FOXA1: Its role and Its Interaction with Prolactin Hormone Signaling in Triple Negative Breast Cancer Tumorigenesis

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Abstract

Triple Negative Breast Cancer (TNBC) is the most resistant subtype of breast cancer. The lack of expression of estrogen, progesterone, and human epidermal growth factor 2 receptors in TNBC leads to the limited use of targeted therapy. Therefore, treatment with chemotherapy remains the main approach for treating TNBC with low therapeutic benefits and increased risk of resistance. Pro-differentiation based therapies by reversing breast cancer cells into an epithelial phenotype have recently been studied as a promising therapeutic approach for TNBC. Indeed, the laboratory of Dr. Ali showed that restoring prolactin pro-differentiation pathway reversed the mesenchymal aggressive phenotype of TNBC into the epithelial form with less aggressive behavior, less invasiveness, and lower metastasis. In addition, they identified a TNBC subgroup of patients that preserved prolactin receptor (PRLR) expression to show better overall survival and better prognosis. By metagenes cluster analysis, the TNBC-PRLR subtype is characterized by the up regulation of luminal-like differentiation genes which are Forkhead Box A1 (FOXA1) and androgen receptor (AR). Our aim is to determine the effect of FOXA1 on the epithelial mesenchymal transition (EMT) of breast cancer cells and how it may modulate the antitumorigenic role of prolactin and its signaling pathway in breast cancer. To achieve our objectives, we measured the expression of FOXA1 at the mRNA and protein levels after treating TNBC-PRLR cell lines with prolactin. We also produced CRISPR knock out of FOXA1 in these cell lines and measured the protein level of both E-cadherin, an epithelial marker and vimentin as a mesenchymal marker. As expected, the knock-out cells showed a decrease in the level of E-cadherin and an increase in vimentin compared to the parental cells. Finally, we conclude that FOXA1 can be a potential pro-differentiation marker in breast cancer cells. Further studies are needed to investigate this role of FOXA1 and its use as a target for personalized medicine in triple negative breast cancer.

Résumé

Le cancer du sein triple négatif (TNBC) est le sous-type de cancer du sein le plus résistant. Le manque d'expression des récepteurs des œstrogènes, de la progestérone et du facteur 2 de croissance épidermique humain dans le TNBC conduit à une utilisation limitée du traitement ciblé. Par conséquent, le traitement par chimiothérapie demeure la principale approche pour le traitement de la TNBC avec de faibles avantages thérapeutiques et un risque accru de résistance. Les thérapies fondées sur la différenciation par inversion des cellules cancéreuses du sein en un phénotype épithélial ont récemment été étudiées comme une approche thérapeutique prometteuse pour le TNBC. En effet, le laboratoire du Dr Ali a montré que le rétablissement de la voie de différenciation de la prolactine a inversé le phénotype mésenchymateux agressif de la TNBC dans la forme épithéliale avec un comportement moins agressif, moins invasif et moins de métastase. De plus, ils ont identifié un sous-groupe de patients TNBC dont l'expression du récepteur de la prolactine (PRLR) est élevée, la survie globale meilleure et le pronostic meilleur. Par l'analyse des groupes de métagènes, le sous-type TNBC-PRLR est caractérisé par la régulation ascendante des gènes de différenciation de type luminal qui sont FOXA1 et AR. Notre objectif est de déterminer l'effet de FOXA1 sur la transition mésenchymateuse épithéliale (EMT) des cellules cancéreuses du sein et comment il peut moduler le rôle antitumorigène de la prolactine et sa voie de signalisation dans le cancer du sein. Pour atteindre nos objectifs, nous avons mesuré l'expression de FOXA1 au niveau de l'ARNm et des protéines après avoir traité les lignées cellulaires TNBC-PRLR avec la prolactine. Nous avons également produit CRISPR knock out de FOXA1 dans ces lignées cellulaires et mesuré le taux de protéines de la E-cadhérine, un marqueur épithélial et de la vimentine comme marqueur mésenchymateux. Comme on s'y attendait, les cellules éliminées ont montré une diminution du taux de E-cadhérine et une augmentation de la vimentine par rapport aux cellules parentales. Enfin, nous concluons que FOXA1 peut être un marqueur pro-différenciation potentiel dans les cellules du cancer du sein. D'autres études sont nécessaires pour étudier ce rôle de FOXA1 et son utilisation comme cible de la médecine personnalisée dans le cancer du sein triple négatif.

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A. List of Abbreviations

ALDH: aldehyde dehydrogenase

BSA: bovine serum albumin

CSC: cancer stem cells

Csn2: Beta Casein

DMEM: dulbecco's modified Eagle's medium

E-Cad: E-Cadherin

EMT: epithelial to mesenchymal transition

ER: estrogen receptor

FBS: fetal bovine serum

FOXA1: Forkhead Box A1

GAS: gamma activated sequence

HDAC: histone deacetylase

HER2: human epidermal growth factor receptor 2

IHC: immunohistochemistry

IL1, 4, 6 and 8: interleukin 1, 4, 6 and 8

Jak2: Janus kinase 2

KM: Kaplan Meier plotter

MAPK: mitogen-activated protein kinase

PRG: progesterone receptor

PRL: prolactin

PRLR: prolactin receptor

RFS: relapse free survival

SDS: sodium dodecyl sulfate

STAT: signal transducer and activator of transcription

TNBC: triple negative breast cancer

Vim: vimentin

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Contribution of Authors

This thesis is presented in a traditional format. This thesis is written by Mounira Ibrahim and revised by Dr. Suhad Ali, the supervisor. It consists of five major chapters. Chapter 1 includes the introduction, a review of related literature background. Chapter 2 contains the materials and methods used. Chapter 3 includes the results and figures. Chapter 4 contains a general discussion of the results. Chapter 5 contains an overall conclusion and suggested future directions. An appendix of copyright permissions for used figures is included.

I wrote the thesis under my supervisor's guidance. I performed most of the experiments described according to the lab protocols and under my supervisor directions. Mr. Julien Boudreault from Dr. Lebrun's laboratory designed and generated the single guide RNAs used for FOXA1 CRISPR Knockouts. Dr. Anwar Shams and Ms. Dana Hamam performed the control immunohistochemistry experiment in MCF-7, T47D and MDA-MB-231 breast cancer cell lines. Dr. Anwar Shams performed the immunohistochemistry experiment in the MCF-7 PRLR knock out cell line and I was following her instructions and helping her in it.

Chapter I- Introduction

1. Prolactin hormone

Prolactin (PRL) hormone is the female lactating hormone and it is produced mainly from the lactotrophs of the anterior pituitary gland (Freeman, Kanyicska et al. 2000). Prolactin is also secreted from extra-pituitary tissues, such as the epithelial cells of the mammary glands (Andersen 1990). During pregnancy, prolactin diffuses into the amniotic fluid to help in stimulating the maturation of the fetus (Riddick and Daly 1982). It is also secreted from other tissues such as the circulating lymphocytes, spleen, lymphoid cells of the bone marrow, brain, decidua, myometrium, thymus, lacrimal gland, tumours, sweat glands, and skin fibroblasts (Horseman, Zhao et al. 1997).

PRL hormone is a polypeptide hormone that is composed of 227 amino acids, where a single peptide chain is composed of 28 amino acids and the rest of the 199 amino acids represent the mature prolactin molecule (Sinha 1995). PRL is composed of a single amino acid chain with three intra molecular disulphide bonds between six cysteine residues (Cooke, Coit et al. 1981). PRL hormone is encoded by the PRL gene which is located on chromosome 6 (Horseman and Yu-Lee 1994). Its size is 10 Kb and it consists of 5 exons and 4 introns (Horseman, Zhao et al. 1997). PRL transcription is directed mainly by a downstream promoter of 5000 bp that is responsible for pituitary PRL synthesis and another upstream region that is responsible for extra pituitary PRL synthesis (Horseman, Zhao et al. 1997).

PRL exerts its roles by binding to prolactin receptors in different parts of the body. Its binding to the receptor activates Jak-Stat signalling pathway and induces the expression of different genes (Bole-Feysot, Goffin et al. 1998, Freeman, Kanyicska et al. 2000).

2. Prolactin Receptors

Prolactin receptors (PRLR) are transmembrane receptors that belong to class I cytokine receptors family (Bole-Feysot, Goffin et al. 1998). It consists of three major receptor domains; the ligand binding extracellular domain (ECD), the transmembrane (TM) domain, and the intracellular domain (ICD) (Bole-Feysot, Goffin et al. 1998). The structure of the PRLR is similar

to other hormone receptors in the same family such as the growth hormone receptor and the erythropoietin receptor (Bazan 1990, Bazan 1990, Goffin and Kelly 1997). The PRLR was found to have different isoforms: short, intermediate and long. They are different in length and size, but all have identical extracellular domains (Bole-Feysot, Goffin et al. 1998). The long form of PRLR in humans is composed of 598 amino acid residues (Bazan 1990, Bazan 1990, Goffin and Kelly 1997). The PRLR gene is located on chromosome 5, composed of 11 exons, and is around 200 Kb in size (Bole-Feysot, Goffin et al. 1998). PRLR are found in various parts of the mammalian tissue with different levels of expression (Bole-Feysot, Goffin et al. 1998, Freeman, Kanyicska et al. 2000). They are highly expressed in the breast, kidney, adrenal cortex, liver, brain and bronchial mucosa, while there is a low level of expression of PRLR in brown adipose tissues, submandibular glands, olfactory neuronal epithelium and bulb, trigeminal and dorsal root ganglia, cochlear duct, and whisker follicles (Bole-Feysot, Goffin et al. 1998, Freeman, Kanyicska et al. 2000).

The extracellular domain (ECD) contains the N-terminus and is the ligand-binding domain in the PRLR (Boutin, Jolicoeur et al. 1988, Boutin, Edery et al. 1989). It consists of around 210 amino acid residues (Boutin, Jolicoeur et al. 1988, Boutin, Edery et al. 1989) and is subdivided into two main subunits, D1 and D2 (can also be called S1 and S2) (Wells and de Vos 1996). The D1 subunit of the ECD contains two disulfide bridges and the D2 subunit contains a duplicated tryptophan–serine called the WS motif in its C-terminus (Wells and de Vos 1996). Both subunits are important for binding and activation of ligands (Rozakis-Adcock and Kelly 1991).

The transmembrane domain has 24 amino acid residues and is anchored by two charged residues; D210 and K235 (Bole-Feysot, Goffin et al. 1998). The function of this domain is not yet clear (Bole-Feysot, Goffin et al. 1998).

The Intracellular domain (ICD) consists of two conserved regions, which are Box1 and Box2 (Kelly, Djiane et al. 1991). Box1 is rich in proline with many hydrophobic residues and is important for the recognition and binding of transducing molecules such as Janus Kinase 2 (Jak2) (Lebrun, Ali et al. 1995, Pezet, Buteau et al. 1997). Box2 is less conserved than Box1 and is absent in short isoforms of PRLR (Bole-Feysot, Goffin et al. 1998, Freeman, Kanyicska et al. 2000).

PRL-PRLR binding leads to the activation and dimerization of PRLR and activation of signaling pathways as the Jak/Stat signaling pathway, which leads to production of milk protein genes (Lesueur, Edery et al. 1991). PRLR has an essential role in lactation as studies showed that PRLR -/+ heterozygous female mice failed to lactate despite the development of mammary glands (Bole-Feysot, Goffin et al. 1998).

3. Jak2 and STAT5 Molecules

Janus Kinase 2 (Jak2) is a tyrosine kinase that is part of the PRLR signaling pathway (Kisseleva, Bhattacharya et al. 2002). It is a member of a tyrosine kinase family that consists of Jak1, Jak2, Jak3, and Tyk2 (Kisseleva, Bhattacharya et al. 2002). Binding of prolactin to its receptor induces receptor dimerization, phosphorylation of the receptor itself and trans phosphorylation and activation of Jak2 molecules (Wagner, Krempler et al. 2004). Activated Jak2 phosphorylates PRLR on the intracellular domain and allows interaction with signaling molecules such as the signal transducer and activator of transcription (STAT) proteins (Wagner, Krempler et al. 2004). Previous studies showed that Jak2 is crucial for the activation of PRL signaling pathway and that its absence in KO mice is embryonically lethal (Wagner, Krempler et al. 2004).

Signal transducer and activator of transcription 5 (STAT5) proteins are the transducer molecules in the PRL signaling pathway (Bole-Feysot, Goffin et al. 1998) (Ihle, Witthuhn et al. 1994, Goffin, Bouchard et al. 1998). Each Stat5 protein is composed of a DNA binding domain, SH3 domain, SH2 domain, a conserved tyrosine, and a C-terminal transactivation domain(Bole-Feysot, Goffin et al. 1998) (Ihle, Witthuhn et al. 1994, Goffin, Bouchard et al. 1998). Activation of PRL signaling pathway by ligand receptor binding leads to phosphorylation of Jak2, that in turn phosphorylates Stat5, then Stat5 dissociates from the receptor and form dimers with another phosphorylated Stat5a/b via their SH2 domains. These dimers translocate to the nucleus, bind to the Interferon- Gamma Activated Sequence (GAS) of the promoter region and induces transcription of the target gene beta casein (Csn2) (Bole-Feysot, Goffin et al. 1998) (Figure 1). Stat5 activation is important for ductal branching during puberty (Bole-Feysot, Goffin et al. 1998, Koptyra, Gupta et al. 2011, Barash 2012). Its activation also induces the proliferation and

differentiation of mammary epithelial cells and production of milk proteins during pregnancy and regulates the number of epithelial cells during lactation and involution (Bole-Feysot, Goffin et al. 1998, Koptyra, Gupta et al. 2011, Barash 2012).

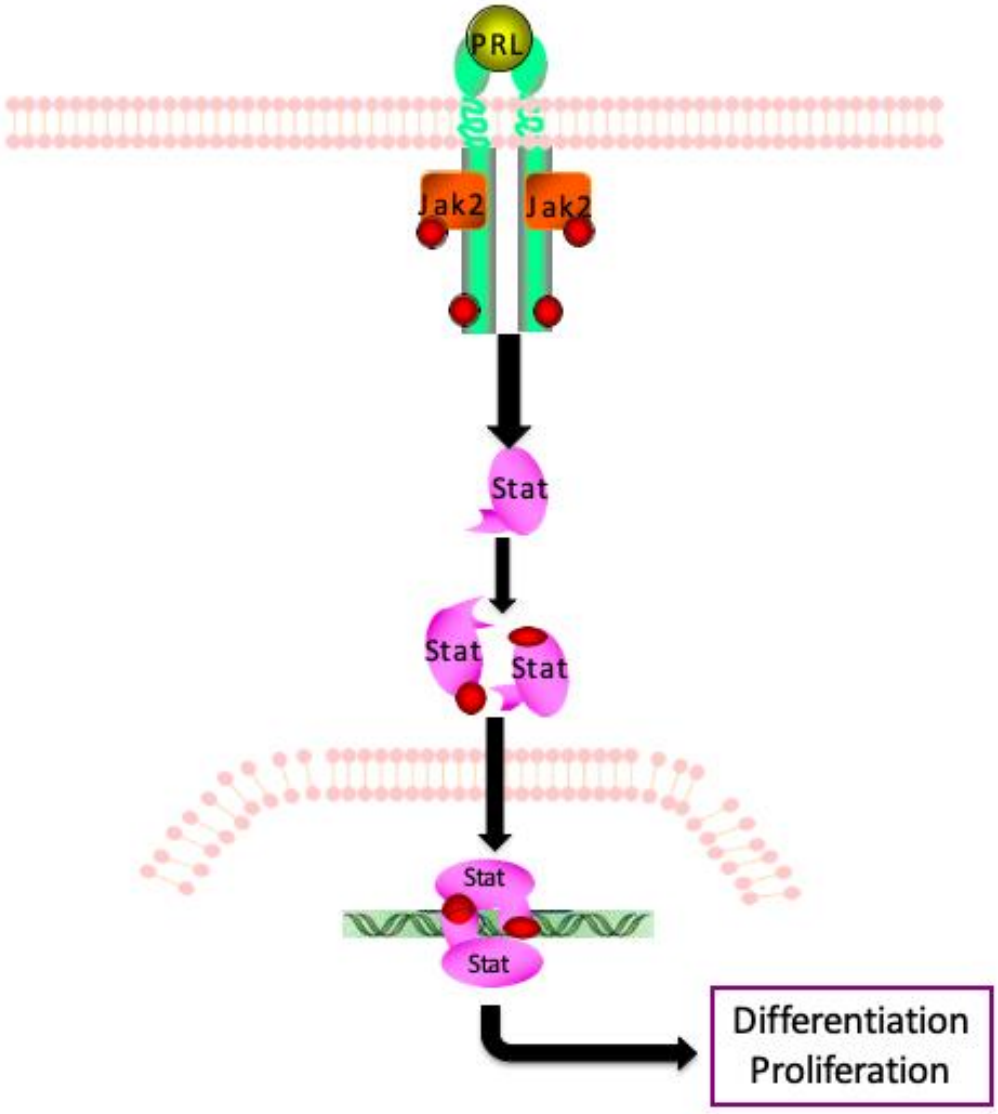


Figure 1: Prolactin Receptor Signaling Pathway. When PRL binds to its receptor, the receptor dimerizes and induces the tyrosine phosphorylation and activation of JAK2, which in turn phosphorylates STAT5a. Phosphorylated Stat5a homodimerizes with another STAT5 and translocate to the nucleus. In the nucleus, it activates the transcription of different target genes as the beta casein gene and other genes responsible for mammary cells differentiation. Modified from *L. Hennighausen et al, prolactin signaling in mammary gland development, Journal of biological chemistry, 1997, doi 10.1074/jbc.272.12.7567.*

4. Biological functions of Prolactin

Prolactin (PRL) hormone has different biological functions, most importantly its role in mammary gland development and lactation. It also has other biological roles as in metabolism, growth and development, reproduction and immune response (Goffin, Bouchard et al. 1998, Goffin, Binart et al. 2002, Marano and Ben-Jonathan 2014). Previous studies showed that PRL is important for regulating both humoral and cellular immunity, and that suppression of PRL secretion reduces the immune response (Nagy and Berczi 1978, Nagy, Berczi et al. 1983). In the ovaries, PRL can have both a luteotropic or luteolytic effect depending mainly on the stage of the estrous cycle (Freeman, Kanyicska et al. 2000). It was shown that PRL is important for keeping the integrity of the corpus luteum in mice six days after mating and inducing progesterone secretion (MORISHIGE and Rothchild 1974). It was also shown that prolactin induces programmed cell death for non-functioning and old corpora lutea by activation of Fas/Fas ligand (FasL) system through CD3-positive lymphocytes (Kuranaga, Kanuka et al. 2000). PRL also has a role in regulating maternal behaviour and knock out of PRLR was found to reduce maternal behaviour in mice in previous studies (Bachelot and Binart 2007).

