastrointestinal side effects by selectively targeting COX-2 [209]. Several preclinical studies in breast cancer have supported "the use of selective COX-2 inhibitors (celecoxib, rofecoxib, etodolac) [194] " to prevent or attenuate "tumor growth and metastasis [194, 210-216] " . Moreover, phase I/II clinical trials also provided evidence for the combined use of COX-2-aromatase inhibitors in the neoadjuvant setting to induce tumor regression in metastatic breast cancer patients [217,218] . Yet disappointingly, "a recent phase III, multicenter, double-blind, randomized trial of celecoxib vs placebo in primary breast cancer patients (REACT trial) showed no benefit in delaying time to progression or overall survival [194, 219] ". We hypothesize that the trial likely failed due to the use of an improper stratification criteria that prevented patients who could have benefitted from celecoxib from receiving it. Thus, efforts are needed to elucidate the molecular basis and associated genes/pathways underlying resistance to celecoxib in breast cancer, in order to identify biomarkers that are predictive of response to anti-COX-2 drugs in breast cancer patients.

2.1.1 Objective

The objective of this study is to identify COX-2 associated genes in TNBC and define their roles and contributions to (1) breast tumorigenesis and (2) response to COX-2 inhibitor.

2.2 Previous work from our lab

We conducted a comprehensive in silico analysis of publicly available genomic, transcriptomic and clinical profiles of breast cancer patients to identify COX-2 associated genes that " harbor high potential to 1) promote TNBC tumorigenesis and 2) resistance to COX-2 inhibitors [194] ".

2.2.1 Identification of COX-2 associated genes in TNBC

First, we manually selected TNBC samples from the TCGA-BRCA dataset according to the IHC status of ER, PR and HER2, and grouped them into COX-2^{high} and COX-2^{low} by comparing their COX-2 mRNA expression levels to the overall distribution using cBioPortal for Cancer Genomics online application (https://www.cbioportal.org/) [223,224]. Then, we used "z-score greater than +1 or less than -0.25 [194] " as the cut-off to select the top 15% of the COX-2^{high} (n=18) and the bottom 15% of the COX-2^{low} (n=19) patient samples for differential expression gene (DEG) analysis. By conducting moderated t-test on patient mRNA expression data using GenePattern web software [225], we shortlisted genes that are differentially enriched in the COX-2^{high} and COX-2^{low} group based on the ranking of their t-values. Finally, to narrow down the range of our selections, we applied additional filters ("fold change > 1.5, p value < 0.05, t-test >2 or < -2, FDR <0.35 [194] ") and identified 43 and 60 genes that are significant and differentially upregulated in the COX-2^{high} and COX-2^{low} group, respectively.

2.2.2 Identification of COX-2 associated genes that display genetic alterations and predict poor prognosis in TNBC

To select for COX-2 associated genes with high tumorigenic potential similar to that of COX-2, we "focused on the 43 DEGs enriched in the COX-2^{high} patient group [194]". First, we used the cBioPortal for Cancer Genomics online application to characterize their genetic alteration profiles in TCGA-BRCA TNBC dataset (n=116). For this, we ranked each of the 43 DEGs according to their genetic mutation rates "([DNA] copy number amplification, mRNA upregulation) [194]"—the sum of the percentage of breast tumor samples in which a given gene is amplified with "copy number status: +2" and upregulated with "z-score greater than +1 [194]", and selected those "that are altered in more than 10%" of the TNBC cohort as "gene list 1 (GL1: 32 genes) [194]". In parallel, we also ranked all 43 DEGs by their "[DNA] copy number amplification rate" in Metastatic Breast Cancer dataset (MBC), and selected those that are "amplified in more than 1% [194]" of MBC patient samples as "gene list 2 (GL2: 36 genes) [194]".

Next, we used the UCSC Cancer Genomics Browser (https://genome-cancer.ucsc.edu) to investigate the expression pattern of each DEG across PAM50 subtypes of breast cancer "from the TCGA-BRCA dataset (n=1247) [194]". Following the analysis, we shortlisted candidates that demonstrate "the highest expression level in basal BC compared with the other subtypes (Luminal A, Luminal B, HER2-enriched, Normal-like) [194]" as "gene list 3 (GL3: 34 genes) [194]". To further identify genes that are specifically upregulated in TNBCs, we used the Breast Cancer Gene-Expression Miner v4.0 (bc-GenExMiner v4.0) online platform to compare the expression level of all 43 DEGs "in TNBC versus non-TNBC patients from a large breast cancer patient cohort (n=5696) [194]". Genes "that are significantly upregulated in TNBCs versus non-TNBCs" are included in "gene list 4 (GL4: 30 genes) [194]".

Last but not least, we used the Kaplan Meier plotter online application (http://kmplot.com/analysis/) [226] to correlate the effect of DEG expression with survival in

patients with basal-like breast cancer. This led us to identify candidates "whose [elevated] expressions significantly associated with poor overall survival and distant metastasis-free survival rates [194]" as "gene list 5 (GL5: 20 genes) [194]".

2.2.3 Identification of COX-2 associated genes that modulate resistance to COX-2 inhibition in breast cancer

Given that all 43 DEGs are enriched "in the COX-2^{high} patient group [194]", we next investigated their roles in mediating breast tumor response to valdecoxib, a selective COX-2 inhibitor. For this, we downloaded drug sensitivity profiles of 37 valdecoxib-treated breast cancer cell lines from the cancer therapeutics response portal v2 (CTRP v2) (http://portals.broadinstitute.org/ctrp/), and ranked them by "their EC50 values[s] for valdecoxib [194]" from low to high. Of the 37 cell lines, we labelled 18 that harbor "the lowest EC50 value[s]" as "more-sensitive (MS) [194]" to valdecoxib, and 19 that harbor the highest EC50 values" as "less-sensitive (LS)" to valdecoxib. We then leveraged the Cell Line Gene Expression (CCLE) dataset in the cBioPortal online application to analyze the expression patterns of each DEG in these 37 cell lines. This led us to eventually shortlist 13 DEGs that are upregulated in "valdecoxib-LS" versus "valdecoxib-MS" cell lines as "gene list 6" (GL6) ".

By cross-referencing "all 6 gene lists (GL1-GL6)", we identified "10 overlapping [DEGs] (TPM4, RGS2, LAMC2, SERPINB5, KLK7, MFGE8, KLK5, ID4, RBP1, SLC2A1) [194]" that are 1) upregulated in COX-2 inhibitor-LS BC cell lines, but also 2) display genetic alterations and predict poor prognosis in patients with aggressive BCs (Table 1). Thus, we conclude that these 10 genes harbor pro-tumorigenic and pro-metastatic potential similar to that of COX-2, and are potential mediators of resistance to COX-2 inhibitors in breast cancer.

2.2.4 Next steps

To confirm our *in silico* predictions, we generated CRISPR/Cas9 knockouts of each of the 10 COX-2 associated candidate genes and validated their functions using cell lines and preclinical models of TNBC. Detailed results are presented in Chapter III.

Chapter 3: Methods

This chapter is reproduced from the "Methods" section of the following published article:

Tian J, **Wang V**, Wang N, Khadang B, Boudreault J, Bakdounes K, et al. Identification of MFGE8 and KLK5/7 as mediators of breast tumorigenesis and resistance to COX-2 inhibition. *Breast Cancer Research*, 2021 Feb 15; 23(1):23 [194].

3.1 Methods

Note: Text used in this section are adapted verbatim from the "Methods" section of the COX-2 paper and cited accordingly

All experimental protocols and procedures were performed in accordance to McGill University regulations. All experimental protocols and procedures were approved by McGill University.

"Cell culture and generation of celecoxib-resistant cells

Human breast cancer cell lines MDA-MB-231 and SUM159 were cultured as previously described [208] . We generated two MDA-MB-231 variant cell lines enriched of celecoxib-resistant cells, using increasing concentrations (40 μM and 80 μM) of celecoxib (pZ0008-5MG, Sigma). Selection pressure was maintained for 3 weeks under these cell culture conditions. At end point, and as a proof-of-principle, COX-2 expression was assessed at both mRNA and protein levels to verify the proper COX-2 increase normally observed in COX-2-resistant cells [222] .

CRISPR-Cas9 sgRNA cloning

Different scrambled sgRNAs and sgRNAs that target COX-2 and 10 candidate genes were cloned into the lentiCRISPR v2 backbone (Addgene plasmid # 52961) individually. All steps were performed according to the protocol provided by Feng Zhang's lab. Briefly, lentiCRISPR v2 plasmid was first digested and phosphorylated with BsmBI and then gel purified. Two oligos of each sgRNA were phosphorylated and annealed to each other using T4 Ligation Buffer. Next, diluted oligos and BsmBI digested lentiCRISPR v2 plasmid were ligated and transformed into Stbl3 bacteria. PCR was performed to confirm the insertion of oligos in the backbone plasmid. Sequences of sgRNAs are shown in Supplementary Table S1.

Lentivirus production and infection

LentiCRISPR v2 plasmids containing scrambled and different sgRNAs sequences were co-transfected into HEK293 cells with the packaging plasmids (psPAX2 and Pmd2.g). Transfection was performed using Opti-MEM (Invitrogen) and bPEI (Sigma). 48 h following transfection, cell culture medium containing lentiviruses were collected. To generate stable gene knockout cell line, MDA-MB-231 cells and SUM159 cells were cultured to 50% confluence and then infected with lentiviruses using 8 μg/ml polybrene. Then, 24 h later, 2 μg/ml of puromycin was added to the medium to select stable cells for a minimum of 1 week.

