Differential Regulation of Cx43 Expression and Gap Junction Formation by Progesterone Receptor A and B in Breast Cancer Cells

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Graduate Department of Physiology University of Toronto

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Master of Science, 2019
Justin Rondeau
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ABSTRACT

Progesterone (P₄) imparts distinct effects in the breast via its progesterone receptors (PRs) PRA and PRB. Since the gap junction and tumour suppressor protein Connexin 43 (Cx43) is differentially regulated by P₄ via PRA/B in human myometrial cells, we sought to delineate the roles of PRA/B on Cx43 in breast cancer cells. We hypothesize that, similar to myometrial cells, the two PRs differentially affect Cx43 expression, trafficking, and gap junction intercellular communication (GJIC) in breast cancer cells. In this project, I have shown that in the luminal A cell line MCF7, PRA promotes Cx43 expression, trafficking (similarly observed in the cell line MFM223), and GJIC whereas PRB inhibits these processes. Conversely, in the basal-like cell line MDA-MB-231, P₄ inhibits Cx43 expression, intracellular trafficking, and GJIC through both PRs. These data provide insight as to how PRs differentially regulate the same gene, Cx43, in contrasting in vitro models of breast cancer.
ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my supervisor, Dr. Stephen Lye, for allowing me to conduct research in his world-renowned laboratory for the past three years. Thank you for placing value in what started as a tiny little side project which has now flourished into my entire MSc thesis! I would also like to extend my gratitude to my Supervisory Committee, Dr. Ted Brown and Dr. Jeff Wrana, for their thoughtful input throughout every stage of my project and for sharing their expertise with me.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>Adriamycin® (Doxorubicin) and Cyclophosphamide</td>
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<td>ACT1</td>
<td>α–Connexin Carboxyl-Terminal Peptide</td>
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<tr>
<td>AF</td>
<td>Activating Function</td>
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<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
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<td>AREG</td>
<td>Amphiregulin</td>
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<tr>
<td>BP</td>
<td>Base Pair</td>
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<tr>
<td>BRCA</td>
<td>Breast Cancer Susceptibility Gene</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CBP</td>
<td>CREB-Binding Protein</td>
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<tr>
<td>CDH1</td>
<td>Cadherin-1</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>Ck2</td>
<td>Casein Kinase II</td>
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<tr>
<td>CMF</td>
<td>Cyclophosphamide, Methotrexate and Fluorouracil</td>
</tr>
<tr>
<td>CPD</td>
<td>Cell Proliferation Dye eFluor 670</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered Regularly Interspaced Short Palindromic Repeats</td>
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<td>dCas9</td>
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<td>DCIS</td>
<td>Ductal Carcinoma in Situ</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ddPCR</td>
<td>Digital Droplet PCR</td>
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<td>1,1'-Dioctadecyl-3,3',3',3'-Tetramethylindocarbocyanine Perchlorate</td>
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</tr>
<tr>
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<td>Double-Stranded Deoxyribonucleic Acid</td>
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<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<td>Endoplasmic Reticulum Associated Degradation</td>
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<td>MCF</td>
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<td>MDA-MB</td>
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<td>MFI</td>
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<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>PRA</td>
<td>Progesterone Receptor A</td>
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<tr>
<td>PRB</td>
<td>Progesterone Receptor B</td>
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<tr>
<td>PRC</td>
<td>Progesterone Receptor C</td>
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<tr>
<td>PRL</td>
<td>Prolactin</td>
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<tr>
<td>PRS</td>
<td>Progesterone Receptor S</td>
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<tr>
<td>PRT</td>
<td>Progesterone Receptor T</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor Activator of Nuclear Factor Kappa-B Ligand</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>RFS</td>
<td>Relapse Free Survival</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNApolIII</td>
<td>RNA Polymerase II</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective Estrogen Receptor Modulator</td>
</tr>
<tr>
<td>SFM</td>
<td>Serum-Free Medium</td>
</tr>
<tr>
<td>sgRNA</td>
<td>Single-Guide RNA</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing-Mediator for Retinoid/Thyroid Hormone Receptors</td>
</tr>
<tr>
<td>SOC</td>
<td>Super Optimal Broth with Catabolite Repression</td>
</tr>
<tr>
<td>SPRM</td>
<td>Selective Progesterone Receptor Modulator</td>
</tr>
<tr>
<td>SRC</td>
<td>Steroid Receptor Coactivator</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate-EDTA</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris Buffered Saline-Tween 20</td>
</tr>
<tr>
<td>TCAG</td>
<td>Centre for Applied Genomics</td>
</tr>
<tr>
<td>TDLU</td>
<td>Terminal Ductal Lobular Unit</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi Network</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour-Node-Metastasis System</td>
</tr>
<tr>
<td>tracrRNA</td>
<td>Trans-Encoded RNA</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcription Start Site</td>
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<td>UCSC</td>
<td>University of California Santa Cruz</td>
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CHAPTER 1: LITERATURE REVIEW
1.1 Epidemiology of Breast Cancer

Breast cancer is the most common cancer amongst women, and overall the second most common cancer in the world. Because breast cancer is more common in the Western world, the incidence of this disease is thought to increase as countries become more westernized\(^1\). In Canada specifically, most women diagnosed with breast cancer are between the ages of 60-69. The incidence of breast cancer is reported to be highest in Newfoundland and Labrador and lowest in New Brunswick\(^2\). Although this disease is quite prevalent amongst the population, incidence rates from the years 1996 to 2012 in Canada have largely stabilized, and this trend is similar in the US due to the increased use of preventative mammography\(^2,3\). Like any disease, breast cancer is a combination of genetics and the environment; however, it is estimated that only 10% of women owe onset of the disease to genetic predisposition, while the majority of cases are due to environmental factors\(^4\).

1.1.1 Types of Breast Cancer

Breast cancer can arise in either the duct, the lobule, or the connective tissue, and breast carcinomas are defined based on their site of origin; ductal carcinomas, for instance, originate in the duct, whereas lobular carcinomas originate in the lobule. The two most common types of breast cancer are invasive ductal carcinoma (IDC) and ductal carcinoma \textit{in situ} (DCIS), both arising from ductal epithelial cells of the breast\(^5\). However, whereas IDC is classified as malignant, that is neoplastic epithelial cells that have invaded through the duct and into the underlying stroma, DCIS is confined to the epithelial layer of the duct itself\(^6\). Invasive lobular carcinoma (ILC) and lobular carcinoma \textit{in situ} (LCIS) are the lobular equivalents to IDC and DCIS, whereas sarcomas originate in the connective tissue of the breast.
1.1.2 Stages and Grades of Breast Cancer

The most common pathological grading system in breast cancer is based on the Nottingham modification of the Bloom-Richardson system and has three parameters: tubule formation, nuclear pleomorphism, and mitotic count. Based on these, a histological grade score of I, II, or III is given, where I is a poorly differentiated tumour and III is a well differentiated tumour. Additionally, the Tumor-Node-Metastasis (TNM) system is an international piece of literature which aids in the anatomical description of breast tumours in order to determine their stage, and unlike the Bloom-Richardson system, is designed for both doctors and patients alike.

The TNM also describes three features of breast tumours: the primary tumour size, a quantitative and qualitative description of the surrounding lymph, and whether there is a metastatic site present. While the Bloom-Richardson system of grading allows for powerful patient prognosis, the TNM system is suited to determine tumour aggressiveness. Since both stage and grade need to be considered when assessing a breast tumour, the American Joint Committee on Cancer (AJCC) has developed a risk score system that incorporates stage, grade, and biological information (such as receptor status) in order to deliver more accurate prognostic information.

1.1.3 Molecular Classifications of Breast Cancer

Recently, molecular consideration has been given to breast tumours to classify them according to expression of key genes. They were first categorized into five distinct subtypes: 1) Luminal A, which is estrogen receptor (ER) and progesterone receptor (PR) positive, human epidermal factor receptor 2 (HER-2) negative, and has low levels of the proliferation marker
Ki67, 2) Luminal B, which is ER/PR positive, HER-2 negative and has high Ki67 expression, 3) Basal-like, which is ER/PR and HER2 negative, 4) HER2-enriched, which is ER/PR negative with HER2 overexpression 5) Normal-like, which is similar to Luminal A but presented with higher tissue-to-tumour ratio. This newer, more robust classification of breast tumours has removed the bias that traditional staging and grading may succumb to. In fact, combining molecular subtype classification with traditional staging practices allows for more prognostic accuracy than traditional breast cancer staging alone, across many groups, proving its benefits in clinic. For this reason, molecular subtyping has been incorporated into TNM staging as a prognostic tool.

Since molecular classification of breast tumours has proven to be useful in clinic, a number of amendments have been proposed as more subtype information has become available. For instance, the genes cytokeratin 5/6 and epithelial growth factor receptor (EGFR) have been added as specific indicators of basal-like subgroup prognosis, which together has proven to be a more accurate survival predictor than the standard “triple negative” (ER/PR/HER negative) genotype alone. Additionally, since its original classification, the HER2-enriched subtype is now further stratified based on the presence of its oncogene 611-CTF, or an alternate form of the receptor which makes tumours more resistant to HER-2 targeted therapy.

Generally, owing to the differing genetic mosaic specific to each subtype, prognosis of luminal A tumours is better than luminal B tumours, and luminal B tumours have a better prognosis than HER-2 enriched tumours and basal-like tumours. This is because luminal tumours are considered ER-responsive, low grade IDC with few genetic mutations in ER-responsive genes, whereas HER-2 enriched and basal-like tumours have mutations in tumour suppressive genes such as p53 and breast cancer susceptibility gene (BRCA), respectively, and
are considered ER-negative, high-grade IDC tumours. However, one should not rule out environmental factors when determining prognosis. Diet and exercise, obesity, metabolic syndrome in general, geographical distribution, quality of life and even medications that are unrelated to breast cancer are all factors that contribute to a woman’s survival and prognosis.

1.2 Therapeutics in Breast Cancer

1.2.1 Hormonal Therapy

1.2.1.1 Rationale for Hormonal Therapy

A major strength of molecular classification is that, in addition to prognostic value, genotyping breast tumours can provide the best course of therapeutic options. For instance, since luminal A and B tumours have hormone receptors, they are responsive to hormonal therapy. This course of action is extremely important in breast tumours because estrogen has been known to contribute to breast cancer for over 100 years. In both pre- and post-menopausal women, increased estrogen levels correlate with an increased risk of breast cancer, and this is compounded in post-menopausal women who are also more likely to be obese. In animals, estrogen has reproducibly shown to induce breast tumours through an ER-mediated pathway. Furthermore, in normal breast cells in vitro, estrogen treatment correlates with an invasive phenotype associated with primary breast carcinogenesis, as well as greater metastatic potential while losing the potential to form normal breast-like structures. Taken together, these results indicate that the blockage of estrogen-mediated pathways may arrest tumour growth and/or induction.

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1.2.1.2 Selective Estrogen Receptor Modulators (SERMs)

Tamoxifen, an anti-estrogen, first became available in the 1970s when it was discovered to be an alternative and preferred therapy to high-dose estrogen in post-menopausal women with breast cancer\textsuperscript{41}. Follow up \textit{in vivo}\textsuperscript{42} and \textit{in vitro}\textsuperscript{43} work concluded that tamoxifen reversed estrogen-mediated effects, and this was accomplished by competitive ER binding\textsuperscript{44}. In fact, the effects of tamoxifen are cell, context, and tissue dependent\textsuperscript{45}, such that it is anti-estrogenic in breast tissue while having estrogenic effects in uterus\textsuperscript{46}. For this reason, tamoxifen is now considered a selective estrogen receptor modulator (SERM)\textsuperscript{47}. Since the introduction of tamoxifen as a therapeutic agent for estrogen-responsive breast tumours, SERMs have also been evaluated as potential chemo-preventive agents. In a clinical trial, the SERM raloxifene, which was given to post-menopausal, osteoarthritic women, was found to inadvertently decrease the risk of invasive breast cancer by 76\%\textsuperscript{48}. Furthermore, in a separate clinical trial, both tamoxifen and raloxifene were found equally effective in reducing the risk of invasive breast cancer\textsuperscript{49}. Since the development of these SERMs, pure estrogen antagonists such as fulvestrant (with no partial agonist effect) have also been approved for clinical use\textsuperscript{50}.

1.2.1.3 Aromatase Inhibitors

Aromatase inhibitors are an alternative option in hormone therapy. Instead of competitively binding to ER like SERMs do, aromatase inhibitors block the action of Cyp19, a cytochrome P-450 enzyme that aromatizes the A ring in 19 carbon androgens to form 18 carbon estrogens\textsuperscript{51}. Thus, these blockers suppress the conversion of androstenedione and testosterone to estradiol\textsuperscript{52}. \textit{In vitro}, the aromatase inhibitor Letrozole induced apoptosis in hormone-responsive
breast cancer cells\textsuperscript{53}, which suggests aromatase inhibitors as a potential therapeutic, and also indicates that breast cancer cells themselves express aromatase. In clinical trials, Letrozole administration was more effective than tamoxifen in disease regression and the time until disease progression in post-menopausal women with hormone-responsive breast cancer\textsuperscript{54}. A similar aromatase inhibitor, Anastrozole, was as therapeutic as tamoxifen in post-menopausal women with hormone-responsive breast cancer; however, Anastrozole was deemed superior due to fewer side effects compared to tamoxifen\textsuperscript{55,56}. This reduction in side effects may arise from the fact that aromatase inhibitors have the same outcome (i.e. reduction in estrogen) irrespective of cell or tissue context\textsuperscript{52}. Additionally, in post-menopausal women with breast cancer, testosterone and androgen content did not increase in aromatase inhibitor users compared to tamoxifen users\textsuperscript{57}, further highlighting the safety of aromatase inhibitors as a therapeutic. Nevertheless, because SERMs and aromatase inhibitors act via separate mechanisms, they can be combined therapeutically\textsuperscript{58} in an adjuvant setting for a potentially more potent effect, although research is limited.

\textbf{1.2.2 Chemotherapy}

In the cases with hormone receptor absence or desensitization, chemotherapy is an alternative systemic therapeutic option for women with breast cancer. Common chemotherapy drug cocktails for breast cancer include a combination of cyclophosphamide, methotrexate and fluorouracil (CMF), or a combination of Adriamycin\textsuperscript{®} (doxorubicin) and cyclophosphamide (AC)\textsuperscript{59}. Often, a combination of drugs is administered that belong to different drug classes; cyclophosphamide is an alkylating agent that interferes with cell function by forming covalent bonds between molecules, fluorouracil and methotrexate are anti-metabolites that block the S
phase of the cell cycle and block DNA replication, and doxorubicin is an anti-metabolite that inhibits topo isomerase 2, an enzyme required for DNA synthesis. Depending upon the grade, receptor status and other treatment options, chemotherapy may be neoadjuvant or adjuvant. Chronologically, in a meta-analysis of clinical trials conducted from 1985 to 2000, the annual death rate of pre-menopausal and post-menopausal women with breast cancer decreased by 38% and 20%, respectively, with adjuvant chemotherapy, regardless of tumour biology. Additionally, among women with ER-positive tumours, mortality rates were further decreased by adjuvant chemotherapy combined with tamoxifen. However, a study conducted from 2000 to 2007 on pre- and post-menopausal women with breast cancer reported an increased rate of overall and relapse-free survival (RFS) of patients after neoadjuvant chemotherapy. In women with triple negative breast cancer, adjuvant chemotherapy increased survival rates while those who did not undergo adjuvant chemotherapy displayed worse prognosis. In fact, even though HER2-enriched breast tumours are considered a vital indicator of poor prognosis, adjuvant therapy with trastuzamab (a monoclonal antibody against HER2 used in targeted molecular therapy) and chemotherapy increased overall survival, despite tumour aggressiveness. These results were replicated in a wide-scale neoadjuvant setting. Taken together, these findings suggest that chemotherapy can be combined with hormonal or targeted molecular therapy in hormone receptor-positive tumours, while in the absence of hormone receptor expression, chemotherapy remains the most viable treatment option.

1.3 Mammary Gland Development

1.3.1 Morphological Changes During Mammary Gland Development
Mammary gland development, or *thelarche*, starts at puberty between the ages of eight to thirteen\(^\text{69,70}\). At this age, mammary epithelium expands and proliferate by branching into the mammary fat pad to allow ductal formation in a process known as ductal morphogenesis\(^\text{71}\). These ducts are composed of a bilayer of cells: the outer layer of cells is composed of myoepithelium whereas the inner layer of cells is composed of alveolar luminal cells or ductal luminal cells\(^\text{72}\). The site of ductal formation and branching is called the terminal end bud, and the stem cell population required for these processes is called cap cells\(^\text{73}\). From their site of origin, primary ducts branch into segmental and subsegmental ducts extending from the nipple\(^\text{72,74}\), where further ductal branching results in terminal branches called acini\(^\text{75}\). The terminal duct lobular unit (TDLU), which is the secretory and transportive unit of the breast, is classified as a group of acini that branch from one terminal duct\(^\text{76}\).

### 1.3.2 Hormonal Regulation in Mammary Gland Development

To drive this host of differentiation, there are many molecular mediators of breast development. Firstly, growth hormone (GH) is essential for ductal branching and has an overarching regulatory effect on mammary gland development\(^\text{77}\). GH also regulates insulin growth factor I (IGF-I), which is imperative in morphogenesis of the TDLU\(^\text{78}\). Another major regulator of ductal morphogenesis is estrogen\(^\text{79}\) through its receptor ER\(^\text{80}\), and this mediator of ductal branching has collaborative capabilities with IGF-I to stimulate greater outgrowth\(^\text{78}\). Additionally, a key regulator of ER-mediated ductal outgrowth is amphiregulin (AREG, a protein of the epidermal growth factor [EGF] family), where both AREG and ER are required for ductal formation and morphogenesis of the TDLU\(^\text{81,82}\). Fibroblast growth factor receptor 2 (FGFR-2) is also required for terminal end branching during mammary gland development at
puberty. Finally, progesterone (P₄) plays a unique role in the developing breast. As well as having a role in the anatomical changes to aid in ductal branching during puberty, P₄ acts synergistically with prolactin (PRL) to stimulate the formation of milk-generating alveoli in preparation for lactation.

1.4 Role of Progesterone in the Development of Mammary Gland

Progesterone (P₄) is an ovarian steroid hormone that first surges during puberty, which facilitates the development of breast tissue. After puberty, there continues to be a periodic spike of P₄ levels during each luteal phase of the menstrual cycle. During this time, P₄ stimulates proliferation in the ducts and lobules of breast tissue. The cyclical nature of P₄-stimulated proliferation in the breast continues until menopause when ovarian production of P₄ ceases and the composition of breast tissue is regressed from epithelial cells to stromal cells and adipose tissue.