The role of prolactin hormone in the mammary gland is intensively studied in the literature. PRL hormone is essential for mammary gland development, mammary epithelial cell differentiation, milk production and milk secretion. Studies in mice illustrated the role of PRL in mammary gland development. The mammary gland in new born mice is composed of small ducts, in which its growth becomes accelerated after puberty (Williams and Daniel 1983). Under the effect of female sex hormones; estrogen and progesterone, these small ducts grow faster to invade almost the whole fat pad, after which alveolar structures start to form in the peripheries (Oakes, Rogers et al. 2008). PRL hormone induces the secretion of progesterone from ovaries (Freeman, Smith et al. 1974, Terkel and Sawyer 1978) and accelerates the formation of alveoli and the terminal differentiation of mammary cells (Neville, McFadden et al. 2002) and its development into secretory phenotype for milk production and secretion (Oakes, Rogers et al. 2008). The knock-out of PRL and/or PRLR in homozygous females was shown that it leads to absence of the secretory alveolar units and losing the ability to produce milk (Horseman, Zhao et al. 1997).

PRL function in stem cell regulation was also illustrated in previous studies. It was shown that PRL and its signaling pathway are crucial for the commitment of luminal progenitor cells in breast tissue into mature secretory cells. In addition, PRL pathway is important for establishing the apical/basal polarity of mammary cells and apical fluid trafficking (Liu, Pawliwec et al. 2015).

5. Role of Prolactin in breast cancer

There is a controversy around the role of prolactin in breast tumorigenesis throughout the literature. Many studies suggested a pro-oncogenic role of prolactin and its receptor. Some studies reported that the autocrine paracrine loop of PRL hormone is increased in breast cancer tissues and correlated it with inducing cancer cell proliferation and tumor progression (Clevenger, Chang et al. 1995, Ginsburg and Vonderhaar 1995, Touraine, Martini et al. 1998). These studies were supported by other authors who showed that PRLR antagonists can inhibit cell growth (Fuh and Wells 1995). Another study in 2003 reported that transgenic mice overexpressing autocrine PRL have higher breast cancer incidence. However, they stated that these tumors have a long latency period of more than one year (Rose-Hellekant, Arendt et al. 2003).

Other studies looked at the expression levels of PRL and its receptor in breast cancer samples, in which some of them reported PRLR expression level in 20-60% of breast cancer samples using radioactive PRL (Holdaway and Friesen 1977, Rae-Venter, Nemoto et al. 1981, Turcot-Lemay and Kelly 1982), while other studies showed an even higher PRLR expression level of 95% in breast cancer samples on the mRNA level using PCR and in-situ hybridization (Ormandy, Hall et al. 1997, Touraine, Martini et al. 1998). Moreover, some studies were able to detect PRLR expression in breast cancer samples at the protein level (Mertani, Garcia-Caballero et al. 1998), but these studies were shown to be using non-specific antibodies for PRLR (Galsgaard, Rasmussen et al. 2009). More recent reports that used specific antibodies of PRLR showed very low levels of PRLR in breast cancer samples (Galsgaard, Rasmussen et al. 2009, Hachim, Hachim et al. 2016).

In addition, some studies suggested using dopamine agonists for treatment of breast cancer (Freeman, Kanyicska et al. 2000) as it showed reduction of circulating PRL levels (Gillam,

Molitch et al. 2006). However, clinical trials testing this hypothesis showed minimal efficacy of these medications in terms of their anti-tumor activity (Bonneterre, Mauriac et al. 1988, Anderson, Ferguson et al. 1993). Other therapeutic molecules were suggested in some reports as PRLR competitive antagonists (Goffin, Kinet et al. 1996) and anti-PRLR antibodies (Damiano, Rendahl et al. 2013, Otto, Särnefält et al. 2015). PRLR competitive antagonists showed a short half-life and low receptor affinity, so they failed to reach clinical trials (Goffin 2017). Anti-PRLR antibodies were tested clinically such as (LFA102) mAb on patients with PRLR-positive breast cancer, but showed minimal antitumor activity as well (Agarwal, Machiels et al. 2016).

On the other hand, other studies have shown PRL and its signaling pathway to be suppressors of breast cancer tumorigenesis (Sultan, Xie et al. 2005, Nouhi, Chughtai et al. 2006). In an invitro study in 2005, PRL and its signaling pathway were shown to be crucial for inducing the luminal phenotype of breast cancer cells by inducing the localization of E-cadherin, an epithelial marker, on cancer cell surface (Sultan, Xie et al. 2005). This showed that PRL plays an important role in inducing cell adhesion and decreasing tumor invasion (Sultan, Xie et al. 2005). Another study that was carried out in our laboratory in 2006 by Nouhi et al showed that the reactivation of PRL signaling pathway in MDA-MB 231, a basal-like breast cancer cell line, decreased its invasiveness, while the suppression of the pathway in T47D, an epithelial-like breast cancer cell line, increased its invasive behavior (Nouhi, Chughtai et al. 2006). In addition, Bratthauer, Strauss et al. associated the lower expression of STAT5 with more invasive behavior in breast carcinomas (Bratthauer, Strauss et al. 2006).

In 2008, a study reported that the hyperactivation of both jak2 and Stat5 that are downstream of PRLR, induced epithelial polarization and luminal properties in breast cancer cells (Sultan, Brim et al. 2008). This shows that PRL signaling pathway has an important role in reversing the epithelial mesenchymal transition (EMT) of breast cancer cells.

In 2014, Sato, Tran et al. proposed that PRL acts as a pro-differentiation factor in breast cancer cells by suppressing basal like Ck5 cells that have stem-like properties and are responsible for chemotherapy resistance (Sato, Tran et al. 2014). This effect of PRL is mediated through its activation of Stat5 that inhibits BCL6, a protein that is highly expressed in poorly differentiated breast cancer cells (Sato, Tran et al. 2014).

Despite what was mentioned before about the oncogenic effect of PRL autocrine/ paracrine loop, more recent studies in 2013 and 2015 showed that it actually has an antiproliferative role by using both cell lines and patient samples (Nitze, Galsgaard et al. 2013, Zhang, Cherifi et al. 2015).

Many studies were interested in examining the association between PRL serum levels and patient outcome. Some studies showed that high PRL serum levels were associated with tumor metastasis (Holtkamp, Nagel et al. 1984, Bhatavdekar, Shah et al. 1990, Mujagic and Mujagic 2004), treatment failure (Bhatavdekar, Patel et al. 1994, Barni, Lissoni et al. 1998), and poor patient survival (Wang, Stepniewska et al. 1995, Patel, Bhatavdekar et al. 1996, Bhatavdekar, Patel et al. 2000). On the contrary, other studies showed an association between high PRL serum levels and favorable patient outcome and prolonged survival as explained in the following. Many reports suggested that expression of PRL and its receptor in patient samples is associated with better tumor properties as being well differentiated tumors, smaller in size and lacking lymph node involvement (Faupel-Badger, Duggan et al. 2014, Hachim, Hachim et al. 2016, Hachim, Shams et al. 2016). They also showed that PRL is expressed higher in less aggressive breast cancer subtypes than in basal triple negative breast cancer samples, and that its expression is associated with prolonged patient survival (Hachim, Hachim et al. 2016). In addition, some reports suggested that low expression or loss of activation of STAT5 was associated with loss of tumour differentiation (Nevalainen, Xie et al. 2004, Bratthauer, Strauss et al. 2006), and more recent studies confirmed that STAT5A loss is associated with poor patient outcome (Peck, Witkiewicz et al. 2012).

Collectively, the previously mentioned studies confirm the antitumorigenic role of PRL hormone and its signaling pathway in breast cancer.

6. Breast Cancer Overview

One in every 8 women in Canada will suffer from breast cancer at some part of her life. These are the most recent statistics that were reported by the Public Health Agency of Canada and Statistics Canada regarding breast cancer incidence in 2018 (Canadian Cancer Statistics Advisory Committee 2018). It is the most common type of cancer in women and is the leading cause of

women cancer-related deaths worldwide (W.H.O 2015). According to an analysis done by the Public Health Agency of Canada, the five-year survival rate of breast cancer patients is 87% and the mortality rate increases with age (Canadian Cancer Statistics Advisory Committee 2018). Breast cancer is a heterogenous disease that comprises different histopathological and molecular profiles. There are different types of classifications for breast cancer. It can be classified according to histological breast cancer type, breast cancer grade, breast cancer stage and gene expression profiling. Histologically, breast cancer tumors are classified as carcinoma in situ (CIS) and invasive breast carcinoma (IBC) (Simpson, Reis-Filho et al. 2005) (Figure 2). Carcinomas in situ (CISs) are lesions that remain in the lobes and ducts of the mammary gland, while invasive breast carcinoma (IBC) are lesions that infilter out of the mammary gland into the connective tissue to spread in different body parts and cause metastasis (Cowell, Weigelt et al. 2013). CISs are divided into lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS), which is more common and has different types which are cribiform, comedo, micro papillary, papillary and solid invasive carcinomas (Li, Uribe et al. 2005, Malhotra, Zhao et al. 2010). IBCs are also divided into several types which are invasive lobular carcinoma (ILC), invasive ductal carcinoma (IDC), adenoid, apocrine, tubular, mucinous, metaplastic, medullary and papillary carcinomas. Invasive Ductal Carcinoma (IDC) is the most common type and is subdivided into well, moderately and poorly differentiated subtypes depending on the level of tubular formation, nuclear polymorphism and mitotic index (Li, Uribe et al. 2005, Malhotra, Zhao et al. 2010).

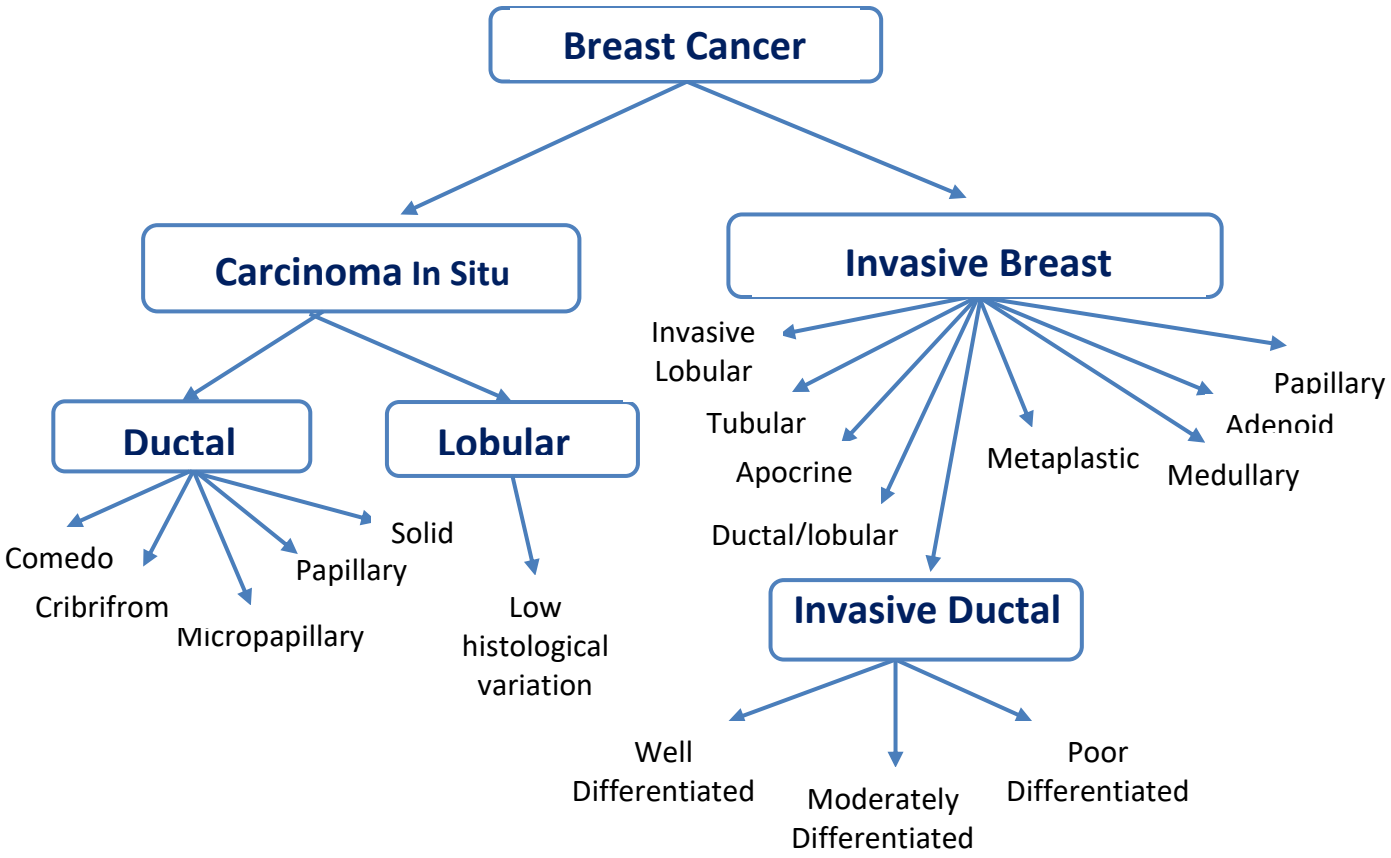


Figure 2: Histological subtypes of breast cancer. Breast cancer can be classified into carcinoma in situ and invasive breast carcinoma. Each type is subclassified into different subgroups according to the histopathological phenotype. Modified from *Gautam K. Malhotra, et al.,* (2010), Histological, molecular and functional subtypes of breast cancers, Cancer Biology & Therapy*10: 10, 955-960*.