Surveyor assay

In total, 200,000 MDA-MB-231 and SUM159 stable KO cells were used for Surveyor assay. All experiments were performed using GeneArt® Genomic Cleavage Detection Kit (life technologies) according to the manufacturer's protocol. Briefly, DNA loci where the gene-specific double-strand breaks occur were PCR amplified. Then these PCR products were denatured and re-annealed so that the mismatches were generated. Next the mismatches were cleaved by Detection Enzyme and detected by gel electrophoresis. Cleavage efficiency was calculated using the following equation: Cleavage efficiency = 1 − [(1 − fraction cleaved) ½]; fraction cleaved = sum of cleaved band intensities/ (sum of the cleaved and parental band intensities). Sequences of PCR primers are shown in Supplementary Table S2.

Distant metastasis mouse model

All animal experiments were conducted in accordance with protocols approved by the McGill University Health Center. Scrambled and candidate gene sgRNA transfected MDA-MB-231 cells (and SUM159 cells) (1×10^6 cells/mouse) were injected into the tail vein of 6-week-old female NOD SCID IL2gammaR knockout (NSG) mice (4 mice per group). Three weeks post injection, mice were sacrificed, and the lungs were collected and fixed in 10% formalin. The lung tissues were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

Quantification of H&E staining

The mean percentage of total lung involvement based on a visual scoring was performed by two pathologists. A variety of patterns were observed in tumor cells including a small nodular, infiltrative, and solid pattern. Where applicable, the largest size of nodule in lung parenchyma was measured microscopically.

Orthotopic xenograft mouse model

The scrambled and candidate gene KO cells generated from MDA-MB-231 cell line were resuspended in serum-free medium and Corning™ Matrigel™ in 1:1 ratio and then transplanted in the mammary gland of 6-week-old female NSG mice (1×10^6 cells per mouse, 8 mice per group). When the tumor size reached 150–200 mm^2, each mice group was randomly subdivided into two groups and treated with either vehicle or celecoxib (7.5 mg/kg/day) through intraperitoneal (IP) injection for up to 3 weeks. Primary mammary tumor size was measured using a caliper (number) times every week and determined according to the formula:

$(4/3) \times \pi \times (\text{Length}/2) \times (\text{width}/2)^2$. The mice were sacrificed when control tumors reach max authorized volume (2.5 cm^3).

Sulforhodamine B (SRB) assay

SRB assay was used to measure growth inhibition in cells. MDA-MB-231 cells (and SUM159 cells) (CRISPR scrambled and stable KO cells) were grown in DMEM complete medium (and F12 HAM's complete medium, respectively) (2500 cells/well) in a 96-well plate and allowed to attach for 24 h. The cells were then treated with a dose range of celecoxib for 96 h. After treatment, the cells were fixed with 50% trichloroacetic acid (TCA) for 2 h at 4 °C, rinsed with water 4 times, stained with 0.4% SRB for 1 h and rinsed with 1% acetic acid. After air dry overnight, the SRB dye was solubilized with 10 mM Tris base and the plates were read at 490 nm using a microplate reader. The results were analyzed and graphed using GraphPad Prism 6.0 (GraphPadSoftware, Inc., San Diego, CA).

Prestoblue assay

MDA-MB-231 (and SUM159 cells) were seeded into 96-well plates with black bottom (2500/well). Then, 24 h later, cells were treated with a dose range of celecoxib for 96 h and then incubated with PrestoBlue™ Cell Viability Reagent (Thermo Fisher Scientific) for 40 min at 37 °C/5% CO_2. Fluorescence measurements (excitation 535 nm, emission 615 nm) were then taken on the 96-well plates and the fluorescence values were recorded and analyzed. Since the fluorescence values have a linear correlation with the cell numbers, the data were used to calculate the percentage of cell viability inhibition following celecoxib treatment.

Western blot

Western blot analysis was performed as previously described [208] . Briefly, human breast cancer cells were lysed in Tris lysis buffer. Lysates containing total protein were separated by SDS-PAGE and transferred to nitrocellulose membrane. COX-2 protein levels were detected using rabbit monoclonal COX-2 antibody (Cell Signalling). Mouse monoclonal β-tubulin antibody (Santa Cruz Biotechnology) was used as loading controls. Each protein was detected using Clarity™ ECL western blotting substrate from Bio-Rad.

Statistics

Student's *t* test or one-way ANOVA was used to evaluate significance between groups. At least three independent experiments were performed and $P < 0.05$ was considered significant [194] ".

Chapter 4: Results

This chapter is presented in a monograph format and adapted from the following published article:

Tian J, **Wang V**, Wang N, Khadang B, Boudreault J, Bakdounes K, et al. Identification of MFGE8 and KLK5/7 as mediators of breast tumorigenesis and resistance to COX-2 inhibition. *Breast Cancer Research*, 2021 Feb 15; 23(1):23 [194].

4.1 Identification of COX-2 associated candidate genes as regulators of TNBC metastasis

To functionally validate each of the 10 candidate genes as regulators of lung metastasis in TNBC, I generated individual CRISPR/Cas9 knockouts of all 10 genes in MDA-MB-231, a TNBC cell line "derived from the pleural effusion of a metastatic breast cancer patient [194,227]" alongside Dr. Jun Tian. Using guides targeting scrambled and COX-2 as negative and positive controls, we designed two single guide RNAs (sgRNAs) targeting site-specific genomic regions for each candidate gene. After cloning specific sgRNAs into the lentiCRISPRv2 backbone and transducing human MDA-MB-231 cells with sgRNA-expressing lentiviruses, we assessed the presence of indel mutations generated by all sgRNAs and selected those with the highest cleavage efficiency (Fig. 1a, b) for subsequent validation studies. We also confirmed by western blot that all 3 COX-2 targeting sgRNAs induced "complete loss of COX-2 protein expression [194]" as compared with non-targeting scrambled sgRNAs (Fig. 1c).

Given the overexpression of COX-2 in invasive breast cancer and its known role in mediating tumor progression, we validated each of the 10 COX-2 associated genes for their contributions to TNBC metastasis, by leveraging an experimental lung metastasis model of breast cancer. For this, we injected MDA-MB-231-derived CRISPR/Cas9 scrambled and knockouts of candidate genes (1×10^6 cells per mouse) into the tail vein of NOD SCID IL2gammaR (NSG) mice to artificially induce the formation of lung metastases. Three weeks post-inoculation, we collected lung tissues of the mice and stained them with hematoxylin and eosin (H&E) to "assess for the presence of metastatic loci". Specifically, we found "mice injected with scrambled MDA-MB-231 cells [194]" to develop "large areas of lung metastases [194]", and "mice injected with COX-2 KO cells" to form "only few micro-metastases (Fig. 2a, b) [194]". These results affirm the pro-metastatic effects of COX-2 in breast cancer, as highlighted by previous reports [205,216]. Among

the 10 COX-2 associated genes, we found individual deletion of 6 genes (TMP4, RGS2, SERPINB5, MFGE8, KLK5, ID4) to result in a ~90% reduction in lung metastatic burden, and KOs of the remaining 4 genes (LAMC2, KLK7, RBP1, SLC2A1) to suppress lung colonization in TNBC by 60-80% (Fig. 2a, b). Thus, we conclude that all 10 COX-2 associated genes have the potential to serve as independent biomarkers of metastasis in TNBC, which validated our *in silico* approach to select DEGs that harbor pro-tumorigenic and pro-metastatic potential similar to that of COX-2.

4.2 Deletion of LAMC2, MFGE8, KLK5, KLK7 and SLC2A1 restores TNBC sensitivity to celecoxib *in vitro*

Based on the observation that all 10 candidate genes are upregulated in valdecoxib-resistant breast cancer cell lines derived from the CTRP dataset, I next validated their roles as potential mediators of resistance to celecoxib, a selective COX-2 inhibitor, in two TNBC cell lines: MDA-MB-231 and SUM159PT, alongside Dr. Jun Tian. Briefly, we treated scrambled and stable candidate gene-KO cell lines generated in MDA-MB-231 "with a dose range of celecoxib [or not] for 4 days [194]", and measured the degree of cell viability inhibition induced by each KO using the sulforhodamine B (SRB) colorimetric assay. We found "gene deletion of *LAMC2, MFGE8, KLK5, KLK7,* or *SLC2A1* [194] " to significantly increase TNBC sensitivity to celecoxib, as shown by a decrease in their IC50 values "compared to [that of] scrambled cells [194]". To the contrary, individual knockouts of "*TPM4*, *RGS2*, *SERPINB5*, *ID4*, or *RBP1* [194]" in MDA-MB-231 cells did not affect TNBC sensitivity to celecoxib (Fig. 3a, b). We then confirmed these results in a Prestoblue assay by treating MDA-MB-231-derived *LAMC2*, *MFGE8*, *KLK5*, and *SLC2A1* KO cells with or without 50 μM celecoxib for 4 days. As expected, we found

silencing the expression of *LAMC2*, *MFGE8*, *KLK5*, and *SLC2A1* to potentiate the effect of celecoxib-induced cytotoxicity as compared to scrambled cells. In fact, celecoxib induced a 51.5%, 49.3%, 47.9%, and 51.3% reduction in cell viability in each of the 4 KO cell lines mentioned above, as compared to a 27.3% reduction in cell viability in scrambled cells (Fig. 3c). To "confirm these findings in a different genetic background [194]", we also generated individual knockouts of *LAMC2*, *MFGE8*, *KLK5*, *KLK7, SLC2A1* in SUM159 PT ("hereafter referred to as SUM159 [194]"), a TNBC cell line that is "derived from a patient with anaplastic breast carcinoma [194]". Interestingly, we found all other candidate-gene KOs, "with the exception of LAMC2 [194]", to restore TNBC sensitivity to celecoxib to variable extent, as demonstrated by their lowered IC50 values in the SRB assay (Fig. S1A, B). Further, I confirmed these results in a Prestoblue assay, and found all four gene KOs including LAMC2 to induce significant inhibition of cell viability as compared to scrambled cells upon celecoxib treatment (Fig. S1C). These results suggest that *LAMC2*, *MFGE8*, *KLK5*, *KLK7, SLC2A1* function as key mediators of celecoxib resistance in TNBC, and knocking out their expression can effectively restore TNBC sensitivity to celecoxib *in vitro*.