1.4.1 Molecular Mechanisms of P₄ in the Breast

At the molecular level, P₄ has established a reputation as a key mediator of proliferation in breast tissue. During puberty and pregnancy, P₄ stimulates ductal side branching required for the expansion of breast epithelium via two signalling waves. Firstly, P₄ acts intracellularly in PR-positive cells to increase proliferation through cyclin D1; this first wave is followed by a second mechanism involving paracrine signaling. The mechanisms of P₄-stimulated proliferation during the second wave remain to be completely elucidated; however, known mediators involve AREG, Wnt4 and receptor activator of NF-κB-ligand (RANKL) and Notch ligands Delta-like 1,3 and Presenillin 2.
1.5 P4 in Breast Cancer

1.5.1 Historical Use of P4 as a Breast Cancer Therapeutic

As mentioned, P4 is mainly secreted from the corpus luteum during the luteal phase of the menstrual cycle, which coincides with proliferation (and the associated stromal changes) in the TDLU of the breast. Since all denominations of breast cancer occur only in the TDLU, it has been conjectured that P4-stimulated proliferation during the luteal phase of a woman’s menstrual cycle contributes to breast cancer. This hypothesis is strengthened with the correlation that breast cancer incidence increases with the cumulative number of menstrual cycles a woman has, which corresponds to the amount of P4 (as well as other ovarian hormones) she is exposed to. Furthermore, women who take combined P4 and estrogen oral contraceptive birth control are at a greater risk of developing breast cancer, and in a number of large-scale studies, post-menopausal women who undergo combination hormone replacement therapy (HRT) with both estrogen and the synthetic P4 medroxyprogesterone acetate (MPA) also have an increased risk of breast cancer. It is still unclear which hormone contributes to increased risk; however, there are substantial proliferative and remodeling changes in normal breast epithelium whilst undergoing this therapy. In accordance with these findings, mammographical density is positively associated with breast cancer risk, and this association was further correlated with P4 exposure. However, in contrast to these studies, post-menopausal women who took natural P4 (instead of MPA) did not have an increased risk of developing breast cancer. These findings could therefore be confounded by MPA having androgenic and glucocorticoid activity, as well as its half-life that is substantially longer than natural P4. Since these publications shed light on the potential of P4
to increase breast cancer risk, there has been a decrease in adding P₄ to combination HRT¹¹⁵, which has seen an overall reduction in risk of IDC and DCIS in women¹¹⁶.

Even though P₄ contributes to carcinogenesis in the breast, it may slow progression of the disease once established. Early clinical trial research found high-dose MPA administration to women with metastatic breast cancer significantly improved RFS compared to women who took low-dose MPA or no MPA¹¹⁷. Additional follow-up studies using the synthetic P₄ megestrol acetate in women with advanced breast cancer also had increased RFS¹¹⁸,¹¹⁹. In fact, megestrol acetate was discovered to be as effective as tamoxifen in treating postmenopausal women with advanced breast cancer¹²⁰. Furthermore, although P₄ has not seen much use in breast cancer treatment, it was discovered to be therapeutic for women who had already been desensitized to aromatase inhibitors¹²¹. Overall, although exposure to P₄ has clearly been implicated as a risk for breast cancer, P₄ may still hold therapeutic value in women that already have the disease, especially in the case modern aromatase inhibitor therapy desensitization.

### 1.5.2 Molecular Mediators of P₄ Signalling

Molecularly, P₄ is known to induce many factors involved in tumorigenesis. In mice, The Wnt signaling pathway, which is induced by P₄, is associated with mammary epithelium proliferation and carcinogenesis¹²². Also in mice, RANKL signaling, activated by P₄, stimulates mammary epithelium proliferation and carcinogenesis. A study using an in vitro model of normal breast epithelial cells also implicated RANKL to promote epithelial to mesenchymal transition and was found to be a potent inducer of carcinogenesis and extravasation¹²³. To correlate this to humans, it was discovered in an ex vivo model that both Wnt and RANKL signaling are induced by P₄¹²⁴, and that serum P₄ levels correlate with serum RANKL levels which further correlates
with the incidence of breast cancer. The inhibitor of differentiation 4 (Id4), which was discovered to be upregulated in response to P4 treatment in the mouse, was also found to be a distinct correlative protein of invasive breast cancer. Furthermore, in an in vivo mouse model, P4 promotes calcitonin expression in the breast, which could have implications in the onset of breast carcinogenesis. Similarly, in an in vivo mouse model, P4 was found to induce AREG expression, which was correlated to breast cancer precursor cell generation. In an in vitro model of breast cancer, P4 upregulated cyclin D1 expression, which in turn increased cell proliferation. Finally, in an in vitro model, P4 contributed to breast cancer cell migration through the increased expression of the cell cycle inhibitor protein p27 and activation of the cytoskeleton-regulating Rho-associated protein kinase. Contrastingly, another in vitro study discovered that although p27 and p21 were upregulated by P4, G1 cellular arrest was also induced. In another study, P4 was found to induce the expression of oncogenic proteins c-myc, c-fos, and EGFR as well as G1 arrest as reported previously. However, these studies were performed in breast cancer cell lines in vitro, and have not been replicated in vivo or ex vivo. These findings implicate a complex role of P4 in regulating breast cancer cell proliferation and survival; however, it is predominantly considered as a pro-proliferation hormone which may hyper-stimulate many proliferative pathways in the breast and may contribute to carcinogenesis.

1.6 Progesterone Receptors (PRs)

1.6.1 PR Regulation and Function

P4 binds to PRs encoded by the PGR/NR3C3 gene found on chromosome 11q22.1. PRs are part of the nuclear receptor superfamily and, once bound to ligand, translocate to the nucleus to regulate transcription and thus gene expression. Once P4 binds to PR, the receptor
undergoes a conformational change which allows it to dimerize\textsuperscript{136} and bind to transcriptional coactivator or co-repressor proteins which participate in gene transcription\textsuperscript{137}. PRs can recruit multiple coactivators of transcription such as the steroid receptor coactivator (SRC) family of proteins and p300/CREB-binding protein (CBP), which in turn can recruit the RNA polymerase II (RNAPolII) complex to initiate transcription\textsuperscript{138}. The specific half-site response element of PR is ‘RGNACA’ (R is either A or G and N is any nucleotide) and is found in the genome as a palindrome\textsuperscript{139} with a spacer of 3 nucleotides. The two major isoforms of PR, PRA and PRB\textsuperscript{134}, are transcribed from the same gene and are identical except for a 164 amino acid truncation of PRA at the N-terminus\textsuperscript{134,140} (Figure 1.1). (There are also shorter isoforms of PR termed PRC, PRS and PRT\textsuperscript{141,142} as well as membrane-bound G-coupled PRs termed mPR\textalpha, mPR\textbeta and mPR\textgamma\textsuperscript{143,144}, although their functions are either unknown or unsubstantiated and have therefore largely remained out of focus.) PRB has an extra transactivation domain compared to PRA that allows for greater binding of transcriptional regulators and therefore PRB is a stronger transcription factor than PRA\textsuperscript{145}. However, an inhibitory consequence of PRB being 164 amino acids greater than PRA is a change in conformation between the two proteins resulting in an N-terminal inhibitory domain being active in PRA while being suppressed in PRB, thus making PRA a dominant inhibitor of PRB action\textsuperscript{146,147}. P\textsubscript{4} bound PRs can activate thousands of genes\textsuperscript{148}; however, the genes they regulate and the extent to which they are regulated are cell-, tissue- and co-activator- dependent\textsuperscript{149}. PRs also display a host of different phosphorylation sites, are regulated based on intracellular cell-cycling events, can interact with scaffolding proteins, and can be involved in chromatin-remodelling events\textsuperscript{150,151}. PRs can also bind to DNA in a ligand-independent manner via activation of protein kinases such as mitogen activated protein kinases (MAPKs) or cAMP-dependent protein kinase A (PKA)\textsuperscript{152} and therefore influence gene
expression without $P_4^{153}$. Furthermore, translocation of PRs from the cytoplasm to the nucleus is a phenomenon observed in many cell types, including the normal breast$^{154}$ and in breast cancer$^{155}$, which directly impacts the extent to which PR-influenced genes are regulated. Interestingly, PRA and PRB each regulate a distinct set of genes that, for the most part, do not coincide with one another$^{156}$.

**1.6.2 PR Expression in the Normal Breast**

It is estimated that 12% of the normal mammary epithelium is PR-positive in pre-menopausal women, with no PR-positive cells residing in the stroma$^{157}$. However, cells that express PR in normal breast tissue do not proliferate under typical circumstances$^{158,159}$, so $P_4$-stimulated effects on proliferation of the population of mammary epithelium as a whole must be affected by PRs either by paracrine signaling or by gap junction intercellular communication (GJIC). In normal breast cells, PRA and PRB are expressed in an equimolar ratio$^{160}$. Furthermore, PRA:PRB heterodimers are the most commonly found in the breast as opposed to PRA or PRB homodimers$^{161}$. Since there is limited knowledge about PRs in the human breast throughout development and pregnancy, the role of PRs in the breast can be inferred from other in vivo models. In murine breast tissue, removal of PRB significantly decreases ductal sidebranching$^{162}$, whereas removal of PRA does not directly affect mammary gland architecture but does affect the structure and function of other reproductive organs such as the uterus$^{163}$. However, even though the effects of PRA on mammary gland development are less clear, microarray analysis showed that PRA signalling in the breast is involved in cell adhesion, immune response, and cell survival-related processes$^{164}$. Furthermore, in the mouse, the PRA:PRB ratio decreases with age and there is an acute PRB dominance during pregnancy,
Figure 1.1: Structures of PRB and PRA. Amino acid composition of the two major PR isoforms, PRB and PRA. Common functional domains are indicated within the region. PRA has identical amino acid composition to PRB with the exception of a 164 amino acid truncation at the N-terminus. PRA has a unique inhibitory domain (ID) which represses PRB action, whereas PRB has an extra activation function (AF), AF3, which allows for greater transcriptional sensitivity. NTD: N-terminal domain, DBD: DNA-binding domain, H: hinge region, LBD: ligand-binding domain.
especially in alveolar epithelium\textsuperscript{165}. However, during lactation there is no PR expression in the murine breast, and after breastfeeding the PRA:PRB ratio returns to the homeostatic, PRB-dominant state\textsuperscript{165}. Additionally, murine mammary stem cells (similar to cap cells in humans), which are PR-negative, require P\textsubscript{4}-stimulated PR-positive cells to secrete downstream factors via paracrine signaling in order to proliferate\textsuperscript{166}. Compared to the mouse the histological structure of rat breast is more similar to the human\textsuperscript{167}. Studies in rats have shown that while both PRA and PRB can be observed throughout puberty and pregnancy, they are not expressed during lactation. Additionally, PRA in the rat breast permanently decreases after regression of alveoli (similar to the mouse) and PRB does not decrease at any other point throughout the life course\textsuperscript{168}. Furthermore, in both the mouse\textsuperscript{165} and rat\textsuperscript{168}, PRB is reported to have pro-proliferative effects in mammary cells, as determined through BrdU incorporation assays.

\textbf{1.6.3 PRs in Breast Cancer}

In breast cancer, the equimolar ratio seen in normal breast cells is lost\textsuperscript{161} and a majority of breast tumours display PRA-dominance\textsuperscript{169} especially in DCIS tumours\textsuperscript{161}. Furthermore, although PR translocation from the cytoplasm to the nucleus can be observed in breast cancer cells\textsuperscript{170}, PRs are often retained in the nucleus rather than recycled to the cytoplasm\textsuperscript{171} suggesting hyper-stimulation of PR-regulated genes. Genetically, PR levels also correlate with \textit{BRCA} mutations. \textit{BRCA1} and 2 are genes involved in double-strand break DNA repair via homologous recombination and are therefore tumour suppressive\textsuperscript{172,173}. Firstly, women with \textit{BRCA1} mutations have increased PR expression in tumour-surrounding tissue\textsuperscript{174} \textit{In vitro}, this correlation is mimicked such that PRs positively correlate with \textit{BRCA1} mutation status in both T47D and MCF7 breast cancer cells\textsuperscript{175}. Furthermore, in breast tumours \textit{in vivo} and in breast cancer cells \textit{in
vitro, the miRNAs miR-181a, miR-23a and miR-26b were found to negatively correlate with PRs, suggesting a repressive effect of these miRNAs on PR expression\textsuperscript{176}. Also, \textit{in vitro} work in breast cancer cells suggests that FGF7 and FGFR-2 promote PR degradation, which leads to a more aggressive phenotype\textsuperscript{177} suggesting a protective role of PRs in the breast. Additional research on the protective effect of PRs \textit{in vitro} suggests PRs directs ER chromatin binding which decreases proliferation\textsuperscript{178}. Conflictingly, blocking PRs \textit{in vitro} in breast cancer cells reveals G2/M cell cycle arrest by suppressing the gene set involved in G2/M cell cycle progression\textsuperscript{179}.

1.6.3.1 PRA-Specific Action

Although PRA has elusive effects in the normal breast, a number of studies have concluded some preliminary findings about the role of PRA in breast cancer. Firstly, women with \textit{BRCA1} and \textit{BRCA2} mutations were shown to express PRA-dominant tumours\textsuperscript{180}. In contrast, an increase in PRA promoter methylation was reported in endocrine-resistant tumours (i.e. unresponsive to hormone therapy), suggesting the PRA gene is more sensitive to epigenetic regulation\textsuperscript{181}, although PRB can also be methylated\textsuperscript{182}. PRA is also observed to be less susceptible to phosphorylation and SUMOylation events\textsuperscript{183}. In a xenograft mouse model of implanted IBH-6 and T47D breast cancer cells, PRA-dominant tumours were inhibited by the anti-P\textsubscript{4} mifepristone (MFP) while stimulated by the synthetic progestin MPA\textsuperscript{184}. In another study, an \textit{ex vivo} model was used to address the same question and showed a decrease in proliferation when PRA-dominant tumours were stimulated with MFP\textsuperscript{185}. Furthermore, the newly developed selective progesterone receptor modulator (SPRM) ulipristal acetate (which was originally designed for leiomyoma treatment\textsuperscript{186} and did not affect normal breast cells \textit{in
vitro\textsuperscript{187}, was discovered to act specifically through PRA in breast cancer cells to inhibit proliferation\textsuperscript{188}. Finally, in a xenograft mouse model using T47D breast cancer cells, as well as additional \textit{in vitro} work using T47D and MCF7 cells, PRA was found to be an inhibitor of ER gene expression and DNA binding compared to PRB\textsuperscript{189,178}. It was also discovered that PRA-rich tumours correlate with poorer survival rates\textsuperscript{189}, a finding that is corroborated with PRA-rich biopsies in women with breast cancer\textsuperscript{199}. Together, these findings implicate PRA as a stable and less transcriptionally active isoform of PR in breast cancer that holds prognostic value of poor survival rates and can have its mechanisms altered by anti-P\textsubscript{4}.

1.6.3.2 PRB-Specific Action

There have been a number of genetic polymorphisms that are linked to differential PRB expression in breast\textsuperscript{191} and other reproductive cancers\textsuperscript{192,193}. Also, in breast cancer PRB is reported to be extensively phosphorylated\textsuperscript{194}. Serine threonine protein kinase casein kinase II (Ck2) mediated PRB phosphorylation\textsuperscript{195} was found to regulate the effects of PRB on breast cancer through the docking protein DUSP6\textsuperscript{196} in order to increase cell proliferation and survival. In women with sporadic breast cancer, the hypermethylation of PRB was correlated with lower grade and higher overall survival\textsuperscript{182}. PRB is also susceptible to SUMOylation which can downregulate its function\textsuperscript{183}. After PRB stimulation with P\textsubscript{4}, it executes many isoform-specific functions in breast cancer. A known downstream effector of PRB activity is the Src/MAPK pathway of cell survival and proliferation which specifically interacts with PRB through its SH3 domain (the part of PR that is absent in PRA)\textsuperscript{197,198}. PRB also upregulates Wnt1 and EGFR2 signalling, two other well-established cell survival markers\textsuperscript{199}. In contrast to the role of PRB as a ligand-bound transcriptional activator of cell survival/proliferation pathways, a xenograft mouse
model of implanted IBH-6 and T47D breast cancer cells discovered that antiP4-stimulated PRB enhanced proliferation via the silencing-mediator for retinoid/thyroid hormone receptors (SMRT), while MPA inhibited proliferation.\textsuperscript{184} Contrastingly, in an \textit{ex vivo} model to address the same question, there were an equal number of tumours that were stimulated and inhibited by antiP4.\textsuperscript{185} It is important to note that since PRB turnover is related to its transcriptional activity\textsuperscript{200,201}, it is difficult to determine when screening for PR protein presence/absence if there is a true loss of PRB protein or if it is being recycled due to its increased activity. Together, these findings implicate PRB as a transcriptionally active and highly turned over isoform of PR that activates many mediators of cell survival, however to address why mechanistic and isolated studies \textit{in vitro} (which suggest P4-stimulated PRB is proliferative) are not in accordance with \textit{in vivo/ex vivo} data (which suggest anti-P4-stimulated PRB is proliferative) remains to be elucidated.

1.7 Connexins

1.7.1 The Life Cycle of a Connexin

Connexins are gap junction proteins that allow for intercellular communication between adjacent cells\textsuperscript{202} and are found in all vertebrates\textsuperscript{203,204}. In humans there are 21 distinct genes that encode for the structurally related and highly conserved family of connexin proteins\textsuperscript{205}. Each connexin consists of four transmembrane $\alpha$-helices, two extracellular domains and one intracellular domain and cytoplasmic C and N- terminal domains\textsuperscript{202} (Figure 1.2). Gap junctions participate in gap junction intercellular communication (GJIC) between two neighbouring cells to facilitate the exchange of ions, small molecules such as secondary messengers, and metabolites that are generally 1 kilodalton (KDa) or less in size\textsuperscript{206}. After transcription, connexins
are translated in the endoplasmic reticulum where they await their hexamerization into connexons; this can either happen in the endoplasmic reticulum or the Golgi, depending on the isoform of connexin\textsuperscript{207,208}. Connexin oligomerization into a hexamer is a pivotal checkpoint in forward trafficking of connexins, as anything other than connexin hexamers is targeted for degradation via the endoplasmic reticulum associated degradation (ERAD) pathway\textsuperscript{209}. In accordance with the secretory pathway, connexons are then moved through the trans-Golginetwork (TGN), packaged into vesicles, and shuttled to the plasma membrane (PM)\textsuperscript{210}. Once inserted into the PM, connexons form gap junction (GJ) plaques (a process involving many connexins colocalizing to form large aggregates), where older connexons are at the centre of the plaque while newer connexons are added along the periphery\textsuperscript{211}. Alternatively, connexons can form hemicannels with the extracellular environment instead of an adjacent cell\textsuperscript{212}. Connexins only remain at the PM for 1 to 3 hours, so an implication of their extremely short half-life is that they are constantly being either recycled or degraded\textsuperscript{213}. Degradation involves internalization of the entire plaque rather than individual hemicannels\textsuperscript{214}. However, whether connexons form plaques or individual GJ hemicannels, and whether GJIC occurs or hemicannel formation with the extracellular environment occurs, all connexons are degraded via either the lysosome or the proteasome pathway\textsuperscript{215,216}. A schematic of the life cycle of a connexon can be found in Figure 1.3.

