The Role of Prolactin Hormone in Triple Negative Breast Cancer Tumorigenicity

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Abstract

Prolactin hormone (PRL) is a key differentiation factor of mammary epithelial cells, mammary gland development and lactation through activation of the Jak2/Stat5 pathway. Triple negative breast cancer (TNBC) accounts for about 15% of all breast cancer cases. These tumors are poorly differentiated and are associated with aggressive pathologic features that lead to metastasis, recurrence and poor patient outcome. In this study we examined the role of prolactin-mediated differentiation pathway in TNBC cell biology and its potential uses in prognosis and therapy.

Our findings showed that PRLR gene expression sub-classifies TNBC patients into a new subgroup (TNBC-PRLR) characterized by epithelial-luminal differentiation with significantly better survival outcomes. To evaluate the role of PRL in TNBC we showed that restoration/activation of PRL pathway in TNBC cells representative of mesenchymal or TNBC-PRLR subgroups led to induction of epithelial phenotype, inhibition of invasiveness and suppression of tumorigenesis *in vitro* and *in vivo*.

Next, we investigated different potential mechanisms though which PRL might produce its anti-tumorigenic effect. Interestingly, we found PRL treatment of basal TNBC cells (MDA-MB-231) as well as TNBC-PRLR cells (MDA-MB-453) is able to suppress the highly tumorigenic cancer stem cell sub-populations ($CD44^+/CD24^-$ and $ALDH^+$) into non-tumorigenic sub-populations ($CD44^-/CD24^-$ and $ALDH^-$). We also found PRL to reduce gene expression of the transcriptional

network (Oct4, Sox2 and Nanog) implicated in self-renewal and stemness and to suppress *in vitro* tumor initiation and capacity of the various breast cancer stem cell subpopulations found in TNBC.

Additionally, PRL was found to induce cellular senescence program through epigenetic regulation of heterochromatin. PRL treatment of TNBC cells (MDA-MB-231 and MD-MB-453) was also able to induce SAβ-galactosidase, regulate different genes/proteins expressed in cellular senescence and formation of senescence associated heterochromatin foci (SAHF). Furthermore, we found PRL to inhibit senescence-associated secretory phenotype. Finally, we found PRL to impede tumor growth and tumour progression in xenograft animal model of MDA-MB-231 cells *in vivo.*

Altogether, our findings provide new insights into the effect of PRL in TNBC and offer potential new modalities for TNBC stratification and development of personalized therapy based on PRL hormone.

Résumé

L'hormone prolactine (PRL), à travers l'activation de la voie de signalisation Jak2/Stat5, est un facteur essentiel de différenciation des cellules épithéliales mammaires, du développement de la glande mammaire et de la lactation. Le cancer du sein triple-négatif (TNBC) constitue environ 15% de tous les cas de cancer du sein. Ces tumeurs sont pauvrement différenciées et sont associées à des caractéristiques pathologiques agressives menant à des métastases, des récurrences ainsi qu'à des résultats décevants pour les patients. Dans la présente étude, nous avons examiné le rôle de la prolactine dans la voie de différenciation des tumeurs TNBC et ses applications potentielles en termes de pronostic et de thérapie.

Nous avons trouvé que l'expression du gène PRLR sous-classifie les patients TNBC dans un nouveau groupe (TNBC-PRLR) caractérisé par une différenciation épithéliale-luminale menant à des résultats significativement meilleurs. Pour évaluer le rôle de PRL dans TNBC, nous avons démontré qu'une restauration/activation de la voie PRL dans les cellules TNBC représentant les sous-groupes mésenchymateux ou TNBC-PRLR a mené à une induction du phénotype épithélial, à une inhibition de l'invasion et à une répression de la tumorigenèse, *in vitro* et *in vivo*.

Ensuite nous avons investigué différents mécanismes potentiels à travers lesquels PRL pourrait jouer son rôle anti-tumorigénique. Nous avons découvert que le traitement PRL des cellules basales TNBC (MDA-MB-231) ainsi que des

cellules TNBC-PRLR (MDA-MB-453) était capable de réprimer les populations de cellules souches cancéreuses hautement tumorigéniques (CD44+/CD24- et ALDH+) en sous-populations non-tumorigéniques (CD44-/CD24- et ALDH-). Nous avons également observé que PRL réduit l'expression génique au niveau transcriptionnel d'un groupe de gènes (Oct4, Sox2 et Nanog) impliqués dans le renouvellement cellulaire et certaines caractéristiques de cellules souches qui inhibent l'initiation de tumeurs *in vitro* ainsi que la capacité de diverses sous-populations de cellules souches cancéreuses trouvées dans TNBC (sentence too long and ununderstandable).

De plus, nous avons démontré que PRL induisait la sénescence à travers une régulation épigénétique de l'hétérochromatineet inhibe le phénotype de sécrétion associé à la sénescence. Finalement, nous avons prouvé que PRL entravait la croissance et la progression tumorale dans des modèles animaux de xénogreffe des cellules MDA-MB-231 *in vivo*.

En résumé, les résultats de nos travaux fournissent de nouvelles informations sur l'effet de PRL dans les TNBC et offrent, potentiellement, de nouvelles avenues pour stratifier et développer des thérapies personnalisées des TNBC basées sur l'hormone PRL.

Acknowledgements

First, I would like to thank my supervisor Dr. Suhad Ali for giving me the opportunity to join her lab and provide me support through all my PhD. Her guidance allowed me to achieve all my goals and improve all my research skills. Her love for science, her patience and dedication spire me to continue my career in the breast cancer field. I would like to thank my advisor committee members: Dr. Jean-Jacques Lebrun for all his attentions, support, guidance and encouragement during all my PhD. Dr. Peter Metrakos, and Dr. Jamil Asselah for their insightful research advice. Dr. Christian O'Flaherty, my academic advisor, for his support and advice during all my graduate study.

Many thanks for Philippe Duquette and Julian Boudreault for translating the thesis abstract to French. Thanks to my friends Ms. Fatima Al Hashmi and Sarah Robins for the English editing of my thesis.

Special thanks to my colleague and unconditional friend Ibrahim Hachim, his advice, research discussions and contributions to my research work, enrich my research development. Without his presence my PhD journey couldn't be as exited and successful as it was.