4.3 Role of MFGE8, KLK5 and KLK7 in regulating primary tumor growth and TNBC resistance to celecoxib *in vivo*

Having previously demonstrated that genetic deletion of MFGE8, KLK5, and KLK7 "not only suppressed tumor metastasis but also restored celecoxib sensitivity to a greater degree compared to other candidate genes [194]", I next evaluated the efficacy of individual KOs of all 3 genes in combination with celecoxib on primary tumor growth using an orthoptic xenograft of breast cancer, alongside Dr. Ni Wang. Briefly, we injected MDA-MB-231-derived scrambled, MFGE8,

KLK5, and KLK7 KO cells "into the mammary fat pad of NSG mice (1×10^6 cells per mouse) [194]" to allow for growth of orthotopic tumors. When mammary tumors become palpable four weeks post-injection, we randomly divided "mice injected with scrambled and [candidate-gene] KO cells [194]" into two groups and treated them "with vehicle or celecoxib (7.5 mg/kg/day) through IP injection for up to 4 weeks [194]". During the course of celecoxib treatment, we measured the size of primary mammary tumors and weighed mice three times a week. We noticed that when celecoxib is used at a dose that is too low to decrease tumor volume in mice injected with scrambled cells ("vehicle vs celecoxib in scrambled KOs, black and gray lines, respectively [194]"), it is able to, however, potentiate the tumor suppression effect in mice injected with MFGE8, KLK5 and KLK7 KO cells. Indeed, mice injected with MFGE8, KLK5 and KLK7 KO cells all experienced significant reductions in tumor volume by 31.3%, 18.6%, and 20.7%, respectively, upon celecoxib treatment as compared with vehicle treatment (Fig. 4a-c, colored lanes).

To determine if inhibition of the COX-2 pathway by celecoxib leads to a compensatory increase in the expression of MFGE8, KLK5, and or KLK7, I generated two MDA-MB-231 variants enriched of celecoxib-resistant clones "by treating parental cells with 40 and 80 μM celecoxib for 3 weeks [194]". Consistent with a previous report that suggests celecoxib-resistant clones present in "aggressive breast cancer cell lines [194]" overexpress COX-2 [28], we observed an upregulation of COX-2 at the mRNA level in celecoxib-resistant variants as opposed to their parental counterparts (Fig. S2A). We also confirmed by western blot that the two celecoxib-resistant variants (40 and 80 μM) derived from MDA-MB-231 produced higher levels of COX-2 protein than the parental cell line itself (Fig. S2B). These results highlight the role of COX-2 as a potential mediator of celecoxib resistance "in certain contexts [194]". In addition, of the 10

candidate genes, we found only LAMC2 to be upregulated at the mRNA level in celecoxib-resistant variants (Fig.S2A). Expression of the 9 other candidate genes remains unaltered in celecoxib-resistant variants (data not shown).

We hypothesize that the discrepancy between results derived from cell-based experiments and information obtained from mining the CTRP database "might be due to [the] various mechanisms of action of different COX-2 inhibitors [194]". Based on our observation, we suspect that these 10 candidate genes (*TPM4*, *RGS2*, *LAMC2*, *SERPINB5*, *KLK7*, *MFGE8*, *KLK5*, *ID4*, *RBP1*, *SLC2A1*) function neither downstream of COX-2 in the COX-2/PGE2 signaling pathway nor in compensatory pathways that are "upregulated in COX-2 inhibitor-resistant cells [194]". On the other hand, we also observed a correlation between the expression of all 10 candidate genes and that of COX-2 in TNBC patients from the TCGA dataset (Fig. S3) through *in silico* work done by Dr. Jun Tian. Given these information, we speculate that the synergistic suppression of TNBC primary tumor growth mediated by COX-2 inhibition and deletion of KLK5/7 and MFGE8 might converge on disrupting "different aspects of [breast] tumorigenesis [194]".

Although the molecular basis underlying resistance to COX-2 inhibition in breast cancer remains elusive, our data suggest that genetic inhibition of KLK5/7 or MFGE8 "can sensitize TNBC to celecoxib [194]" in both *in vitro* and *in vivo* models of human breast cancer. This provides a rationale for establishing KLK5/7 and/or MFGE8 as a treatment-selection biomarker in TNBC to identify groups of patients who are more likely to benefit from celecoxib than without. In order to translate this finding to the clinic, all three genes need to be assessed individually with respect to their ability to differentiate treatment outcomes among TNBC patients treated with celecoxib or standard of care in a randomized trial. Currently, we are unable to estimate the magnitude of

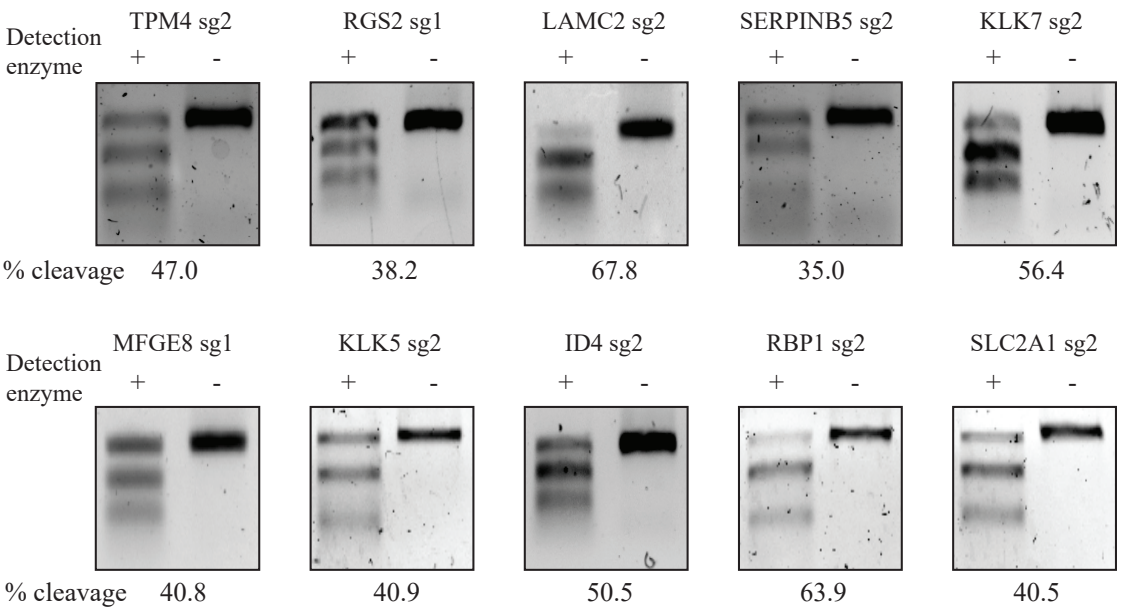
clinical benefits celecoxib will confer those who test negative for expression of KLK5/7 and/or MFGE8 based on preclinical data alone. However, our results provide a starting point for designing randomized clinical trials that are guided by the use of "all (or some of) the identified genes [194]" as potential biomarkers or therapeutic targets in combination with COX-2 inhibitors for TNBC management.

Taken together, we performed function validation studies of 10 candidates identified from *in silico* analysis of multi-omics data generated from various breast cancer patient cohorts. Of the 10 candidates, we identified 3 genes—KLK5, KLK7, and MFGE8—whose deletion can 1) suppress TNBC metastasis to lung and 2) increase TNBC sensitivity to celecoxib *in vivo*. We also observed that combined inhibition of COX-2 with MFGE8 or KLK5/7 induced synergistic suppression of breast tumor growth. These results provide an initial proof-of-concept to support the development of "COX-2 inhibitor-based combination therapies for breast cancer patients [194]".