Breast cancer can also be classified in a molecular pattern according to the gene expression profiling done by microarray analysis (Perou, Sørlie et al. 2000) (Sørlie, Perou et al. 2001). This method classifies the disease based on the expression of different intrinsic genes that determine the tumor phenotype and show its expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (Perou, Sørlie et al. 2000) (Sørlie, Perou et al. 2001). Each molecular subtype has different patient outcome and respond differently to therapy (Parker, Mullins et al. 2009) (Hu, Fan et al. 2006). These subtypes are Luminal A, Luminal B, HER2+, Normal-like and Basal-like, and are also known as breast cancer intrinsic subtypes (Dai, Li et al. 2015). The Luminal A group is

the most common type representing 50 % to 60 % of all breast tumors and is characterised by positive expression of both ER and PR. It is also characterised by low expression of proliferation genes as ki67 and is considered a low histological grade with good patient outcome. The luminal B group represents 10-20% of breast tumors and is characterised by being ER, PR positive and HER2 negative with high expression of proliferation genes (ki67) or ER, PR and HER2 positive. It is considered a higher histological grade and of worse patient outcome in comparison to the Luminal A subtype. The HER2+ subtype, which represents 15 to 20 % of breast cancer types, is characterised by the overexpression of HER2 and has a high proliferation rate and shows highly aggressive behaviour (Sørlie, Perou et al. 2001) (Sørlie, Tibshirani et al. 2003) (Parker, Mullins et al. 2009). The normal-like subtype has similar features to normal breast tissue and is characterised by having fibroadenoma genes (Peppercorn, Perou et al. 2007). The basal-like subtype, also known as Triple Negative, represents 10 to 20 % of breast cancer types and is characterised by the lack of ER, PR and HER2 expression and the expression of basal cytokeratin genes CK5/6 (Rakha, Reis-Filho et al. 2008), CK14, CK17, CD44 gene and epidermal growth factor receptor (EGFR) gene (Klingbeil, Natrajan et al. 2010). It has a high histological grade and is associated with poor patient outcome (Turner and Reis-Filho 2006). This type is also associated with BRCA1 gene mutations (Turner and Reis-Filho 2006), in which studies showed that BRCA1 dysfunction can be a driver of basal-like breast cancer types (Gorski, James et al. 2010). Although basal-like and Triple Negative tumors are considered the same in clinical practice, there is a 30% discordance between the two subtypes (Kreike, van Kouwenhove et al. 2007). Different immunohistochemistry markers are used to identify each type, where ER, PR, HER2, EGFR and CK5/6 are considered the gold standard for basal-like subtype identification, while ER, PR and HER2 are the standard identification molecules for the Triple Negative subtype (Nielsen, Hsu et al. 2004). The Claudin-Low subtype, representing 10 to 14 % of all breast tumors, is characterised by expression of tight junction and intracellular adhesion genes as Claudin 3,4, and 7, Ocludin and E-cadherin. It is also identified with the expression of epithelial-mesenchymal transition genes and a cancer stem cell phenotype. This breast

cancer subtype is known for having the worst patient outcome (Herschkowitz, Simin et al. 2007, Prat, Parker et al. 2010).

7. Triple Negative Breast Cancer

Triple Negative Breast Cancer (TNBC) represents 15% of all breast cancer cases and is characterized by the lack of expression of Estrogen Receptor (ER), Progesterone Receptor (PR) and Human Epidermal Growth Factor Receptor 2 (HER2) (Carey, Perou et al. 2006). This is why it is challenging to develop a targeted therapy for it and chemotherapy remains its main treatment strategy with many side effects and low therapeutic benefits (Foulkes, Smith et al. 2010). It is reported as the most aggressive breast cancer subtype with the highest recurrence rate and shortest overall patients' survival (Foulkes, Smith et al. 2010). Many risk factors play a role in developing the disease such as age, genetic susceptibility, race, breastfeeding and maternity. Population-based studies showed that women of African-American ethnicity develop TNBC at a younger age (Lund, Trivers et al. 2009). This was shown to be due to a mutational change in their BRCA-1 that leads to hereditary breast cancer development (Foulkes, Stefansson et al. 2003). Some studies also showed that the young age at first pregnancy can increase the risk of developing TNBC (Millikan, Newman et al. 2008). On the other hand, breastfeeding for a longer duration and the increased number of breastfed children are two factors that were shown to decrease the risk of developing TNBC (ElShmay 2016, Ma, Ursin et al. 2017). TNBC is a heterogenous disease with six different subtypes according to the gene expression profiling (Lehmann, Bauer et al. 2011). These subtypes are two basal-like groups (BL1 and BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM) and luminal androgen receptor (LAR) (Lehmann, Bauer et al. 2011) (Figure 3).

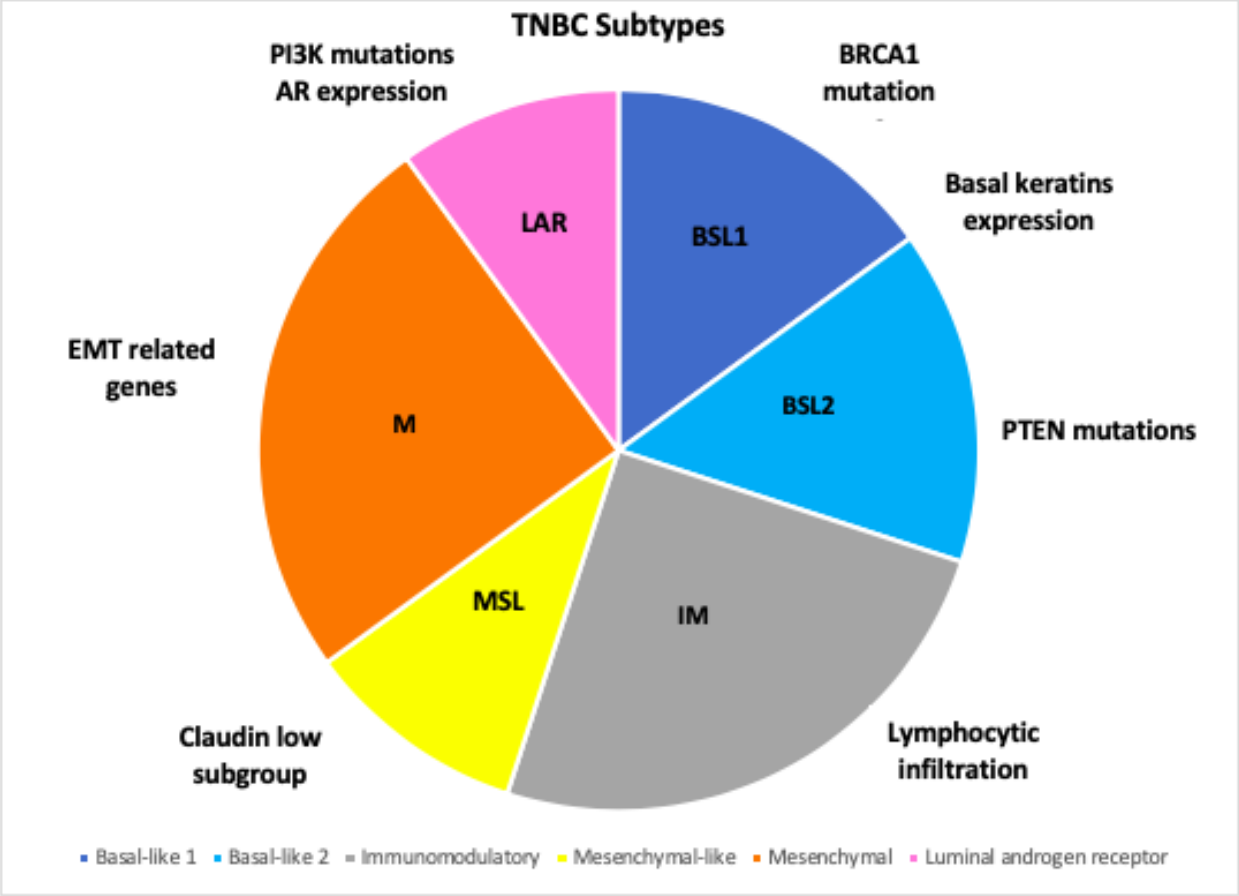


Figure 3: Subtypes of triple negative breast cancer according to Lehmann classification. There are six subtypes with different genetic profiles: BL1: basal-like 1, BL2: basal-like 2, IM: Immunomodulatory, ML: mesenchymal-like, MSL: mesenchymal stem-like and LAR: luminal androgen receptor. Modified from *Nicholas C. Turner, et al. (2013), Tackling the Diversity of Triple-Negative Breast Cancer, Clin Cancer Res; 19(23).*

The Basal Like1 subtype is characterised by increased cell proliferation, cell cycle components, genes related to DNA damage and BRCA1 mutations. The Basal Like2 is characterised by epidermal growth factor (EGF) signaling and PTEN mutations. The immunomodulatory subtype is associated with lymphocytic infiltration, immune cell signaling, antigen processing and presentation and signaling through immune signal transduction pathways (Lehmann, Bauer et al. 2011). The mesenchymal subtype is characterised by increased cell motility, cell differentiation and genes associated with epithelial-mesenchymal transition (EMT). The mesenchymal stem-like subgroup is associated with low levels of proliferation genes and high levels of stem cells. The Luminal Androgen

Receptor subtype is associated with the expression of luminal genes and is enriched with androgen receptor signaling and PI3K mutations and represents only 10% of total TNBC subgroups (Lehmann, Bauer et al. 2011). Many genetic drivers were found to be associated with TNBC. TP53 is the most common mutation found in 80% of TNBC tumors (Shah, Roth et al. 2012). PTEN mutations are found in 10% of TNBC tumors (Shah, Roth et al. 2012), PIK3CA mutations are found in 7-10% of TNBC tumors (Stemke-Hale, Gonzalez-Angulo et al. 2008), RB1 mutations are found in 20% of TNBC tumors (Network 2012), while BRCA1 mutations are the most predominant, found in 80% of TNBC tumors (Lehmann, Bauer et al. 2011, Turner and Reis-Filho 2013). The molecular classification of TNBC according to the genetic profiling helps in the discovery of new molecular targets and developing new therapeutic approaches to improve patients' outcome. Recently, another classification of TNBC has emerged according to the mRNA expression and DNA profiling, in which four subtypes were identified: luminal AR (LAR), Mesenchymal (MES), Basal-like immunosuppressed (BLIS), and Basal-like immune-activated (BLIA) (Burstein, Tsimelzon et al. 2015). The LAR subtype is associated with the expression of AR, prolactin and ErbB4 signaling. It is also associated with the expression of estrogen-related genes such as FOXA1, GATA3, PGR and XBP1. The MES subtype is associated with the overexpression of ADH1B, CD36, GHR, PPARG, ADRB2 and IL1R1. The BLIS subgroup is characterised by the expression of SOX family transcription factors and the down regulation of immune-regulated pathways. The BLIA subtype is associated with the upregulation of immune-related genes and the expression of STAT genes, CD2, CTLA4, CXCL9, CXCL10, CXCL11 and TOP2A (Burstein, Tsimelzon et al. 2015). This classification aids in the development of new prognostic markers and targeted therapies.

8. Stemness of Triple Negative Breast Cancer

TNBC is a poorly differentiated type of breast cancer, characterized by its enrichment with cancer stem cells (CSCs) that causes disease recurrence and metastasis (Anders and Carey 2009) (Kim, Joo et al. 2009) (Phillips, McBride et al. 2006). Cancer stem cells are tumor-initiating cells that have the ability to self-renew, differentiate and form a new tumor (Luo, Brooks et al. 2015). They are regulated by a group of transcription factors mainly SOX2, OCT4 and NANOG

(Ben-Porath, Thomson et al. 2008). OCT4 is responsible for the self-renewal of embryonic stem cells (Niwa, Miyazaki et al. 2000) and binds with SOX2 to form a heterodimer, in which together they regulate NANOG by binding to its promoter (Rodda, Chew et al. 2005). SOX2 is also responsible for the basal-like phenotype in some breast cancer subtypes (Rodriguez-Pinilla, Sarrio et al. 2007). NANOG is responsible for maintaining pluripotency in embryonic stem cells and has a role in cell cycle progression (Gawlik-Rzemieniewska and Bednarek 2016). Studies showed that the overexpression of OCT4 and NANOG increased the invasiveness of CSCs, while their knockdown inhibited CSCs migration in-vitro, concluding that both transcription factors are important for regulating the epithelial-mesenchymal transition (EMT) of breast cancer cells (Wang, Lu et al. 2014). Cancer stem cells (CSCs) are also recognized by the expression of other markers such as CD44, CD24 and ALDH1 (Al-Hajj, Wicha et al. 2003, Ginestier, Hur et al. 2007) . CD44 is a transmembrane receptor for hyaluronic acid and acts as a cofactor for many cytokines and growth factors. It is important for cell adhesion and is associated with tumor dissemination (Paulis, Huijbers et al. 2015). CD24 is a surface glycoprotein that is important for signal transduction. Its presence indicates a more differentiated epithelial phenotype of cells (Kim, Kim et al. 2011). Both CD44 and CD24 are used as prognostic markers to indicate the CD44+/CD24- highly aggressive subpopulation, that is characterised by poor patient outcome and resistance to therapy (Honeth, Bendahl et al. 2008) (Buess, Rajski et al. 2009). It was also shown that this subpopulation has a higher proliferation rate (Ma, Li et al. 2014) and higher risk of recurrence (Wang, Wang et al. 2017). The knock down of CD44 in combination with chemotherapy was reported to decrease cell proliferation and increase cell apoptosis (Van Phuc, Nhan et al. 2011). In addition, its knock out in-vivo was shown to terminally differentiate CSCs and decrease the expression of stem cell-related genes (Pham, Phan et al. 2011). This paves the way for new therapeutic approaches for TNBC such as gene therapy and differentiation therapy (Pham, Phan et al. 2011). Finally, the last marker for CSCs is Aldehyde dehydrogenase (ALDH), which is an enzyme responsible for the detoxification of both exogenous and endogenous aldehydes (Tomita, Tanaka et al. 2016). ALDH1 specifically is the marker for CSCs and its presence indicates bad prognosis. It has three different isotypes ALDH1A1, ALDH1A2 and ALDH1A3 (Tomita, Tanaka et al. 2016). ALDH1A1 is associated with

higher grades of tumor, metastasis and poor patient outcome (Ginestier, Hur et al. 2007). ALDHA1A3 is associated with higher grade of the disease and metastasis (Marcato, Dean et al. 2011). The combination of CSCs markers expression (CD44+, CD24-) with ALDH1 high activity increase the metastasis of breast cancer stem cells and enhance tumor growth (Croker, Goodale et al. 2009) (Ginestier, Hur et al. 2007).

9. Therapeutic approaches for Triple Negative Breast Cancer

As previously mentioned, TNBC lacks the expression of Estrogen Receptor (ER), Progesterone Receptor (PR) and Human Epidermal Growth Factor Receptor 2 (HER2) (Carey, Perou et al. 2006). Therefore, there is no targeted therapy for it and chemotherapy remains the main treatment used nowadays for TNBC patients with low therapeutic benefits and numerous side effects. There is high rate of disease recurrence after chemotherapy due to the heterogenic nature of TNBC and its diverse response to different treatments (Foulkes, Smith et al. 2010). We will discuss some of the current therapeutic approaches.

One category of treatments is the platinum agents, in which Carboplatin and Cisplatin are the most commonly used. These are platinum salts that bind directly to DNA to form DNA-platinum adducts that inhibit cell division and cause cell apoptosis (Tian, Zhong et al. 2015). Other treatments that are non-platinum based are Doxorubicin, anthracycline and taxanes. Anthracycline is used mainly in the metastatic setting of the disease as it has an increased toxicity risk. However, taxane-based treatments are effective in all settings of the disease and are recommended as a first-line treatment option for TNBC patients (Liedtke, Mazouni et al. 2008). Some targeted therapies have developed recently with the hope of increasing the efficacy of treatment. Some examples of these targeted therapies are Poly (ADP-Ribose) polymerase inhibitors (PARP), EGFR inhibitors, AR/ PI3k inhibitors, Src inhibitors and cancer stem cell inhibitors. Poly ADP-Ribose polymerase (PARP) is an enzyme that repairs the DNA by removing the damaged DNA sequences using excision repair mechanisms. In TNBC patients with BRCA1/2 mutations, inhibition of this enzyme leads to the accumulation of single strand breaks that leads to cell death in a process known as “synthetic lethality”. Examples of PARP inhibitors are olaparib, niraparib, veliparib, rucaparib and talazoparib (Tutt, Robson et al. 2010,

Dwadasi, Tong et al. 2014). Anti-EGFR monoclonal antibodies are still under investigation, but have already shown an improvement in the patients' response and progression-free survival in metastatic TNBC patients (Carey, Rugo et al. 2012, Trédan, Campone et al. 2015). AR inhibitors such as Enzalutamide and Bicalutamide are mainly used for LAR subtype patients. This subtype is associated with PI3K mutations, so PI3K inhibitors are used in combination with AR inhibitors to stabilize the disease (Gonzalez-Angulo, Stemke-Hale et al. 2009) (Bendell, Rodon et al. 2012). Src inhibitors such as Desatinib are used in combination with chemotherapy in metastatic TNBC tumors and have shown a synergistic effect on reducing the tumor growth (Tryfonopoulos, O'Donovan et al. 2009). Cancer stem cell inhibitors are still under investigation with new therapeutic options emerging with time. The combination of Vitamin D3 with androgen receptor agonist was found to inhibit CSC phenotype, induce CSCs differentiation and increasing the epithelial phenotype by decreasing ALDH+ cells, up regulation claudins and down regulating vimentin (Thakkar, Wang et al. 2016). The CDK4 inhibitor Palbociclib was recently discovered and was shown to prevent CSCs from self-renewal and proliferation (Dai, Zhang et al. 2016).