Finally, I would like to thank my parents, Mr. Jose López and Mrs. Carmen Ozuna, for their love, unconditional support and for encourage me all the time to be motivated and persevere until I accomplished what I dream. Thanks to my brother Ariel López for his love and unconditional support. Many thanks to my husband Omar Saldaña for his love, unconditional support and for made this experience meaningful.

Preface & Contribution of Authors

This thesis is presented in a manuscript-based format. This thesis is written by Vanessa M. López Ozuna and revised by Dr. Suhad Ali, the supervisor. It consists of four major chapters. Chapter 1 includes the introduction, a review of related literature background. Chapter 2 contains a published article. Chapter 3 comprises a manuscript under submission; Chapter 4 includes a general discussion and conclusion. An appendix of extended materials and methods is included.

Chapter 2 was published in Scientific Reports (Nature Publishing Group). I wrote the manuscript under my supervisor's guidance. I designed and performed all the experiments described under my supervisor directions. Dr. Ibrahim Hachim performed analyses of immunohistochemistry (IHC) experiments and Dr. Mahmood Hachim performed bioinformatics data analyses. Dr. Jean Jacques Lebrun contributed design and revising the article. We collaborate with the Small Animal Imaging labs Platform (SAIL) of the Research Institute of the McGill University Health Centre to perform the PET/SPECT/CT scan images and its analysis**.**

Chapter 3 is a manuscript under submission. I wrote the manuscript under my supervisor guidance. I designed and performed all the experiments described under my supervisor directions. Dr. Ibrahim Hachim performed analyses of immunohistochemistry (IHC) experiments and Dr. Mahmood Hachim performed bioinformatics data analyses. Dr. Jean Jacques Lebrun contributed design and revising the manuscript.

I have collaborated on, and published or submitted the following articles as first author or coauthor:

1 Hachim IY, Hachim MY, **Lopez VM**, Lebrun JJ, Ali S., Prolactin Receptor Expression is an Independent Favorable Prognostic Marker in Human Breast Cancer, Appl Immunohistochem Mol Morphol. 2016 Apr; 24 (4): 238-45.

2 Hachim IY, Hachim MY, **López-Ozuna VM**, Ali S, Lebrun JJ., A dual prognostic role for the TGFβ receptors in human breast cancer, Hum Pathol. 2016 Nov; 57:140-151.

3 **López-Ozuna VM**, Hachim IY, Hachim MY, Lebrun JJ, Ali S., Prolactin Pro-Differentiation Pathway in Triple Negative Breast Cancer: Impact on Prognosis and Potential Therapy, Nature Scientific Reports, 2016 Aug 2;6: 30934.

4 **López-Ozuna VM**, Hachim IY, Hachim MY, Lebrun JJ, Ali S., Prolactin pro-differentiation pathway activation depletes cancer stem cells and inhibits tumorigenesis through the induction of cellular senescence in triple negative breast cancer *(under submission).*

5 Hachim IY, **López-Ozuna VM**, Hachim MY, Ali S., Lebrun JJ., New therapeutic application of Prolactin hormone in HER-2 overexpressing tumors through the suppression of HER- 2 enriched ALDH positive stem cells subpopulation *(under submission).*

Contribution to knowledge

- In this original study, I describe for the first time the role of PRL in TNBC biology.
- I found that PRL signaling pathway components individually (PRL, PRLR, Jak2 and Stat5a), or as a gene signature is able to predict TNBC patients with significantly better outcomes.
- My results identified PRLR as a novel and relevant sub-classifier, able to detect a new subgroup (TNBC-PRLR) with better overall survival.
- This study provide for the first time more detailed information about the mechanism through which PRL induces its anti-tumorigenic and anti-tumor progression effects in vitro and in vivo.
- Altogether, these findings led us to propose a new management strategy for TNBC patients. This approach is based on screening for PRLR expression in patients that may benefit from the use of PRL hormone as potential therapy.
- All those elements of the thesis are considered original scholarship and distinct contributions to knowledge.

List of Abbreviations

ALDH, aldehyde dehydrogenase;

BSA, bovine serum albumin;

CSC, cancer stem cells;

DMEM, dulbecco's modified Eagle's medium;

EAD, entinostat, all trans retinoic acid, doxorubicin;

Elf5, E74-like factor 5;

EMT, epithelial to mesenchymal transition;

ER, estrogen receptor;

FACS, fluorescence-activated cell sorting;

FBS, fetal bovine serum;

HDAC, histone deacetylase;

HER2, human epidermal growth factor receptor 2;

IHC, immunohistochemistry;

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

MAPK, mitogen-activated protein kinase;

MMP, matrix metalloproteinase;

PI3K, phosphatidylinositol-3 kinase;

PRG, progesterone receptor;

PRL, prolactin;

PRLR, prolactin receptor;

PTEN, phosphatase and tensing homolog deleted on chromosome ten;

RB, retinoblastoma;

RANKL, Receptor activator of nuclear factor κ-b ligand;

SAβ-galactosidase, senescence associated β-galactosidase;

SASP, senescence associated secretory phenotype;

SDS, sodium dodecyl sulfate;

SAHF, senescence associated heterochromatin foci;

STAT, signal transducer and activator of transcription;

TNBC, triple negative breast cancer;

Wnt, wingless-type mammary tumor virus integration;

Rationale and Objectives

Prolactin (PRL) is a major regulator of mammary gland development and terminal differentiation of mammary epithelial cells through activation of the Jak2/STAT5 pathway. However, its role in breast cancer development/progression is not fully elucidated. Previous work in this lab demonstrated a potential ant-tumorigenic role for PRL in breast carcinogenesis. Indeed, PRL through PRLR/Jak2 signaling was found to suppress epithelial-mesenchymal-transition (EMT) and reduces the invasive properties of breast cancer cells. In addition, we also found that expression of PRLR and PRL in human breast cancer clinical cases to correlate with favorable prognosis and better patient outcome. Together, these findings provide compelling evidence regarding the role of PRL pathway in maintaining tissue differentiation and to exert anti-tumorigenic effects in breast carcinogenesis. This suppressive role of PRL is still emerging and needs to be further elaborated. In addition, the role of PRL in TNBC has not yet been investigated. The aim of this thesis is to detail the mechanism and provide further insights into the anti-tumorigenic role of PRL in TNBC. The principal objectives of this study include: (1) elucidate the role of PRL and its signaling pathway in regulating TNBC biology, (2) dissect the prognostic value of PRL differentiation pathway in TNBC patients, and (3) evaluate PRL as a potential novel pro-differentiation therapy in TNBC.