Selection criteria	*High genetic alteration in TCGA-TNBC patients (**GL1**)*	*High genetic alteration in MBC patients (**GL2**)*	*High mRNA expression in PAM50 basal BC patients (**GL3**)*	*High Mrna expression in TNBC patients (**GL4**)*	*High expression correlates with poor survival outcomes (**GL5**)*	*High expression in COX-2 inhibitor resistant BC cell lines (**GL6**)*
Gene symbol	MDFI EPHB3 FBXO32 MSN SFRP1 ID4 ACTN4 TNFRSF21 MFGE8 TFAP2C RGS2 ITGB4 SLC44A2 ITGA6 S100B KLK7 DEGS1 GNAS PTGS2 LAMC2 KLK5 TACSTD2 SLC2A1 CTNNB1 KLK6 CD55 TPM4 FURIN RBP1 KRT6B PPP1R1B SERPINB5	FBXO32 PPP1R1B THBS1 LAMC2 STAC2 ATP1B1 DEGS1 IER3 OGFRL1 GNAS TFAP2C CD55 SFRP1 ITGB4 MFGE8 EPHB3 ACTN4 RGS2 PTGS2 SLC2A1 COL9A2 DSP MDFI PTP4A1 TNFRSF21 ID4 FURIN CTNNB1 KLK7 TPM4 TACSTD2 FBLN2 SERPINB5 KLK5 KLK6 RBP1	TPM4 TNFRSF21 RGS2 TFAP2C ITGA6 COL9A1 SFRP1 FURIN LAMC2 TACSTD2 MSN S100B FBXO32 STAC2 DSP KRT6B SERPINB5 ITGB4 FOXI1 EPHB3 MDFI KLK7 OGFRL1 MFGE8 ACTN4 KLK5 SLC44A2 KLK6 ID4 RBP1 PTP4A1 TMX4 SLC2A1 GNAS	TPM4 TNFRSF21 RGS2 TFAP2C ITGA6 SFRP1 FURIN LAMC2 CD55 MSN S100B FBXO32 STAC2 KRT6B FBLN2 SERPINB5 ITGB4 EPHB3 MDFI KLK7 OGFRL1 MFGE8 ACTN4 KLK5 SLC44A2 KLK6 ID4 RBP1 TMX4 SLC2A1 GNAS	TPM4 RGS2 TFAP2C ITGA6 ATP1B1 LAMC2 CD55 TACSTD2 CTNNB1 KRT6B SERPINB5 IER3 ITGB4 KLK7 MFGE8 KLK5 ID4 RBP1 PTP4A1 SLC2A1	TPM4 RGS2 COL9A2 SFRP1 LAMC2 FBLN2 SERPINB5 KLK7 MFGE8 KLK5 ID4 RBP1 SLC2A1

Table 1: "Gene lists based on various selection criteria from the 43 DEGs in COX-2 high patients [194] "

A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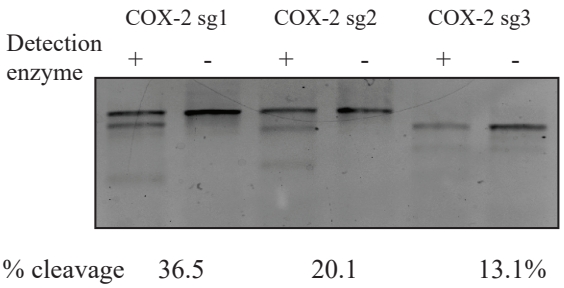
TPM4 sg2
RGS2 sg1
LAMC2 sg2
SERPINB5 sg2
KLK7 sg2
+ -
% cleavage 47.0 38.2 67.8 35.0 56.4
MFGE8 sg1
KLK5 sg2
ID4 sg2
RBP1 sg2
SLC2A1 sg2
% cleavage 40.8 40.9 50.5 63.9 40.5

B


Detection enzyme
COX-2 sg1
COX-2 sg2
COX-2 sg3
+ -
% cleavage 36.5 20.1 13.1%

C

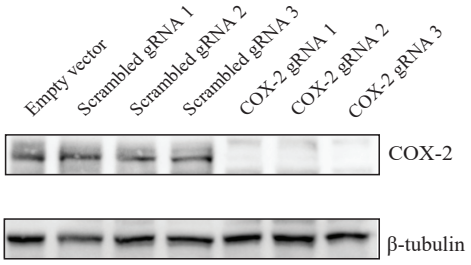
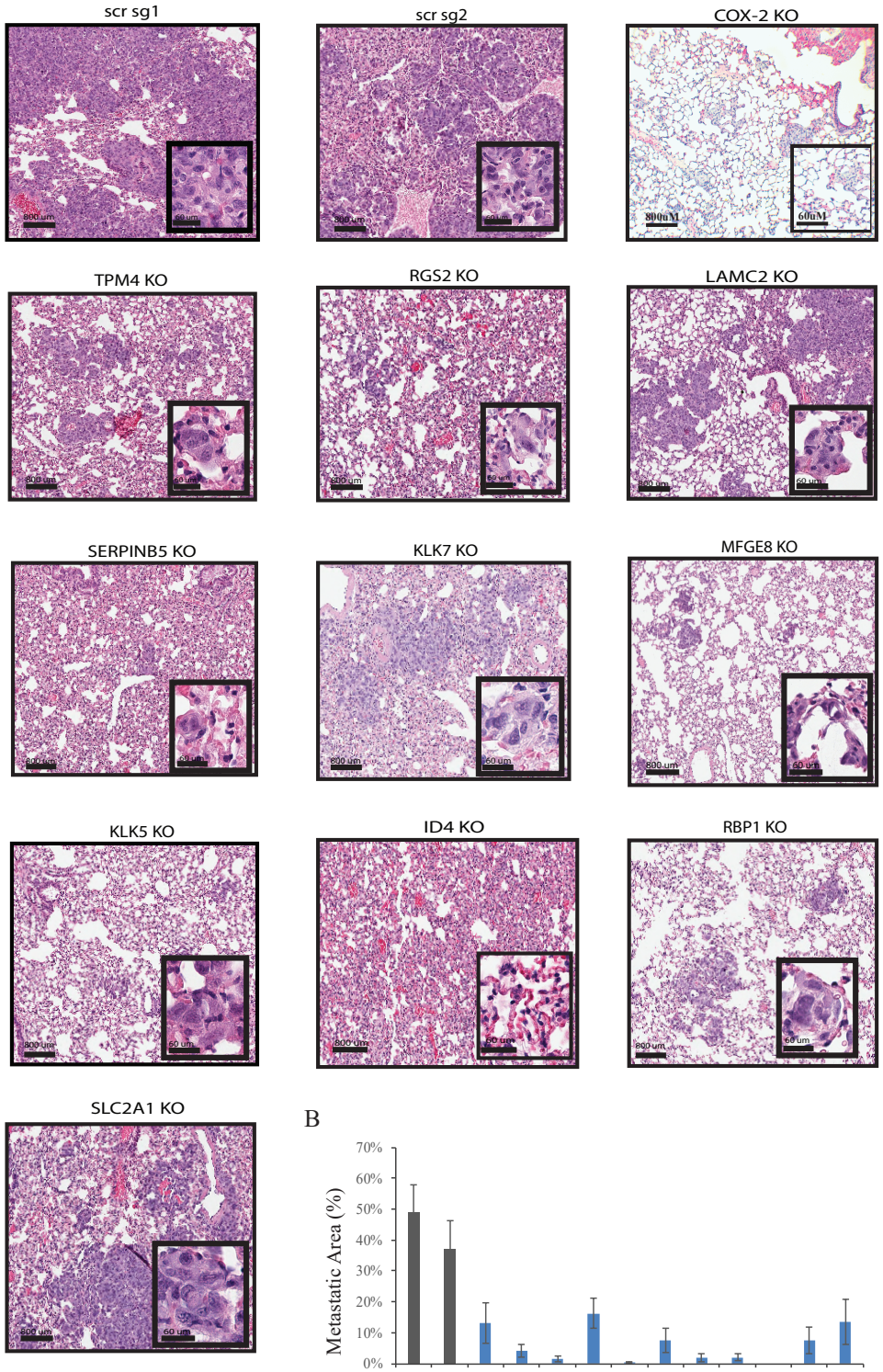

Empty vector
Scrambled gRNA 1
Scrambled gRNA 2
Scrambled gRNA 3
COX-2 gRNA 1
COX-2 gRNA 2
COX-2 gRNA 3
COX-2
β-tubulin

Figure 1: "CRISPR knock out of COX-2 and 10 candidate genes in TNBC cells

A,B MDA-MB-231 cells were infected by lentivirus containing various gRNA sequences targeting 10 candidate genes (**A**) and COX-2 (**B**), and then subjected to Surveyor nuclease assay. Cleavage efficiency for each KO was calculated. **C,** COX-2 KO in MDA-MB-231 cells was validated by western blot [194]".