\textbf{1.7.2 Connexins Expressed in the Breast}

In the human breast, Connexin 26 (Cx26) and Connexin 43 (Cx43) are principally expressed\textsuperscript{217,218}. Specifically, Cx26 is expressed in ductal and alveolar luminal cells, whereas Cx43 is expressed in stromal and myoepithelial compartments that surround the Cx26-expressing
Figure 1.2: Tertiary structure of one Cx43 monomer inserted into the plasma membrane. Each Cx43 monomer consists of four transmembrane α-helices, two extracellular domains, and one intracellular domain. The N-terminal domain (NTD) and C-terminal domain (CTD) are also intracellular, and Cx43 possesses a long CTD tail that can be post-translationally modified to alter Cx43 function.
cells. Even though both connexins are expressed in the breast, Cx43 is found to have constitutive expression compared to Cx26 (whose intracellular expression is low and variable in comparison), and displays GJIC formation in breast epithelial cells. However, due to the limitations of obtaining breast samples throughout development and pregnancy, the extent to which other connexins are expressed throughout the life course remains to be elucidated. For this reason, *in vivo* models have provided insight into the temporal and spatial expression and function of connexins in the mammary gland.

1.7.2.1 Cx26

Cx26 is present in mouse mammary tissue in all stages of life where they connect ductal luminal cells, and during lactation Cx26 mRNA and protein have high expression levels in the milk-secreting alveolar cells. To determine the role of Cx26 in ductal and alveolar cells, a preliminary study using Cx26 KO mice found ductal and alveolar architecture was preserved and these mice were able to produce milk proteins normally, which suggested either a potential functional overlap between connexins or a non-specific role of Cx26 in ductal/alveolar structure and function. In accordance with this potential overlap, Cx26 KO mice showed compensatory Cx30 overexpression; however, since Cx30 has only recently been found in human mammary tissue, it is difficult to extrapolate functions specific to Cx26. Similarly in studies where Cx26 was overexpressed in mice, lactating females showed no ductal and alveolar structural changes and were able to produce milk proteins normally; however, they were unable to feed their litters due to defective contractility of myoepithelial cells. Furthermore, overexpression of Cx26 inhibited Cx43 expression in the myoepithelial compartments of the
Figure 1.3: The Life Cycle of Cx43: translation, insertion into the PM, and degradation. After transcription, Cx43 monomers are translated in the endoplasmic reticulum (ER) [1]. Misfolded monomers are degraded by the proteasome [2], while correctly folded monomers progress through the Golgi apparatus to become Cx43 hexamers, also known as connexons [3]. At the trans-Golgi network (TGN), connexons await forward trafficking to the PM via vesicular transport [4]. After PM insertion, connexons can regulate the flux of small molecules with the extracellular environment [5], or they can diffuse through the PM laterally to create connexon aggregates, or GJ plaques, in order to increase GJIC with adjacent cells [6]. Connexons are then shuttled to the endosome [7] where they are either recycled back to the PM or shuttled to the lysosome for degradation [8].
breast and Cx43-mediated contractility\textsuperscript{225}, suggesting a potential cross-talk mechanism between Cx26 and Cx43 specifically. Together, these \textit{in vivo} findings suggest that Cx26 is not required for ductal branching or alveologenesis in the mouse and that Cx26 is also endogenously expressed at low levels in the mouse mammary gland which is substantiated in humans.

1.7.2.2 Cx43

Cx43 is also present in mouse mammary tissue in all stages of the life course forming GJIC within the myoepithelium\textsuperscript{217}; however, in contrast to Cx26, Cx43 expression decreases during pregnancy, further decreases during lactation, and increases again after involution\textsuperscript{226}. In both the mouse and rat mammary gland, Cx43 phosphorylation status increases as pregnancy progresses and is highest during lactation\textsuperscript{222,227}. To understand the function of Cx43 in breast development, many allele-specific KO studies of Cx43 have been conducted. In mice with one allele of Cx43 KO (Cx43\textsuperscript{+/−}), no structural or functional defect was found pertaining to the breast\textsuperscript{228}. Similar observations were made when one allele of Cx43 was substituted for one Cx40 allele (Cx43\textsuperscript{Cx40+/−})\textsuperscript{229}. However a substitution with one allele of Cx43 for one of Cx32 (Cx43\textsuperscript{Cx32+/−}) resulted in murine mothers being unable to lactate, suggesting a contractility defect\textsuperscript{229}. Interestingly, a conflicting follow-up study noted no structural or functional defect pertaining to the breast in these mice\textsuperscript{230}. Importantly, the significance of these findings cannot currently be extrapolated to humans since the literature of human Cx32 and Cx40 in mammary tissue remains sparse\textsuperscript{224}. Another study reported decreased ductal branching in mice when one allele of Cx43 was substituted for one of Cx26 (Cx43\textsuperscript{Cx26+/−})\textsuperscript{231}, confirming the above notion that increased Cx26 may act as an inhibitor of Cx43-specific action in the breast\textsuperscript{225}. In a point mutation study, mice with an isoleucine to threonine substitution in the cytoplasmic domain of
one allele of Cx43 \((\text{Cx}43^{1130T/+})\) had stalled ductal branching, but had no functional defect pertaining to lactation\(^232\). Finally, a similar point mutation study in mice with a glycine to serine substitution in the extracellular domain of one allele of Cx43 \((\text{Cx}43^{G60S/+})\) reported stalled ductal branching as well as a lactation defect due to inability to secrete milk from alveoli\(^233, 234\). These studies suggest that the extracellular domain may be crucial for GJIC coupling and/or myoepithelial contractility. However, this remains to be elucidated. Overall, these \textit{in vivo} findings suggest that Cx43 is imperative for murine breast development in terms of both structure and function, and implicate that in humans, Cx43 cannot be replaced by Cx26. However a loss of one allele of Cx43 may not be enough to cause a significant functional or structural defect in the mammary gland.

1.8 Connexin Dysregulation in Breast Cancer

It is generally regarded that connexins act as tumour suppressors by increasing GJIC and therefore decreasing potential tumourigenesis\(^235,236\). Furthermore, it is speculated that in addition to an increased GJIC leading to the spread of pro-apoptotic or anti-tumourigenic signals between cells, a loss of GJIC leads to cells becoming a heterogeneous population, thereby losing their sense of “self”, and therefore making it easier for mutations to accumulate\(^237\).

1.8.1 Cx43-Specific Dysregulation in Breast Cancer

1.8.1.1 Clinical and Ex Vivo Evidence

In support of the above theory, many studies have discovered a link between Cx43 status and breast cancer biopsies in women. In one study, DCIS, IDC and ILC tumours expressed reduced Cx43 compared to normal breast tissue (possibly due to the decrease in non-cancerous
myoepithelium\textsuperscript{220}, but there was no difference between stage, grade, or hormone receptor status\textsuperscript{238}. A similar study found that there was reduced Cx43 expression in ductal carcinomas; however, the Cx43 that was expressed in invasive breast cancer was localized in the cytoplasm rather than at the PM\textsuperscript{221}. Another study discovered that Cx43 increased as a function of ER expression and decreased as a function of HER2 expression, suggesting that as cells acquire a more invasive genotype, Cx43 is lost\textsuperscript{239}. Finally, in a follow-up study, Cx43 correlated to a significantly greater distant metastasis-free survival (DMFS), suggesting Cx43 held tumour-suppressive properties\textsuperscript{224}.

However, other studies correlate Cx43 to either a tumour-initiating protein or an oncoprotein. Firstly, two separate studies noted the aberrant localization of Cx43 to the stromal compartment of breast tissue instead of the myoepithelium in invasive breast tumours, suggesting Cx43 is upregulated specifically in this cell type\textsuperscript{220,240}. Additionally, as mentioned, breast tumours also display aberrant localization within the myoepithelium, where Cx43 was localized to the cytoplasm instead of the PM\textsuperscript{221}. Using the same microarray data described above, higher Cx43 mRNA expression correlated to a lower RFS and overall survival (OS) in women with ER-negative tumours\textsuperscript{224}. A follow up study suggested that increased Cx43 mRNA expression was associated with higher death and RFS\textsuperscript{241}. Taken together, these conflicting results suggest that loss of Cx43 may occur early in tumorigenesis, and the re-expression of aberrantly localized Cx43 may be a function of the tumour’s metastatic capacity.

1.8.1.2 In Vitro Evidence
Since no connexin-based therapeutic is currently approved for clinical use, *in vitro* work has been employed in breast cancer cell lines to help elucidate the mechanistic role of Cx43 on tumourigenesis.

1.8.1.2.1 Cx43-Mediated Gap Junction Dependent Functions

It has been established that Cx43 overexpression results in increased GJIC\(^{242,243}\) while its knockdown results in decreased GJIC\(^{244}\). In studies where Cx43 alteration directly affected GJIC, Cx43 had conflicting roles in the contribution to carcinogenesis. In a study using a cell line with primary tumour characteristics (HBL100), Cx43 overexpression was found to increase diapedesis\(^{242}\), while in another study using a cell line with basal-like characteristics (MDA-MB-231-MET), Cx43 overexpression was found to decrease cell invasion\(^{243}\). In the Cx43 knockdown study, both proliferation and migration were increased in a cell line with basal-like (Hs578T)\(^{244}\).

In another series of experiments, manipulators of Cx43 expression and GJ formation were used to assess Cx43-mediated gap junction dependent functions. A study reported inhibition of tumour cell extravasation when Cx43 expression knockdown was performed using RNA interference or when GJ formation was blocked using carbenoloxone\(^{241}\). Furthermore, in this study, GJIC was increased and extravasation was decreased upon activation of the metastatic gene *twist*, which is known to increase Cx43 expression\(^{241}\). Additionally, the Cx43-mediated GJ enhancer 6-methoxy-8-[(3-aminopropyl)amino]-4-methyl-5-(3-trifluoromethylphenyloxy)quinoline (PQ1) increased GJIC in the epithelial-like breast cancer cell line T47D\(^{245,246}\). This finding was replicated in a mouse model of spontaneous mammary carcinoma\(^{247}\). Cx43-mediated GJIC is increased by the α–connexin carboxyl-terminal peptide (ACT1), which mimics the cytoplasmic regulatory domain of Cx43. A study reported that
treatment of three breast cancer cell lines MCF7, MDA-MB-231, and BT474 with ACT1 resulted in decreased proliferation and in BT474 it also decreased cell survival\(^{248}\). Finally, in the luminal A breast cancer cell line MCF7, all-trans retinoic acid, which increases Cx43 expression, was found to increase GJIC and the bystander effect (which was GJ-mediated) while decreasing survival\(^{249}\).

Together, these results suggest that Cx43 plays a tumour-suppressive role in \textit{in vitro} models of breast cancer, where Cx43 expression and carcinogenesis display an inverse relationship, and manipulating GJIC with Cx43-specific modulators could be effective in mimicking the tumour suppressive nature of Cx43 itself.

\subsection{1.8.1.2.2 Cx43-Mediated Gap Junction Independent Functions}

Cx43 is also known to modulate cellular tumour-suppressive properties in a number of cancerous cell types independently of GJIC, including prostate cancer\(^{250}\), hepatocarcinoma\(^{251}\), cervical cancer\(^{252}\), keratinocyte cancer\(^{253}\), glioma\(^{254}\) and pancreatic cancer\(^{255}\). In breast cancer specifically, Cx43 has been reported to act independent of GJ formation in the basal-like cell line MDA-MB-231 to decrease the angiogenic molecule thrombospondin-1 in a 2-D model\(^{244}\), as well as decrease migration, angiogenesis and EMT in a 3-D model\(^{256}\). In \textit{ex vivo} microarray data of invasive breast cancer, Cx43 was found to regulate proliferation independently of GJ formation\(^{257}\). Specific molecules that are hypothesized to be regulated by Cx43 via a GJIC-independent mechanism are the cell adhesion molecules \(\alpha\)-catenin, \(\beta\)-catenin and ZO-2\(^{258}\) and the pro-apoptotic protein Bak\(^{259}\). Finally, in the basal-like breast cancer cell line MDA-MB-231, the antiangiogenic drugs genistein and quercetin increased Cx43 expression and decreased proliferation but did not increase GJIC, suggesting Cx43 may regulate cell proliferation through
a GJIC-independent pathway\textsuperscript{260}. In summary, these results suggest that Cx43 can also suppress many parameters of tumorigenesis via GJIC-independent mechanisms.

1.9 Rationale and Hypothesis

1.9.1 Rationale, Objective and Aims

In the endometrium, Cx43 was found to be differentially regulated by the ovarian hormones estradiol and P\textsubscript{4}\textsuperscript{261,262,263}. In the myometrium, the relationship between P\textsubscript{4} and Cx43 was further elucidated where PR isoforms PRA and PRB were found to differentially regulate Cx43 expression and intracellular trafficking\textsuperscript{264}. In an \textit{ex vivo} study of breast cancer, Cx43 expression correlated positively with both PR and ER expression in invasive breast cancer\textsuperscript{257}. However, whether or not P\textsubscript{4}/PR isoforms regulate Cx43 expression, intracellular trafficking and GJIC mediated cell connectivity in breast cancer cells has not been previously examined.

1.10.2 Objective, Aims and Hypothesis

For this study, my objective was to determine the role of PR isoforms PRA and PRB on the regulation of the gap junction and tumour suppressor gene Cx43 in breast cancer cells. I hypothesize that PRA and PRB differentially regulate Cx43 in breast cancer cells. Specifically, I aim to examine (1) if PR isoforms differentially regulate Cx43 expression, (2) if PR isoforms differentially regulate Cx243 intracellular trafficking and (3) if PR isoforms differentially regulate gap junction intercellular communication (GJIC).
CHAPTER 2: GENERATION OF MCF7-PRBKO AND MCF7-PRABKO VIA CRISPR/CAS9, AND MDA-MB-231-PRA AND MDA-MB-231-PRB VIA STABLE TRANSFECTION
2.1 Introduction

The PR isoforms, PRA and PRB, are transcribed from the same gene and a large portion of their translated product is identical\textsuperscript{134,140}, therefore manipulating the genome at this locus requires a precise gene editing tool such as CRISPR.

CRISPR is an acronym for clustered regularly interspaced short palindromic repeats. This particular arrangement of DNA, where palindromic sequences (termed CRISPR repeats) are interspaced with variable sequences of similar nucleotide length (termed CRISPR spacers) and flanked by associated CRISPR-associated (cas) genes was first discovered in 2002 in bacteria\textsuperscript{265}. However, their functional significance in the bacterial genome was discovered three years later, when the variable sequences (CRISPR spacers) were analyzed to be homologous to viral and plasmid DNA that was integrated into the bacterial genome\textsuperscript{266,267,268}. In 2007 it was posited that CRISPR sequences function as a bacterial immune system\textsuperscript{269} to primarily target foreign DNA\textsuperscript{270}. Once it was discovered that the majority of bacteria and archaea contain CRISPRs, CRISPR repeats were noted to typically be 29-37 nucleotides (nt) in length, while CRISPR spacers were noted to typically be 32-38 nt in length\textsuperscript{271}.

Overall, CRISPR-cas systems are classified into the different cas genes and proteins they encode into three types, where Type 1 systems encode the endoribonuclease cas3, Type 2 systems encode cas9, and Type 3 systems encode cas10\textsuperscript{272,273}. Although these three systems provide CRISPR-cas immunity through alternative biochemical and functional mechanisms, each system performs the same three steps in order to protect themselves from foreign DNA: adaptation, biogenesis, and targeting\textsuperscript{274}. In adaptation, CRISPR spacers that are generated post-infection are incorporated into the bacterial genome that are flanked by newly-generated
CRISPR repeats (as stated above, palindromic sequences that are not derived from pathogenic DNA). In biogenesis, premature CRISPR RNA (crRNA), which is RNA that encodes multiple spacers each flanked by repeats is transcribed from an initial leader sequence. Upstream of the leader sequence, Cas endoribonucleases are also transcribed. Once transcribed, Cas endoribonucleases cleave the premature crRNA to form mature crRNAs which encode a single CRISPR spacer. In targeting, mature crRNAs align with foreign complementary sequence DNA and this newly-formed double-stranded sequence (one strand pertaining to the crRNA and the other pertaining to the foreign DNA) is cleaved by Cas proteins once more, thereby preventing invading DNA from incorporating into the host genome.

Considering our understanding of CRISPR-Cas systems has drastically improved since they were first discovered, CRISPR-Cas has since been exploited for biotechnical and genome editing applications. Particularly, type II systems have been used in genome editing, since unlike type I and type III systems which require a complex of enzymes, type II systems require only the endoribonuclease Cas9 to introduce predictable double stranded DNA (dsDNA) breaks. Additionally, dsDNA breakage via Cas9 in bacteria requires a trans-encoded CRISPR RNA (tracrRNA), which is partially homologous to the foreign DNA integrated in CRISPR repeats. However, since mammalian cells do not encode tracrRNAs, using Cas9 to introduce a dsDNA break into the mammalian genome requires a single guide RNA (sgRNA). sgRNAs mimic the duty of the tracrRNA by having sequence homology to the crRNA (or in the case of genome editing, any locus of interest) and allow for the recruitment of Cas9 (Figure 1.4). Once a dsDNA break is introduced via Cas9 in mammalian cells, DNA can be repaired by non-homologous end joining (NHEJ), a process which adjoins two blunt ends from the same strand of DNA that Cas9 cleaved. Alternatively, if a piece of DNA is supplied that is
Figure 1.4: CRISPR/Cas9 genetic engineering schematic. Cas9 is supplied with a guide RNA (sgRNA) that consists of a Cas9-recognizable guide RNA backbone (green) and a sequence of DNA that is homologous to the proposed DNA cut site (red) [1]. In the endogenous piece of DNA, Cas9 first recognizes the protospacer adjacent motif (PAM, yellow) [2]. The sgRNA then binds to its homologous genomic locus, where RuvC and HNH domains of Cas9 cut 3 nucleotides upstream of the PAM (white) to introduce a dsDNA breakage [3]. The DNA will then repair itself to introduce a frameshift mutation known as non-homologous end joining (NHEJ) [4], or if an editing template is provided, extraneous DNA can be incorporated into the genome to allow integration via homology directed repair (HDR) [5].
homologous to the Cas9 cleavage site, this extraneous DNA can be incorporated into the corresponding genome via a process known as homology-directed repair (HDR) \(^{283,284}\).