Chapter 1: Introduction - Literature Review

1. Prolactin hormone

1.1 Chemistry and molecular biology

Prolactin (PRL) hormone is produced and secreted mainly from the anterior pituitary gland by specialized cells called lactotrophs (Freeman, Kanyicska et al. 2000). However, it is also secreted in other extra-pituitary tissues (Andersen 1990). PRL gene in humans, is found in chromosome 6 and is a member of the prolactin/growth hormone/placental lactogen family (Horseman and Yu-Lee 1994) (Cooke, Coit et al. 1981, Owerbach, Rutter et al. 1981, Truong, Duez et al. 1984). Importantly, two independent promoter regions regulate the pituitary and extra-pituitary expression of PRL gene. One is proximal to a 5k-bp region that regulates the pituitary-specific expression (Berwaer, Monget et al. 1991) and the other is an upstream promoter that regulates the extra-pituitary expression (Berwaer, Martial et al. 1994).

PRL is a single polypeptide hormone that is produced as a pro-hormone of 227 amino acids, with a signal peptide of 28 amino acids. The remaining 199 amino acids represent the mature human prolactin molecule (Sinha 1995). PRL is organized in a single amino acid chain with three intra molecular disulfide bonds between six cysteine residues (Cooke, Coit et al. 1981).

1.2 Sites of PRL synthesis and secretions

The main site of PRL secretion is the lactotrophs in the anterior pituitary gland (Herlant 1964). However, it is also secreted in extra-pituitary tissues. Mammary epithelial cells represent the most significant source for extra-pituitary PRL production during lactation (Nolin and Witorsch 1976, Steinmetz, Grant et al. 1993). Decidual tissue (Andersen 1990) produces PRL identical to the one in the pituitary tissue (Riddick, Luciano et al. 1978, Clements, Whitfeld et al. 1983). PRL diffuses into the amniotic fluid during pregnancy and is believed to stimulates maturation and osmoregulation of the fetus (Riddick and Daly 1982). Other tissues such as myometrium and endometrium are also sources of PRL.

However, their functions are still unclear. (Gellersen, Bonhoff et al. 1991) (Walters, Daly et al. 1983). Moreover, PRL secretion was also found in other tissues including the brain (Fuxe, Hokfelt et al. 1977), spinal cord (Harlan, Shivers et al. 1989), prostate (Nevalainen, Valve et al. 1997)(37), urethral gland (Tsubura, Morii et al. 1986), gut and adipose tissue (Brandebourg, Hugo et al. 2007). Additionally, PRL was shown to be produced by lymphocytes and immune system tissues, including the thymus, spleen and lymph nodes (Wu, Devi et al. 1996).

1.3 Prolactin variants

The size of PRL hormone is 23kDa. However variants of PRL were also described as a result of alternative splicing, proteolytic cleavage and posttranslational modifications (Freeman, Kanyicska et al. 2000) Figure 1.1.

1.3.1 The splice variants of PRL

Alternative splicing is not regarded as a major mechanism of PRL variants. However, there is some evidence that alternatively spliced variants were detected both in brain tissue and the pituitary gland (Emanuele, Jurgens et al. 1992).

1.3.2 Proteolytic cleavage

There are several well-studied forms of cleaved PRL hormone including 14-, 16-, and 22-kDa prolactin variants (Freeman, Kanyicska et al. 2000). The 16-kDa fragment was shown to be present in human pituitary gland as well as the plasma (Sinha, Gilligan et al. 1985). This isoform is a product of the enzymatic activity of kallikrein, which is a subgroup of serine proteases with trypsin like activity located in the Golgi apparatus, and lactotrophs secretory granules (Powers 1986).

Interestingly, no receptors for the16-kDa fragment as well as other PRL cleavage products had been described (Lkhider, Seddiki et al. 2010). However, the anti-angiogenic effect of this fragment is mediated through high affinity binding sites on endothelial cells (Clapp, Martial et al. 1993).

Cleavage by Kallikrin can lead to the production of another PRL variant, the 22-kDa prolactin fragment [prolactin- (1—173)], through clipping the PRL molecule in a thiol-dependent manner (Freeman, Kanyicska et al. 2000). The thiol will alter the folding states of the PRL molecule that is recognized by kallikrein as a substrate (Anthony and Powers 1993), The production and release

of this form was found to be sex-specific (restricted to female rats) and inhibited by dopamine (Anthony, Stoltz et al. 1993).

1.3.3 Post-translational modifications

Post-transcriptional modifications represent the major source for prolactin variants and can result from the processing of the mature PRL molecule. These modifications include phosphorylation, dimerization, polymerization and glycosylation (Sun, Lou et al. 1996)

1.3.3.1 Phosphorylation

This process occurs in the lactotrophs and involves esterification of hydroxyl groups of serine and threonine residues (Greenan, Balden et al. 1989). The importance of PRL phosphorylation is highlighted by the significant difference observed in biological activity between phosphorylated and non-phosphorylated PRL (Hansson, Waters et al. 1995). Non-phosphorylated PRL was shown to have much higher biological activity than the phosphorylated form (Wang and Walker 1993). However, in spite of this lower biological activity, the phosphorylated PRL was shown to play an important role in modulating the autocrine PRL, secretion through suppression of the release of the non-phosphorylated form (Ho, Greenan et al. 1989).

1.3.3.2 Dimerization and polymerization

This process results in the formation of high-molecular-weight PRL termed as macro prolactin, and is characterized by lower biological activity (Sinha 1995).

1.3.3.3 Glycosylation

The glycosylated PRL was previously discovered in variable proportions in different species including mammals (Sinha 1995). The glycosilation can be either N-glycosylation, where the attachment of the sugar molecule is though nitrogen, or O-glycosylation, where the attachment of the carbohydrate molecule is through the oxygen atom (Freeman, Kanyicska et al. 2000). Similar to the phosphorylated and dimerized PRL, glycosylated PRL is also characterized by reduced biological activity (Markoff, Sigel et al. 1988). In addition, the affinity of this form to bind PRLR was also found to be reduced (Haro, Lee et al. 1990).