A


scr sg1
scr sg2
COX-2 KO
TPM4 KO
RGS2 KO
LAMC2 KO
SERPINB5 KO
KLK7 KO
MFGE8 KO
KLK5 KO
ID4 KO
RBP1 KO
SLC2A1 KO

B

Metastatic Area (%)
70%
60%
50%
40%
30%
20%
10%
0%
Scr sg1
Scr sg2
COX-2 KO
TPM4 KO
RGS2 KO
LAMC2 KO
SERPINB5 KO
KLK7 KO
MFGE8 KO
KLK5 KO
ID4 KO
RBP1 KO
SLC2A1 KO

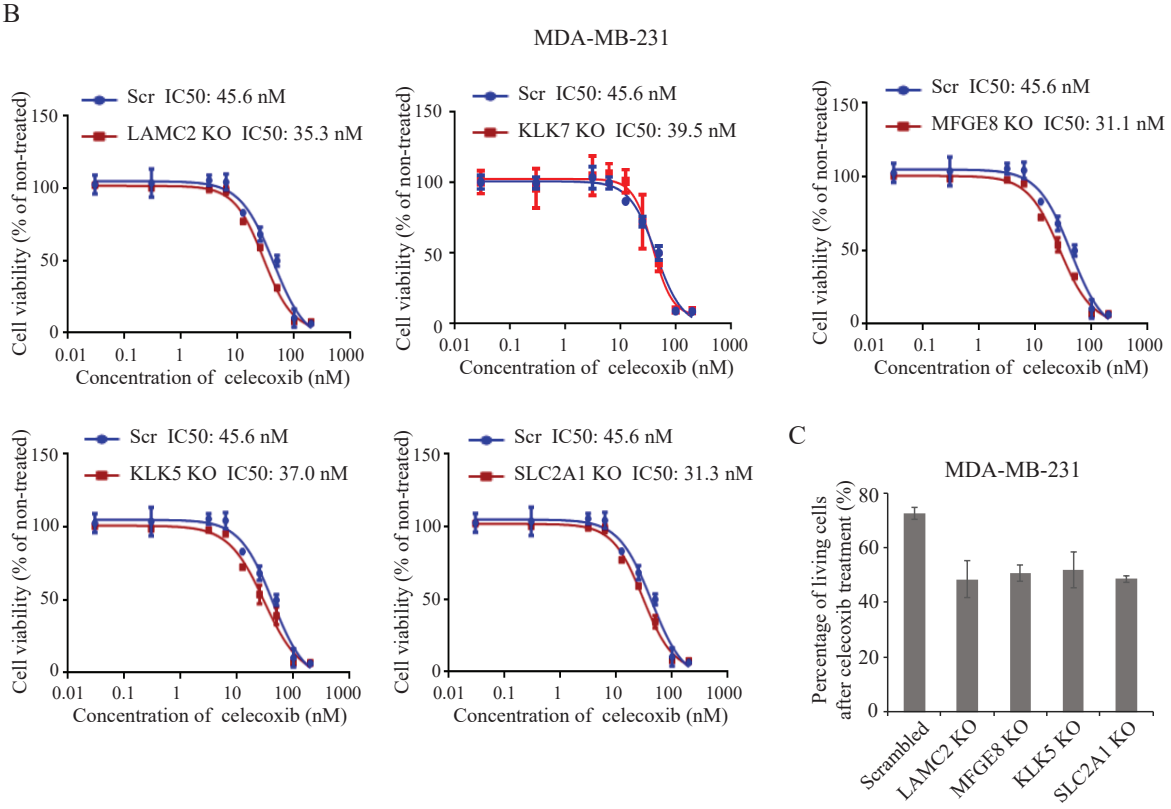
Figure 2: "Identification of COX-2 associated genes regulating breast tumor lung metastasis

A, H&E staining of the lung tissues from NSG mice injected with MDA-MB-231 control (scrambled) cells, COX-2 KO cells, and each of the 10 candidate genes KO cells. **B,** Percentage of metastatic area of the control and KO tumors was calculated by two pathologists [194] ".

A

CRISPR knock out in MDA-MB-231 cells	*IC50 +/- SEM (uM)*	*P value*
Scramble	45.63 +/- 0.95	
TPM4	41.75 +/- 0.6	0.097
RGS2	42.69 +/- 1	0.343
LAMC2	35.28 +/- 2.52	0.046
SERPINB5	40.13 +/- 0.51	0.068
KLK7	39.53 +/- 0.44	0.016
MFGE8	31.11 +/- 1.24	0.0003
KLK5	36.96 +/- 2.31	0.044
ID4	41.74 +/- 0.55	0.083
RBP1	46.53 +/- 2.23	0.508
SLC2A1	31.27 +/- 1.27	0.0004

B



C

Figure 3: "Identification of COX-2 associated genes contributing to COX-2 inhibitor resistance in TNBC cells

A, MDA-MB-231 control and KO cell lines were treated with a dose rage of celecoxib for 4 days and subjected to cell viability test using SRB assay. IC50 values of control and each KO cells were indicated. **B,** Celecoxib dose response curve in MDA-MB-231 control, LAMC2, KLK7, MFGE8, KLK5, and SLC2A1 KO cells. **C,** MDA-MB-231 control, LAMC2, MFGE8, KLK5, and SLC2A1 KO cells were treated with 50 μM celecoxib for 4 days and then subjected to Prestoblue cell viability assay. Percentage of living cells after celecoxib treatment was calculated [194]".

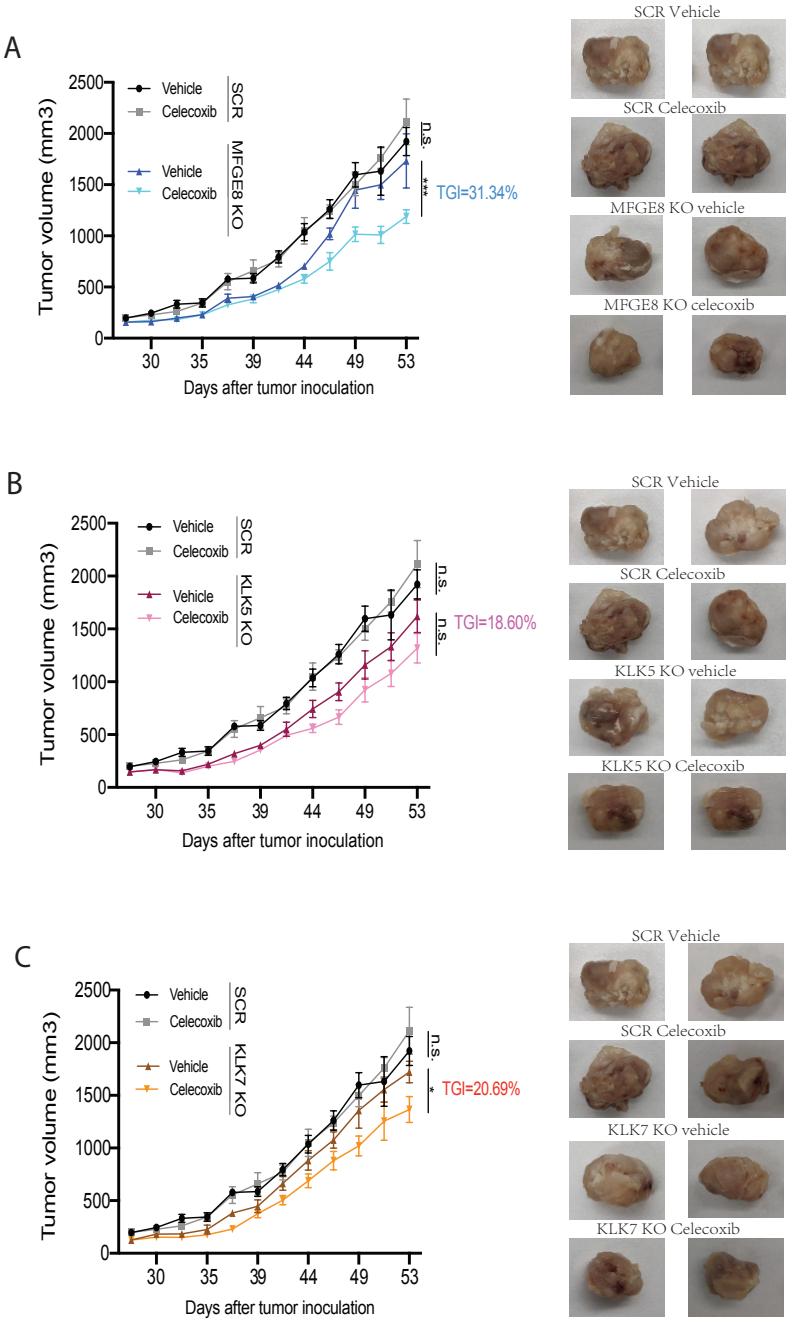

A
Tumor volume (mm3)
2500
2000
1500
1000
500
0
30
35
39
44
49
53
Days after tumor inoculation
Vehicle
Celecoxib
SCR
Vehicle
Celecoxib
MFGE8 KO
n.s.
***
TGI=31.34%
SCR Vehicle
SCR Celecoxib
MFGE8 KO vehicle
MFGE8 KO celecoxib
B
Tumor volume (mm3)
Days after tumor inoculation
Vehicle
Celecoxib
SCR
Vehicle
Celecoxib
KLK5 KO
n.s.
n.s.
TGI=18.60%
SCR Vehicle
SCR Celecoxib
KLK5 KO vehicle
KLK5 KO Celecoxib
C
Tumor volume (mm3)
Days after tumor inoculation
Vehicle
Celecoxib
SCR
Vehicle
Celecoxib
KLK7 KO
n.s.
*
TGI=20.69%
SCR Vehicle
SCR Celecoxib
KLK7 KO vehicle
KLK7 KO Celecoxib

Figure 4: "Role of COX-2-associated genes in regulating celecoxib resistance in vivo

Primary tumor growth curves and tumor images from mice inoculated with MDA-MB-231 control, MFGE8 (**A**), KLK5 (**B**), and KLK7 (**C**) KO cells and randomly grouped (n = 8/group). Celecoxib treatment was performed as described in the "Methods" section [194] ".