Since its discovery, using CRISPR as a genome editing tool has expanded rapidly. In fact, it is projected that in 2018 alone over 5000 publications will use CRISPR or Cas9 in their title\(^ {285}\). Additionally, researchers have evolved CRISPR-Cas systems into second-generation gene editing tools. For instance, a catalytically-dead Cas9 (dCas9) can facilitate binding to DNA without the dsDNA break, therefore preventing transcriptional machinery to bind\(^ {286,287}\). Also, nickase Cas9 has been used to introduce single nucleotide changes without dsDNA breakage, therefore adding immense precision compared to standard Cas9 gene editing\(^ {288,289}\). Finally, any variant of Cas9 can be fused to a reporter gene to analyze expression/genome location, or dCas9 can be fused to another protein such as a DNA methyl transferase to analyze site-specific (as directed by Cas9’s affinity for the supplementary sgRNA) methylation patterns\(^ {290,291}\). Overall, CRISPR-Cas systems have proven to be diverse and powerful tools for genome editing, with much promise for the future.

In this study, we chose to use CRISPR/Cas9 on the cell line MCF7. In addition to being the most studied model of breast cancer in vitro\(^ {292}\), MCF7 is classified as a luminal A subtype of breast cancer and therefore expresses both PRA and PRB (as well as ER)\(^ {293}\). Also, a previous study using MCF7 discovered that these cells have a heterozygous deletion of the PGR gene\(^ {178}\), thus increasing the chances that CRISPR/Cas9 will result in a successful knockout. In contrast, we chose to stably integrate PRs into the cell line MDA-MB-231. MDA-MB-231 is considered a claudin-low subtype, which is similar to basal-like molecular classification in that it does not express ER, PR, or HER2, however claudin-low subtypes also have low expression of E-cadherin and claudin-3, claudin-4, and claudin-7\(^ {293}\). Since MDA-MB-231 does not express ER or
PR, we chose this model to analyze the effect of individual PR isoforms in isolation, without potential crosstalk between ER and PR.

2.2 METHODS

2.2.1 Materials

All buffers used, such as Tween 20-Tris buffered saline (TBST) and Tris-acetate-EDTA (TAE) were prepared in facility at the Lunenfeld-Tanenbaum Research Institute (LTRI).

2.2.2 Cell lines and cell culture

The human breast adenocarcinoma cell lines MCF7, MDA-MB-231, and MFM223 were obtained from American Type Culture Collection (ATCC, VA, USA), and STR profiling of cell lines was verified via the Centre for Applied Genomics (TCAG) DNA/Sequencing Facility (Toronto, ON, Canada). All cell lines were maintained in complete medium: DMEM (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS, Wisent, QC, Canada), 0.6 units/ml recombinant insulin (Lilly, IN, USA), 1% Penicillin/Streptomycin (Pen/Strep, Lonza, Basel, Switzerland) and 0.1% Normocin (Invivogen, CA, USA).

2.2.3 CRISPR/Cas9 KO of PRA, PRB, and PRAB in MCF7

The CRISPR/Cas9 gene editing, including the sequence confirmation of selection regions at the PR gene locus in MCF7 cells, strategy of KO, guide design, and guide generation was performed in collaboration with Virlana Shchuka, PhD candidate, in the laboratory of Professor Jennifer Mitchell (Ramsay Wright Zoological Laboratories, University of Toronto, ON, Canada).
2.2.3.1 Strategy for PRAKO, PRBKO and PRABKO using CRISPR/Cas9

To delete PRB and both PRA and PRB, CRISPR/Cas9 deletion via non-homologous end joining (NHEJ) was chosen to delete the PRB and PRAB transcription start sites (TSS), respectively. Since the PRA gene is a subset of the PRB gene, to delete only PRA, we chose to mutate the start methionine of the region encoding the PRA protein from ATG (methionine) to GCG (alanine) using CRISPR/Cas9 homology directed repair (HDR). A schematic for the insert oligo can be found in Figure 2.1.

![Figure 2.1: Inset oligo for PRA KO HDR strategy in MCF7 cells.](image)

This oligo incorporates a PRB untranslated region (UTR) for sequence homology required for HDR, the sequence for Cerulean protein for easy visualization of integration, porcine 2A self-cleavage peptide to liberate Cerulean from PR, and the PR open reading frame (ORF). To disrupt PRA protein translation, a substitution two nucleotides AT to GC will be incorporated into the oligo at the first methionine which encodes PRA.

2.2.3.2 Designing guides for PRB, PRAB, and PRA KO

Unique 20 base pair sequences were identified based on the Zhang CRISPR guide track as displayed on the UCSC Human Genome Browser. A schematic of chosen guides can be found in Figure 2.2, and guide sequences (which include sequences of the gRNA backbone as well as 19 bases that precede the PAM used can be found in Table 2.1. To disrupt PRB transcription, two guides flanking the annotated PRB TSS were selected, here described as “PRBguideL” and “PRBguideR”. To disrupt PRA and PRB transcription, the same “PRBguideL”, was selected (upstream of the PRB TSS) and a guide downstream of the PRA TSS was selected, here described as “PRABguideR”. To disrupt PRA translation, a guide was selected to overlap the start methionine of the region that encoded the PRA protein, here described as “PRAguide”.

![Figure 2.2: Schematic of chosen CRISPR guide sequences.](image)
Figure 2.2: Guides were chosen to delete PR transcription/translation start sites. Unique 20 base pair sequences retrieved from the Zhang Guide Track referenced using BLAT to the UCSC Human Browser Genome.

Table 2.1: Combination and sequences of guides used in CRISPR/Cas9 KO of PRs in MCF7 cells.

<table>
<thead>
<tr>
<th>Guide Name</th>
<th>sgRNA + backbone sequence</th>
<th>Targeted Gene Deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRBguideL</td>
<td>F: TTT CTT GGC TTT ATA TAT CTT GTG GAA AGG ACG AAA CAC CGT TCA GAT CCT ACC GGT AAT R: GAC TAG CCT TAT TTT AAC TTG CTA TTT CTA GCT CTA AAA CAT TAC CGG TAG GAT CTG AAT</td>
<td>PRB KO, PRAB KO</td>
</tr>
<tr>
<td>PRBguideR</td>
<td>F: TTT CTT GGC TTT ATA TAT CTT GTG GAA AGG ACG AAA CAC CGA GAG GTC CGA CTA GCC CCA R: GAC TAG CCT TAT TTT AAC TTG CTA TTT CTA GCT CTA AAA CTG GGG CTA GTC GGA CCT CTC</td>
<td>PRB KO</td>
</tr>
<tr>
<td>PRABguideR</td>
<td>F: TTT CTT GGC TTT ATA TAT CTT GTG GAA AGG ACG AAA CAC CGA CCA ATA ATA AAG GAT CAG R: GAC TAG CCT TAT TTT AAC TTG CTA TTT CTA GCT CTA AAA CCT GAT CCT TTA TTA TTG GTC</td>
<td>PRAB KO</td>
</tr>
<tr>
<td>PRAguide</td>
<td>F: TTT CTT GGC TTT ATA TAT CTT GTG GAA AGG ACG AAA CAC CGT CAG CTC AGT CAT GAC GAC R: GAC TAG CCT TAT TTT AAC TTG CTA TTT CTA GCT CTA AAA CGT CGT CAT GAC TGA GCT GAC</td>
<td>PRA KO</td>
</tr>
</tbody>
</table>

2.2.3.3 PR gene sequence verification

Before KO using CRISPR/Cas9 could be conducted, sequences between the MCF7 genome and the human UCSC reference genome needed to be verified. MCF7 cells were grown
in a 10 cm culture dish. For DNA extraction, cells were washed with 1x PBS (Wisent), scraped with a 25 cm cell scraper (Starstedt, Germany) and collected in 200 µl of 1x PBS in a labeled 1.5 ml Eppendorf tube. A QIAmp DNA Mini Kit (Qiagen, MD, USA) was used to extract and purify DNA according to the manufacturer’s instructions. DNA concentration was measured by Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., USA). For PCR amplification using MCF7 genomic DNA as template, primers were designed flanking the potential sgRNA target regions. A list of primers is presented in Table 2.2. HIFI Platinum Taq DNA polymerase kit (Invitrogen) was used according to the manufacturer’s protocol on a thermal cycler (Eppendorf) to generate amplicons. The cycling protocol is described as follows: initial denaturation at 94°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C and extension at 72°C for 30 seconds. Amplicons were then sent for DNA sequencing at TCAG according to TCAG guidelines using the forward and reverse primers shown in Table 2.2.

Table 2.2: Primer pair sequences used in PCR to verify the PR gene in MCF7 flanking the proposed sgRNA target regions for PRBKO.

<table>
<thead>
<tr>
<th>Proposed sgRNA target region</th>
<th>Primer Sequence 5’-3’</th>
</tr>
</thead>
</table>
| PRBguideL                   | F: GCA CAA ATA CAA CAA GGC TTA CC  
                            | R: AGC AGG AGA AAC TTG AAA GCA T |
| PRBguideR                   | F: GGG AGC GCA AGA AAA AGT AGT AA  
                            | R: GGT AAG CCT TGT TGT ATT TGT GC |

2.2.3.4 PR gene copy number assay

DNA from MCF7 cells was extracted and purified as described above (see section 2.2.6). To test for copies of the PR gene in the MCF7 genome, DNA was sent to TCAG, which uses digital droplet PCR (ddPCR) to assess copy number of the PR gene compared to the standard
reference gene RNaseP (which is known to exist in two copies).

2.2.3.5 Cloning of guide vector constructs

All guides were assembled, cloned, and sequenced in accordance with protocols from the Mitchell Lab. CRISPR gRNA vector assembly method. Lyophilized forward and reverse 60 base pair inserts for sgRNA assembly in Table 2.2 were ordered (Eurofins MWG Operon, USA) and reconstituted in sterile water to a stock concentration of 100 µM. Each sgRNA sequence insert was then further diluted by mixing 50 µl of 100 µM sgRNA in 450 µl sterile water to make a working concentration of 10 µM. To anneal the 3’ and 5’ ends of each gRNA, 10 µl of 10 µM forward primer and 10 µl of 10 µM reverse primer were combined in a 1.5 ml Eppendorf tube and placed in a beaker of boiling water to stimulate denaturation. Each combination of forward and reverse primers was left in the beaker until the water dropped to 50°C for 5 minutes to stimulate annealing. To each tube, 0.5 µl of Phusion polymerase (New England Biolabs Inc. [NEB], MA, USA), 10 µl 5x HF buffer (NEB), 1 µl of 10 mM dNTP mix (NEB) and 18.5 µl purified water were added for a total solution volume of 50 µl. Each tube was placed in an Eppendorf thermal cycler (Eppendorf, Germany) at 72°C for 30 minutes to allow guides to extend. To put each gRNA into a parent vector, a Gibson Assembly was set up using 1 µl of linearized gRNA vector backbone (Addgene, Catalog #41824, MA, USA), 0.6 µl of gRNA product, 10 µl of 2x Gibson Assembly Master Mix (NEB) and 8.5 µl purified water. Each reaction was placed at 50°C for 60 minutes to allow gRNA vectors to be incorporated into the gRNA vector backbone. After assembly, 2 µl of each vector was combined with 50 µl DH5α Escherichia coli (E. coli, Thermo Fisher Scientific) on ice for 30 minutes, heat shocked at 42°C for 40 seconds, incubated on ice for 2 minutes, and then incubated at 37°C on a shaking incubator (Eppendorf).
for 1 hour in 450 µl super optimal broth with catabolite repression (SOC, Sigma-Aldrich). 100 µl of *E. coli* were then spread on to LB agar plates containing Kanamycin (prepared by LTRI) and incubated at 37°C overnight. The next day, 5-10 colonies were picked and placed into 5 ml Lysogeny Broth (LB) supplemented with 5 µl Kanamycin Sulfate (Invitrogen) and incubated at 37°C shaking incubator (Eppendorf) for 6 hours. All LB cultures were then stored at 4°C until further use, in which 1 ml of each colony in LB underwent vector isolation via Plasmid DNA Miniprep Kit (Bio Basic, ON, Canada) according to the manufacturer’s instructions. To evaluate if a) the gRNA was incorporated into the gRNA vector backbone and b) *E. coli* received the vector, diagnostic digests were run on a 2% agarose gel. The extracted plasmids underwent enzymatic digestion with EcoR1 (NEB). The digestion of native sgRNA plasmid backbone using EcoR1 (NEB) yields a 414 bp product by cutting at two sites, while the recombinant plasmid with guide insert was expected to yield a 474 bp product (414 plus 60 bp of guide oligos). To digest the plasmid DNA, 0.5 µg of EcoR1 (NEB) was added to 0.5 µg DNA, 2.5 µl 10x NEBuffer (NEB) and topped up with sterile water to a total volume of 25 µl in a labelled Eppendorf tube. After gently pipetting to mix contents, the reagents were incubated at 37°C for 1 hour to allow digestion. Digested plasmids were then gel purified through electrolysis of the digest in a 2% agarose gel (2 mg of agarose powder [Thermo Fisher Scientific] in 100 ml TAE buffer, microwaved for 3 minutes and supplemented with 0.01% Sybrsafe DNA gel stain [Thermo Fisher Scientific] before casting). For electrophoresis, 10 µl of each digested plasmid was mixed with 2 µl 10x FastDigest Green Buffer (Thermo Fisher Scientific) and loaded beside 10 µl of 100 bp ladder (Frogga Bio, ON, Canada) to interpret results. Each gel was run at 100 volts until proper separation of bands was attained. sgRNA-containing plasmids were then sent for DNA sequencing using the T7 primer at TCAG according to TCAG guidelines for sequence
confirmation. After verification of sequence between the plasmid integrated into the *E. coli* colonies and the original gRNA sequence, the mini-cultures containing bacterial cells carrying the appropriate vectors were expanded and plasmids were extracted via Plasmid DNA Maxiprep Kit (Bio Basic) to obtain enough DNA suitable for transfection.

2.2.3.6 Electroporation of guides and Cas9-GFP

Guide vectors (5 µg) in combinations in Table 1 along with 5 µg Cas9-GFP (Addgene, Catalog # 44719) were transfected using electroporation via Neon® Transfection System (Thermo Fisher Scientific) according to the manufacturer’s instructions. Electroporation parameters were set according to one of Neon®’s previously tested parameters for MCF7 cells: 5 x 10^6 were loaded into a 100 ul tip and subjected to 2 pulses at 1100 volts for 30 milliseconds (ms) each.

2.2.3.7 Sorting based on GFP fluorescence

Two days after the transfection protocol outlined in section 2.2.3.6, cells were removed from the plate using 0.05 % trypsin-EDTA (Thermo Fisher Scientific) for 5 minutes. After neutralization of trypsin by the addition of 5 ml of complete medium, cells were spun at 500 x g for 5 minutes. Cells were then re-suspended in 1 ml of sorting buffer consisting of 1x PBS (Wisent), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Wisent), and 1% FBS (Wisent) in a labelled Eppendorf tube. All sorting, including initialization of the instrument and gating parameters, was performed by Annie Bang, co-ordinator at the Flow Cytometry facility in LTRI. Single cells were sorted on the MoFlo Astrios EQ Cell Sorter (Beckman Coulter, CA, USA) based on GFP fluorescence into 96 well plates. All cells were
initially sorted into DMEM (Invitrogen) media containing 50% FBS (Wisent) and 10 µg/ml insulin (Lilly) without antibiotic until discrete colonies could be seen in many of the wells of 96 well plates, at which time media was changed to regular growth medium outlined in 2.2.2. Once cells formed discrete colonies, each clone was split into 3 wells of 3 separate 96 well plates to use in 1) maintaining the clone in culture, 2) freeze as a stock clone, and 3) use for DNA and protein extraction for analysis.

2.2.3.8 Screening of clones for PGR disruption using PCR

Crude DNA extraction was performed using prepGEM® DNA extraction kit (Zygem, VA, USA) according to the manufacturer’s instructions. To test for clones that carried possible deletions of target regions, primers flanking the proposed deleted region were used in a qPCR run with the rationale that an amplification signal will only be detected if the target region has been deleted since the undeleted region will be too large for amplification. External primers flanking each proposed deletion site can be found in Table 3.

Table 2.3: Primer pair sequences used in PCR to examine KO status of clones

<table>
<thead>
<tr>
<th>Name of Primer Pair</th>
<th>Primer Sequence 5’-3’</th>
<th>Testing For</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRBKO_ext F: TTC TCC AAG AGA GTT CTC CAA CTT R: CTT ATG AGG TTC CAT CCC AAA GA</td>
<td>PRB deletion</td>
<td></td>
</tr>
<tr>
<td>PRABKO_ext F: CTT ATG AGG TTC CAT CCC AAA GA R: ACT CAG AGC CAT CCT CCT CCT</td>
<td>PRAB deletion</td>
<td></td>
</tr>
</tbody>
</table>

For PCR amplification, HIFI Platinum Taq DNA polymerase kit (Invitrogen) was used according to the manufacturer’s protocol on a thermal cycler (Eppendorf) to generate amplicons. The cycling protocol is described as follows: initial denaturation at 94°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 59.9°C for PRBKO_ext and
61.5°C for PRABKO_ext for 30 seconds, and extension at 72°C for 1 minute/kilobase. Amplicons were then sent for DNA sequencing at TCAG according to TCAG guidelines.