10. New Modalities in TNBC Treatment

The understanding of the molecular mechanisms of TNBC has led to the development of new therapeutic modalities that are targeting the main carcinogenic pathways in TNBC. These modalities are immunity modulation, epigenetics modulation, inhibition of histone deacetylase, induction of cellular differentiation and senescence. Immune-checkpoint inhibitors such as anti-CTL4 antibodies and PDL1/PD1 inhibitors have been recently under clinical trials investigations as single agents or in combination with chemotherapy, in which the trials reported an overall response rate of 19% and tolerable safety (Mittendorf, Philips et al. 2014) (Nanda, Chow et al. 2016). Modulating epigenetics in TNBC has also been studied recently and some studies focused on the DAPK and ID4 genes as TNBC prognostic markers (Gheibi, Kazemi et al. 2012). DAPK is hyper methylated in TNBC and its hypermethylation is associated with the size and tumor grade of TNBC (Hafez, Al-Shabanah et al. 2015). Therefore, studies showed that DAPK inhibition suppresses tumor growth (Zhao, Zhao et al. 2015). ID4 are basic transcription factors

that act as tumor suppressors and are hyper methylated in TNBC and are also associated with the tumor size in TNBC, so can be used as prognostic biomarkers for estimating the aggressiveness of the disease (Hafez, Al-Shabanah et al. 2015). Agents acting on post-translational modifications (PTMs) such as acetylation have been studied recently to induce cell apoptosis and cell cycle arrest (Damaskos, Garmpis et al. 2017). Studies showed an anti-tumor activity of histone deacetylase agents such as Vorinostat which is one of the most advanced in its category. It acts by inducing changes in acetylating motif and producing downstream effects on the apoptotic pathways (Luu, Morgan et al. 2008). Although clinical trials showed its high toxic effect with a modest clinical benefit (Luu, Morgan et al. 2008), preclinical studies showed that it can be used to reactivate estrogen receptors and inhibit metastasis in TNBC (Palmieri, Lockman et al. 2009, Stark, Burger et al. 2013). Panobinostat is another histone deacetylase inhibitor that was shown to reduce tumor growth by inhibiting the cell cycle and inducing apoptosis and EMT reversal in TNBC preclinical studies (Rao, Balusu et al. 2012, Tate, Rhodes et al. 2012, Rhodes, Tate et al. 2014). Another novel treatment strategy that has been under investigation is the induction of cellular differentiation in the aim of restoring the mature phenotype that resembles the normal cells. This helps in stopping tumor growth and allows chemotherapy to kill malignant cells (Cruz and Matushansky 2012). Recent studies showed that the cyclin-dependant kinase 4 (CDK4) acts as a stem cell regulator and a novel TNBC prognostic marker. Therefore, CDk4 inhibition using palbociclib was shown to inhibit CSC renewal and reverse the mesenchymal-like phenotype in TNBC to a luminal, epithelial-like phenotype with a better patient outcome (Samarut and Rochette-Egly 2012). Other studies investigated the induction of cellular senescence as a novel approach for TNBC treatment. Cellular senescence can be induced by different stimuli such as DNA damage, telomere shortening and increase of mitogens or oncogenic proteins (Munoz-Espin and Serrano 2014). Senescent cells are quiescent, non-dividing and terminally differentiated cells, but are metabolically active and resistant to apoptosis (Takahashi, Ohtani et al. 2007). However, they can develop into an immunogenic phenotype known as senescent associated secretory phenotype (SASP) that forms an inflammatory microenvironment causing senescent-cells eradication (Krtolica, Parrinello et al. 2001, Parrinello, Coppe et al. 2005, Coppé, Kauser et al. 2006, Laberge, Awad et

al. 2012, Lee and Lee 2014). Although cellular senescence is suggested to be a potent tumor suppressing mechanism, it may induce an immune response that causes chronic inflammation and stimulates tumor growth and angiogenesis. Therefore, further investigations are needed to explore the consequences of cellular senescence, the mechanisms causing the SASP and how it can affect cancer progression.

11. FOXA1 and breast cancer

FOXA1 is a member of the forkhead class of DNA-binding proteins that functions as a transcriptional activator for liver-specific transcripts such as albumin and transthyretin and acts as a pioneer factor that interacts with chromatin, in which it opens the compacted chromatin of other proteins by interacting with nucleosomal core histones, so replacing linker histones at target enhancer or promotor sites (Bingle and Gowan 1996) (Figure 4). In breast cancer, FOXA1 acts as a pioneer factor for Estrogen receptor (ER) and its expression in ER+ breast cancer may indicate poor patient outcomes (Ross-Innes, Stark et al. 2012). On the contrary, in ER- breast cancer, FOXA1 is considered a favorable biomarker associated with low grade of the disease and improved disease-free survival (Albergaria, Paredes et al. 2009). In addition, FOXA1 is considered a distinct biomarker for non-metastatic TNBC as its expression correlates with two EMT markers, E-cadherin and Twist1 (BenAyed-Guerfali, Dabbèche-Bouricha et al. 2019). Therefore, FOXA1 is considered an antagonist of the epithelial-to-mesenchymal transition (EMT) by its positive regulation of E-cadherin and maintenance of the epithelial phenotype of cancer cells (Song, Washington et al. 2010). It is involved in the regulation of apoptosis by inhibiting the expression of BCL2 (Song, Wei et al. 2009). It is also involved in the regulation of the cell cycle by activating the expression of CDKN1B, alone or with BRCA1 (Williamson, Wolf et al. 2006). According to Guiu and colleagues, co-expression of AR and FOXA1 is recorded in TNBC cases that behave like luminal tumors with a morphology distinct than other TNBC subtypes (Guiu, Charon-Barra et al. 2015). Jiang and colleagues reported that “among the 102 TNBC cases from the TCGA cohort with available exome sequencing data, 19 had at least one functional somatic mutation in the AR/FOXA1 pathway and 35 had at least one functional

somatic mutation in the AR/FOXA1 pathway or at least one germline BRCA1 mutation" (Jiang, Shi et al. 2016). Excellent overall survival was reported in patients with at least one mutation in one of the genes in this pathway. (Jiang, Shi et al. 2016).

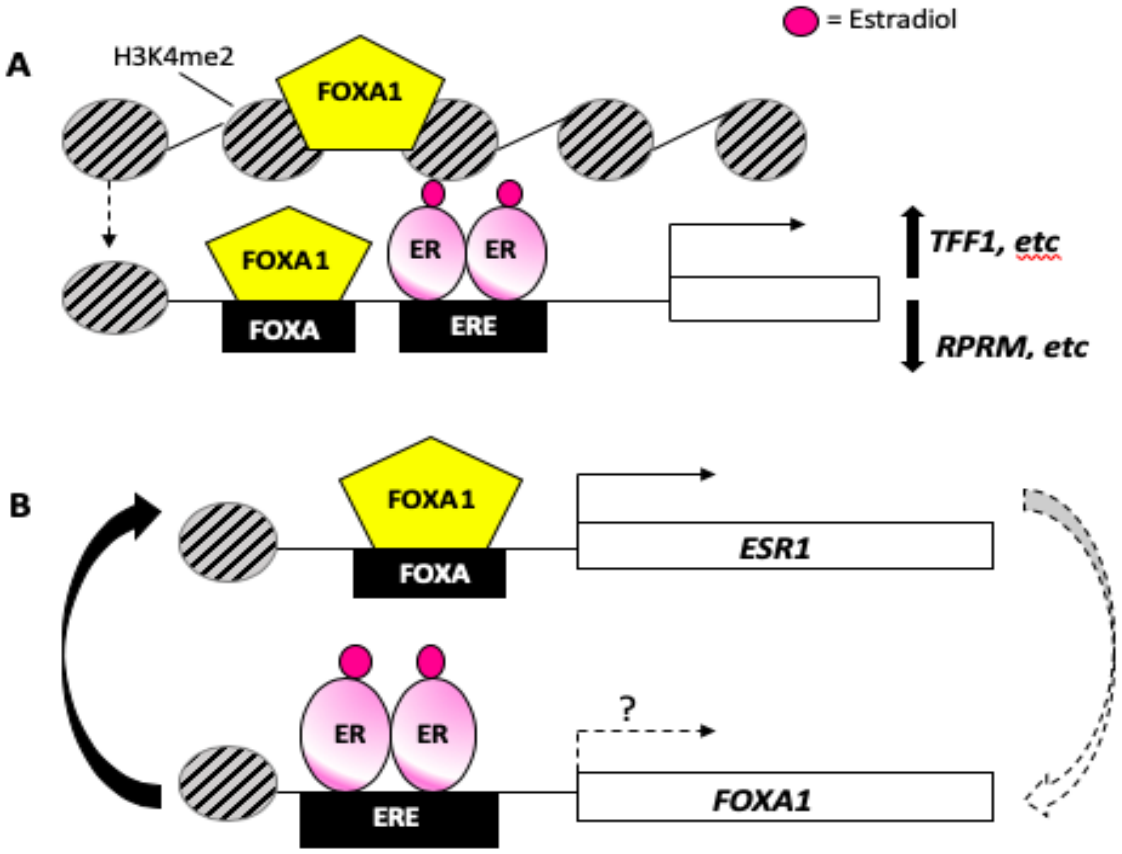


Figure 4: FOXA1 acts as a pioneer transcription factor that modulates the chromatin structure. FOXA1 binding to the DNA is associated with open chromatin state and subsequent binding and activation of ER gene, so it is responsible for the estrogen mediated cell cycle progression in Breast cancer. Adapted from *Sizemore, Gina & A Keri, Ruth*. (2012), Bioscience reports. 32. 113-30. 10.1042/BSR20110046.

12.Rationale, Hypothesis and Objectives:

At the physiological level, prolactin is essential for lobuloalveolar formation of the mammary ducts during pregnancy, terminal differentiation of the mammary epithelial cells, and for synthesis of milk components during lactation (Ormandy, Naylor et al. 2003). Knock-out mice of PRL, its receptor, and components of the Janus-activated kinase-2 (JAK2)/signal transducer and activator of transcription-5a (STAT5a), the main signaling cascade downstream of the PRLR, confirmed the critical role of PRL in mammary gland alveolar differentiation as these mice showed limited alveolar growth and were unable to lactate (Ormandy, Naylor et al. 2003) (Liu, Robinson et al. 1997). In addition, the laboratory of Dr. Ali experimentally proved that the reactivation of PRL/PRLR signaling pathway in mesenchymal invasive cancer cells suppresses epithelial-mesenchymal transition (EMT) and decreases the invasive properties of breast cancer cells (Nouhi, Chughtai et al. 2006). One other aspect of prolactin antitumorigenic effect is its induction of terminal differentiation of cancer stem cells populations in breast cancer (López-Ozuna, Hachim et al. 2019). Breast cancer stem cells are causative for high metastasis and recurrence in TNBC (Anders and Carey 2009) (Kim, Joo et al. 2009) (Phillips, McBride et al. 2006). PRL/PRLR signaling pathway converts the tumorigenic CD44+/CD24- cancer stem cells into the non-tumorigenic CD44-/CD24- phenotype (López-Ozuna, Hachim et al. 2019).

Using KM plotter database of 580 TNBC patients, Lopez et al. in our lab showed that patients with higher expression of PRL signaling pathway consisting of PRL, PRLR, JAK2 and STAT5a gene signature, have prolonged relapse free survival (RFS) and are associated with better prognosis and favorable outcome (López-Ozuna, Hachim et al. 2016) (Figure 5).

By robust molecular subtype predictor classification (RMSPC) in bc-GenExMiner 3.0, PRLR gene expression showed significant positive correlation with luminal-like genes associated with LAR signaling (FOXA1 and AR) and with epithelial differentiation genes (Claudin-CD24), with ELF3, EPHB3 and BIRC5 genes (López-Ozuna, Hachim et al. 2016) (Figure 6).

The role of these metagenes in TNBC tumorigenesis and how they may interact with prolactin antitumorigenic effect is yet to be discovered. We decided to focus on studying FOXA1, as it has a high correlation score with Prolactin, and to investigate its interaction with PRL antitumorigenic role in breast cancer.

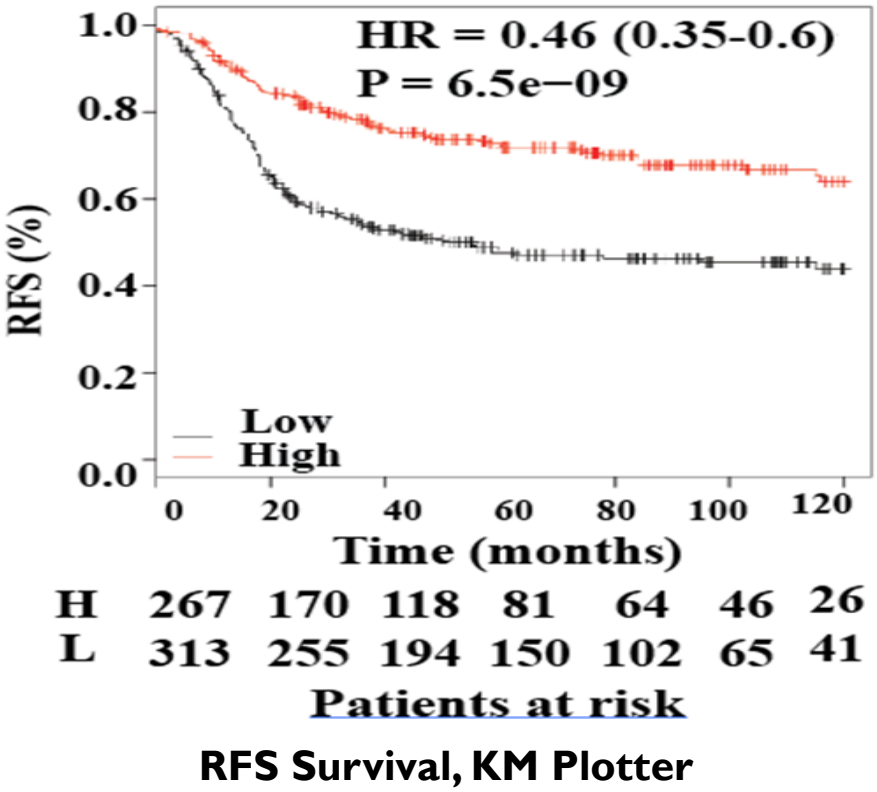
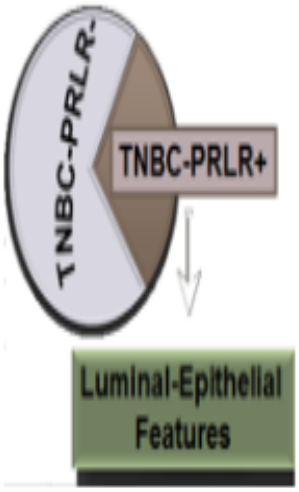


Figure 5: Kaplan Meier Survival Analysis of 580 TNBC patients. Patients with higher expression of PRL signaling pathway consisting of PRL, PRLR, JAK2 and STAT5a gene signatures, have prolonged relapse free survival (RFS) and are associated with better prognosis and favorable outcome. From *López-Ozuna, Hachim et al,* Scientific Reports, 2016.