A

CRISPR knock out in SUM159 cells	*IC50 +/- SEM (uM)*	*P value*
Scramble	44.19 +/- 2.05	
LAMC2	43.57 +/- 4.29	0.924
KLK7	32.5 +/- 0.44	0.026
MFGE8	33.01 +/- 1.73	0.017
KLK5	35.62 +/- 3.53	0.119
SLC2A1	36.68 +/- 0.51	0.068

B

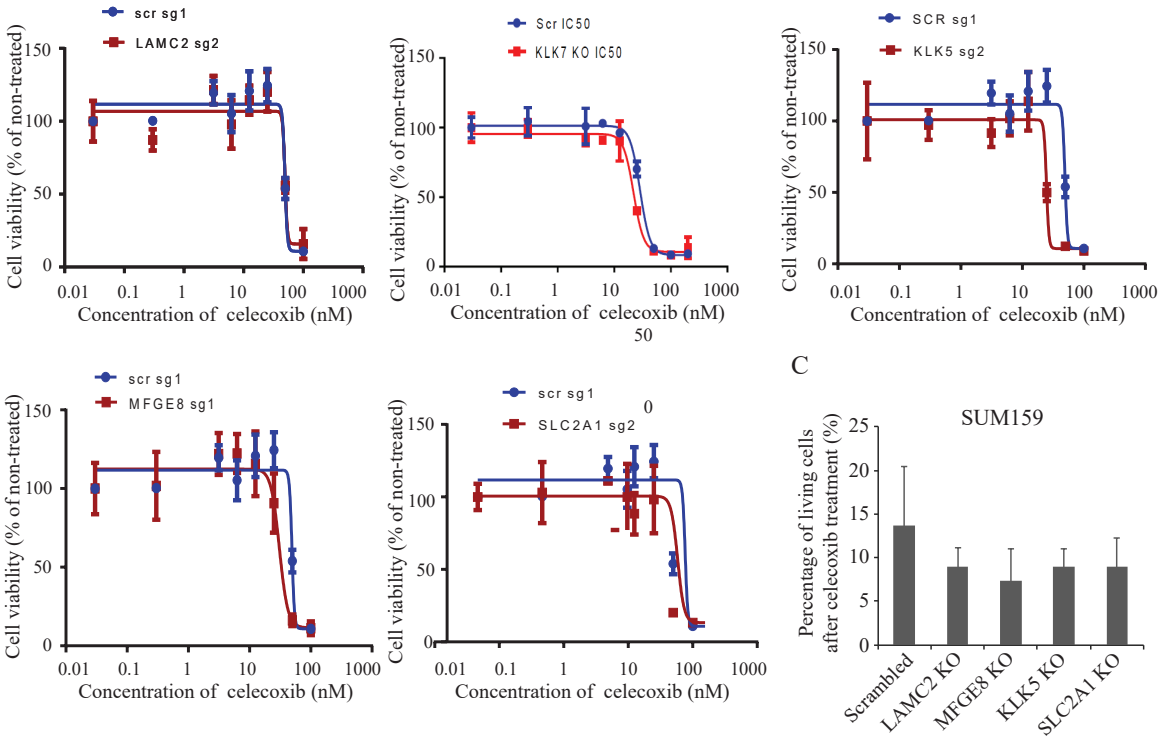
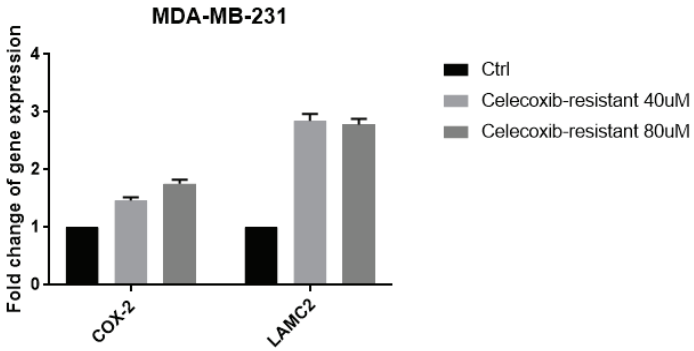


Figure S1: "**A**, SUM159 control and KO cell lines were treated with a dose rage of celecoxib for 4 days and subjected to cell viability test using SRB assay. IC50 values of control and each KO cells were indicated. **B**, Celecoxib dose response curve in SUM159 control, LAMC2, KLK7, MFGE8, KLK5, and SLC2A1 KO cells. **C**, SUM159 control, LAMC2, MFGE8, KLK5, and SLC2A1 KO cells were treated with 50 μM celecoxib for 4 days and then subjected to Prestoblue cell viability assay. Percentage of living cells after celecoxib treatment was calculated [194] ".

A



B

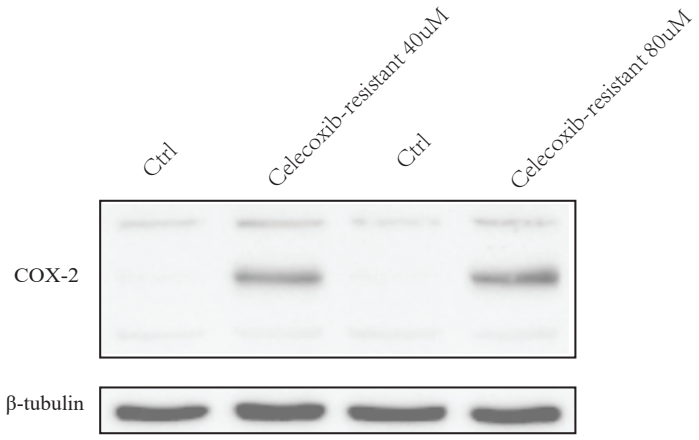


Figure S2: "**A**, mRNA expression levels of COX-2 and LAMC2 were assessed in MDA-MB-231 parental cells and two celecoxib-resistant variant cell lines by qPCR. **B**, COX-2 protein levels were measured in MDA-MB-231 parental cells and celecoxib-resistant cells by western blot [194] ".

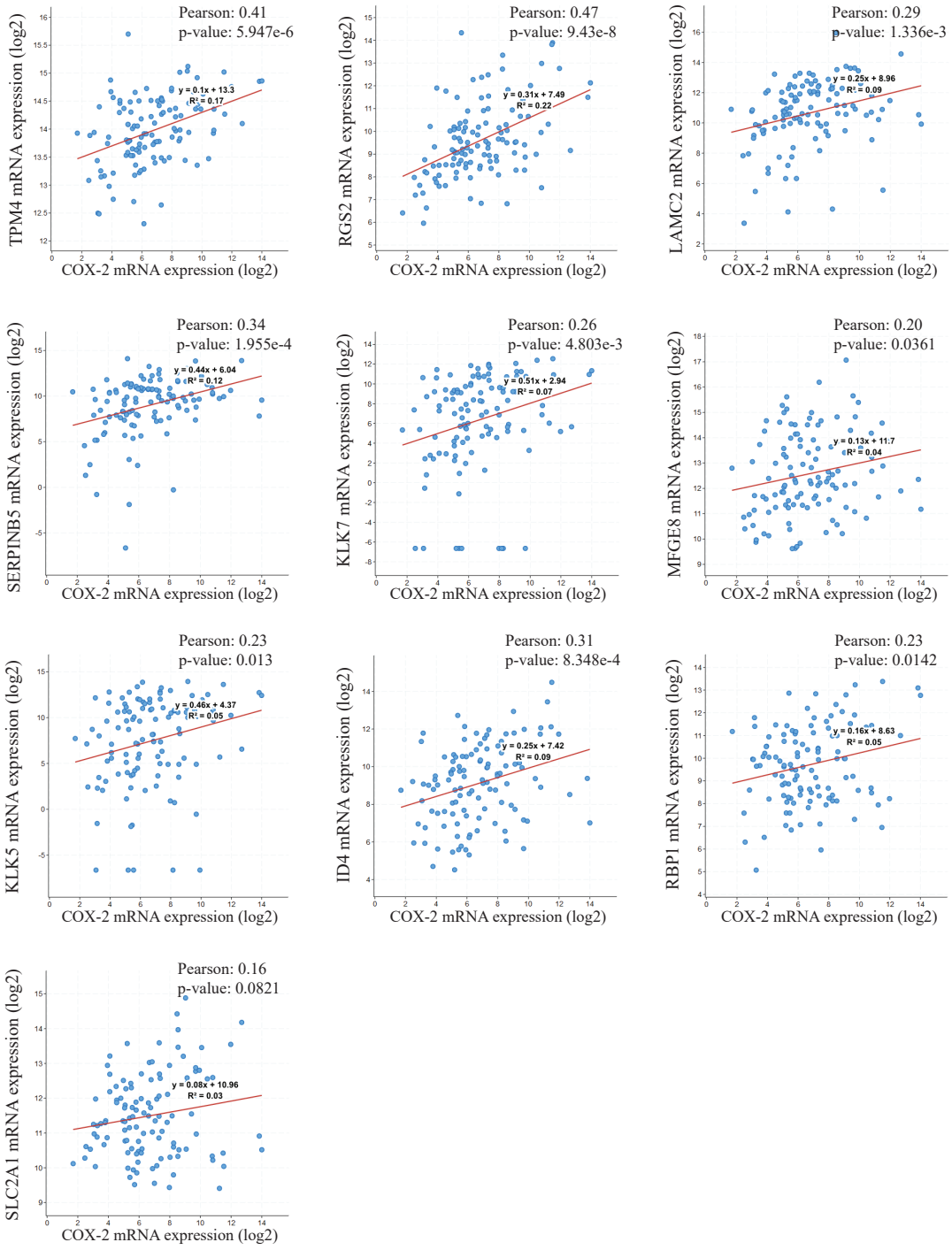


Figure S3: "Pearson's correlation analysis of 10 candidate genes expression with COX-2 expression in TNBC patients from TCGA dataset [194] "