2.2.3.9 Screening of clones for PGR disruption using immunofluorescence

Cells were seeded and cultured in 6-well plates on top of glass coverslips for 24 hours. Cells were then fixed with ice-cold 1:1 methanol/acetone (methanol: Sigma Aldrich, acetone: Thermo Fisher), washed 3 times with PBS (Wisent), permeabilized with 0.02% Triton X (Sigma Aldrich) in PBS (Wisent) and then washed 3 times with PBS (Wisent). Nonspecific binding was blocked with 1% bovine serum albumin (BSA, Wisent) in PBS (Wisent) for 1 hour. Primary antibodies were then incubated overnight at 4°C in 1% BSA. A list of antibodies used for immunofluorescence, their respective dilutions and species can be found in Table 4. For KO screening, PR (Santa Cruz, TX, USA) and PRB (Cell Signal, MA, USA) antibodies were used. After overnight incubation, cells were washed three times with PBS, incubated with fluorescence conjugated secondary antibodies in PBS (Wisent) for one hour, washed three times with PBS (Wisent), and then incubated with 5 µg/ml 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 15 minutes in PBS (Wisent). A list of secondary antibodies, their respective dilutions and species can be found in Table 2.5. After subsequent washing 3 times, glass coverslips were mounted on to slides using Immu-Mount mounting solution (Thermo Fisher Scientific) and kept in the dark at 4°C until imaging. Images were taken on a Leica DMIL LED inverted microscope (Leica, Wetzlar, Germany).

Table 2.4: List of antibodies used in immunofluorescence analysis

<table>
<thead>
<tr>
<th>Targeted Protein</th>
<th>Species</th>
<th>Dilution</th>
<th>Company</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR</td>
<td>Rabbit polyclonal</td>
<td>1:100</td>
<td>Santa Cruz</td>
<td>H-190</td>
</tr>
<tr>
<td>PRB</td>
<td>Rabbit monoclonal</td>
<td>1:100</td>
<td>Cell Signaling</td>
<td>C1A2</td>
</tr>
<tr>
<td>Cx43</td>
<td>Rabbit polyclonal</td>
<td>1:100</td>
<td>Millipore</td>
<td>AB1728</td>
</tr>
<tr>
<td>Phalloidin</td>
<td>N/A</td>
<td>1:100</td>
<td>Thermo Fisher Scientific</td>
<td>A12381</td>
</tr>
</tbody>
</table>
Table 2.5: List of secondary antibodies used in immunofluorescence analysis

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Company</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor 488</td>
<td>Donkey anti-rabbit</td>
<td>1:300</td>
<td>Thermo Fisher Scientific</td>
<td>A21206</td>
</tr>
<tr>
<td>Alexa Fluor 594</td>
<td>Donkey anti-goat</td>
<td>1:300</td>
<td>Thermo Fisher Scientific</td>
<td>A11058</td>
</tr>
</tbody>
</table>

2.2.3.10 Screening of clones for PGR disruption using Western Blotting

Cells were seeded in 6 cm plates for 24 hours. Cells were then collected in 100 µl lysis buffer, a buffer containing 1M Tris/HCl pH 6.8, 10% sodium dodecyl sulfate (SDS), 5% glycerol, 38% sterile water with added 1% 100x Halt protease and phosphatase inhibitor (Thermo Fisher) and placed into labelled Eppendorf tubes on ice. For membrane disruption and protein extraction, cells were sonicated at 2.5 decibel (dB) using a XL-2000 Qsonica (Misonix, NY, USA), for 15 seconds twice, placed on ice for 5 minutes, heated at 95°C for 10 minutes, placed back on ice for 15 minutes, and then centrifuged at max speed for 15 minutes at 4°C. The supernatant was then removed and placed into a new labelled Eppendorf tube, while the precipitate was discarded. The supernatant was subject to western blot analysis. Protein concentration was measured using the Pierce BCA protein assay kit (Thermo Fisher) according to the manufacturer’s instructions by a uQuant microplate reader (Bio-Tek, VT, USA). To test for PRB and PRAB expression, 50 µg of total protein was combined with 25% NuPAGE LDS 4x Sample Buffer (Thermo Fisher Scientific) and topped up with lysis buffer until a final volume of 50 µl was obtained. Samples were boiled for 10 minutes to allow denaturation and then placed on ice until loading into the gel. To make the 7% resolving gel, the following reagents were combined in a 50 ml Falcon tube and placed between two glass slides: 10 ml sterile water, 4.7 ml 30% acrylamide/bis solution (Bio-Rad, CA, USA), 5 ml 1.5M Tris/HCl pH 8.6, 200 µl of 10% SDS, 100 µl 10% ammonium
persulfate (APS, Sigma Aldrich) and 20 µl tetramethylethylenediamine (TEMED, Invitrogen).

Once set, the following reagents were combined in a 15 ml Falcon tube to make a 4% stacking gel and placed on top of the resolving gel: 4223 µl sterile water, 667 µl 30% acrylamide/bis solution, 625 µl 1M Tris/HCl pH6.8, 50 µl 10% SDS, 50 µl 10% APS, and 10 µl TEMED. A 10-well comb was added to the stacking gel to allow sample loading. Once the stacking gel was set, glass slides were placed in a Western Blot cassette (Bio-Rad) and connected to a power supply. Samples were added with the first lane reserved for BLUelf Prestained Protein Ladder (GeneDireX, USA) and run at 100 volts until ladder separation could be seen. Once finished running, gels were transferred to Trans-Blot Turbo Midi PVDF membranes (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad) for 10 minutes using the “high molecular weight” setting. After transfer, membranes were blocked in 5% milk (Wisent) in TBST for 1 hour on a rocker. Primary antibodies were incubated at 4°C overnight. A list of primary antibodies and their dilutions used in western blotting can be found in Table 6. The next day, membranes were washed 3 times in TBST for 10 minutes each, secondary antibodies were incubated in 5% milk for one hour at room temperature with gentle rocking, and membranes were washed 3 times with TBST after incubation. A list of secondary antibodies used in western blotting can be found in Table 7. Chemiluminescence detection was performed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) according to the manufacturer’s instructions. All western blotting was imaged on the ChemiDoc Imaging System (Bio-Rad).

Table 2.6: List of antibodies used in Western Blotting analysis

<table>
<thead>
<tr>
<th>Targeted Protein</th>
<th>Species</th>
<th>Dilution</th>
<th>Company</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR</td>
<td>Rabbit polyclonal</td>
<td>1:500 in milk</td>
<td>Santa Cruz</td>
<td>H-190</td>
</tr>
<tr>
<td>Cx43</td>
<td>Rabbit polyclonal</td>
<td>1:1000 in BSA</td>
<td>Millipore</td>
<td>AB1728</td>
</tr>
<tr>
<td>ERK2</td>
<td>Rabbit polyclonal</td>
<td>1:2500 in milk</td>
<td>Santa Cruz</td>
<td>C-14</td>
</tr>
<tr>
<td>Alpha Tubulin</td>
<td>Mouse monoclonal</td>
<td>1:2500 in milk</td>
<td>Abcam</td>
<td>AB7291</td>
</tr>
</tbody>
</table>
Table 2.7: List of secondary antibodies used in Western Blotting analysis

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Company</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit HRP</td>
<td>Donkey anti-rabbit</td>
<td>1:5000</td>
<td>GE Life Sciences</td>
<td>NA934V</td>
</tr>
<tr>
<td>Mouse HRP</td>
<td>Donkey anti-goat</td>
<td>1:5000</td>
<td>GE Life Sciences</td>
<td>NA931V</td>
</tr>
</tbody>
</table>

2.2.4 Stable transfection of EV, PRA, and PRB in MDA-MB-231

2.2.4.1 Strategy for stable transfection

To integrate PR isoforms into the PR-negative cell line MDA-MB-231, stable transfection and antibiotic selection was performed. This method allows for quick and efficient selection of clones that have the vectors integrated into the genome.

2.2.4.2 pSG5-EV, pSG5-PRA, pSG5-PRB, and linear hygromycin resistance plasmid acquisition

PRA and PRB expression vectors in a pSG5 backbone (as well as just the backbone itself) were a kind gift from Dr. P Chambon, University of Strasbourg, France. The linear hygromycin resistance gene was purchased from Clontech Laboratories (CA, USA).

2.2.4.3 Electroporation of vectors

Each individual plasmid (5 µg, pSG5-EV or pSG5-PRA or pSG5-PRB), along with 0.5 µg linear hygromycin resistance gene (Clontech) were transfected using electroporation via Neon® Transfection System according to the manufacturer’s instructions. Electroporation parameters were executed according to one of Neon®’s pre-set parameters for MDA-MB-231 with some modifications: 5 x 10^6 cells were loaded into a 100 ul tip and subjected to 2 pulses at 1350 volts for 20 ms each.
2.2.4.4 Selecting and maintaining clones with antibiotic containing media

After MDA-MB-231 electroporation, cells were plated in a 10 cm dish in regular growth medium. Non-transfected MDA-MB-231 were also plated in an adjacent 10 cm dish to serve as a negative control for antibiotic selection. Two days after electroporation, both 10 cm dishes were subjected to antibiotic selection with regular growth medium supplemented with 1 mg/ml hygromycin (Wisent). When no viable cells could be seen in the non-transfected MDA-MB-231 negative control cells, the 10 cm dish was discarded and transfected cells were maintained in regular growth medium supplemented with 100 µg/ml hygromycin (Wisent).

2.2.4.5 Isolation of clones into identical colonies

When discrete colonies could be seen with the naked eye, clones were mechanically detached from the plate using a p200 pipette tip and placed in one well of a 96 well plate. Each clone was then expanded into two wells of a 6-well plate. One 6-well plate was used to maintain the clone in culture, and the other 6-well plate was used for protein extraction to examine expression of PRs via western blotting.

2.2.4.6 Screening of clones of PR expression using western blotting

Clones were screened for PR expression via Western Blotting as described above (see section 2.2.10).
2.3 RESULTS

2.3.1 Generation of PRBK0 and PRABKO MCF7 cells

To determine PR isoform-specific effects on Cx43 modulation, the ER, PRA/B-positive, Cx43-expressing MCF7 cell line was chosen to perform CRISPR/Cas9-mediated KO of PRB, PRA and both PRs (PRAB). This breast cancer model represents a PR-positive tumour with cells having an epithelial-like morphology. As well as an epithelial gene profile such as expression of ER, PR, HER2 and CDH1 (E-cadherin) these cells are also luminal A subtype²⁹³.

2.3.1.1 Genotyping results at the PGR locus confer 100% sequence homology between MCF7 and the UCSC human reference genome

Primers flanking the PRB KO target regions were designed and amplicons were generated using MCF7 genomic DNA. This was executed to ensure there were no unannotated SNPs, insertions or deletions relative to the UCSC reference genome. TCAG-sequenced amplicons were then mapped to the UCSC reference genome using BLAST-like alignment tool (BLAT), and 100% sequence homology was observed at the PGR locus sequenced between the MCF7 genome and the UCSC reference genome (Figure 2).³

2.3.1.2 TCAG ddPCR confirms 1 copy of the PR gene in MCF7 cells

MCF7 cells were genotyped to determine the exact copy number of the PR gene. TCAG sequencing of the MCF7 genome at the PR locus determined that compared to RNAse P, a
standard reference gene with 2 copies in the human genome (as defined by TCAG), there is only 1 copy of the PR gene in MCF7 cells (Figure 2.4).

2.3.1.3 Guide vector constructs were successfully cloned into the backbone plasmid

Diagnostic digests were run on agarose gels to ensure the sgRNA guides were incorporated into the sgRNA plasmid backbone. Size analysis of the resolved products on agarose gels showed successful cloning of the gRNA (474 bp) in all but one ‘PRB guide L’ bacterial clone as indicated by its size (414 bp) (Figure 2.5). One clone from each guide was expanded and plasmids were extracted by Maxiprep.

2.3.1.4 Guides and Cas9-GFP were successfully transfected into MCF7

After generation and verification, guides were transfected along with Cas9-GFP into MCF7 cells and then subsequently sorted for GFP expression. GFP acted as the reporter and was indicative of a successful transfection and thereby a potential incision by Cas9 at the sgRNA loci. Since MCF7 cells exhibit autofluorescence in the same channel as GFP detection, cells were gaited into a GFP-negative population (denoted .neg), a GFP-low/potentially-autofluorescent population (denoted .GFP) and a GFP-high population (denoted .sort). In both PRB KO and PRAB KO transfections, only the GFP-high populations were sorted and maintained (Figure 2.6).
Figure 2.3: Genotyping results of MCF7 confer 100% sequence homology. Regions of DNA flanking each guide were PCR amplified and sequenced for sequence homology using the UCSC Human Reference Genome. Purple/blue bars represent UCSC reference genome. Bars labelled PRB_KO_guideL and PRB_KO_guideR represent location of the guides. PRB_R_R, PRB_R_F, PRB_L_R, and PRB_L_F represent amplicons generated, and their black status indicates sequence homology.
Figure 2.4: **Copy number results of MCF7 confirm 1 copy of PGR gene.** TCAG Digital droplet PCR using primers within the PR gene suggest that PR (depicted in blue) has an average of 1 copy, while RNAseP (depicted in brown) has an average of 2 copies. Triplicates were performed on each gene.
Figure 2.5: Screening of recombinant bacterial clones. Plasmids digested with EcoR1 were run on 2% agarose gels to determine insertion of guides. Detection of 474 bp indicates successful insertion of guides in the plasmid, whereas 414 bp indicates plasmid without inserted guides. 1-8: PRB guide L. 9-12: PRB guide R. 13-24: PRAB guide R. 25-29: PRA guide.
Figure 2.6: Sort reports for CRISPR KO cell lines. PRB KO: Cells were transfected with Cas9-GFP, PRB guide R, PRB guide L and cells in the .sort gait (outlined in red at the bottom of the dot plot statistics) were interpreted to have guide integration based on GFP fluorescence (10.69% efficiency). PRAB KO: Cells were transfected with Cas9-GFP, PRB guide L, PRAB guide R and cells in the .sort gait (outlined in red at the bottom of the dot plot statistics) were interpreted to have guide integration based on GFP fluorescence (10.35% efficiency). Y-axis measures GFP fluorescence at 488 nm, while X-axis measures a channel where there is minimal fluorescence difference between cells (in this case far-red) at 640 nm.
2.3.1.5 PCR using primers flanking deletions confirms deletion of PRB and PRAB

Primers were designed flanking the proposed target regions and then subjected to a PCR protocol that would only allow for amplicon generation in the instance of a successful deletion. Since amplicons in PR-gene deleted clones (284 bp for PRBKO and 258 bp for PRABKO) were much smaller than amplicons in non-PR-gene deleted clones (607 bp for PRBKO and 1593 bp for PRABKO), PCR extension times were strictly regulated to be no more than 1 minute/kb. After PCR, amplicons were sent to TCAG to be sequenced, and sequencing results were mapped to the UCSC reference genome via BLAT. In one PRBKO and one PRABKO clone, amplicons were generated that flanked the proposed deleted region (PRB_ext and PRAB_ext for PRBKO and PRABKO, respectively) suggesting a deletion at the PR locus (Figure 2.7). In the PRB KO clone, the deletion flanked the PRB TSS (top) and in the PRAB KO clone, the deletion flanked both the PRB and PRA TSSs (bottom).

2.3.1.6 Western blotting confirms deletion of PRB and PRAB

In order to examine the deletion of PRB protein in MCF7-PRBKO and of both PR isoforms in MCF7-PRAB KO and to further verify PCR and immunofluorescence results, western blotting was conducted for protein analysis. Compared to MCF7-WT cells, which displayed two prominent PR-reactive bands at 114 kDa (PRB) and 94 kDa (PRA), MCF7-PRBKO cells displayed only one prominent PR-reactive band at 94 kDa (PRA), confirming the selective deletion of PRB. Furthermore, compared to MCF7-WT cells, MCF7-PRABKO cells displayed no PR-reactive bands, confirming the deletion of both PRA and PRB. Tubulin shows protein content loaded in each lane (Figure 2.8).
Figure 2.7: Sequencing results detect PRB TSS deletion in PRB KO clone and PRA/B TSS deletion in PRAB KO clone. Amplicons from PCR using crude DNA extraction were sent to TCAG to be sequenced. Sequencing results using the amplicon created by external primers flanking the proposed deleted region suggest that the deletions were successfully achieved in the TSS region of PRB (top) in the PRB KO clone and PRA/B TSS (bottom) in the PRAB KO clone.
Figure 2.8: Western Blot analysis confirms PRB KO and PRAB KO in MCF7 cells. After probing with a PR antibody, MCF7 WT (lane 3) displayed two bands at 114 and 94 kDa detecting PRB and PRA, respectively. Conversely, MCF7 PRAB KO (lane 1) had an absence of bands at these two molecular weights, confirming there is no PRA and PRB protein made in this clone. Furthermore, MCF7 PRB KO (lane 4) had an absence of 114 kDa band but a prominent 94 kDa band, confirming absence of PRB protein. Lane 2 represents a clone with only partial PRB KO and was therefore not analyzed further. Tubulin was used as a loading control.
2.3.1.7 Immunofluorescence analysis confirms deletion of PRB and PRAB

To verify the PCR results of successful KO of PRB and PRAB, immunofluorescence analysis was performed to detect total PRs in KO cells compared to wild type (WT) MCF7 cells. Two antibodies were used: one that detects total PR protein (PRA and PRB) and one that detects specifically PRB alone. DAPI was also used for nuclear staining. In the PRB KO clone, total PR protein was detected in both WT and PRBKO cells; however, PRB protein was only detected in WT cells (Figure 2.9A). By contrast, in the PRAB KO clone, neither total PR antibody nor PRB antibody binding was detected (Figure 2.9B). These results further verified the deletion of PRB and PRAB in PRBKO and PRABKO clones, respectively.

2.3.2 Generation of MDA-MB-231-EV, MDA-MB-231-PRA, and MDA-MB-231-PRB

To determine the effects of PR isoforms on Cx43 in an ER/PR-negative, Cx43-positive breast cancer cell line, MDA-MB-231 was chosen which represents an aggressive and invasive form of breast cancer. As well as having an invasive breast cancer gene signature including no expression of ER, PR, HER2 and CDH1, this cell line has a mesenchymal morphology and is considered a basal-like subtype.

2.3.2.1 Western blotting confirms stable expression of PRA and PRB in MDA-MB-231-PRA and MDA-MB-231-PRB cells, respectively

To verify stable expression of PRA and PRB, MDA-MB-231-EV, MDA-MB-231-PRA and MDA-MB-231-PRB cell lines were subjected to western blot analysis. Compared to EV controls, two clones exhibited expression of PRA protein (94 kDa), while one clone exhibited PRB expression (114 kDa). MDA-MB-231-EV displayed no PR immunoreactivity (Figure 2.10).
Figure 2.9: Immunofluorescence confirms A) PRB KO and B) PRAB KO in MCF7 cells. Green fluorescence denotes total PR/PRB, blue fluorescence denotes DAPI. All images were taken at a constant exposure time at 400x magnification.
Figure 2.10: Western Blot analysis confirms generation of PRA- and PRB- stable MDA-MB-231 cells. After probing with a PR antibody, MDA-MB-231-EV (lane 1) displayed no PR bands, MDA-MB-231-PRA (lane 2) displayed a prominent PRA band at 94 kDa, and MDA-MB-231-PRB (lane 3) displayed one prominent band at 114 kDa (PRB), and a lower band at 94 kDa to suggest low PRA expression. Tubulin was used as a loading control.
2.4 DISCUSSION

The PRs PRA and PRB are two homologous nuclear steroid receptor isoforms that only differ in the 164 additional amino acids at the N-terminus of PRB. However, although they share sequence homology, their functions are contrasting and distinct. Their high sequence homology, as well as to the fact that they are transcribed from the same gene, makes it difficult to manipulate one PR (either PRA or PRB) without disrupting the other. Since our study attempts to address the effects of individual PR isoforms on Cx43 expression, trafficking, and function, we used CRISPR/Cas9 to selectively KO PRB and both PRs (PRA and PRB) from the PR-positive cell line MCF7. Furthermore, we used transfection via electroporation to stably integrate PRA and PRB expression constructs into the PR-negative cell line MDA-MB-231.