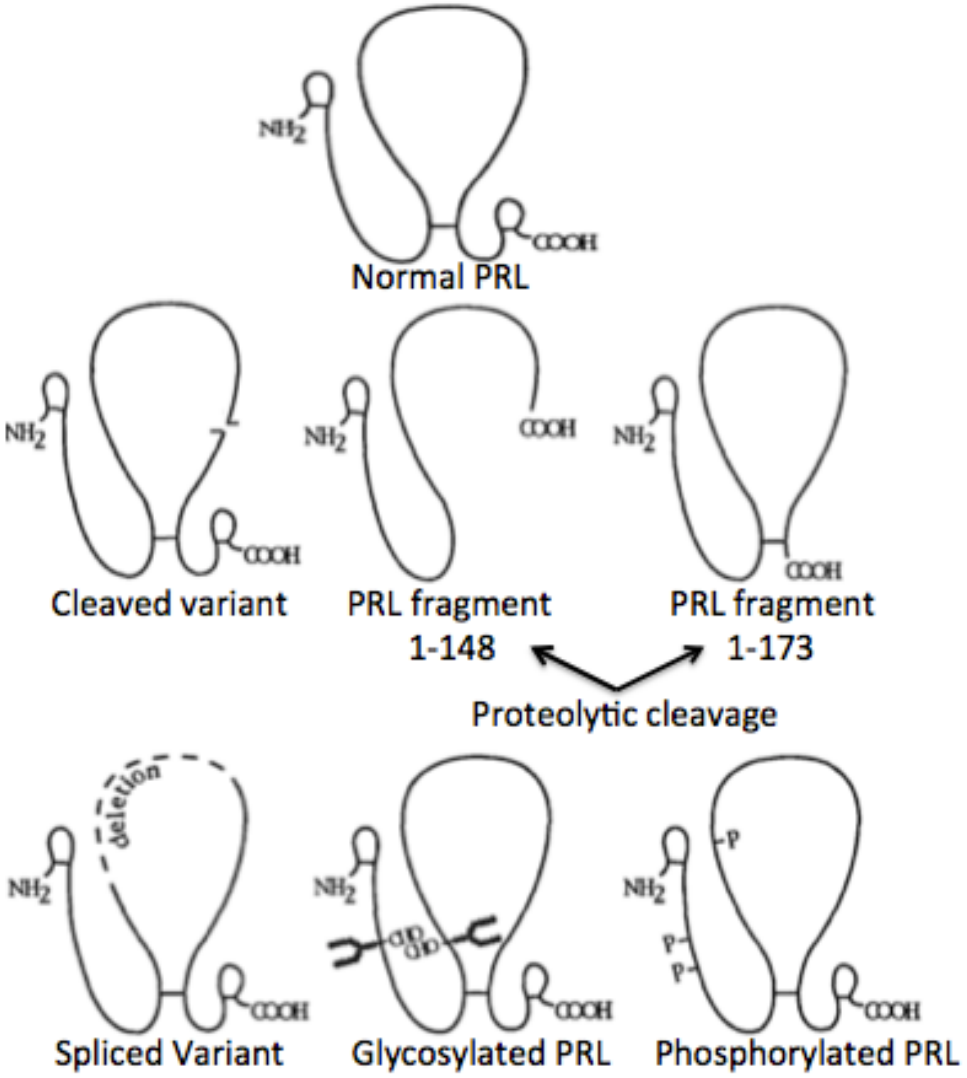

Figure 1.1 Schematic diagram of PRL structure and its variants. PRL variants depicted are resulted of an alternative splicing, proteolytic cleavage and posttranslational modifications (phosphorylation, dimerization, polymerization and glycosylation). Modified from Sinha YN (1995), structural variants of prolactin: occurrence and physiological significance, Endocrine Reviews, Volume 16, Issue 3, 354–369.

1.4 Prolactin receptor

PRL produces its function through the prolactin receptor (PRLR). PRLR is a trans membranous class 1 cytokine receptor (Bazan 1990) that consists of three major domains. These domains are the ligand-binding extracellular (EC) domain, the transmembrane (TM) domain and the intracellular domain (IC). In humans, the long form of the PRLR consists of 598 amino acid residues. The structure of PRLR shows a great similarity with other hormone receptors belonging to the same family, including growth hormone receptor (hGHr) and erythropoietin receptor (hEPOr). (Bazan 1990, Bazan 1990, Goffin and Kelly 1997). PRLR gene is present on chromosome 5 in (5p13-14). It is formed of 11 exons and is over 200kb in length (Bole-Feysot, Goffin et al. 1998).

1.4.1 Ligand-binding extracellular (EC) domain

This part of the PRLR contains the N-terminus and consists of 210 amino acids in both rat and human species (Boutin, Jolicoeur et al. 1988, Boutin, Edery et al. 1989). In addition, it shows resemblance in sequence with other cytokine receptors (Wells and de Vos 1996). It is usually divided into two subdomains, the NH2-terminal D1 (membrane-distal) and membrane-proximal D2 subdomain (these domains can alternatively be called S1 and S2 respectively). Each of these two domains consists of 100 residues and show a great similarity to the fibronectin type III molecule, and they are essential for the receptor-ligand interaction (Wells and de Vos 1996). D1 subdomain of the extracellular portion consist of two disulphide bridges (Cys12-Cys22 and Cys51-Cys62), and a

duplicated tryptophan–serine (WS) motif (Tpr-Ser-x-Trp-Ser) in the D2 domain (Wells and de Vos 1996). Both are essential for the binding of ligands as well as activation (Rozakis-Adcock and Kelly 1991).

1.4.2 Transmembrane domain

This portion of the PRLR is 24-amino acids long and anchored by two charged residues; D210 and K235 (Bole-Feysot, Goffin et al. 1998). Ligand-independent hPRLR dimers are formed due to the interaction between the transmembrane domains even when both EC and IC domains were removed, However the role of this region in PRLR functional activity is not well understood (Bole-Feysot, Goffin et al. 1998).