		gRNA sequences
TPM4	sg1	CTCACCAGAGAAACTTGCCC
	sg2	GCTTCTCCTGCGCGTCCTTC
RGS2	sg1	AATTCTGACTCCAAGAAACG
	sg2	CAGATGGTCTTGCTGCATTC
LAMC2	sg1	TGGCAGACATCGGTCGCATC
	sg2	AGTGCTCGATGTGACAACTC
SERPINB5	sg1	CGACCAGACCAAAATCCTTG
	sg2	ATTTGATAGGGCCACTCCCT
KLK7	sg1	GCCGCAAGGGAAAGTTCCCC
	sg2	AGGGTACCTCTGCACACCAA
MFGE8	sg1	TGACCATGCCTGCGCGGTTC
	sg2	GTAGCCCTTAAGGCACGTGC
KLK5	sg1	GGCCGGGGAAGACGCCCGGT
	sg2	ACAGGGAGTAGTGGCCGAGA
ID4	sg1	GGCCGGCGCGGTGAACAAGC
	sg2	CACTGCGCTCAACACCGACC
RBP1	sg1	GCTCATCACCCTCGATCCAC
	sg2	CCCCACCGCAGACGTCAATG
SLC2A1	sg1	CCTCGTTGCGGTTGATGAGC
	sg2	CTTCGTGTCCGCCGTGCTCA

Supplementary Table 1

Supplementary Table 1: "CRISPR/Cas9 sgRNA sequences targeting 10 COX-2 associated genes [194] ".

Gene name		Forward primer	Reverse primer
TPM4	sg1	AAGATCGCGCCACTGCACTC	GAGAGCTGAAAAAGCTGGTGCC
	sg2	CGCCTTCTCCTCCTCCTCTT	TGGTCCTCAGGACGAGGGAA
RGS2	sg1	CAAGGTCAGTCTTTATGGCAGGTC	AGGAGGGGTAAAAAGTCCCTCCA
	sg2	CTAGCCCGCTTTGTCCTTGATTAC	CAGGTGGGGAAGAAAATCAGCC
LAMC2	sg1	GGTGATAGTTGCTTCCAATGCCG	CTCACCGGTAACATCAGGCAAC
	sg2	GCAGCAGATGGTGCTTCTTACTTC	TCCCAACCTCTTAGGGGTATTGTG
SERPINB5	sg1	ATGCCCCACTCTGTCCCTATC	CTTCCTTTCTTCCCTGCTCCTTC
	sg2	ACCATGGCCAATGCCAAGGTC	GGCTTCCTGATCCAGCAACATTAG
KLK7	sg1	GCGTCCTCACTCCTGTGCAT	CCCCTTCTCTGCAATTGGTCTC
	sg2	AACTCAGTGTGGCGTTAGCGATG	GGAGCAGGGTCCTTAACATTGG
MFGE8	sg1	AACCCCAGTGATGAACCCTCC	TGCCTTCATTGTCCCTTTGTGGTC
	sg2	TCTCACCTGTGTCCCCAGCT	TGCATCAGCATCAGGCCTGG
KLK5	sg1	ACTTCTTCCTGCAGTGGGCG	TACCTGAGCCTGGGCTCTGT
	sg2	GGGTCTGACATCTTTAGTGGGACG	GGTGGGGTTGGAGATGGTTG
ID4	sg1	TGCTCTCAGAAACGCTGGGG	TCAACACCGACCCGGTGAGA
	sg2	CGTTATCGACTACATCCTGGACC	TCCTCGGAAATCAGGCTGGC
RBP1	sg1	TATCAAGTTTGGGAGCTGCCCCCT	GCAAAAGGGCTTAGCTCATTGCTG
	sg2	TCTATGCCTGTCAGATCCTCCTC	ATGTCTGCTCGTTGGCCCTG
SLC2A1	sg1	TGTCCCGCGCAGCTTCTTTAG	CATGTGACCGATGAGGAAACTGAG
	sg2	GTGGGAGGTAGGGGAGACTT	TGGGCGGAAGAGAAACTCTGC

Supplementary Table 2

Supplementary Table 2: "qPCR Primers for 10 COX-2 associated genes [194] ".

Chapter 5: Discussion and Conclusion

This chapter is presented in a monograph format and adapted from the following published article:

Tian J, **Wang V**, Wang N, Khadang B, Boudreault J, Bakdounes K, et al. Identification of MFGE8 and KLK5/7 as mediators of breast tumorigenesis and resistance to COX-2 inhibition. *Breast Cancer Research*, 2021 Feb 15; 23(1):23 [194].

5.1 Discussion

COX-2 plays an important role in various aspects of tumorigenesis of breast cancer—from the initial development of primary tumors to the subsequent dissemination of cancer cells to distant organ sites— and is "overexpressed in 40% of invasive breast carcinoma[s] [194] ". Although preclinical studies suggest that targeted inhibition of COX-2 by celecoxib, a selective COX-2 inhibitor, can effectively suppress "breast tumor growth and metastasis [194] ", subsequent clinical studies in breast cancer patients yielded mixed results. On one hand, several phase II trials provided direct evidence to support: 1) the pre-operative use of celecoxib in primary breast cancer patients [230], and 2) the use of celecoxib in combination with aromatase inhibitors in the neoadjuvant and metastatic settings for postmenopausal hormone receptor-positive patients [217,218,231]. On the other hand, a phase III REACT trial failed to provide clear-cut evidence in favor of the use of celecoxib to delay "time to progression [194] " or prolong "overall survival in primary breast cancer patients [194, 219] " . Since gene expression profiling studies were not performed on these patients, we hypothesize that an insufficient understanding of the molecular mechanisms governing celecoxib sensitivity/resistance in breast cancer, leading to poor patient stratification, likely contributed to the failure of the trial. Thus, we designed this study with the objective to elucidate the role of COX-2 and its associated genes in mediating breast tumorigenesis and resistance to COX-2 inhibitor.

In particular, we focused on TNBC—a highly aggressive yet heterogeneous subtype of breast cancer—due to its invasiveness, tendency to relapse, poor prognosis, and limited treatment options. By analyzing RNA-seq data of TNBC samples and mining "their associated genomic, transcriptomic and clinical profiles" from various patient datasets, we identified 10 COX-2 associated genes

(*TPM4*, *RGS2*, *LAMC2*, *SERPINB5*, *KLK7*, *MFGE8*, *KLK5*, *ID4*, *RBP1*, *SLC2A1*) that are highly altered and expressed in aggressive BCs and BC cell lines classified as "less-sensitive" to COX-2 inhibitor, and that predict poor prognosis in BC patients. We then generated CRISPR/Cas9 knockouts of each of these candidate genes in two TNBC cell lines—MDA-MB-231 and SUM159—to validate their functions in regulating 1) TNBC metastasis and 2) TNBC resistance to celecoxib. Of the 10 candidate genes, we found deletion of *TPM4*, *RGS2*, *SERPINB5*, *MFGE8*, *KLK5*, *ID4* to result in a ~90% reduction in lung metastatic burden, and deletion of *MFGE8, KLK5, KLK7* to restore TNBC sensitivity to celecoxib "both *in vitro* and *in vivo* [194] ".

MFGE8 is "a secreted glycoprotein that mediates adhesion to integrin-expressing cells [232] ", and is encoded by the "milk fat globule-EGF factor 8 [194] " gene. Several mechanisms by which *MFGE8* promotes tumorigenesis have been reported, including via upregulation of Akt and twist signaling to induce EMT [235]; enhancement of vascular endothelial growth factor (VEGF)-dependent angiogenesis [236]; and promotion of phagocytosis of apoptotic and aged red blood cells [233,234] . Given that we did not observe a change in phenotype nor in the proliferative capacity between MFGE8-KO cell lines and its parental counterparts, we hypothesize that the reduced metastatic spread to the lung mediated by MFGE8 deletion in TNBC cells might be due to "impaired *MFGE8*-mediated angiogenesis [194] ". Previous studies have shown that COX-2/PGE2 signaling mediates resistance to inhibition of the VEGF pathway [237] , and blocking COX-2 can potentiate the anti-angiogenic effect resulting from suppression of the VEGF/VEGFR2 signaling in preclinical models of breast and colon cancer [238] . Given that we did not observe an upregulation of MFGE8 in celecoxib-resistant TNBC cells, we speculate that the synergistic suppression of "TNBC primary tumor growth [194] " mediated by dual inhibition of COX-2 and

MFGE8 might be due to their convergent actions on the induction of an anti-angiogenic response that is potentially greater than targeting either MFGE8 or COX-2 alone, as opposed to an overcoming of resistance to celecoxib. The precise mechanisms by which MFGE8 and COX-2 cooperate to regulate TNBC tumorigenesis and resistance to celecoxib is worthy of further investigation. Our results suggest that combinatorial targeting of COX-2 and MFGE8 represents a promising therapeutic strategy for TNBC management. Given the overexpression of MFGE8 in TNBC versus non-TNBC patients [239], and the essential role of the gene in mediating normal mammary gland development [46], knowing when to target MFGE8 becomes critical as to prevent any abnormalities from occurring during remodelling of the gland.