This study marks the first time PRs have been deleted using CRISPR/Cas9 genetic engineering in the luminal A cell line MCF7. Previous in vitro work used MCF7 and the luminal A cell line T47D to delineate the roles of PRA and PRB via the overexpression of one isoform, thereby altering the PRA:PRB ratio. Although useful, this approach does not address the functions of each individual isoform; since PRA and PRB readily form heterodimers over homodimers, a disturbance in PRA:PRB ratio may mask minute differences that homodimers impose. Our study used CRISPR/Cas9 gene editing to design a ER/PR+ model in the cell line MCF7 in order to delineate the differential roles of the individual PRs.

Due to the deletion strategy we initiated for MCF7-PRBKO, this study confirms the putative location of the PRB TSS. Furthermore, since designing the PRABKO_guideR well downstream of the putative PRA TSS resulted in PRA and PRB KO, it can be assumed that the location of the putative PRA TSS is also correct. However, although both CRISPR/Cas9 strategies to use NHEJ to delete PRB and PRAB proved to be successful, we were unable to
successfully use CRISPR/Cas9-mediated HDR to introduce a deletion into the start methionine of PRA in MCF7 cells. However, our lab has successfully generated a PRB expression vector with a deletion in the start methionine of PRA (unpublished), allowing for the potential to stably transfect PRABKO cells with this construct. This construct differs from the current pSG5-PRB plasmid used, which does not have the start methionine of PRA mutated and therefore has leaky expression of PRA. This will generate a new cell line with only PRB protein with no PRA protein made. In addition to our preliminary results using this construct, an additional study has also verified that this is the sole start methionine of PRA, and that changing its sequence effectively obliterates PRA translation\textsuperscript{163}. Overall, this study has successfully used CRISPR/Cas9 technology in MCF7 cells to manipulate the PR gene using NHEJ, with the potential to also manipulate PRs using HDR in the future.

We also used separate PRA and PRB expression vectors to create two PR stable cell lines: MDA-MB-231-PRA and MDA-MB-231-PRB. A previous study using a bi-inducible model of MDA-MB-231-PRA/B discovered that PRA and PRB differentially regulate focal adhesion kinases (FAK) and therefore differentially impact cell migration\textsuperscript{295}. This study adds to the growing literature of how PRs regulate a distinct set of genes. Importantly, in their model, the authors in this paper also see PRA protein expression upon stimulation of PRB protein expression. This was also noticed in our work, where MDA-MB-231-PRB cells did exhibit some PRA protein. This is because, due to the nature of the pSG5-PRB vector construct, the entire ORF of PRB was cloned into the vector backbone. Since the PRA gene is a subset the PRB gene, whenever PRB is expressed, the PRA TSS (as well as the start methionine of PRA) will also be expressed. However, since the expression of PRA in MDA-MB-231-PRB is considerably lower than PRB (almost undetectable), this clone can still model PRB-dominant breast tumour.
Pertaining to our study, although the bi-inducible model of PR expression in MDA-MB-231 exists, no study has examined PR isoform-specific regulation of Cx43 using this model.

In summary, our current work on the widely-used MCF7 and MDA-MB-231 cells have introduced two new models (one with luminal A subtype and one with basal-like subtype). These cell line provide the means to study the effects of individual PR isoforms on any candidate gene.
CHAPTER 3: REGULATION OF CX43 EXPRESSION, INTRACELLULAR TRAFFICKING, AND GAP JUNCTION FORMATION BY PROGESTERONE RECEPTOR ISOFORMS IN BREAST CANCER CELL LINES MCF7 AND MDA-MB-231
3.1 INTRODUCTION

In the breast, P₄ signalling works synergistically with estrogen for mammary gland expansion, which is different to the action of P₄ in other female reproductive organs, such as uterus, where during the menstrual cycle, P₄ inhibits estrogen-stimulated proliferation²⁹⁶. Nonetheless, expression of hormone receptors is indicative of a better prognosis in women with breast cancer (largely because they have functional ERs and thus are good candidates for antiestrogen hormone therapy). Moreover, since 100% of PR-positive tumours are also ER-positive²⁹⁷, P₄ may play a fundamental role in regulating tumorigenesis in breast cancer that is yet to be elucidated.

P₄ acts primarily via its two nuclear receptors, PRA and PRB, to regulate P₄-mediated effects. However, in breast cancer there is frequently an altered PR expression profile, aberrant localization of PRs, or a disruption in the PRA:PRB ratio. Still, in clinical practice, testing for PR status in breast biopsies does not discriminate between PRA and PRB, even though PRA and PRB regulate distinct sets of genes¹⁵⁶ in the breast and PRA antagonizes PRB¹⁴⁶,¹⁴⁷. The first prominent association between the differential effects that PRA and PRB impart on the regulation of the gap junction facilitator and tumour suppressor protein Cx43 was in the myometrium, where PRA promoted Cx43 expression and trafficking to the PM whereas PRB restricted these Cx43-related processes²⁶⁴. Cx43 is also an important regulator of tumourigenesis, as studies pertaining to breast cancer have discovered that many breast tumours display a decreased expression²²¹,²²⁴,²³⁸,²³⁹ or aberrant localization²²⁰,²²¹,²⁴⁰ of Cx43, thus impairing cells to participate in functional GJIC. However, although ER expression has been correlated with Cx43 expression in breast biopsies, no such correlation has been reported for PRs. Furthermore, the extent to which PRs can differentially regulate Cx43 in breast cancer remains to be elucidated.
In experiments presented in this chapter, I hoped to elucidate the extent to which PRs differentially contribute to the role of Cx43 function in breast cancer cells. For all MCF7 experiments in this chapter, MCF7-PRABKO was excluded from analysis due to its generation and validation being much later than MCF7-PRBKO.

3.2 METHODS

3.2.1 mRNA collection and isolation

Cells were seeded in 6-well plates for 24 hours. The next day, cells were treated with either vehicle (0.01% ethanol in sterile water) or P₄ (Sigma Aldrich, 100 nM, diluted in 0.01% ethanol) for 24 hours after starvation with serum-free medium (SFM) supplemented with 0.6 units/ml recombinant insulin (Lilly) for 4 hours. To extract RNA, cells were washed with PBS (Wisent), lysed with 350 µl Buffer RLT Plus (Qiagen) and collected in labelled 1.5 ml Eppendorf tubes on ice. mRNA isolation was performed using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer’s instructions. RNA concentration was measured by NanoDrop 1000 spectrophotometer. RNA was stored at -80°C until use.

3.2.2 Quantitative real time PCR (qRT-PCR) analysis

After mRNA isolation and purification, 10 µl of 50 ng/µl RNA were converted to cDNA to a stock solution of 50 ng/µl via the iScript gDNA Clear cDNA Synthesis kit (Bio-Rad) in a thermal cycler using the following protocol: 25°C for 5 minutes, 46°C for 20 minutes, 95°C for 1 minute. cDNA stocks were diluted 10 x (10 µl of cDNA in 90 µl of sterile water) to make a working
cDNA concentration of 5 ng/µl which was used in all qRT-PCR experiments. All cDNA was stored at -20°C until analysis. A list of primer pairs used for qRT-PCR can be found in Table 3.1.

Table 3.1: Primer pair sequences used in q-RT-PCR to test for Cx43 mRNA expression

<table>
<thead>
<tr>
<th>Gene Target</th>
<th>Primer Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx43</td>
<td>F: TTG CTG CGA ACC TAC AT CAT CAG T</td>
</tr>
<tr>
<td></td>
<td>R: GCC AGG GAC ACC AAG GAC AC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: AGA TCA TCA GCA A</td>
</tr>
<tr>
<td></td>
<td>R: CAT GAG TCC TCC C</td>
</tr>
<tr>
<td>18S</td>
<td>F: GTA ACC CGT TGA ACC CCA ATT</td>
</tr>
<tr>
<td></td>
<td>R: CCA TCC AAT CGG TAG TAG CG</td>
</tr>
<tr>
<td>YWHAZ</td>
<td>F: ACT TTT GGT ACA TTG TGG CTT CAA</td>
</tr>
<tr>
<td></td>
<td>R: CCG CCA GGA CAA ACC AGT AT</td>
</tr>
</tbody>
</table>

To assess gene expression, each cDNA sample was analyzed in triplicate in a 384-well plate. Each reaction consisted of 1 µg cDNA, 2.5 µg SYBR-Green (Sigma Aldrich), 0.5 µl primer pair, and 1 µl sterile water. All analyses were conducted on the CFX384 Real-Time System C1000 Thermal Cycler (Bio-Rad, USA) using the CFX Manager software 3.1 (Bio-Rad), using the following cycling protocol: initial denaturation at 95°C for 20 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing/extension at 54°C for 20 seconds. Replicates with a quantification cycle standard deviation (ΔCq) ≤ 0.4 were excluded from analysis.

3.2.3 Protein collection and isolation

Cells were seeded in 6 cm plates for 24 hours. The next day, cells were treated with either vehicle or P₄ for 24 hours after culture with serum-free media (SFM) supplemented with 0.6 units/ml recombinant insulin (Lilly) for 4 hours. Protein was collected and isolated as described above (see section 2.2.3.10).
3.2.4 Western blot analysis

Cx43 protein expression was analyzed via western blotting as described in section 2.2.3.10, and normalized to extracellular signal-regulated kinase (ERK2) as a housekeeping gene. Quantification of Cx43 expression was conducted using Image Lab Software (Bio-Rad).

3.2.5 Immunofluorescence analysis

Immunofluorescence was conducted as described in section 2.2.3.9. Information on sources and dilutions of primary and secondary antibodies are provided in Table 2.4 and Table 2.5. Images were taken on TCS SP8 four-laser inverted confocal microscope (Leica, Wetzlar, Germany) and a DMI 6000 spinning disc confocal microscope (Leica, Wetzlar, Germany). 3D imaging of cells was taken and analyzed on TCS SP8 four-laser inverted confocal microscope using Leica software. To generate 3D images, 40 stacked images were compiled 0.25 µm apart over a total Z-area of 10 µm.

3.2.6 Transient transfection

Cells were seeded in 6-well plates in regular growth media outlined in 2.2.3. The next day, media was changed to serum-free Opti-MEM (Invitrogen) for one hour. TransIT-LT1 Reagent (Mirus Bio LLC, WI, USA) was used for transient transfection as per company’s protocol using the plasmids described in section 2.2.3.2. Briefly, 250 µl of Opti-MEM (Invitrogen) were combined with 2.5 µg of each plasmid DNA needed for transfection and 7.5 µl of TransIT-LT1 Reagent in each separate labelled Eppendorf tube. The reagents were gently mixed by pipetting and left to sit at room temperature for 15 minutes. The mixture was then gently pipetted into each well of the 6-well plate and left to incorporate into the cells overnight. As well as the vectors
included in section 2.2.4.2, the pDEST-Cx43-pEGFP expression construct was obtained via the Addgene website (Catalog # 40907).

3.2.7 Live cell imaging and analysis

After the Cx43 and PR overexpression by chemical transfection protocol described in 3.2.6, cells were treated with either vehicle or P₄ and immediately monitored on the IN Cell Analyzer 2000 by Mikhail Bashkurov at the S.M.A.R.T. High Content Screening Facility at LTRI. Cells were imaged every 20 minutes over the course of 8 hours, and still-frames were compiled to create motion-pictures of each treatment.

3.2.8 Quantification of gap junction intercellular communication using flow cytometric analysis

Cells were seeded in 12-well plates at a density of 300,000 cells/well (MCF7) or 250,000 cells/well (MDA-MB-231) overnight before treatment with either vehicle or P₄ for 24 hours. The next day, half of the cells from each treatment were stained with the gap junction permeable dye Cell Proliferation Dye eFluor 670 (CPD, Thermo Fisher, denoted as the “donor” cells), and the other half was stained with the gap junction impermeable dye Vybrant 1,1'-Dioctadecyl-3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI, Thermo Fisher, denoted as the “acceptor” cells). To stain, cells were detached from the culture plate using Accumax (Innovative Cell Technologies, CA, USA) for 5 minutes. Accumax (Innovative Cell Technologies) reagent was removed by dilution with complete growth medium (1:1) and centrifugation (500 x g for 5 minutes). Cells were then re-suspended and washed in PBS (Wisent) and then subsequently centrifuged at 500 x g for 5 minutes. After removal of PBS, donor cells were stained with 2 µM (diluted in PBS) CPD for 15 minutes at 37°C, while the acceptor cells were stained with 3.5 µl/ml of DiI for 10 minutes at
37°C. After their respective incubation times, cold growth medium that amounted to 10 times the volume of dye used was added to each Eppendorf tube to stop further cell labelling. Cells were then centrifuged at 500 x g for 5 minutes, the solution was aspirated, and cells were further washed twice to remove all traces of extracellular dye. Donor and acceptor cells from each treatment were then co-cultured together in 12-well plates overnight. To ensure maximal cell-cell connections between “donor” and “acceptor” populations, each well was mixed thoroughly by pipetting before overnight co-culture. As a positive control, 100,000 donor and acceptor cells were plated separately in different wells while 100,00 unstained cells were plated as negative control. A representation of the staining and co-culture dye-transfer assay protocol is shown in Figure 3.1. The next day, cells were detached using Accumax, centrifuged at 500 x g for 15 minutes, and re-suspended in 100 µl of 0.4 µg/ml DAPI (Sigma Aldrich, USA) diluted in PBS. Cells were then transferred to a labelled 5 ml Falcon round-bottom tube (BD, NJ, USA) or a labelled 1.5 ml Eppendorf tube and then placed on ice until Flow Cytometric analysis. Cells in 5 ml Falcon round-bottom tubes (BD) were analyzed on the Gallios Flow Cytometer (Beckman Coulter, CA, USA) and cells in the 1.5 ml Eppendorf tubes were analyzed on the Amnis ImageStreamTM Mark II (Amnis Corporation, WA, USA). Flow cytometric analysis on the Gallios Flow Cytometer and the Amnis ImagestreamTM Mark II was conducted by Annie Bang at the Flow Cytometry facility in LTRI. Gallios analysis was conducted on Kaluza software, and Amnis ImagestreamTM Mark II analysis was conducted on IDEAS software. GJIC was measured as mean fluorescence intensity (MFI) of “donor” (CPD) dye in “acceptor” (DiI)-stained cells.

3.2.9 Statistical Analysis
Statistical analysis was performed using Prism 5 software (GraphPad Software Inc., CA, USA). Two-way ANOVA using multiple comparisons and Bonferroni post-hoc test was performed to determine significance between groups. Statistical significance was accepted at $p \leq 0.05$ (*), $p \leq 0.01$ (**).
Figure 3.1: Co-culture dye transfer assay allows quantification of dye transfer between cells. Representative images of individually stained MCF7 cells and dye transfer upon contact. A) Half of each treatment receives staining with CPD eFluor 670, a gap junction permeable dye. B) Half of each treatment received staining with CM-DiI, a gap junction impermeable dye. C) Cells were co-cultured overnight to allow gap junction formation. D) The gap junction permeable dye transfers through gap junctions to neighbouring cells. E) MFI of gap junction permeable dye is measured in gap junction impermeable dye stained cells. All images taken at 400x magnification on the Amnis ImagestreamTM Mark II (Amnis Corporation).
3.3 RESULTS

3.3.1 Analysis of Cx43 mRNA expression via qRT-PCR in breast cancer cells

3.3.1.1 Cx43 mRNA expression is increased in P₄-stimulated MCF7-PRBKO compared to P₄ and Vehicle-stimulated MCF7-WT cells

To examine the regulation of Cx43 mRNA by PR isoforms, MCF7-WT (expressing both PRs) and MCF7-PRBKO cells (expressing PRA alone) were treated with either vehicle or P₄ for 24 hours. qRT-PCR analysis revealed that PRBKO cells treated with P₄ have a significantly higher gap junction α-1 protein (GJA1, the gene that transcribes Cx43) mRNA expression compared to WT cells treated with P₄ (p<0.05) or vehicle (p<0.01) (Figure 3.2).

3.3.1.2 Cx43 mRNA expression is increased in MDA-MB-231-PRA and MDA-MB-231-PRB compared to MDA-MB-231-EV cells; however, P₄ stimulation significantly reduces Cx43 induction in MDA-MB-231-PRA cells

To further examine the regulation of Cx43 mRNA expression by PRs in the PR/ER-negative cell line MDA-MB-231, MDA-MB-231-EV (expressing no PRs), MDA-MB-231-PRA (expressing PRA) and MDA-MB-231-PRB (expressing PRB) cell lines were treated with either vehicle or P₄ (100 nM) for 24 hours. qRT-PCR analysis revealed that while the PR expressing stable cells exhibited higher levels of Cx43 mRNA compared to EV control cells (p<0.05), P₄ treatment significantly inhibited Cx43 expression in PRA, but not PRB, expressing cells (p<0.05) (Figure 3.3).
Figure 3.2: P₄-stimulated MCF7-BKO cells have significantly increased Cx43 mRNA expression compared to MCF7-WT cells treated with P₄ or vehicle. For each experiment the average of GAPDH, 18S, and YWHAZ were used as housekeeping genes. Statistical significance was determined using two-way ANOVA with multiple comparisons and Bonferroni post-hoc test. Statistical significance of '*' represents $p \leq 0.05$, '***' represents $p \leq 0.01$. n=4.
Figure 3.3: MDA-MB-231-PRA and MDA-MB-231-PRB cells have significantly increased Cx43 mRNA expression compared to MDA-MB-231-EV cells, however P$_4$-stimulation significantly decreases Cx43 mRNA expression in both PRA expressing cells. For each experiment the average of GAPDH, 18S, and YWHAZ were used as housekeeping genes. Statistical significance was determined using two-way ANOVA with multiple comparisons and Bonferroni post-hoc test. Statistical significance of ‘*’ represents $p \leq 0.05$, ‘**’ represents $p \leq 0.01$. n=4.
3.3.2 Analysis of Cx43 protein expression via western blotting in breast cancer cells

3.3.2.1 Cx43 protein expression is increased in P4-stimulated MCF7-PRBKO compared to P4-stimulated MCF7-WT

To examine the regulation of Cx43 protein by PR isoforms, MCF7-WT (expressing both PRs) and MCF7-PRB KO cells (expressing PRA alone) were treated with either vehicle or P4. Western blot analysis revealed that MCF7-PRBKO cells treated with P4 have a significantly higher Cx43 protein expression compared to MCF7-WT cells treated with P4 (p<0.05) consistent with the qPCR data from this cell line (Figure 3.4).