Biological Component	Key Markers	PRLR Expression					
Apocrine/ Androgen Receptor Signaling	AR, FOXA1	Correlation	Parameters	FOXA1	AR		
		PRLR	r	0.2	0.22		
		PRLR	p	<0.0001	<0.0001		
Claudin-CD24	CLDN3, CLDN4, CD24' ELF3	Correlation	Parameters	CLDN3	CLDN4	CD24	ELF3
		PRLR	r	0.14	0.11	0.12	0.09
		PRLR	p	0.0008	0.106	0.0033	0.0378

Figure 6: PRLR-associated Metagenes. By Robust Molecular Subtype Predictor Classification (RMSPC) in bc-GenExMiner 3.0, PRLR gene expression shows significant positive correlation with luminal-like genes associated with LAR signaling (FOXA1 and AR), with epithelial differentiation genes (Claudin-CD24), with ELF3, EPHB3 and BIRC5 genes. Adapted from *López-Ozuna, Hachim et al,* Scientific Reports, 2016.

Hypothesis: FOXA1 modulates Prolactin anti-tumorigenic effect, and Prolactin-induced mesenchymal to epithelial/ luminal transition of triple negative breast cancer.

Objectives:

1. To analyze how PRL and its signaling pathway change the patient survival outcome in comparison to its associated metagenes e.g. FOXA1.
2. To determine the effect of PRLR stimulation on the expression of FOXA1 in TNBC.
3. To investigate the effect of PRLR knockout on the expression of FOXA1 in MCF-7, an ER+ breast cancer subtype.

4. To explore the role of FOXA1 in the epithelial mesenchymal transition of cells in TNBC-LAR subtype.

Chapter II- Materials and Methods:

All experimental protocols were done in accordance with McGill University Health Centre, McGill University guidelines and regulations.

1. **Cell culture:** Human triple-negative breast cancer cell line (MDA-MB-453) was purchased and maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 µg/ml streptomycin. All cell lines were grown at 37 °C in 5% CO2. The cells were collected using 0.025% trypsin EDTA following the specified incubation period. The reagents used in this are DMEM (Multicell, Cat. Number 095-150) and fetal bovine serum (FBS) (ATCC Cat. Number 30-2008).
2. **PRLR stimulation and PRL treatment:** Recombinant human prolactin (rhPRL) (150 ng/mL and 250 ng/mL) was used to treat MDA-MB-453, which has epithelial phenotype, for 12 hrs & 24 hrs. Recombinant Human Prolactin (rhPRL) was purchased from Abcam (ab51703).
3. **RNA extraction and qRT-PCR:** Total RNA from MDA-MB-453 treated with rhPRL for 12 & 24 hrs was isolated, reverse transcribed and used for qRT-PCR amplification. Total RNA was extracted from cells using Trizol (Invitrogen). Samples were quantified by absorbance at 260 nm. The cDNA from the extracted total RNA was obtained using iScript (Biorad Cat. Number 170-8840). RT-qPCR was performed using Rotorgene (Corbett) and SsoAdvanced Universal SYBR Green Supermix (Biorad Cat. Number 172-5270) according to the manufacturer's recommendation.

4. **Gene Targets Cloning:** Generation of CRISPR/CAS9 single guide RNA for FOXA1 as in the following table (Table 1) by Escherchia Coli bacterial cloning according to the addgene CRISPR cloning protocol (http://www.addgene.org/crispr/guide/).

Gene Name	Small-Guide RNA number	Gene sequence of the sgRNA targeting the corresponding gene	Chromosome position of sgRNA	Cut sites of the sgRNA	Exon number
FOXA1	sg1	GTTGGACGGCGCGTACGCCA	Chr14:37592343-37592365	37592359	Exon 2/2
FOXA1	sg2	GTAGCTGCGCTTGAACGTCT	Chr14:37592286-37592308	37592302	Exon 2/2
FOXA1	sg3	CAGCTACTACGCAGACACGC	Chr14:37594903-37594925	37594909	Exon 1/2

Table 1: Cut sites of sgRNAs used to induce indel mutations and generate CRISPR – mediated knockouts of FOXA1 in MDA-MB-453 Breast cancer cells.

5. **Database Analysis:** I used Kaplan-Meier plotter database to evaluate the expression of FOXA1 and PRL signaling pathway components as a single gene signature, using the following probeset ID (Affimetrix): PRL (205445_at), PRLR (206346_at), Jak2 (205841_at), Stat5a (203010_at) and FOXA1 (204667_at) in relation to patient outcome. This database provides information about 4,142 breast cancer patients containing 339 TNBC patients. Patients were divided by median into high and low expressers. Relapse free survival (RFS) was used as an end point to predict patient outcome. The number of patients at risk is indicated in the corresponding figures.
6. **Immunohistochemistry:** Slides were incubated with a rabbit polyclonal antibody to PRLR-L (H300) (Santa Cruz Cat. Number #sc-20992) and with a rabbit polyclonal antibody to FOXA1 (Abcam Cat. Number #ab23738) using positive and negative controls for both as

previously described (Hachim, Shams et al. 2016). Slides were baked for 30 minutes at 55C, followed by deparafinization and rehydration. Antigen retrieval was performed in sodium citrate 10mM, pH 6.0 buffers. Slides were incubated with a rabbit polyclonal antibody against PRLR-L (H300) (Santa Cruz Cat. Number #sc-20992), or a rabbit polyclonal antibody to FOXA1. UltraVision LP Detection System HRP Polymer & DAP Plus Chromogen (Thermo Fisher Scientific, Fremont CA) was used for detection. The slides were scanned using Aperio XT slide scanner (Leica Biosystems).

7. **Lentivirus Production:** HIV-based viruses were produced in HEK293T cells using polyethylenimine (PEI) according to the addgene CRISPR cloning protocol (http://www.addgene.org/crispr/guide/ and collected after 48 hrs.
8. **MDA-MB-453 Cell Transfection:** The cells were infected with the produced lentivirus by 1 vector CRISPR-mediated system according to the addgene CRISPR cloning protocol (http://www.addgene.org/crispr/guide/ and cells were selected in growth media containing 2µg/ml of Puromycin (InvivoGen #ant-pr-1, ant-pr-5).
9. **Cell lysis and Western Blotting:** For whole cell lysates and western blotting, cells were lysed in a RIPA lysis buffer as described previously (Ali and Ali 1998). I then ran the proteins on SDS-PAGE and transferred them to nitrocellulose membrane for western blotting analysis using a rabbit polyclonal antibody to FOXA1 (Abcam Cat. Number #ab23738), a mouse monoclonal antibody to GAPDH (Santa Cruz Cat. Number #sc-365062), a mouse monoclonal antibody to vimentin (Santa Cruz Cat. Number #sc-373717) and a mouse monoclonal antibody to E-cadherin (BD Sciences Cat. Number #610182).

Chapter III-Results

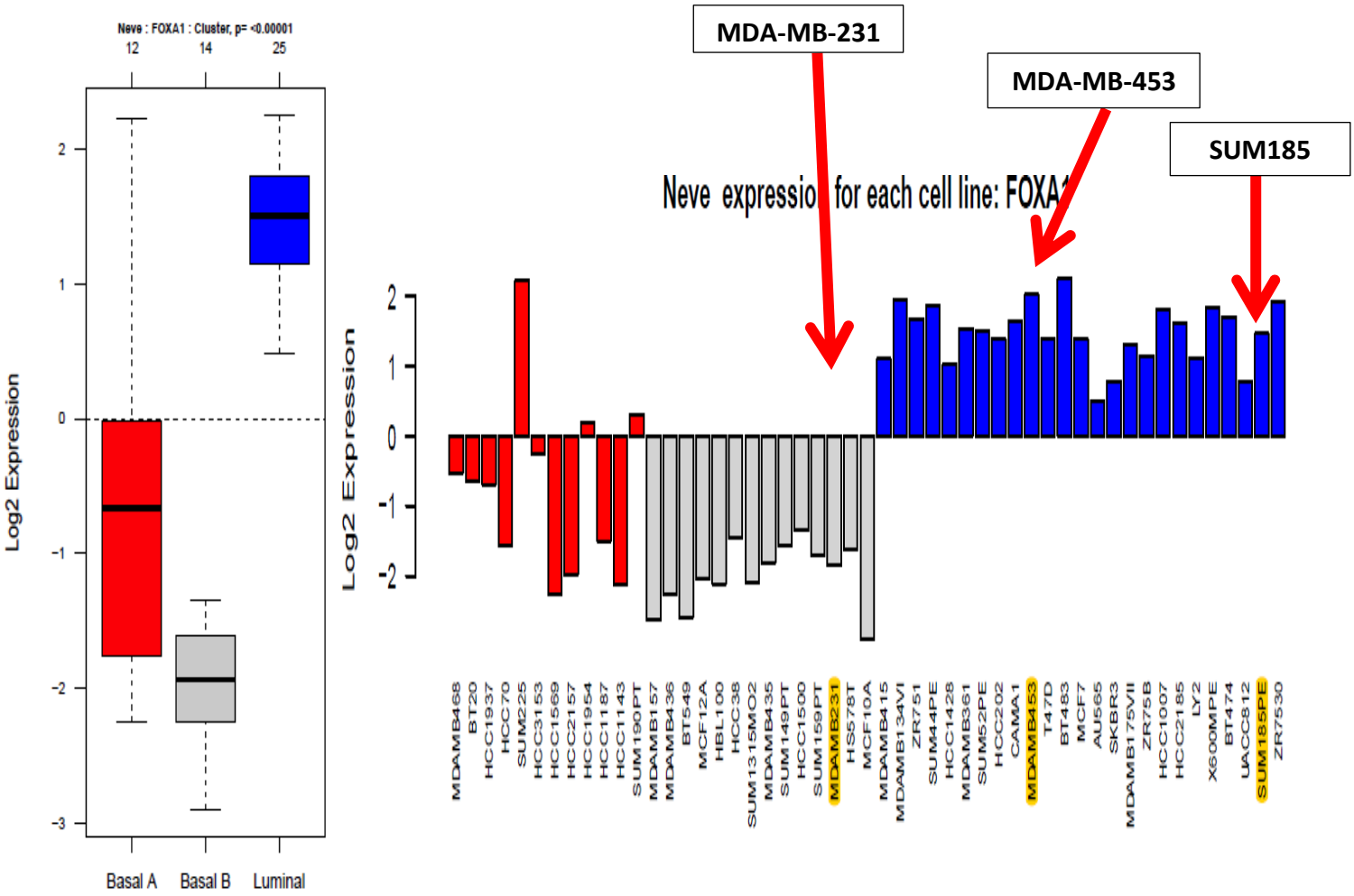
1. FOXA1 gene expression analysis in GOBO in breast cancer cell lines and patient samples

I first started with a screening of the expression of FOXA1 and other PRL-associated metagenes in the GOBO database in different breast cancer cell lines and patients' tumor samples. GOBO database is the Gene Expression-Based Outcome for Breast Cancer Online database. It is a publicly available database that can be used to investigate the prognostic value of one gene or a group of genes in a dataset of 1881 breast cancer patients (Ringnér, Fredlund et al. 2011). It can also be used to investigate the levels of gene expression in 51 breast cancer cell lines that represent the different subtypes of breast cancer (Neve, Chin et al. 2006), and make an assessment between the gene expression levels and a set of clinicopathological parameters such as molecular subtypes, grade of the disease and the ER state of the tumor (Ringnér, Fredlund et al. 2011). In addition, it allows users to associate between the levels of gene expression levels and patient outcome using both relapse free survival (RFS) and disease metastasis free survival (DMFS) as endpoints (Ringnér, Fredlund et al. 2011).

I found out that FOXA1 is higher expressed in both MDA-MB-453 and SUM185 cell lines, which represent luminal epithelial TNBC subtype, compared to MDA-MB231 that represents the basal mesenchymal TNBC cell line (Figure 7A). Furthermore, FOXA1 is higher expressed in luminal patient tumors and tumors of low grade of the disease compared to mesenchymal basal tumors and tumors of high grade of the disease (Figure 7B).

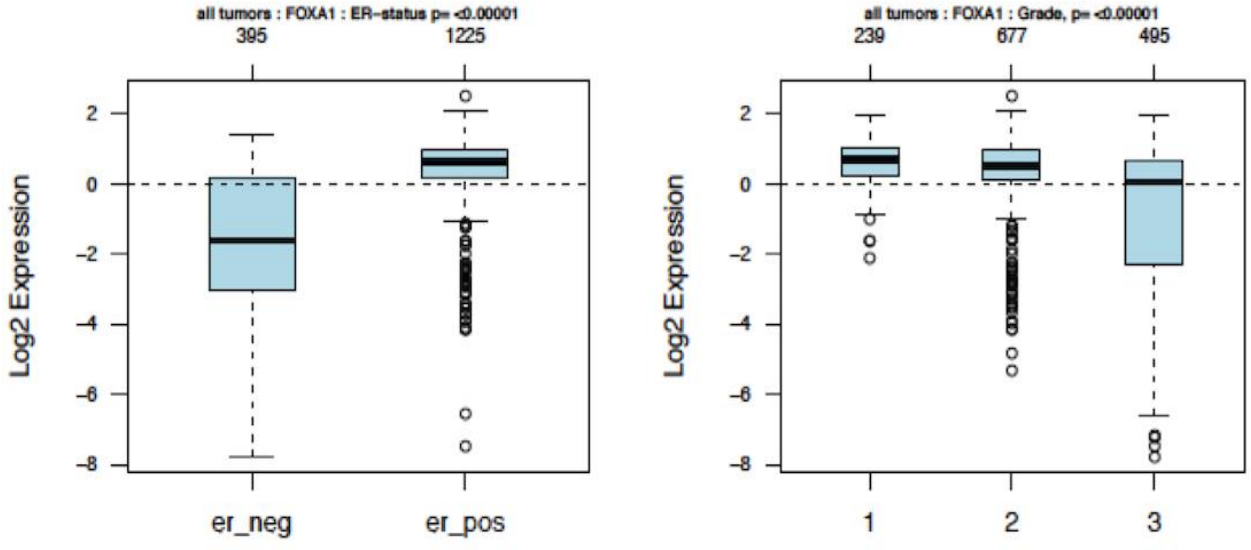
A

FOXA1


Neve : FOXA1 : Cluster, p= <0.00001
12
14
25
Log2 Expression
Basal A
Basal B
Luminal
MDA-MB-231
MDA-MB-453
SUM185
Neve expressio for each cell line: FOXA1
Log2 Expression
MDAMB468
BT20
HCC1937
HCC70
SUM225
HCC3153
HCC1569
HCC2157
HCC1954
HCC1187
HCC1143
SUM190PT
MDAMB157
MDAMB436
BT549
MCF12A
HBL100
HCC38
SUM1315MO2
MDAMB435
SUM149PT
HCC1500
SUM159PT
MDAMB231
HS578T
MCF10A
MDAMB415
MDAMB134VI
ZR751
SUM44PE
HCC1428
MDAMB361
SUM52PE
HCC202
CAMA1
MDAMB453
T47D
BT483
MCF7
AU565
SKBR3
MDAMB175VII
ZR75B
HCC1007
HCC2185
LY2
X600MPE
BT474
UACC812
SUM185PE
ZR7530