Kallikrein-related peptidases 5 and 7 (KLK5 and KLK7) are "members of a subgroup of 15 homologous secreted serine proteases [194]" and are upregulated "in endocrine or hormone-responsive tissues [such as] breast, ovary, and skin [194, 241,242]". *In vitro* studies suggest that KLK5, when auto-activated, initiates a proteolytic cascade that involves activation of downstream pro-KLK7, and is thus considered a "physiological activator of KLK7 [194,243]". In this study, we found elevated expression of KLK5 and KLK7 to correlate with aggressive features of TNBC and poor prognosis in TNBC patients. This is consistent with previous reports highlighting the role of KLK5/7 as tissue/serological biomarkers and negative survival outcome indicators in various human malignancies, including those of the breast and ovary [244-248]. Existing literatures on KLK5 and KLK7 also suggest that these kallikreins play critical roles in the regulation of "tumor cell growth, invasion, metastasis, and angiogenesis [194,249]" during the course of cancer progression. Here, we demonstrated for the first time that individual knockouts of KLK5 and KLK7 not only suppressed TNBC metastasis to lung, but also increased TNBC sensitivity to celecoxib *in vitro* and *in vivo*. Given the lack of information on the existence of a

crosstalk between COX-2 and KLK signaling and their convergent actions on common downstream targets, it is necessary to investigate the mechanisms by which dual inhibition of COX-2 and KLK5/7 sensitize TNBC tumors to celecoxib, which we believe will be critical to the "future design and personalization of novel COX-2 inhibitor-based combination therapies in clinical settings [194] ".

As a next step in this research to validate the use of KLK5/7 and MFGE8 as a treatment-selection or predictive biomarker in TNBC, early-phase trials that randomize patients to receive the experimental (celecoxib) or standard therapy (placebo/standard of care) regardless of their biomarker status need to be conducted. Normally, the expression level of a gene, or its biomarker value, should predict a benefit, or the lack of it, among patients treated with celecoxib or not in the clinical setting. Given our preclinical data, we hypothesize that patients with low or negative expression of KLK5/7 or MFGE8 are more likely to experience a favorable outcome (i.e. prolonged survival) with celecoxib treatment than without, whereas patients with high expression of KLK5/7 or MFGE8 are more likely to experience the same outcome regardless of intervention or an unfavorable outcome with celecoxib treatment than without. However, such assumptions are made on the premise that performance indices (i.e. sensitivity, specificity, positive predictive value, and negative predictive value) for a given marker can be directly measured or estimated from trial data, which is not the case for predictive biomarkers [252] . This is because we have no way of knowing or even predicting the outcome of a patient for a treatment that he or she is not subscribed to. Instead, we can only observe the outcome of that individual under the treatment he or she received. Therefore, it is fundamentally challenging to assess the probability that a single biomarker value can predict treatment benefit at the individual level. Nevertheless, studies claim that we can still evaluate the population impact of a biomarker on patient outcomes observed

under treatment or no treatment by extrapolating data from the randomized controlled trial [252]. Moving forward, we envision that results obtained from a stratified biomarker trial should inform clinical decisions accordingly and provide recommendations for or against the establishment of KLK5/7 and MFGE8 as a predictive biomarker of response to celecoxib in the context of TNBC.

Apart from its application in the biomarker setting, the kallikreins also have potential to serve as therapeutic targets in the preclinical development of KLK-targeting drugs. In particular, small molecule inhibitors against several KLKs, including KLK5, KLK7 and KLK14, have been identified via high-throughput screening of large chemical libraries, as in the case of triazole derivatives [253]. These compounds are designed to covalently modify the hydroxyl group on Ser 195, which forms part of the catalytic triad (His 57, Asp 102, Ser 195) that is conserved across all active sites of the multi-membered kallikrein family [254]. Owing to their small surface interaction with the active pocket of the target enzyme, small-molecule KLK inhibitors often exhibit promiscuity and lack of selectivity against one KLK. In order to stabilize drug-protein interactions and harness the intrinsic KLK-inhibiting activity of naturally-occurring protease inhibitors, pharmacological efforts have largely been directed toward the development of peptide/protein-based KLK inhibitors [255-257]. As of current, no kallikrein-targeted therapies have been clinically approved to treat KLK-associated pathologies. However, it is anticipated that in the near future, a new generation of selective and potent KLK inhibitors will be developed as our knowledge of the various biological roles played by human kallikreins in select disease settings expands.

While the present discussion focuses on the potential of KLK5/7 and MFGE8 as treatment-selection biomarkers and therapeutic targets for COX-2 inhibitor-based combination therapies in TNBC, we believe that other candidates identified in our list of 10 COX-2 associated genes may also be targeted to uncover novel therapeutic vulnerabilities in a variety of human malignancies.

For example, LAMC2 has been identified as a metastatic marker in lung adenocarcinoma. A study suggests that high expression of LAMC2 predicts poor prognosis in patients with lung adenocarcinoma and enhances the metastatic capacity of lung adenocarcinoma cells via the induction of EMT [258] . In addition, upregulation of RGS2 has been found to induce dormancy-like phenotypes and tumor relapse in non-small cell lung cancer via translational control of eIF4a and ATF4 [259] . Further, a recent paper published in Nature Communications suggests that pharmacological and genetic inhibition of glucose transporter 1 (GLUT1), encoded by the SLC2A1 gene, can effectively block the growth of RB1-positive TNBC cells by suppressing their glucose uptake, thus forcing these cancer cells to adapt to an OXPHOS metabolic profile [260] .

In conclusion, systematic screening of 10 COX-2 associated genes through combined *in silico, in vitro* and *in vivo* analysis led us to identify KLK5/7 and MFGE8 as key mediators of breast tumorigenesis and resistance to COX-2 inhibition. Deletion of KLK5/7 and MFGE8 not only significantly suppressed TNBC metastasis to the lung, but also restored TNBC sensitivity to celecoxib *in vivo*, resulting in synergistic inhibition of mammary primary tumor growth. These results highlight the potential clinical utility of KLK5/7 and MFGE8 as predictive biomarkers of sensitivity/resistance to anti-COX-2 drugs in breast cancer patients.

5.2 Conclusion

COX-2 is a key enzyme involved in the conversion of arachidonic acid to prostaglandins, which bind to their associated receptors to mediate signaling responses in inflammation, blood pressure regulation, clotting, and gastrointestinal (GI) protection [159]. Due to the close link between inflammation and tumorigenesis, COX-2 and its derived PGE2, a major source of prostaglandin, are aberrantly upregulated at sites of inflammation and in many cancer types, including those of

the breast [194,198-200]. Given the involvement of both COX-2 and PGE2 in the regulation of tumor growth, invasion and metastasis, selective inhibitors of COX-2 including celecoxib have shown potent anti-tumorigenic effects in preclinical studies of breast cancer [210-216].

Previous work from our lab identified COX-2 as a potent inducer of stemness and a critical mediator of chemoresistance in TNBC. We have shown that COX-2 expression is upregulated in TNBC and corresponds to poor survival outcomes in patients with basal-like TNBC [208]. Despite its promise as a therapeutic target in TNBC, targeted inhibition of COX-2 by selective COX-2 inhibitors failed to elicit clear-cut benefits in clinical studies of breast cancer. In particular, a randomized phase III REACT trial showed that the use of celecoxib as an adjuvant therapy in patients with primary breast cancer neither extended their overall survival nor delayed disease progression [219]. These results highlight an insufficient understanding of the molecular mechanisms governing response to COX-2 inhibitors.

In this study, we conducted a systematic screening of 10 COX-2 associated genes through combined *in silico*, *in vitro* and *in vivo* analysis. First, we "[interrogated] human breast cancer RNA-seq data with their associated transcriptomic, genomic, and clinical profiles [194]" in public patient databases to identify 10 COX-2 associated genes (TPM4, RGS2, LAMC2, SERPINB5, KLK7, MFGE8, KLK5, ID4, RBP1, SLC2A1) that are highly altered and expressed in aggressive BCs and BC cell lines classified as "less-sensitive" to COX-2 inhibitor, and that also predict poor prognosis in BC patients. Then, we generated CRISPR/Cas9 knockouts of each of the 10 candidate genes and validated their "contributions to TNBC metastasis and resistance to COX-2 inhibitors [194]" using cell lines and preclinical models of TNBC. Specifically, we found individual deletion of all 10 genes to significantly suppress lung colonization in TNBC by variable extent, and genetic inhibition of KLK5, KLK7, and MFGE8 to effectively restore TNBC

sensitivity to celecoxib both *in vitro* and *in vivo*. Taken together, "our study supports the establishment and use of novel COX-2 inhibitor-based combination therapies as future strategies for TNBC treatment [194] ".

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