3.3.2.2 P4 decreases Cx43 protein expression MDA-MB-231-PRA and MDA-MB-231-PRB compared to their respective vehicle-treated controls

To assess the regulation of Cx43 protein by PR isoforms in the basal-like breast cancer cell lines MDA-MB-231-EV (expressing no PRs), MDA-MB-231-PRA (expressing PRA alone) and MDA-MB-231-PRB (expressing PRB alone) were treated with either vehicle or P4. Western blot analysis revealed that similar to mRNA expression, Cx43 protein was higher in PRA- and PRB-expressing cells compared to EV control cells; however, P4 inhibited the expression of Cx43 protein in both PRA- or PRB-expressing cells (Figure 3.5).

3.3.3 Cx43 localization analysis via immunofluorescence and live cell imaging

3.3.3.1 MCF7-PRBKO displayed increased Cx43 co-localization with actin at the plasma membrane compared to MCF7-WT

To examine Cx43 localization, MCF7-WT and MCF7-PRBKO cells were treated with vehicle or P4 for 24 hours. Cells were then probed with anti-Cx43 followed by Alexafluor 488 (green) and anti-actin followed by Alexafluor 594 (red) and counterstained with DAPI (blue) for
nuclear visualization. In MCF7-WT cells treated with vehicle, Cx43 was found evenly distributed throughout the cell. Treatment with P4 in these cells did not seem to alter Cx43 distribution. In MCF7-PRBKO cells Cx43 was found to co-localized with actin at the plasma membrane (as indicated by yellow fluorescence) in vehicle-treated cells; Cx43 distribution and its co-localization with actin was also not affected by P4 treatment (Figure 3.6). These results suggest that PRA alone may regulate Cx43 trafficking to the plasma membrane, and that PRB inhibits this trafficking regardless of P4 stimulation.

3.3.3.2 P4-stimulated MDA-MB-231-PRA displayed nuclear Cx43 staining

To examine Cx43 localization, MDA-MB-231-EV, MDA-MB-231-PRA and MDA-MB-231-PRB cells were treated with vehicle or P4 for 24 hours and then processed as described in the previous section. In contrast to MCF7 cells, the MDA-MB-231-EV cells did not exhibit Cx43 co-localization with actin, and Cx43 localization was predominantly cytoplasmic (perinuclear) independent of P4 treatment, suggesting that Cx43 does not traffic to the plasma membrane of MDA-MB-231-EV cells. MDA-MB-231-PRA cells treated with vehicle showed increased trafficking of Cx43 towards the plasma membrane: however, neither its incorporation into the plasma membrane nor its co-localization with actin was observed. P4 treatment in MDA-MB-231-PRA cells resulted in partial staining of Cx43 into the nucleus. MDA-MB-231-PRB cells showed Cx43 intracellular distribution similar to EV cells where Cx43 was mostly localized to the perinuclear region, and this effect seemed to be independent of P4 treatment (Figure 3.7). These results suggest that only PRA directly affects Cx43 localization in the basal-like cell line MDA-MB-231 when stimulated with P4.
Figure 3.4: A) Representative Western Blot of MCF7 WT versus BKO cells treated with either P₄ or its vehicle control. B) P₄-stimulated MCF7-BKO cells have significantly increased Cx43 protein expression compared to P₄-stimulated WT cells. ERK2 was used as a loading control. Statistical significance of B) was determined using two-way ANOVA with multiple comparisons and Bonferroni post-hoc test. ‘*’ represents p≤0.05. n=3.
Figure 3.5: MDA-MB-231-PRA and MDA-MB-231-PRB cells have increased Cx43 protein expression compared to MDA-MB-231-EV cells, however P4-stimulation decreases Cx43 expression in both PRA and PRB expressing cells. ERK2 was used as a loading control. Representative blot of 2 independent experimental replicates is shown.
Figure 3.6: Cx43 co-localizes with Actin at the plasma membrane in MCF7-PRBKO cells.
Representative immunofluorescence images of MCF7 WT and MCF7 BKO cells showing Cx43 in green color, Actin in red and their co-localization in yellow. MCF7 WT and MCF7 BKO cells display differential Cx43 localization patterns such that MCF WT displays cytoplasmic expression of Cx43 whereas MCF7 BKO traffics some Cx43 to the plasma membrane. Additionally, MCF7 BKO cells stain for Cx43 with greater intensity. In both WT and KO cells, P₄ did not alter Cx43 localization or staining intensity. All images taken at 1260x magnification.
Figure 3.7: PRA and PRB promote differential intracellular distribution of Cx43 in MDA-MB-231 cells. EV cells display cytoplasmic Cx43 and does not change with \( P_4 \) treatment. PRA cells treated with \( P_4 \) partially intensify Cx43 staining in the nucleus. PRB cells treated with vehicle and \( P_4 \) display a Cx43 localization pattern similar to EV cells. All images taken at 630x magnification.
3.3.3.3 3D image analysis reveals nuclear Cx43 staining in P4-stimulated MDA-MB-231-PRA cells

To examine Cx43 localization in 3-dimensional space, MDA-MB-231-EV, MDA-MB-231-PRA and MDA-MB-231-PRB cells were treated with vehicle or P$_4$ for 24 hours and then processed as described in the previous section. Similar to the 2D image analysis in this cell line, MDA-MB-231-EV cells did not exhibit Cx43 co-localization with F-actin, and Cx43 localization was predominantly cytoplasmic (perinuclear) independent of P$_4$ treatment. MDA-MB-231-PRA cells treated with vehicle showed a similar trafficking pattern to MDA-MB-231-EV cells, however P$_4$ treatment in MDA-MB-231-PRA cells resulted in partial staining of Cx43 in the nucleus. 3D image analysis revealed Cx43 in the same plane as the nucleus. Similar to the 2D image analysis in this cell line, 3D image analysis of MDA-MB-231-PRB cells showed Cx43 intracellular distribution similar to EV cells (Figure 3.8). These results suggest that only PRA directly affects Cx43 localization in the basal-like cell line MDA-MB-231 when stimulated with P$_4$. More detailed analysis using 3D projections can be found in the supplementary attachment “Figure 3.8 Expanded”.

3.3.3.4 PRA expression restored Cx43 trafficking in endogenous PRB expressing MFM223 when stimulated with P$_4$.

To determine if exogenous PRA expression could restore Cx43 intracellular trafficking in breast cancer cells, I used the PRB-positive, Cx43-negative cell line MFM223. MFM223 cells were transiently co-transfected with pDEST-Cx43-pEGFP and pSG5-PRA or its empty vector (EV, pSG5, to account for endogenous expression of PRB) expression constructs and treated with vehicle or P$_4$ for 24 hours. Intracellular localization of Cx43 was determined via GFP imaging. In
both vehicle- and P4-stimulated MFM223 cells transfected with EV (endogenous PRB expression), Cx43 was found to be retained in the cytoplasm at the perinuclear space. However, in only P4-stimulated MFM223 cells transfected with PRA, Cx43 was observed to be distributed throughout the cell, while vehicle-treated PRA cells did not display forward trafficking of Cx43 (Figure 3.9) These results further suggest that P4 stimulated PRB restricts Cx43 intracellular forward trafficking while P4-stimulated PRA promotes it.

3.3.3.5 Time lapse imaging suggests differential trafficking pattern over time of PRA versus PRB expressing MFM223

To analyze the duration at which differential Cx43 trafficking can be observed between PRA- and PRB-expressing cells, live cell imaging analysis was performed. The PRB-expressing cell line MFM223 was co-transfected with pDEST-Cx43-pEGFP and pSG5-PRA or its empty vector (EV, pSG5, to account for endogenous expression of PRB) expression constructs and then treated with vehicle or P4. Immediately after treatment, cells were analyzed over time using the IN Cell Analyzer 2000. In P4-stimulated MFM223 cells transfected with EV (endogenous PRB expression), Cx43 remained perinuclear throughout the 8-hour duration, while in cells transfected with PRA Cx43 was seen diffused across the cytoplasm over the course of 8 hours (Figure 3.10).

3.3.4 Assessment of gap junction intercellular communication via dye transfer assay

3.3.4.1 Dye transfer capacity is significantly increased in MCF7-PRBK0 compared to vehicle-treated WT MCF7-WT

To assess if differential expression and trafficking of Cx43 by PRs results in differential gap junction formation and hence affects cell connectivity, a co-culture dye transfer assay was
Figure 3.8: 3D imaging analysis in MDA-MB-231 cells reveals PRA and PRB promote differential intracellular distribution of Cx43. V- and P₄-stimulated EV and PRB cells display cytoplasmic distribution of Cx43, however P₄-stimulated PRA cells display nuclear Cx43 immunoreactivity. All images taken at 630x magnification.
Figure 3.9: P₄-stimulated PRA restores Cx43-GFP trafficking in MFM223 cells. Representative images of GFP imaging from P₄-stimulated MFM223 cells co-transfected with Cx43-GFP and PRA or its EV. Cx43 distribution is localized to the perinuclear region in EV cells which endogenously express PRB, while Cx43 intracellular trafficking is restored when PRA is overexpressed. All images taken at 630x magnification.
Figure 3.10: Time lapse imaging suggests the differential trafficking pattern of P_{4r}-stimulated PRA versus P_{4r}-stimulated PRB expressing breast cancer cells in MFM223 cells. Cx43-GFP trafficking was only seen in PRA expressing cells, as their EV control retained Cx43 in the perinuclear space. All images taken at 400x magnification.
performed on MCF7-WT versus MCF7-PRBKO cells. After treatment with vehicle or P₄ for 24 hours, 50% of the cells from each treatment were stained with a GJ permeable dye (CPD; red) and 50% with a GJ impermeable dye (DiI; yellow) which were then co-cultured for 24 hours to allow dye transfer between cells. The mean fluorescence intensity (MFI) of “donor” dye (CPD-stained) in “acceptor” cells (DiI-stained) was used as a measure of GJIC using flow cytometric analysis. Dye transfer in V- and P₄-stimulated MCF7-PRB KO cells was significantly increased compared to MCF7-WT cells treated with vehicle (p<0.01 and p<0.05, respectively). However, dye transfer within MCF7 WT and MCF7 PRB KO cells did not significantly change with P₄ treatment (Figure 3.10). These results suggest that although P₄ treatment in PRA expressing cells increases Cx43 expression and trafficking in MCF7 cells, differential GJIC may be a ligand-independent phenomenon.

3.3.4.2 Dye transfer capacity appears to be decreased in P₄-stimulated MDA-MB-231-PRA and MDA-MB-231-PRB compared to their respective vehicle-treated controls.

To determine if differential expression and trafficking of Cx43 by PRs results in differential gap junction formation and hence differential GJIC in MDA-MB-231, the same co-culture assay described in the previous section was performed on MDA-MB-231-PRA, MDA-MB-231-PRB and MDA-MB-231-EV. Cells were treated with vehicle or P₄ and stained with CPD and DiI as described in the previous section. Flow cytometric analysis revealed that both P₄-stimulated MDA-MB-231-PRA and MDA-MB-231-PRB cells showed a decrease in MFI of donor dye, suggesting a decrease in GJIC (Figure 3.11). Although an n of 1, this reflects a decrease in expression as shown by qPCR and western blotting for this cell line. These results suggest that, in contrast to MCF7, PRA negatively affects GJIC and also acts in a ligand-dependent manner.
Figure 3.11: MCF7-PRBKO cells have significantly increased GJIC compared to vehicle-treated MCF7-WT cells. All values expressed as fold change MFI of Log base10 values compared to ‘WT V’, and is the quantification of CPD dye in DiI cells. Statistical significance was determined using two-way ANOVA and multiple comparisons and Bonferroni post-hoc test. Statistical significance of ‘*’ represents $p \leq 0.05$ and of ‘**’ represents $p \leq 0.01$. n=3.
Figure 3.12: A decrease in GJIC is observed in both MDA-MB-231-PRA and MDA-MB-231-PRB upon P₄ stimulation compared to their vehicle controls. All values expressed as fold change MFI compared to ‘EV V’. This difference was greater in PRA cells. n=1
3.4 Discussion

3.4.1 Cx43 expression in MCF7 and MDA-MB-231 cells

3.4.1.1 Cx43 expression pattern in MCF7 compared to MDA-MB-231

It is generally regarded that hormone receptor loss is associated with a worse prognosis. Furthermore, in a study using microarray analysis of Cx proteins as potential breast tumour prognostic markers, PR and ER expression was found to correlate positively with Cx43 ex vivo in invasive breast tumours. Similarly, a study using an in vitro model of breast cancer in the luminal A cell line MCF7 reported that downregulation of ER results in ubiquitination and proteasome mediated degradation of Cx43. However, the relationship between PR isoforms and Cx43 in breast cancer cells has yet to be defined. Our current results suggest that the role in regulating Cx43 expression in a luminal A versus basal-like setting is dependent upon the specific PR isoforms present. In the luminal A cell line MCF7, P4-stimulated PRA induced Cx43 expression, while in the basal-like MDA-MB-231, both PR isoforms inhibited Cx43 expression when stimulated with P4.

P4 regulates a distinct set of genes through its receptor isoforms PRA and PRB in normal mammary cells as well as in breast cancer cells. Although PRB is considered as pro-proliferative in the breast, PRA dominance is reported in the majority of breast tumours and is associated with a worse prognosis. However, previous in vitro work using MCF7 cells only attempted to study PRs via the overexpression of one isoform to alter the PRA:PRB ratio, not by the assessment of individual PR isoforms in isolation. In another in vitro study using MDA-MB-231 cells, although individual PR isoforms were stably integrated into the genome to analyze metastasis, PR isoform-specific regulation of Cx43 was not investigated. Our study addresses how PR isoforms affect Cx43 expression both in a luminal A and basal-like breast cancer model.
through the expression of specific PR isoforms.

Whereas Cx43 expression is known to be differentially regulated by PR isoforms in the myometrium\textsuperscript{264}, differential regulation in the breast has yet to be elucidated. Since P\textsubscript{4} acts synergistically with estrogen in breast development, whereas P\textsubscript{4} opposes estrogen action in the uterus during the menstrual cycle\textsuperscript{296}, previous studies of the differential regulation of Cx43 by PR isoforms in the myometrium may not be applicable to the breast. Here we show that in the luminal A cell line MCF7, PRA and PRB can differentially regulate both Cx43 mRNA and Cx43 protein expression, where P\textsubscript{4}-liganded PRA significantly increases Cx43 mRNA and protein expression compared to P\textsubscript{4}-stimulated cells expressing both receptors. This supports previous findings in the myometrium, where PRA-expressing cells also significantly increased Cx43 expression levels compared to PRB-expressing cells\textsuperscript{264}. However, unlike previous findings in the myometrium, P\textsubscript{4}-stimulation of PRA in MCF7 was required to significantly increase Cx43 mRNA and protein expression.

In the basal-like MDA-MB-231, stable transfection of PRA or PRB resulted in an increase of Cx43 expression compared to EV (PR-negative) control cells. However, P\textsubscript{4}-stimulation of both PRA and PRB expressing cells resulted in a significant decrease in Cx43 expression compared to EV control cells. Additionally, in PRA-expressing cells, P\textsubscript{4}-stimulation significantly decreased Cx43 expression compared to vehicle-treated cells. These results suggest that in basal-like breast cancer, the differential regulation of Cx43 by PRA and PRB is perhaps lost. This result is supported by a study that found PRs lose their sensitivity to translocate to the nucleus in breast cancer cells\textsuperscript{171} suggesting differential sensitivity to PR-stimulated genes is lost. Also, the stability of PRA and its relative inability to be post-transcriptionally altered by phosphorylation\textsuperscript{183} could explain why P\textsubscript{4}-stimulated PRA tumours decreased Cx43 expression.
compared to P$_4$-stimulated PRB tumours, although this remains to be further elucidated.

### 3.4.2 Cx43 trafficking in MCF7 and MDA-MB-231 cells

Since Cx43 is the most widely studied connexin, its localization has been analyzed extensively using immunofluorescence analysis. In breast cancer, two trends have been observed regarding Cx43 immunoreactivity: either there is an overall reduction of Cx43 in breast tumour tissues and cancer cells$^{221,224,238,239}$, or there is an aberrant subcellular localization of Cx43 where it is restricted to the cytoplasm away from the PM$^{220,221,240}$. Since Cx43 and Cx43-mediated GJIC is regarded as tumour-suppressive, restoring Cx43 trafficking to the PM could be beneficial to arrest tumourigenesis.

### 3.4.2.1 The role of PR isoforms on Cx43 trafficking in MCF7 and MFM223 cells

The current *in vitro* study examines the effect of PRA or both PRs on Cx43 trafficking in the luminal A cell line MCF7 cell line. In this study we have demonstrated that in the absence of PRB, PRA promotes Cx43 trafficking to the PM, as indicated by the co-localization of Cx43 with actin at the cell periphery. In contrast, in WT cells where both PR isoforms are expressed, there was negligible localization of Cx43 at the PM, no co-localization with actin and an overall decrease in Cx43 reactivity. These data suggest that in luminal A-like cells, P$_4$ favours the normal course of Cx43 intracellular trafficking via PRA, while it not only inhibits Cx43 expression via PRB but also blocks its forward trafficking to the PM. We further confirmed these findings in another luminal A breast cancer cell line MFM223, which expresses only PRB and is Cx43-negative. We used exogenous expression of Cx43 in this cell line and found that P$_4$ stimulation restricts the forward trafficking of Cx43 and confines it to the perinuclear region
even when Cx43 is overexpressed, confirming that the PRB receptor blocks the forward trafficking of Cx43 to the PM. In this cell line the re-introduction of P₄-stimulated PRA expression and dominance restored Cx43 trafficking, confirming that PRA promotes Cx43 forward trafficking. Taken together these data suggest that in PRA dominant luminal A tumours P₄ may facilitate proper Cx43-mediated GJIC via more Cx43 that is trafficked to the PM while in PRB dominant tumours P₄ may have the opposite effects. These data confirm the western blotting data from MCF7, which demonstrate that PRA promotes Cx43 protein expression. Previous studies have shown that PRB has a pro-proliferative role in mammary cells since it regulates the expression of cell cycle regulatory genes¹⁹⁷,¹⁹⁸,¹⁹⁹. Since Cx43 is also regarded as tumour suppressive, PRB may also exhibit a proliferative role here in luminal A tumour cells via inhibition of Cx43.