B

Histologic Grades



Molecular Subtypes

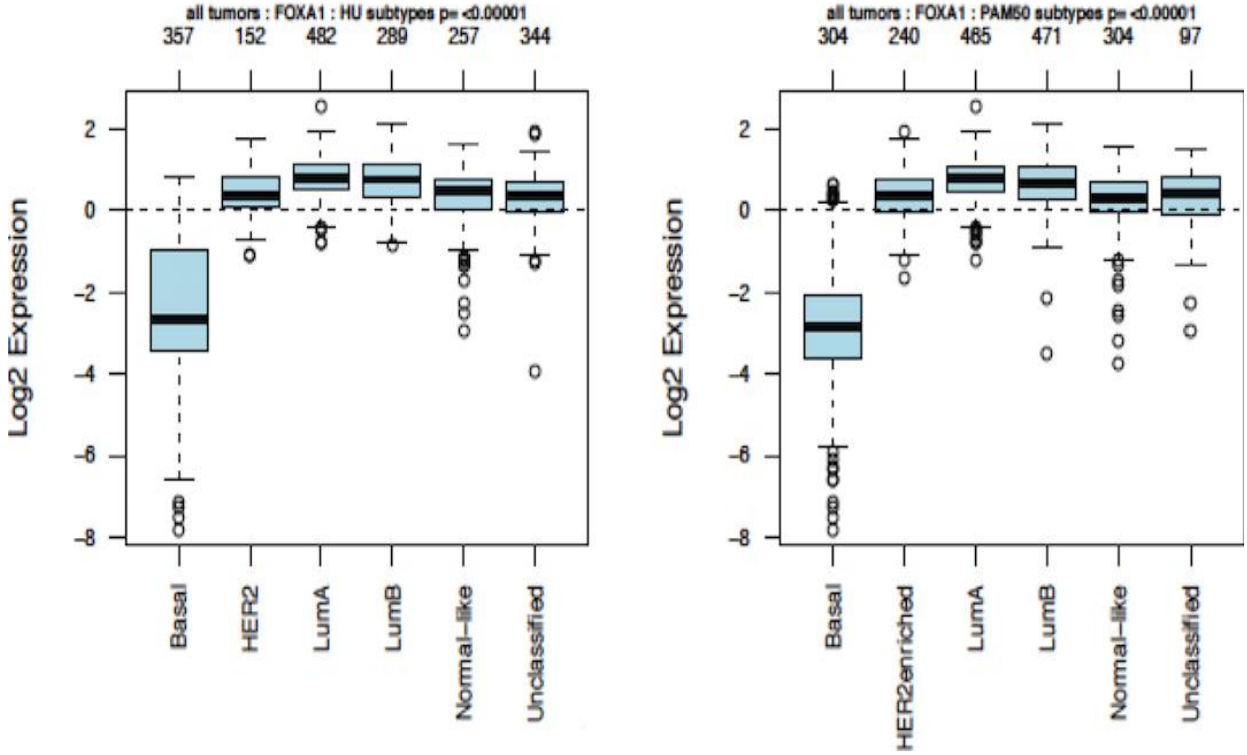
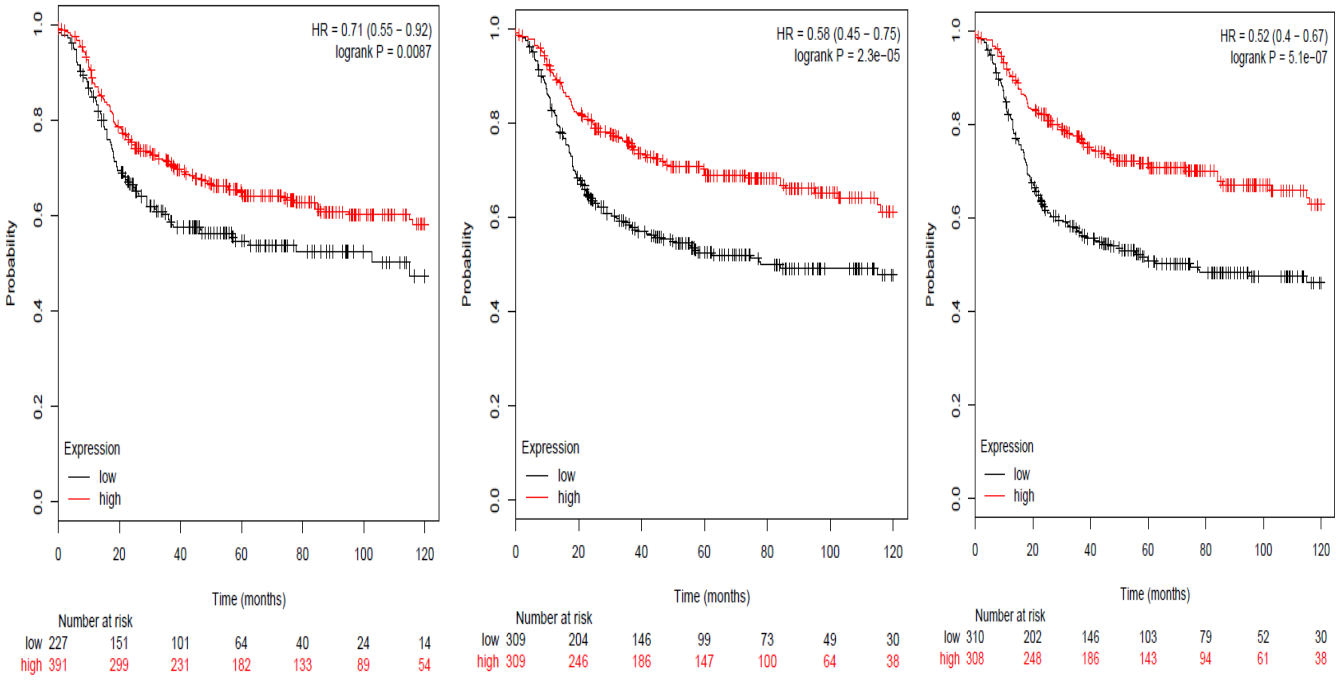


Figure 7: Level of FOXA1 Gene expression using GOBO database: **A)** In breast cancer cell lines of different subtypes. **B)** In 1881 patient's tumor samples according to the molecular subtypes and the histologic grades of the tumors.

2. Kaplan Meier Survival Analysis of FOXA1 vs PRLR signaling pathway

I carried out a survival analysis using Kaplan Meier plotter database of a group of 580 triple negative patients to check the prognostic value of FOXA1 on its own and with the prolactin-signaling pathway. I found that patients with high expression of both prolactin receptor signaling pathway and FOXA1 have better survival and prognosis than patients with high expression of FOXA1 only (Figure 8). This indicates that there might be a cross talk mechanism between prolactin receptor signaling and FOXA1 that affects the disease prognosis.



FOXA1 RELAPSE FREE Survival

FOXA1_PRL_PRLR_JAK2_STAT5 RELAPSE FREE SURVIVAL

PRL_PRLR_JAK2_STAT5 RELAPSE FREE SURVIVAL

Figure 8: FOXA1 gene expression in association with patient's outcome using Kaplan- Meier analysis. Incorporating PRLR signaling pathway with FOXA1 shows higher RFS in a 10-years censoring than FOXA1 alone.

3. FOXA1 protein expression in breast cancer cells following long term treatment by prolactin

All the previously mentioned findings encouraged us to check if prolactin can have a regulatory effect on FOXA1 expression in the luminal TNBC subtype. To fulfill this objective, I treated the MDA-MB-453 cell line with prolactin hormone with 2 different concentrations 150ng/ml and 250ng/ml. I checked FOXA1 expression both on the protein level by western blotting and on the mRNA level by RT-qPCR. At the protein level, FOXA1 protein showed a decrease in expression after 12 and 24 hours of 250ng/ml PRL treatment and a decrease in expression after 12 hours of 150 ng/ml PRL treatment (Figure 9).

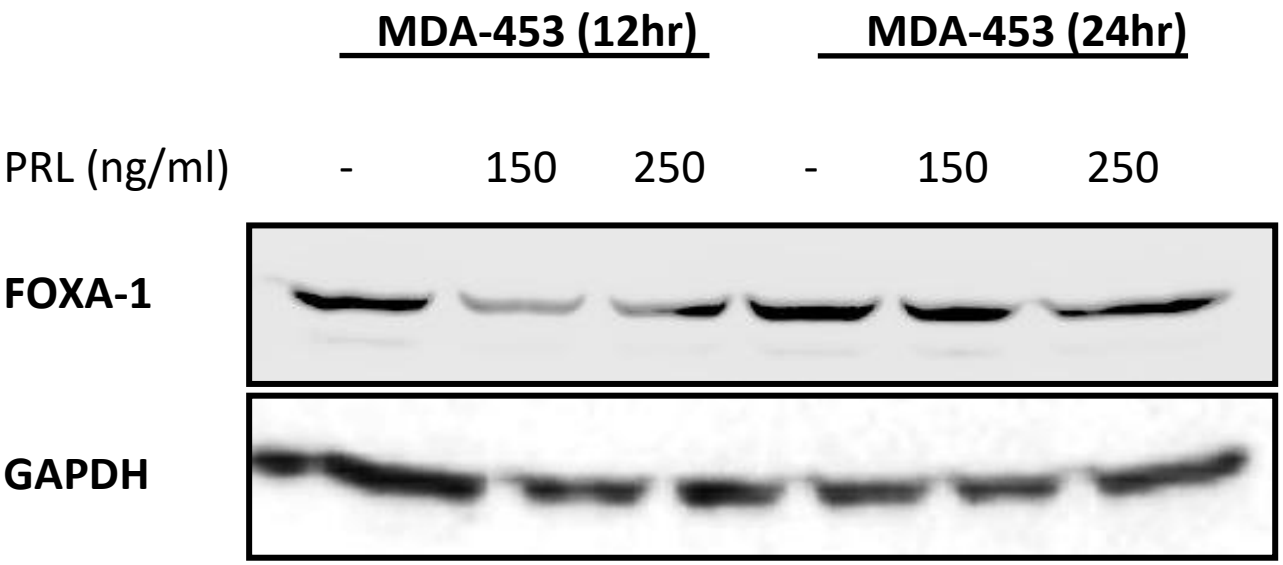
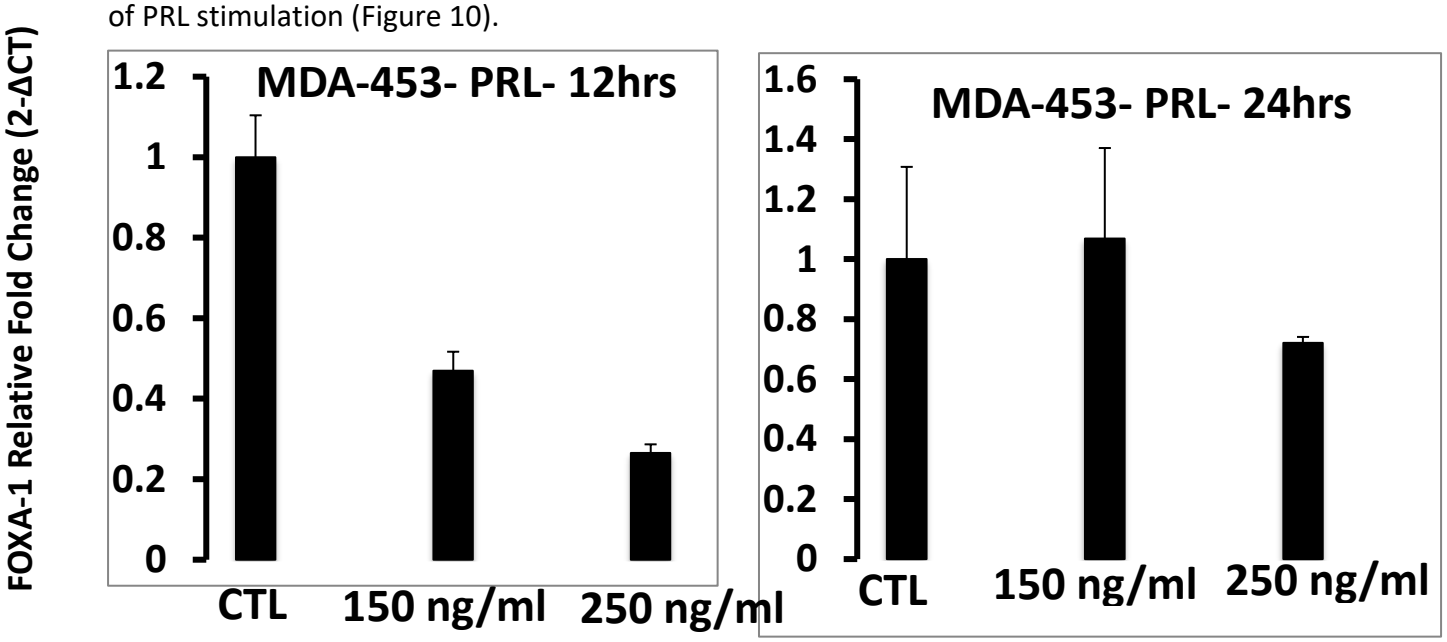


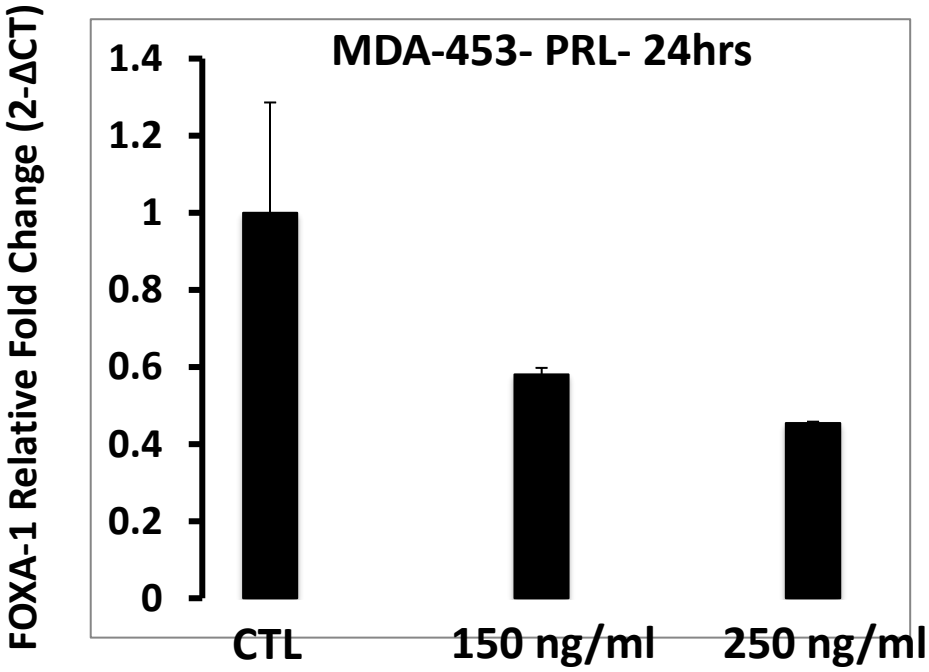
Figure 9: Level of FOXA1 Gene expression decreases by treating the MDA-MB-453 cells with prolactin hormone. The cells were treated with 150ng/ml and 250ng/ml hPRL for 12 hrs and 24hrs then a total lysate of the cells was extracted after the given time point and the FOXA1 protein was detected using anti-FOXA1 polyclonal antibody by western blot.

4. FOXA1 m-RNA expression in breast cancer cells following long term treatment by prolactin

To check at which time point PRL can exert its main regulatory effect on FOXA1 expression, I applied both long term and short term PRL treatment to the cells and checked FOXA1

expression on the mRNA level by RT-qPCR. By the long-term treatment of the cells with 250ng/ml PRL, FOXA1 expression decreased after 12hrs. However, I obtained variable results at the time point of prolactin treatment for 24hrs and the effect diminished after 48 and 72 hours of PRL stimulation (Figure 10).





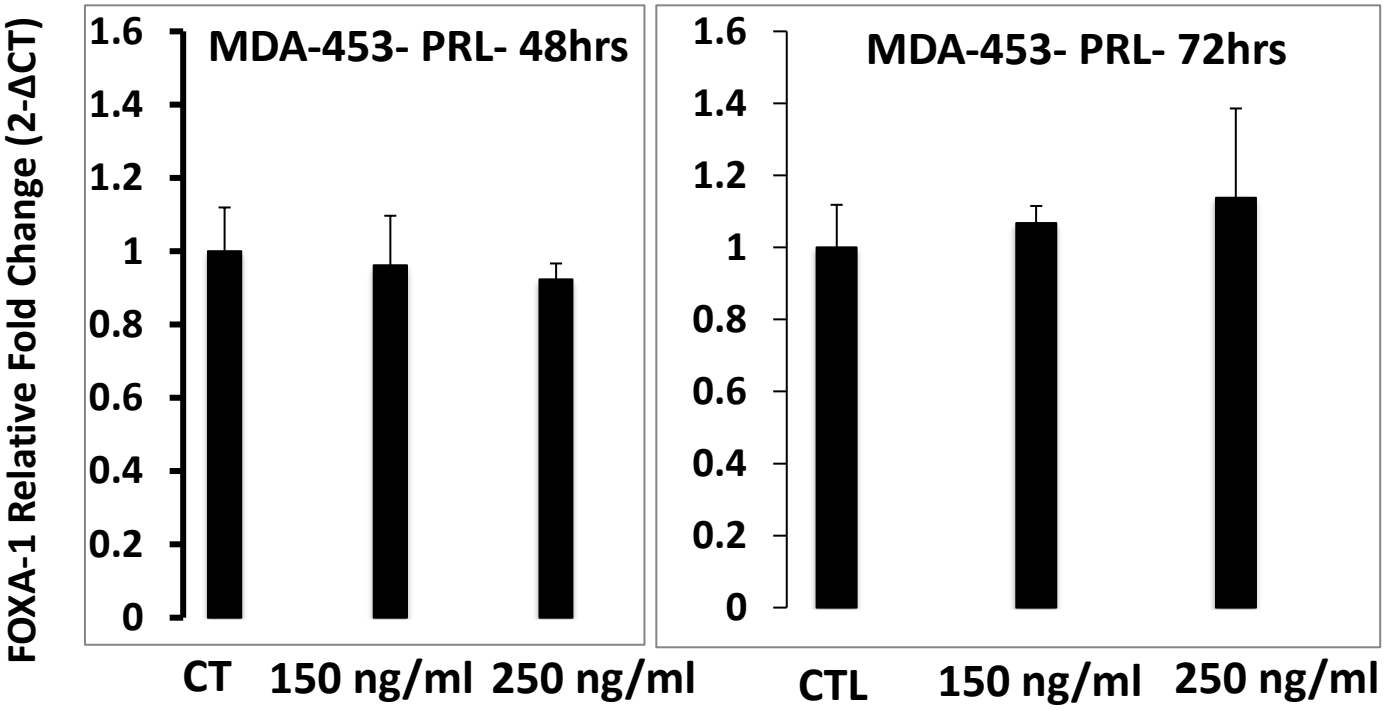


Figure 10: Level of FOXA1 gene expression decreases by treating MDA-MB-453 cells with prolactin hormone, and effect diminishes after > 24 hrs. The cells were treated with 150ng/ml and 250ng/ml of hPRL for 12, 24, 48 and 72 hours for each hPRL concentration and mRNA was collected and expression measured by qRT-PCR.