1.4.3 Intracellular domain

This domain in the long form is composed of 364 C-terminal residues. The IC domain contains Box 1 and Box 2 conserved regions (Kelly, Djiane et al. 1991). Box 1 is a membrane-proximal region and contains 8 amino acids between 243 and 250 residues. This area is proline-rich with hydrophobic residues and is essential for the recognition and binding of transducing molecules such as Janus kinase 2 (JAK2) (Lebrun, Ali et al. 1995, Pezet, Buteau et al. 1997). In contrast, the box 2 region (288-298 amino acids), is less conserved and is composed of series of hydrophobic and acidic residues (Clevenger and Kline 2001). Additional regions in the IC domain are the V Box and X Box. The V Box is located between Box 1 and Box 2, while the X Box

region is located directly to C-terminal and Box 2. The function of these two regions is still not well understood (Clevenger and Kline 2001).

1.4.4 Jak2 and Stat5 molecules

Jak2 is a tyrosine kinase that is constitutively associated with the proximal region of the intracellular domain. After PRL binds and receptor dimerization occurs, detectable phosphorylation of jak2 takes ~1 min. This allows two jak2 molecules to be close enough to each other to be trans-phosphorylated and phosphorylates the PRLR tyrosine residues. Importantly, phosphotyrosines constitute a docking site for other molecules that contain SH2 domains (Rillema, Campbell et al. 1992, Brooks, Dai et al. 2014).

STAT (signal transducer and activator of transcription) proteins are transducer molecules. STAT5a and STAT5b were identified as transducer molecules of the PRLR. The STAT structure consists of a DNA binding domain, SH3 and SH2-like domains, and an NH2- and a COOH- terminal transactivating domain, when PRL binding activates the PRLR signalling, STAT5 SH2-domain will bind to the tyrosine residues of the PRLR and will be phosphorylated by Jak2. Then STAT5 will dissociate from the receptor and will form dimers through the SH2 domain with another phosphorylated STAT5 molecule. These dimers will translocate into the nucleus at the GAS (γ-interferon activated sequence) binding region to induce gene transcription (Bole-Feysot, Goffin et al. 1998) (Ihle, Witthuhn et al. 1994, Goffin, Bouchard et al. 1998).

1.4.5 PRLR isoforms

As mentioned above, the long form of the PRLR contains 11 exons (Bole-Feysot, Goffin et al. 1998). However, some reports show that there are other PRLR isoforms that originate from alternative splicing (Figure 1.2):

1.4.5.1 ΔS1 isoform: This isoform, lack exons 4 and 5 as well as encoding the D1 subdomain of the extracellular domain. This isoform usually has less affinity for PRL compared to the long form (Kline, Rycyzyn et al. 2002).

1.4.5.2 ΔS2 isoform: The ΔS2 isoform is characterized by the lack of the S2 subdomain that was found to cause constitutive activation of the receptor (Tan, Huang et al. 2008).

1.4.5.3 intermediate isoform: This isoform usually results from a 573-base pair deletion, located in exon 10. This results in a truncated intra cytoplasmic domain and a frameshift causing the addition of 13 residues. This isoform was shown to be functional, however, it displays a distinct preference in signaling pathways and different tissue specific expression compared to the PRLR long form (Kline, Roehrs et al. 1999).

1.4.5.4 Short isoforms: Two naturally occurring short isoforms of PRLR are believed to occur due to alternative splicing of exons 10 and 11 (Hu, Meng et al. 2001). These two isoforms are named S1a and S1b. S1a encodes 376 amino

acids with a partial deletion of exon 10, lacks X Box region and has a unique C-terminal region with 39 additional residues (Hu, Meng et al. 2001). S1b is only 288 amino acids in length and has a complete deletion of exon 10. It is terminated shortly after the Box1 region and contains three additional amino acids in the C terminus region that are believed to be originated from exon 11 (Hu, Meng et al. 2001). These two isoforms were found in various normal tissues as well as some breast cancer cell lines. Interestingly, while both of the short forms showed a similar binding affinity compared with the long form, they were also shown to lack the ability to mediate the activation of PRL-induced β-casein gene promoter (Hu, Meng et al. 2001).

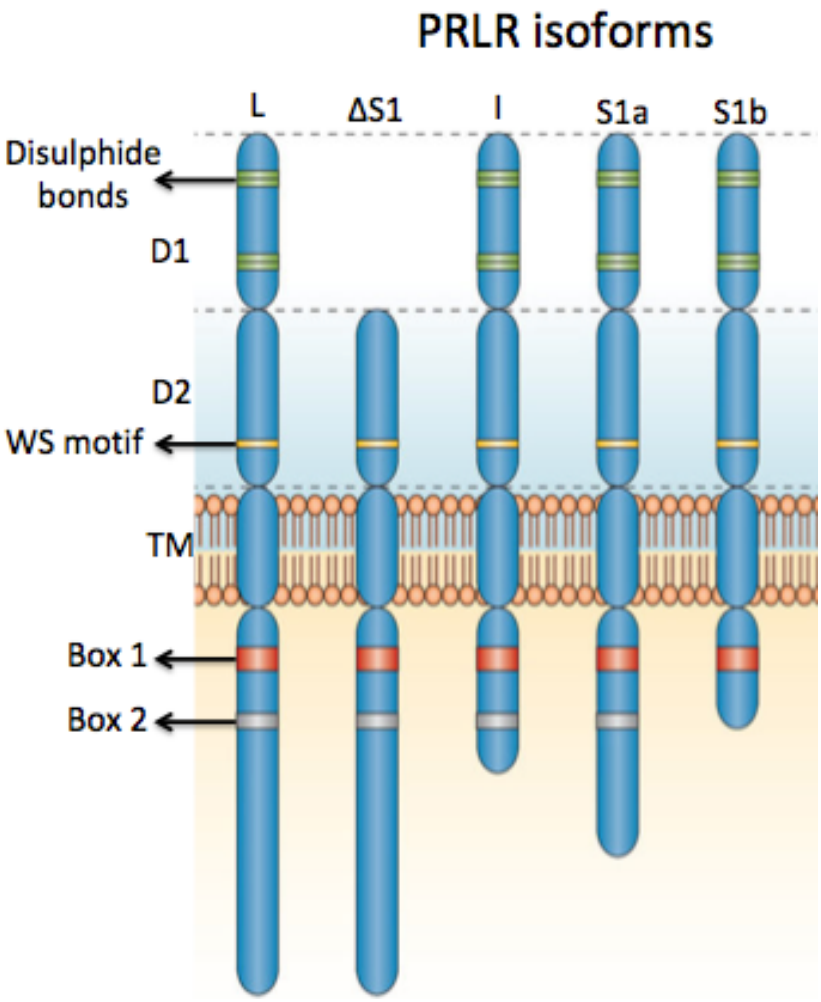

Figure 1.2 PRLR structure and isoforms. The PRLR include an extracellular domain (includes tryptophan–serine motif domain (WS motif)) with two binding domains (D1 and D2), a transmembrane domain and a cytoplasmic domain that includes Box 1 and Box 2. Long (L), short (S) and intermediate (I) isoforms have been characterized. However, other PRLR isoforms are originated from alternative splicing: ΔS1 isoform and short isoform 1a and 1b. Modified from Bernard V et al. (2015), New insights in prolactin: pathological implications, Nature Reviews Endocrinology 11, 265–275.