Currently it is accepted that as cells lose the expression of hormone receptors (ER or PR), they become more aggressive. Additionally, as cells become more aggressive, Cx43 is lost or aberrantly localized²³⁹. Our data provide a mechanism for these two observations and suggest that, in low-grade tumours, it is the dominance of PRB that reflects decreased Cx43 localization, whereas PRA may promote or restore functional Cx43 at the PM.

3.4.2.2 The role of PR isoforms on Cx43 trafficking in MDA-MB-231 cells

Next, we focused on the basal-like, PR-negative breast cancer cell line MDA-MB-231 and examined the trafficking patterns of Cx43 when either PRA or PRB was stably expressed. EV controls displayed equal distribution of Cx43 throughout the cytoplasm. Vehicle-treated PRA cells displayed increased forward trafficking of Cx43 throughout the cytoplasm, however Cx43 localization at the PM was not observed. An increase of Cx43 immunoreactivity was also
observed compared to EV controls. Treatment with P₄ in PRA cells resulted in an overall decrease of Cx43 immunoreactivity and an increase in nuclear Cx43 localization. Vehicle-treated PRB cells displayed increased Cx43 immunoreactivity compared to EV controls, and also displayed increased cytoplasmic localization of Cx43. Treatment with P₄ in PRB cells resulted in more diffuse Cx43 throughout the cytoplasm, with some nuclear staining, however trafficking to the PM was not observed.

Trafficking data in MDA-MB-231 conflicts with the expression data in this cell line. Whereas vehicle-treated PRA and PRB cells exhibit increased Cx43 expression compared to EV controls, P₄ stimulation decreased Cx43 expression compared to their respective vehicle-treated controls. Also, the differential trafficking of Cx43 by PRA and PRB in this cell line is in contrast with the MCF7 and MFM223 data, as well as in stark contrast with previous myometrial data observed in our lab²⁶⁴, all of which showed PRA promoted forward Cx43 trafficking whereas PRB restricted Cx43 trafficking. The differential trafficking pattern observed in MDA-MB-231, and the observation that under any circumstance Cx43 does not actually reach the PM, may be due to its basal-like characteristics, which has also been observed in invasive breast tumour biopsies²²¹. Additionally, a previous study using MDA-MB-231 in vitro found that Cx43 was targeted for lysosomes without ever reaching the PM²⁹⁹. Although an antibody against a lysosomal marker was not tested here, this too is consistent with our observation that Cx43 never reached the PM. However, since the re-expression of Cx43 in MDA-MB-231 was associated with a decrease in proliferation in a 2D model²⁴⁴ and a decrease in metastasis in a 3D model²⁵⁶, increased Cx43 expression by PRs may still prove to be tumour-suppressive through GJIC-independent pathways.

When PRA cells were stimulated with P₄, a drastic change in Cx43 localization to the
nucleus was observed. Nuclear Cx43 is a topic that has been sparsely reported, and functions of nuclear Cx43 remain to be elucidated. However, nuclear Cx43 was first noted in rat liver epithelial cells\textsuperscript{300}, and has since been noted in many cell types including chick lens\textsuperscript{301}, heart fibroblasts\textsuperscript{302}, and chondrocytes\textsuperscript{303}, however these findings were purely observational. Recent studies have suggested that nuclear Cx43 staining is also present in some colorectal cancer biopsies and was associated with a chance of better prognosis\textsuperscript{304}. However, because there is limited research on nuclear Cx43, especially in ex vivo and in vivo studies, the authors concluded that more research is needed. In cardiomyocytes and HeLa cells, the C-terminal domain (CTD) of Cx43 was observed to translocate to the nucleus to inhibit proliferation\textsuperscript{305}, a finding that coincides with the finding that the Cx43 CTD has a nuclear localization sequence (NLS). The Cx43 CTD has been found to interact with numerous proteins and can regulate gene expression independently of the remainder of of Cx43 protein\textsuperscript{306}. In a study using MDA-MB-231 cells, over-expression of Cx43 in vivo inhibited tumor growth but did not increase GJIC. The authors concluded that the Cx43 CTD may be regulating proliferative genes via translocation to the nucleus\textsuperscript{307}, however they did not explicitly observe Cx43 nuclear staining. While it remains to be determined whether the full length Cx43 or only the Cx43 CTD translocates to the nucleus to regulate gene expression, these results do suggest that Cx43 might impact breast cancer cell proliferation through GJIC-independent pathways.

Based on the above literature of nuclear Cx43, we speculate that in P\textsubscript{4}-stimulated PRA-expressing MDA-MB-231 cells, Cx43 staining in the nucleus may regulate gene transcription. The presence of Cx43 within the nucleus would also suggests that Cx43 is acting through GJIC-independent mechanisms. Importantly, our observation of nuclear Cx43 staining in MDA-MB-231 cells was performed using a C-terminal antibody against Cx43, therefore the current study is
not able to distinguish whether full length or only the CTD of Cx43 may exhibit nuclear staining. Our failure to observe co-localization of Cx43 with F-actin suggests that in MDA-MB-231 cells actin does not regulate Cx43 nuclear transport. Although Cx43-actin binding has been discovered to regulate Cx43 transport dynamics previously, the mechanism by which Cx43 (or the Cx43 CTD) translocates to the nucleus and how PRA but not PRB influences this change in MDA-MB-231 cells remains to be elucidated.

3.4.3 Dye transfer as a measure to assess Cx43 at the plasma membrane

This study examines Cx43 GJIC indirectly via the intrinsic capacity of cells to transfer different colour dyes. Since PM localization of Cx43 does not necessarily equate to functional channel capacity at the PM, using flow cytometry to assess dye transfer provides an unbiased assessment of GJIC.

3.4.3.1 Dye transfer in MCF7 cells

The current study shows that in V- and P₄-stimulated BKO MCF7 cells, there is a significant increase in dye transfer compared to V-treated WT MCF7 cells, although a more significant difference was observed in V-treated BKO MCF7 cells compared to V-treated WT MCF7 cells. This is in accordance with expression and immunofluorescence data from this cell line, where MCF7-PRBKO cells have higher levels of Cx43 mRNA and protein compared to MCF7-WT cells. However, P₄ stimulation did not affect the dye transfer ability of MCF7-PRBKO cells suggesting that P₄ can enhance Cx43 expression levels in this cell line and may not have a direct role in regulation of GJIC. This suggests that although P₄-stimulated PRA may stimulate Cx43 expression, it is not necessarily functional at the PM. Furthermore, this points to
a P₄-independent role of PRA in this cell line, where PRA does not need P4 stimulation in order to affect dye transfer capacity.

3.4.3.2 Dye transfer in MDA-MB-231 cells

In MDA-MB-231 cells, we found that all P₄-stimulated cells (EV, PRA, and PRB) had a decreased dye transfer compared to their vehicle controls, and in accordance with expression (and trafficking) data, PRA cells had the least dye transfer. Overall, it is important to consider that these results represent an n=1 and should be repeated before definitive conclusions can be made.

Interestingly, many previous studies using MDA-MB-231 found that an increase of Cx43 did not correlate to an increase in GJIC, but did decrease proliferation\textsuperscript{244}, the rate of mitosis\textsuperscript{256}, and differentiation\textsuperscript{260} suggesting that an increase in Cx43 expression may not correlate with an increase in dye transfer in this study. An previous study also referred to MDA-MB-231 as gap junction incompetent, as Cx43 overexpression never reached the PM\textsuperscript{299}. However, although an n=1, our preliminary data suggest that P₄-stimulation in both PRA and PRB expressing MDA-MB-231 cells decreases GJIC, which correlates with Cx43 expression data from this study. The dye transfer data from this study also correlates to the trafficking data in PRA expressing cells, which exhibits nuclear translocation of Cx43, therefore allowing less Cx43 to be forward trafficked. A decrease in dye transfer proportionate to the decrease in Cx43 expression and trafficking would implicate Cx43-mediated GJIC in this cell line, however currently more dye transfer assay data is required to asses Cx43-mediated GJIC in this cell line.

4.1 Summary of Results
In summary, we present two *in vitro* models of breast cancer with which we assessed the role of PR isoforms PRA and PRB on the regulation of the gap junction and tumour suppressor protein Cx43. Firstly, we used CRISPR/Cas9 genetic engineering to delete PRB in the PRA/B-positive luminal A cell line MCF7, and then compared MCF7-PRBKO (only PRA expressing) cells with MCF7-WT cells (both PRA and PRB expressing). Secondly, we used stable integration of PRA and PRB expression constructs in the basal-like cell line MDA-MB-231, and then compared MDA-MB-231-EV controls with MDA-MB-231-PRA and MDA-MB-231-PRB. Using these two models, our data demonstrate that: 1) Cx43 mRNA and protein expression is increased in MCF7-PRBKO cells compared to MCF7-WT cells, whereas MDA-MB-231-PRA and MDA-MB-231-PRB have increased Cx43 mRNA and protein expression compared to EV controls, however P4 reduces Cx43 this expression; 2) Cx43 exhibits more prominent PM immunoreactivity in MCF7-PRBKO cells compared to MCF7-WT cells, whereas no PM Cx43 staining is observed in MDA-MB-231; 3) In MDA-MB-231-PRA, Cx43 appears to be localized within the nucleus upon P4 stimulation; 4) MCF7-PRBKO cells exhibit more dye transfer between cells than MCF7-WT which is indicative of increased GJIC, whereas P4-stimulated MDA-MB-231-PRA and MDA-MB-231-PRB cells both exhibit decreased dye transfer and therefore decreased GJIC compared to their respective vehicle controls. A schematic of summary results can be found in Figure 4.1.
Figure 4.1: Model of PR regulation on Cx43 expression, trafficking and GJIC in breast cancer cells. In MCF7 cells, MCF7-PRBKO increases Cx43 expression, trafficking and GJIC compared to MCF7-WT. Thus, PRA is directly implicated in regulating Cx43 in this cell line. Conversely, in MDA-MB-231 cells, both MDA-MB-231-PRA and MDA-MB-231-PRB increase Cx43 expression compared to MDA-MB-231-EV cells. However, specifically in MDA-MB-231-PRA cells, $P_4$-stimulation decreases Cx43 expression. Preliminary results suggest $P_4$-stimulation in MDA-MB-231-PRA cells, however further investigation is required.
4.2 Significance of Study

This is the first study to establish a differential relationship between PR isoforms and Cx43 expression, intracellular trafficking and GJIC in vitro in breast cancer cells. Current clinical practice only screens women for the presence or absence of total PR using immunohistochemistry\textsuperscript{11,257} and does not discriminate between individual PR isoforms. However, with the knowledge that PRA and PRB regulate Cx43 differentially, this study provides additional incentive to screen for individual PR isoforms in women with breast cancer.

Although not tested here, Cx43 is regarded as a tumour suppressive protein\textsuperscript{235,236}. Our study suggests that PRs affect Cx43 expression, trafficking, and function differently depending on if the tumour displays luminal A characteristics or basal-like characteristics as indicated by the differing results obtained in MCF7 and MDA-MB-231 cells. Based on the study using MCF7 cells, we propose that P\textsubscript{4} stimulation may be beneficial to women with PRA-dominant, low grade tumours in order to stimulate increased Cx43 expression, trafficking to the PM, and GJIC. However, it is also known that as a tumour becomes more invasive, cells lose the expression of Cx43 and GJIC\textsuperscript{221}. For MDA-MB-231, there is conflicting evidence. Due to the decrease in Cx43 expression, trafficking, and GJIC in MDA-MB-231-PRA and MDA-MB-231-PRB, P\textsubscript{4} administration may be harmful in PRA-dominant or PRB-dominant high-grade tumours. However, P\textsubscript{4}-stimulated PRA intensifies Cx43 staining in the nucleus, and previous studies suggest that nuclear Cx43 (or its CTD) decreased cell proliferation and was correlated with a better overall survival\textsuperscript{304,305}. This suggests that although P\textsubscript{4}-stimulated PRA decreases GJIC, it may suppress tumourigenesis through a GJIC-independent mechanism, although this needs to be further elucidated.

This study demonstrates differential regulation of a putative tumour suppressor protein,
Cx43, by PR isoforms. However, because P₄ was implicated as a contributor to breast cancer risk⁹⁰,⁹¹,⁹², P₄ is not used in hormonal therapy¹¹⁵ and has largely fell out of focus for consideration as a therapeutic hormone for breast cancer. It may be that P₄ is not inherently mitogenic, but depends upon the PRs that are present within the tumour. With the potential of PRs to maintain ER expression in breast cancer³⁰⁸, our work has the potential to uncover the differential roles of PRs and suggest that P₄ therapy may be appropriate in some circumstances as an alternative, or in addition to, estrogen/ER therapy. P₄ has already been implicated in the reduction of tumour metastasis and proliferation in vitro³⁰⁹, and P₄/PRs have already been shown to modulate ER binding (thereby imposing direct effects on tamoxifen treatment) and ER-related tumour growth¹⁷⁸, therefore our study contributes to the literature of the potential tumour-suppressive effects of P₄ treatment.

4.3 Limitations

There are a number of limitations to our study that must be considered in forming our conclusions. Firstly, although a model of stable PRA and PRB expression was established in the PR-negative cell line MDA-MB-231, we were unsuccessful in establishing a model of PRAKO (expressing PRB alone) to compare with PRBKO (expressing PRA alone) and PRAB KO in the PRA/B-positive cell line MCF7. Although a previous group has utilized the same strategy as us to successfully mutate the start methionine of PRA to only express PRB¹⁶³, all potential PRAKO clones tested negative for the mutation. While preliminary conclusions can be made about the effect of P₄ on Cx43 expression and GJIC in MCF7 cells solely expressing PRA (i.e. PRB KO), we are unable to draw conclusions about the effect on MCF7 cells solely expressing PRB (PRA KO) and therefore could not conclude on PRB-specific effects in this cell line.
Additionally, while one MCF7-PRBKO and one MCF7-PRABKO clone were established, additional clones could not be established due to poor survival rates of MCF7 cells after sorting. A previous study also using our strategy was also only able to verify one clone after sorting, and therefore could only come to preliminary conclusions. However, considering off-target effects of using the CRISPR/Cas9 system are still highly controversial additional clones would be useful to solidify the relationship between PRs and Cx43 MCF7.

Thirdly, although MCF7 was genotyped at the PgR locus to reveal only one copy of the gene, the copy number of PgR genes that were stably transfected into MDA-MB-231 was not analyzed. A previous study confirmed that MCF7 cells have a heterozygous loss of the PgR gene (indicating 2 copies of the gene is the normal genotype in humans), so copy number analysis in MDA-MB-231 would provide additional support to address the physiological relevance of this model.

Furthermore, although in previous work Cx43 has been shown to increase the bystander effect (the transfer of molecules between adjacent cells, and thus pertaining to our current study, dye transfer), an increase in dye transfer in our current work is not necessarily contingent upon Cx43. Thus we do not rule out a role for other connexins (such as Cx26 which is the other connexin majorly expressed in the breast) that could possibly be influenced by P4 or PRs.

Finally, although statistics were performed on each study where an n ≥ 3, each experimental n was too small to determine if the data was normally distributed and therefore significance values can be assumed only as indicators of change. For this reason, conclusions inferred in this study will need to be further substantiated once a larger n can be generated.

4.4 Future Directions
Our current work suggests that in breast cancer cells, PRA and PRB have the capacity to differentially affect Cx43 expression, localization, and GJIC. However, we have not shown whether PR isoforms can differentially affect the ability of Cx43 to modulate any parameter of carcinogenesis through its capacity to form gap junctions including, but not limited to, proliferation, metastasis, cell cycle regulation, or survival. This is an important element that will require further research.

A potentially important finding of this study is the observation of apparent nuclear localization of Cx43. While there have been some limited reports in the literature in other tissues, the significance of this finding for breast carcinogenesis has yet to be determined. These data were obtained using an antibody directed to the C-terminus of Cx43. A next step would be to use an N-terminal antibody (i.e. an antibody that does not probe the Cx43 CTD) to gain insight as to whether the full length Cx43 is translocating to the nucleus or only the CTD. Since the Cx43 CTD is known to regulate gene expression, independently of GJ formation, a follow-up immunofluorescence study would be useful to address this issue. siRNA-mediated knockdown of Cx43 or the use of monoclonal antibodies would also help to determine whether Cx43 is truly localized to the nucleus or whether there is this represents non-specific binding.

In all of experiments, we used P₄ in the absence of estrogen or anti-estrogen. However, to fully address the role of differential regulation of Cx43 by PRs, the administration of an anti-estrogen should also be considered. Since ERs regulate PR expression and PRs regulate ER binding, the inextricable link between these two receptors should be further elucidated, especially considering tamoxifen is the current standard of care for women with hormone-responsive tumours. For this reason, once a complete MCF7 model is established, a logical next step would be to stimulate our MCF7 KO model with anti-estrogen with P₄ treatment. This will
address the effect of estrogen on Cx43 in breast cancer, as well as the interplay between ER and PR to modulate Cx43.

Our results provide a novel insight as to how PRs differentially affect Cx43 in vitro. However, scaling this work up to incorporate animal models will allow us to assess if PRs differentially affect Cx43 in vivo. Additionally, explants can be harvested and cultured to assess how PR expression can relate to known downstream effectors of PR-action, including proliferation, apoptosis, and cell cycle regulators. If the same trends are seen in these two models, PR isoforms can be thought of as key regulators of Cx43 in breast cancer, a finding which may help to bring P₄ as a therapy back into the limelight.

Finally, in the MDA-MB-231 protein analysis and dye transfer assay, there is only an \( n \) of 1. Results should be confirmed with a greater \( n \) value in order to elucidate the pattern of Cx43 translation and GJIC in this cell type.

REFERENCES

9. Cserni, G., Chmielik, E., Cserni, B. & Tot, T. The new TNM-based staging of breast


64. Steponaviciene, L. *et al.* Triple negative breast cancer: adjuvant chemotherapy effect on


92. Amphiregulin is an essential mediator of estrogen receptor function in mammary gland development. 1–6 (2007).


171. Arnett-Mansfield, R. L. *et al.* Focal Subnuclear Distribution of Progesterone Receptor Is