5. FOXA1 expression following short term treatment by prolactin

By the short-term treatment, FOXA1 expression first increased by 2 hours treatment of 250ng/ml PRL, decreased by 4 hours treatment, then almost remained unchanged by a 6 hours prolactin treatment (Figure 11). Altogether, the data from different time point of PRL treatment shows that Prolactin maybe playing a role in regulating the expression of FOXA1 in LAR subtype breast cancer cells.

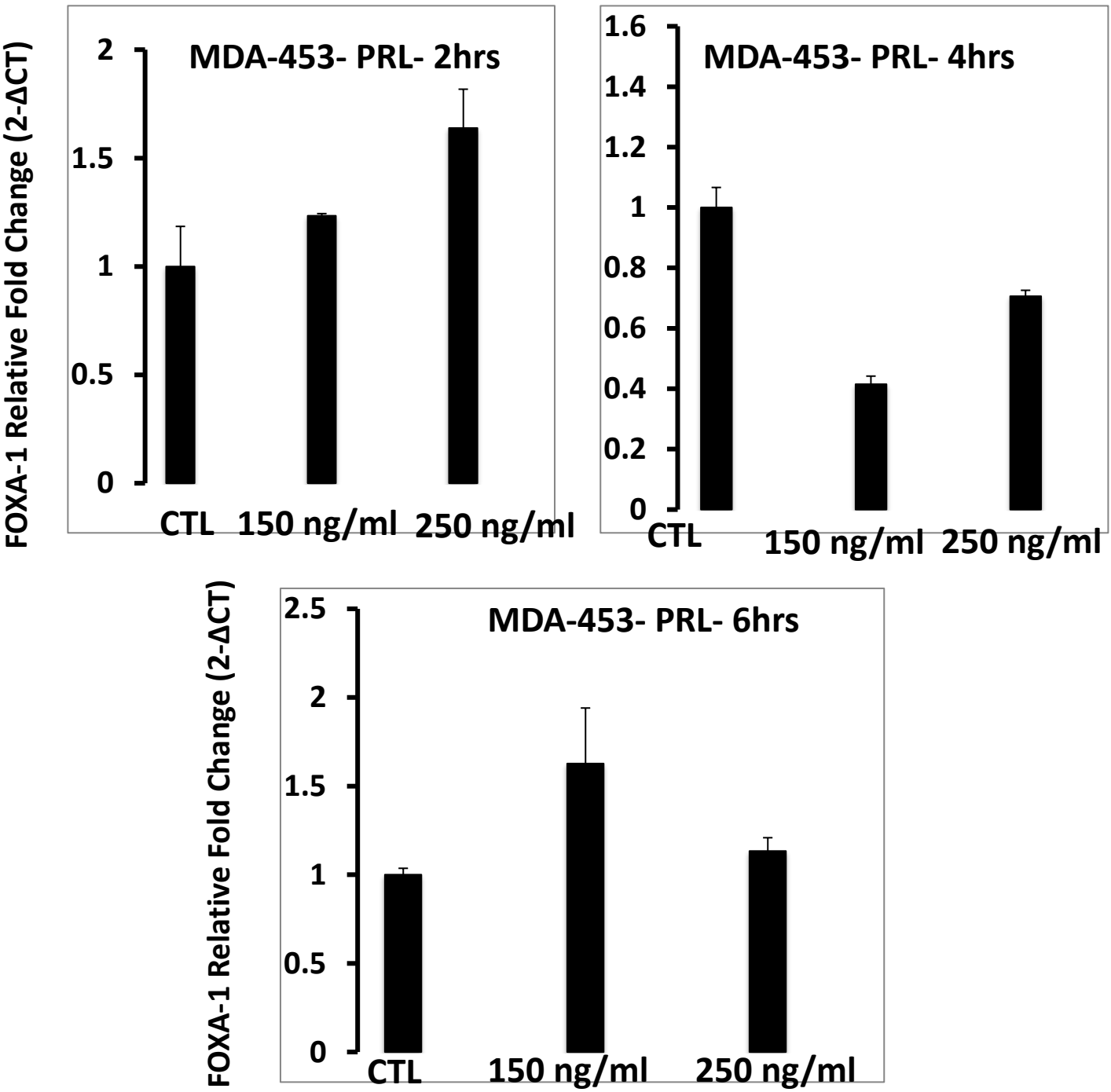
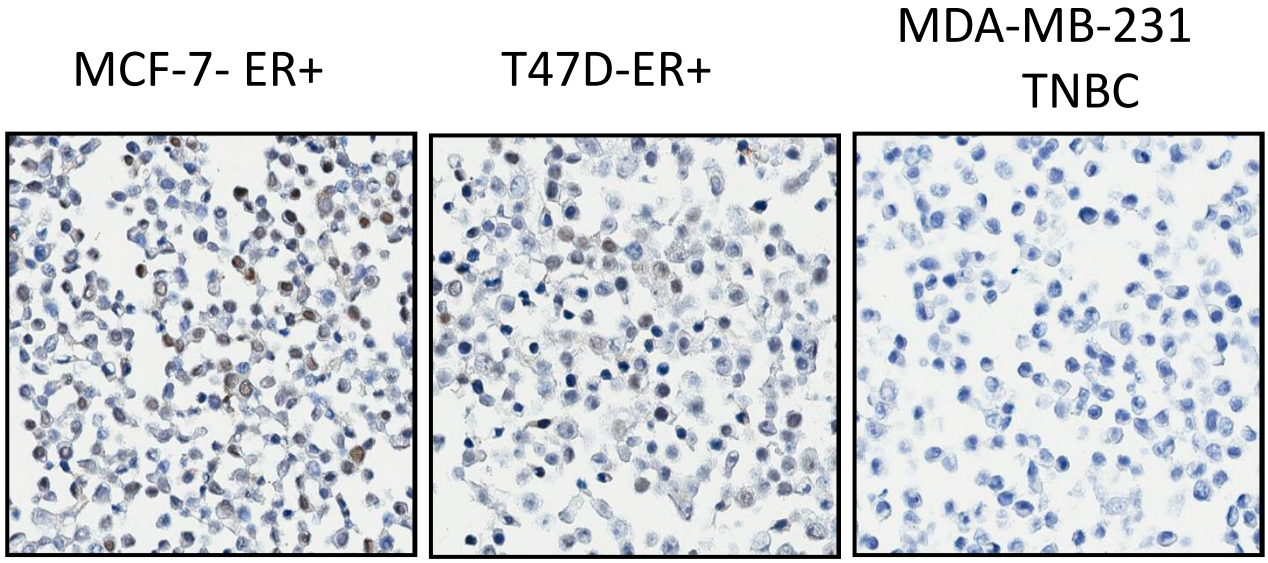


Figure 11: Level of FOXA1 gene expression after short-term treatment. The cells were treated with 150ng/ml and 250ng/ml of hPRL for 2, 4 and 6hr for each hPRL concentration and mRNA was collected and expression measured by qRT-PCR.

6. PRLR expression is essential for FOXA1 expression

Our next objective was to investigate whether PRLR expression is essential for FOXA1 expression and whether PRLR knockout can affect the expression of FOXA1 in breast cancer cells. FOXA1 is normally expressed in MCF7 and T47D, ER2+ cancer cell lines, in which both have a luminal epithelial phenotype, while MDA-MB231, which shows a mesenchymal metastatic phenotype, normally lacks the expression of FOXA1 (Figure 12A). The experiment was performed by Dr. Anwar shams and Ms. Dana Hamam in the laboratory of Dr. Ali. In addition, Dr Anwar Shams managed to generate a CRISPR-mediated knockout of PRLR in MCF-7 breast cancer mouse xenograft model. With the help of Dr Anwar, I carried out an Immunohistochemistry on those knockouts. In contrast to MCF7 cells, IHC analysis of MCF-7/PRLR KO xenograft showed a loss of expression of FOXA1 (Figure 12B). This result indicates that PRLR expression is important for FOXA1 expression and both of them play a significant role in maintaining the luminal epithelial differentiation of breast cancer cells.

A



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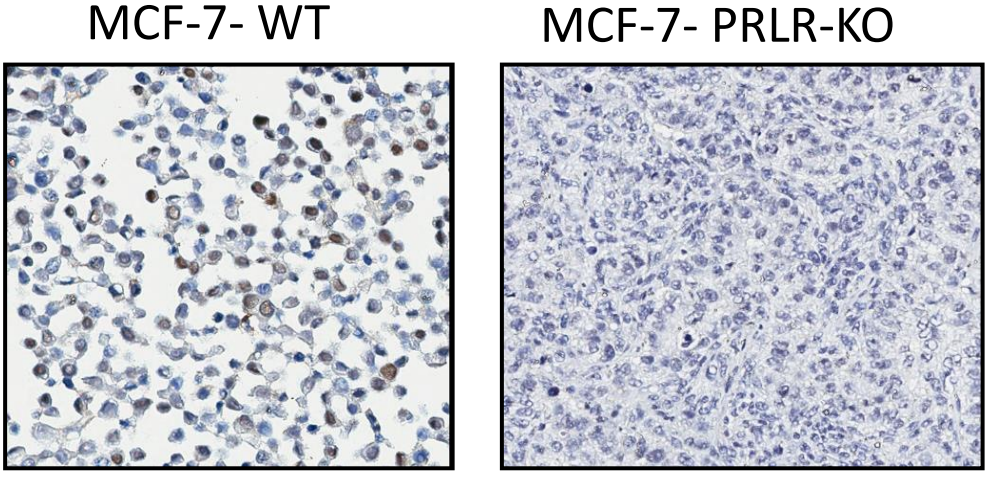


Figure 12: A) FOXA1 protein expression in different breast cancer cell lines. Positive immunohistochemical nuclear staining of FOXA1 in MCF7, and T47D cells both with an epithelial/ luminal-like phenotype. Negative immunohistochemical nuclear staining of FOXA1 in MDA-MB-231, triple negative breast cancer cell line with a mesenchymal phenotype.
B) FOXA1 protein expression in MCF7 parental cell line (left) compared to its expression in a tumor mass of a PRLR knock out xenograft NOD/SCID mouse (right). Knock out of PRLR in MCF7 tumors leads to the loss of FOXA1 protein expression.

7. FOXA1 expression diminishes in a CRISPR-mediated knockout in LAR subtype breast cancer cells

Our last objective was to explore the role of FOXA1 in breast cancer tumorigenesis. This was done by a CRISPR-cas9 knock out of FOXA1 gene in MDA-MB-453 by lentivirus transduction. The position that each single guide RNA targets for different genes is indicated in the methods section. I confirmed the results of the knockout by carrying out a western blot for FOXA1 in the transfected cells. It showed a knock-out of FOXA1 in cells edited by sgRNA2 in comparison to parental cells and the non-targeting (NT) scrambled sequence transduced cells (Figure 13). As well, I observed a minor loss of FOXA1 expression in cells infected with sgRNA1.

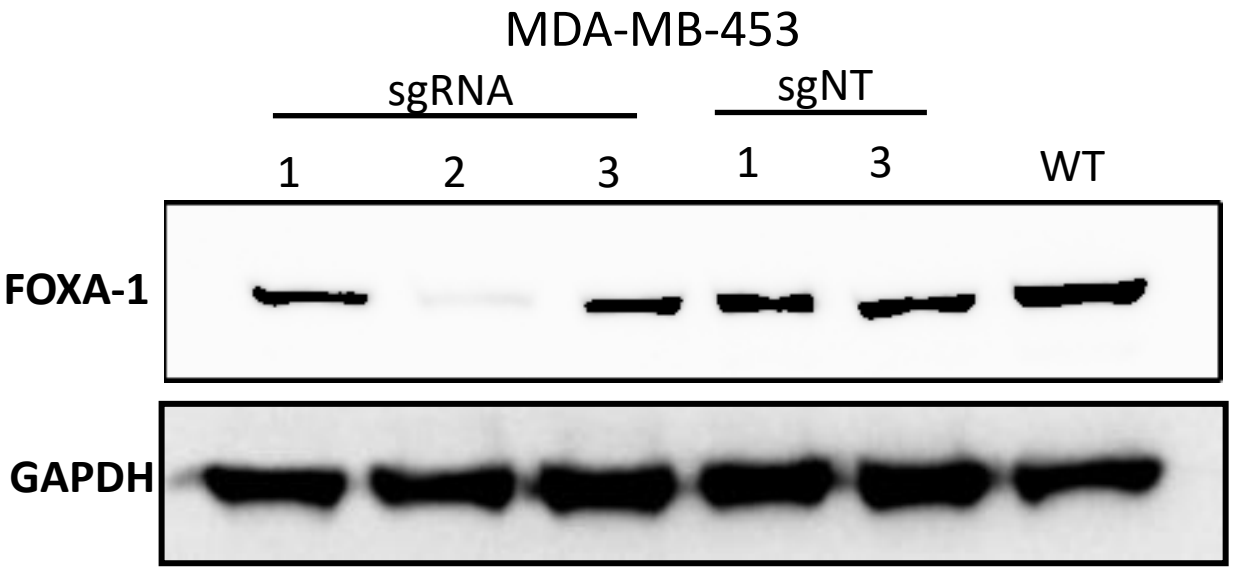
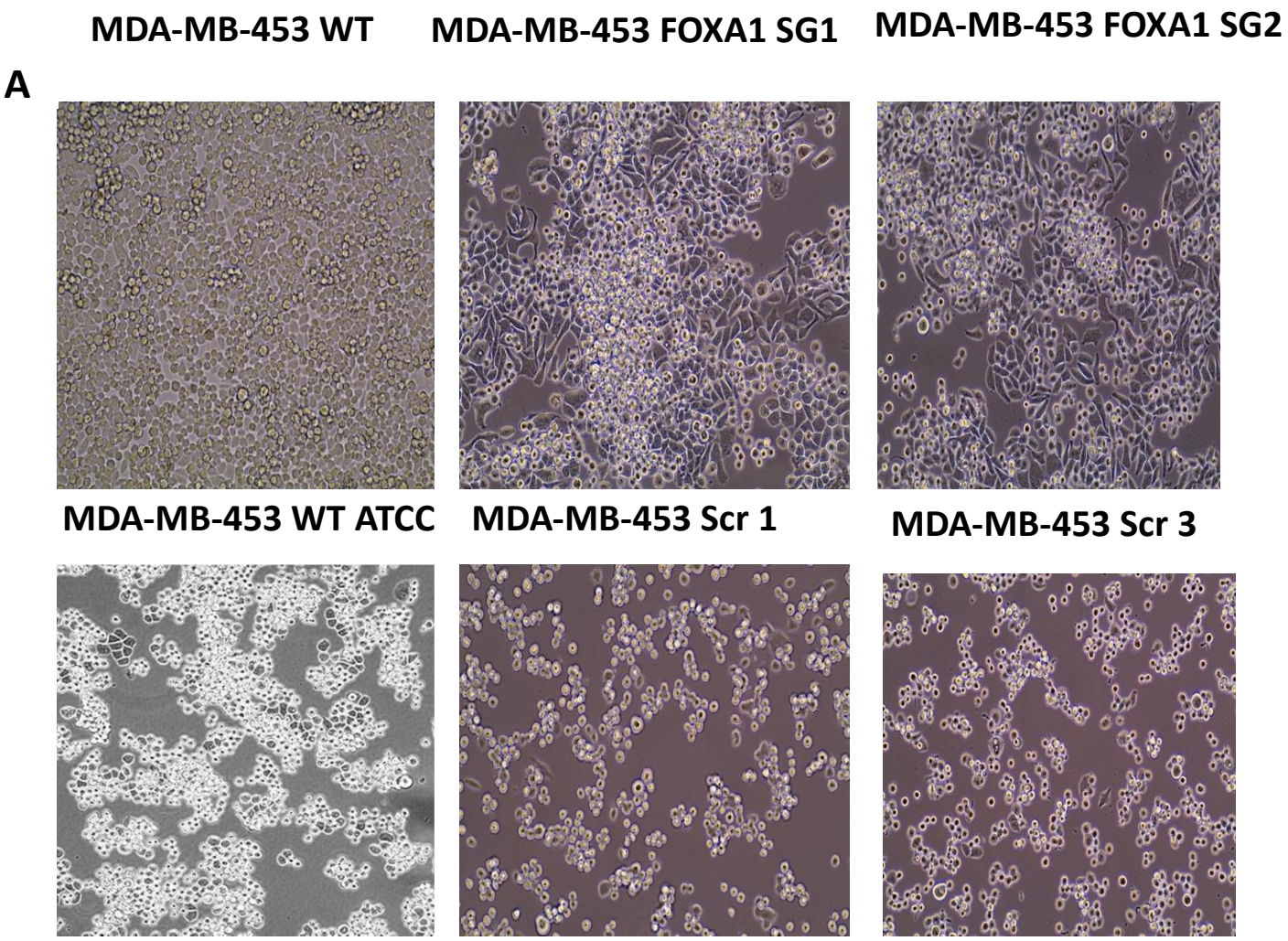


Figure 13: FOXA1 protein expression in a CRISPR knockout in an MDA-MB-453 cell line compared to the parental and scrambled sequence edited cells. Loss of FOXA1 expression in cells edited with guide RNA2 is clear compared to the parental and scrambled sequence cells.