1.6 PRL signaling pathway

The initial step in PRL pathway activation involves structural changes in the EC following the binding of PRL to the PRL. These structural changes in the EC domain that are mediated by the ligand/receptor complex will also play a role in transmitting structural changes in the IC domain (Brooks 2012).

Indeed, PRL should bind to two PRLRs through two distinct biding sites present in PRL molecule (Broutin, Jomain et al. 2010, van Agthoven, Zhang et al. 2010). Initially, PRL will interact with PRLR through binding site 1 and this will result in the formation of the initial hormone-receptor complex. This step is needed for the consecutive interaction of site 2 of the same PRL molecule with another PRLR, leading to the formation of heterotrimeric complex (2 receptors, 1 hormone) (Sivaprasad, Canfield et al. 2004). As mentioned above, the intracellular domain of the PRLR lacks intrinsic kinase activity, and associated-protein kinases are used to transmit signals after ligand binding (Rillema, Campbell et al. 1992). Jak2, was found to be constitutively associated with PRLR in the proximal membrane region of the IC domain (Pezet, Buteau et al. 1997). Usually, after one minute of PRL binding to the PRLR, JAK2 auto phosphorylation is observed, as well as phosphorylation of the tyrosine residues of the PRLR itself (Lebrun, Ali et al. 1995) (Rui, Djeu et al. 1992). The tyrosine residues act as potential SH2 docking sites for STAT5 that is phosphorylated by the receptor-associated JAK2 kinase after docking with the receptor. This is followed by the dissociation of STAT5 from the receptor and the formation of

homodimers or heterodimers between two phosphorylated STAT molecules that then translocate into the nucleus bind specific DNA motifs and induce gene transcription (Rui, Djeu et al. 1992, Ihle 1996, Bole-Feysot, Goffin et al. 1998) (Figure 1.3).

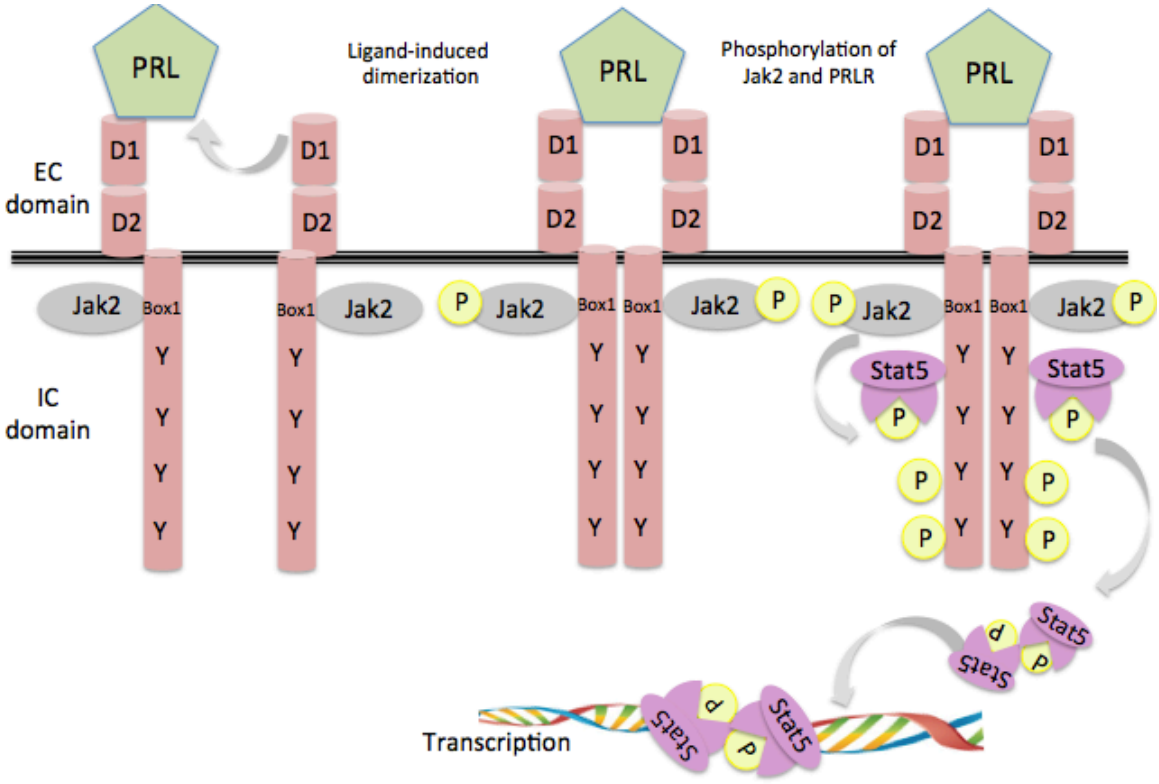

Figure 1.3 Mechanisms of prolactin receptor activation. When the hormone-receptor complex is formed, JAK2 auto phosphorylation is observed, as well as the tyrosine residues of the PRLR itself. These tyrosine residues act as potential SH2 docking sites for STAT5 that is phosphorylated by JAK2 after docking with the receptor. This is followed by the dissociation of STAT5 from the receptor and the formation of homodimers between two phosphorylated STAT molecules that then translocate into the nucleus and bind specific DNA motifs to induce gene transcription. Modified from Maria M (2016), The Role of Prolactin in Men, Endocrinol Metab Syndr 5:22

1.7 Biological functions of prolactin hormone:

PRL is known for its diverse biological functions, including growth and development, reproduction, metabolism, immune system modulation, osmoregulation and behavior. These are addition to its well-known role in mammary gland development and lactation (Bole-Feysot, Goffin et al. 1998, Goffin, Binart et al. 2002, Marano and Ben-Jonathan 2014).