8. Changes in cellular phenotype to the mesenchymal form in FOXA1 CRISPR knocked out cells

After knocking out FOXA1, I noticed a change in the cells' phenotype in both guide RNA1 and guide RNA2 plates from being epithelial to look more mesenchymal, resembling the MDA-MB-231 cell line which is a basal subtype of TNBC (Figure 14). Therefore, I wanted to determine the effect of FOXA1 knockout on the epithelial mesenchymal transition (EMT) of the cells.



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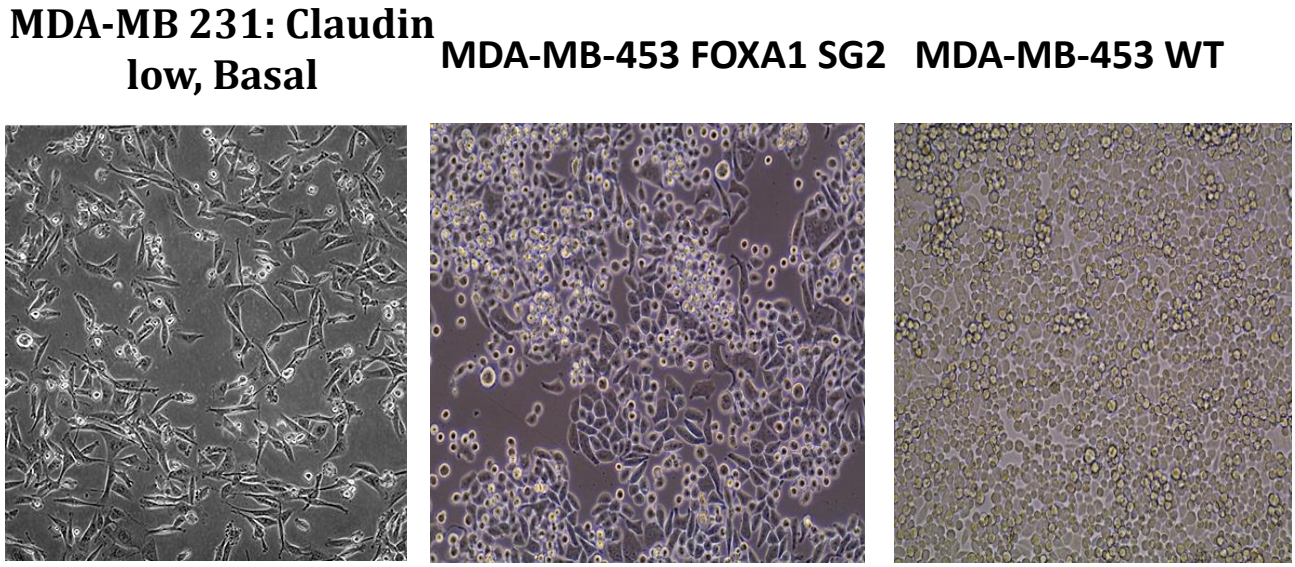

Figure 14: A) Changes in the cellular phenotype to the mesenchymal form in FOXA1 CRISPR knock out cells.

B) Knockout of FOXA1 changes the cell morphology to resemble the basal mesenchymal subtype of TNBC.

9. Gain of Vimentin and loss of E-cadherin expression in FOXA1 knocked out cells

I checked for the expression level of the two EMT markers; vimentin and E-cadherin by western blot. Indeed, as can be seen vimentin protein levels were marginally increased in all FOXA1 sgRNA infected cells compared to parental and scramble cells (Figure 15A). On the other hand, it showed a decrease in E-cadherin protein levels in both sg1RNA and sg2RNA knocked-out cells compared to the parental and the scrambled sequence breast cancer cells (Figure 15B).

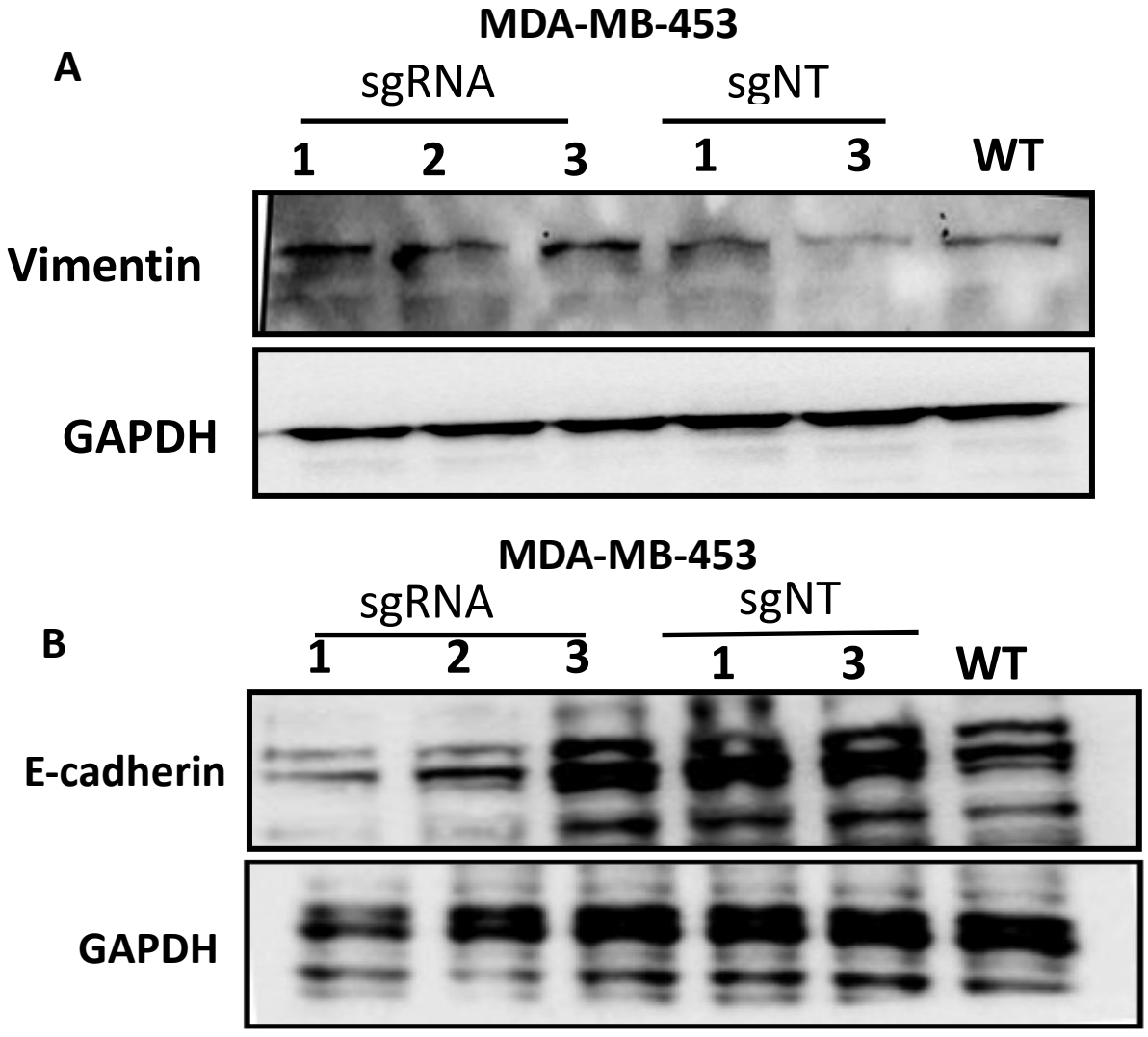

Figure 15: A) Gain of Vimentin expression in cells edited with guide RNA1 and guide RNA2 compared to the parental and scrambled sequence cells. Vimentin is a filament protein expressed in the mesenchymal cells and a marker of EMT.
B) Loss of E-cadherin expression in cells edited with guide RNA1 and guide RNA2 is clear compared to the parental and scrambled sequence cells. E-cadherin is a cell-cell adhesion protein in epithelial cells and an epithelial marker.

Chapter IV- Discussion:

Triple Negative Breast Cancer (TNBC) is the most challenging type of breast cancer due to its heterogeneity, resistance and lack of targeted therapy (Foulkes, Smith et al. 2010). It is characterized by poor patient survival due to its aggressiveness and high metastatic and recurrence rates (Foulkes, Smith et al. 2010). Despite the recent advances in subclassifying TNBC into six molecular subtypes, chemotherapy remains the main approach for treating TNBC with low therapeutic benefits (Lehmann, Bauer et al. 2011) (Foulkes, Smith et al. 2010). Therefore, novel targets and prognostic biomarkers are needed for developing effective therapeutic strategies for TNBC. A main reason for the aggressive behavior of TNBC is the loss of cellular differentiation and the mesenchymal phenotype of its cells and their high invasive abilities (Anders and Carey 2009) (Kim, Joo et al. 2009) (Phillips, McBride et al. 2006). For this reason, activation of mammary differentiation pathways and reprogramming the cells into the epithelial phenotype can all be used as novel approaches for improving TNBC prognosis and treatment.

Many studies elucidated the role of PRL hormone and its signaling pathway in mammary gland development and terminal differentiation of mammary epithelial cells (Hennighausen and Robinson 2005). However, the role of PRL hormone in breast cancer progression is still controversial in the literature. Although some previous studies reported that PRL can lead to the development of mammary tumors (Wennbo, Gebre-Medhin et al. 1997, Chen, Ramamoorthy et al. 1999, Clevenger, Furth et al. 2003, Rose-Hellekant, Arendt et al. 2003), more recent studies actually emphasized the antitumorigenic role of prolactin and its signaling pathway in breast cancer (Nouhi, Chughtai et al. 2006, Haines, Minoo et al. 2009). In addition, PRL and PRLR signaling pathway were shown to be correlated with good prognosis and better patient outcome (Galsgaard, Rasmussen et al. 2009, Nitze, Galsgaard et al. 2013, Hachim, Hachim et al. 2016, Hachim, Shams et al. 2016). Indeed, our lab recently showed that 30% of TNBC cases express intermediate/high PRLR mRNA levels and identified this subgroup as a TNBC-PRLR+ subtype with unique characteristics. This TNBC-PRLR subtype showed improved patient overall survival outcomes in comparison to other TNBC subtypes (López-Ozuna, Hachim et al. 2016). Using metagene cluster analysis, this subtype is also characterized by the up regulation of luminal-like differentiation genes which are FOXA1 and AR (López-Ozuna, Hachim et al. 2016).

In my study, I wanted to investigate how FOXA1 can modulate the antitumorigenic role of PRL and whether it can have a role in the reversal of the epithelial mesenchymal transition of TNBC cells. Our results showed that FOXA1 is higher expressed in the epithelial luminal subtypes of TNBC than the basal subtype and that FOXA1 is associated with lower grades of the disease. However, incorporating PRLR signaling pathway with FOXA1 still showed better patient outcome and improved prognosis than FOXA1 alone. We also showed that Prolactin regulates the expression of FOXA1 in TNBC LAR subtype and that PRLR expression is essential for FOXA1 expression. Using the CRISPR-Cas9 mediated gene editing technology, we showed how the loss of FOXA1 expression in the TNBC luminal breast cancer cells can lead to the increase of vimentin expression as an EMT marker and the decrease of the epithelial marker E-cadherin, which concludes that FOXA1 has an essential role in inhibiting the epithelial mesenchymal transition of breast cancer cells. This aligns with a recent study that showed that FOXA1 induces E-Cadherin expression at the protein level in breast cancer cells and decreases its suppressor Slug both on the protein level and the mRNA level (Anzai, Hirata et al. 2017).

Altogether, these results highlight the importance of FOXA1 in maintaining the epithelial phenotype of breast cancer cells and how it can be a potential pro-differentiation marker.

Although cytotoxic chemotherapy is still the present mainstay for treating TNBC patients, other targeted therapies of better efficacy should be considered. Different targets such as DNA repair molecules (Farmer, McCabe et al. 2005), epidermal growth factor receptors (EGFR) (Baselga, Gómez et al. 2013) and AR inhibitors (Gucalp, Tolaney et al. 2013) were recently studied to develop new treatment strategies against TNBC, but mostly showed high toxic effects and tumor recurrence. Pro-differentiation based therapy is a new approach suggested for TNBC treatment, in which cancer cells are reprogrammed into a less aggressive phenotype (Fenaux, Chevret et al. 2000, Yan and Liu 2016). In this study, we showed that FOXA1 can be used a target for reversing the mesenchymal invasive cell phenotype into an epithelial, non-invasive one with better disease prognosis. This study paves the road for further investigation into the usage of FOXA1 in personalized medicine for improving TNBC patient outcome.

Chapter V- Conclusions and Future Directions:

Recent studies showed that Prolactin hormone (PRL) and its signaling pathway exhibit an antitumorigenic effect in breast cancer by reversing the epithelial mesenchymal transition of cells (Nouhi, Chughtai et al. 2006). In addition, Prolactin receptor (PRLR) was shown to be used as an independent marker that distinguishes a unique TNBC subgroup with better patient overall survival outcomes (López-Ozuna, Hachim et al. 2016). This TNBC-PRLR+ subtype is characterized by the high expression of luminal-like differentiation genes such as FOXA1 (López-Ozuna, Hachim et al. 2016). Our study showed how PRL can regulate the expression of FOXA1 in luminal TNBC cells and how it is essential for FOXA1 expression. We also showed that FOXA1 is important for maintaining the epithelial phenotype of TNBC cells, thus can be considered a potential pro-differentiation marker and a potential target for personalized medicine in triple negative breast cancer.

Our study paves the way for more testing of FOXA1 antitumorigenic role in breast cancer. The reversal of epithelial mesenchymal transition in breast cancer can be tested by invasion assays both invitro and in animal models. The rate of cell proliferation can also be studied by MTT assays. Measuring the degree of stemness of TNBC cells after FOXA1 knockout can also be investigated by tumor sphere formation assays and measuring the expression of self-renewal markers such as OCT4, SOX2 and NANOG. Animal testing can be used to measure the degree of tumorigenesis and angiogenesis using xenograft mice models. Clinical trials can be organized to test the effectiveness of FOXA1 upregulation in improving the disease prognosis in TNBC patients, both alone and in combination with the conventional chemotherapeutic agents.

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