Here we will mention some examples of these functions:

1.7.1 Water and electrolyte balance

Previous reports showed that PRL plays a role in the regulation of electrolyte balance promoting sodium, potassium and water accumulation. Also it is related to the control of the osmoregulation in fish through the regulation of water and salts balance in the gills (Ogawa, Yagasaki et al. 1973).

1.7.2 Immune response

PRL hormone plays a role in different aspects of the immune response, including its role in regulating both humoral and cellular immunity. This effect is observed in physiological as well as pathological conditions (Nagy and Berczi 1981, Berczi and Nagy 1982). This was supported by early reports that found that removal of the pituitary gland or suppression of PRL secretion was sufficient to reduce both humoral and cell mediated immunity (Nagy and Berczi 1978, Nagy, Berczi et al. 1983).

1.7.3 Ovary

The most important role of PRL in the ovary is on the luteal function. Its effect is variable and can be either luteotropic or luteolytic depending mainly on the stage of estrous cycle in different species of animals (Freeman, Kanyicska et al. 2000). In rodents, PRL plays a role in sustaining corpus luteum integrity 6 days after mating (Morishige and Rothchild 1974). In addition, it also plays a role in enhancing progesterone production by the luteal cells, which is crucial for the fertilized ovum implantation, inhibition of ovulation and pregnancy maintenance. This action is orchestrated with LH/hCG hormone (Cecim, Kerr et al. 1995). In addition, the luteolytic action is mainly mediated thought regulation of programmed cell death in corpus luteum. This PRL-induced program cell death is mainly mediated though CD3-positive lymphocytes which cause the activation of Fas/Fas ligand (FasL) system leading to apoptosis (Kuranaga, Kanuka et al. 2000). It is believed that this luteolytic action eliminates the old and non-functioning corpora lutea (Freeman, Kanyicska et al. 2000).

1.7.4 Reproductive behavior

The role of PRL hormone in regulating reproductive behavior includes a variety of aspects such as female receptivity and parental behavior. This role includes the regulation of gathering, nest building and taking care of offspring by their mother (Bridges, DiBiase et al. 1985, Bridges, Robertson et al. 1996, Lucas, Ormandy et al. 1998). Indeed, pup-induced maternal behavior was found significantly reduced in virgin and pregnant PRLR−/− or PRLR+/−mice. This

reduction of maternal behavior was also found to be associated with lactation failure (Bachelot and Binart 2007).

1.7.5 Mammary gland

The role of PRL hormone in the mammary gland is one of the best-studied and well-illustrated functions. This role includes mammary gland development, mammary epithelial cell differentiation, milk production and maintenance of milk secretion.

In mice, it was shown that the ductal system in newborn mice is composed of slow growing small ducts. These will persist until puberty, when the rate of the ductal growth is accelerated. After puberty and at the end of the duct tips, terminal end buds (TEBs) start to develop (Williams and Daniel 1983). Under the effect of estrogen and progesterone hormone, the duct system starts to invade the fat pad until they reach near the periphery of the fat pad. This is followed by the formation of more alveolar structures. With each estrous cycle more buds appear from the ducts, leading to the formation of a more branched ductal tree with secondary and tertiary ductal side branches (Oakes, Rogers et al. 2008).

After coitus, PRL hormone secretion is induced from the anterior pituitary gland. This helps in maintaining the progesterone hormone secretion from the ovaries (Freeman, Smith et al. 1974),(Terkel and Sawyer 1978). In addition, under the influence of PRL, it causes acceleration of the alveolar formation its cell differentiation and polarization (Neville, McFadden et al. 2002). All these

together, leads to the change of alveolar epithelium into secretory phenotype essential for milk production during lactation (Oakes, Rogers et al. 2008).

Additional modifications during the secretory phase activation are induced by progesterone hormone withdrawal, these include tight junctions closure and milk and lipid movement to the lumens of the alveoli (Neville, McFadden et al. 2002).

The role of PRL hormone in regulating mammary gland development was illustrated in a panel of reports using a group of mouse models with altered PRL signaling (Horseman, Zhao et al. 1997, Liu, Robinson et al. 1997, Ormandy, Binart et al. 1997, Wagner, Krempler et al. 2004). These reports showed that, while knockout of PRL or PRLR in adult homozygous females was sufficient to cause complete absence of the lobulo-alveolar units (Horseman, Zhao et al. 1997, Ormandy, Camus et al. 1997), PRL knockout heterozygotes showed only minimal changes compared to normal (Horseman, Zhao et al. 1997).

Additional reports showed that, while the levels of phosphorylated Stat5a and 5b are extremely low in virgin and early pregnancy mice, these levels are dramatically increased after 14 days of pregnancy. Moreover, high levels of phosphorylated Stat5 were also observed in functional postpartum gland (Liu, Robinson et al. 1996).

The important role of pituitary PRL in milk production and lactogenesis was illustrated when subsequent lactation was stopped following removal of pituitary gland during pregnancy. Moreover, homozygous PRL or PRLR knockout mice were unable to produce milk (Horseman, Zhao et al. 1997) Figure 1.4.

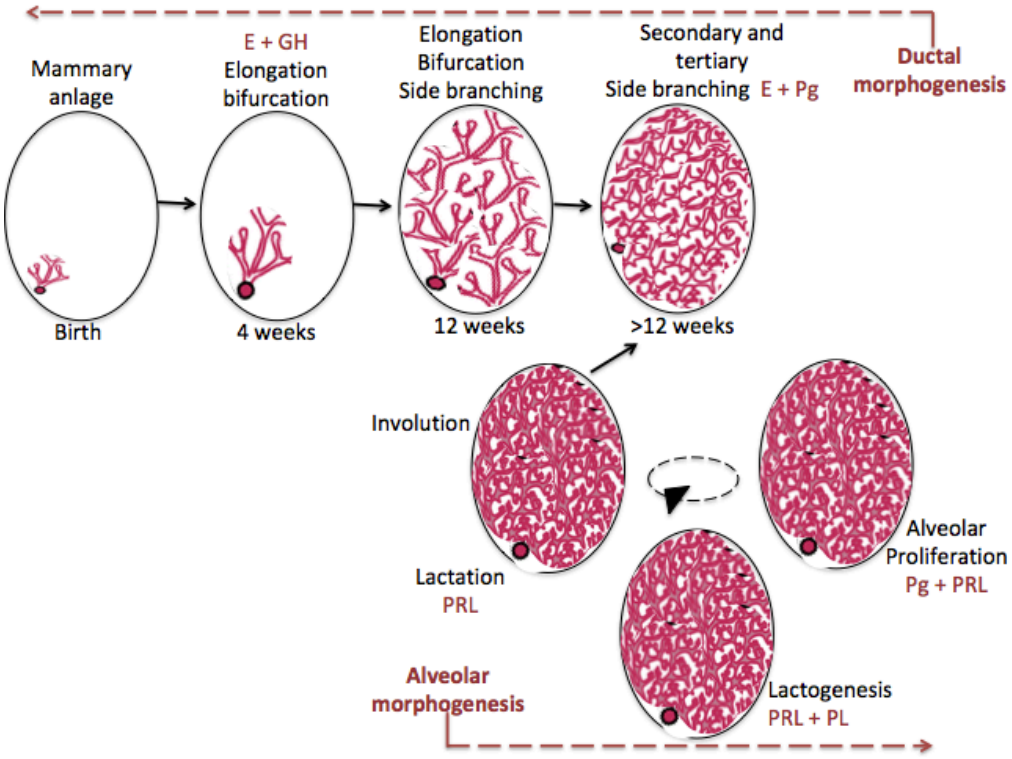

Figure 1.4 Prolactin regulation of mammary gland development. Different stages of mammary gland development in the adult mouse: birth, puberty, lactation and involution. Some of the important factors in these developmental processes are highlighted. E: estrogen, GH: growth hormone, Pg: progesterone, PRL: prolactin, PL: placental lactogen hormone. Modified from Watson C et al. (2008), Mammary development in the embryo and adult: a journey of morphogenesis and commitment. Development, 135: 995-1003.

1.7.6 Stem cell regulation

There are a limited number of reports highlighting the role of PRL in regulating adult tissue stem cells, however, recently, more findings suggest that PRL and its signaling pathway play a role in regulating stem populations in different tissues (Sackmann-Sala, Guidotti et al. 2015).

In breast tissue, it was shown that PRL orchestrates mammary gland differentiation in association with estrogens and progesterone (Hennighausen and Robinson 2005, Brisken and O'Malley 2010). This essential role was supported by results in PRLR knockout mice, which were unable to form functional alveolar structures during pregnancy despite the normal ductal system formation)Ormandy, Camus et al. 1997, Binart, Helloco et al. 2000(

During lactation, PRL was shown to play an important role in determination of luminal progenitor cells commitment into secretory cells (Lee and Ormandy 2012). Also, it was proposed, that the receptor activator of nuclear factor κ-B, Rank and its ligand (RankL) are produced by mature luminal cells under the effect of progesterone and PRL (Srivastava, Matsuda et al. 2003, Asselin-Labat, Vaillant et al. 2010). This causes the induction of ELF5 factor that mediates their alveolar differentiation during pregnancy (Rios, Fu et al. 2014).

Furthermore, PRLR is essential for the flux of stem/progenitor cells and its conversion into alveolar secretory lineage (Schramek, Leibbrandt et al. 2010).

The role of PRL in regulating stem cell differentiation was substantiated by a more recent report, which showed that PRL and its signaling pathway is essential for the induction of mature luminal cells. In addition, the same report

found that the PRL pathway also mediates the establishment of apical/basal polarity and basal/lateral localization of E-cadherin, as well as apical lipid droplet trafficking (Liu, Pawliwec et al. 2015). However, other reports showed an opposite finding with a suggestion that PRL might alter mammary epithelium hierarchy though increasing the progenitor cell populations (O'Leary, Shea et al. 2017). For that reason, more studies are needed to investigate the role of PRL in regulating stem cell populations.

1.8 Role of PRL in breast cancer

1.8.1 The tumor promoter role

1.8.1.1 Circulating PRL

Previous reports showed an association between breast cancer and PRL serum levels (Zumoff 1988), as well as higher risk of breast cancer development (Kwa, De Jong-Bakker et al. 1974). Moreover, a large prospective cohort study found that high circulating PRL was associated with increased breast cancer risk in general and in postmenopausal women (Hankinson, Willett et al. 1999) (Tikk et al., 2014)(Berinder, Akre et al. 2011). However, therapeutic options based on pharmacological inhibition of circulating PRL, including dopamine agonists like bromocriptine, have failed (Goffin 2017).

1.8.1.2 Autocrine/paracrine loop

As mentioned previously, PRL secretion is not restricted to the pituitary gland, but rather extra-pituitary PRL secretion is observed in a variety of tissues, including breast tissue (Nolin and Witorsch 1976, Steinmetz, Grant et al. 1993).

Some authors suggest that might be due to an autocrine/paracrine loop (Chen, Stairs et al. 2012). Moreover, it was proposed that this autocrine/paracrine loop might be increased in breast cancer and was attributed to breast tumorigenesis (Clevenger, Chang et al. 1995, Ginsburg and Vonderhaar 1995, Touraine, Martini et al. 1998). These findings are supported by a panel of *in vitro* studies, which demonstrate that the PRL pathway can activate mechanisms that lead to tumor progression (O'Leary, Shea et al. 2015). This includes the activation of breast cancer cell proliferation and survival pathways (Clevenger, Chang et al. 1995, Ginsburg and Vonderhaar 1995) and motility (Maus, Reilly et al. 1999). This notion was supported by other reports that found that PRLR antagonist can reverse this action leading to cell growth inhibition and even induction of apoptosis (Fuh and Wells 1995). Also, some reports hihglight that the PRL pathway can activate some of the oncogenic pathways including Ras-Raf-MAPK pathway (Clevenger, Torigoe et al. 1994) (Das and Vonderhaar 1996, Das and Vonderhaar 1996, Llovera, Pichard et al. 2000) as well as PI3K pathway (Acosta, Munoz et al. 2003, Chakravarti, Henry et al. 2005).

In addition, a panel of transgenic mice models showed that over expression of autocrine PRL shown to have higher incidence of mammary tumors (Rose-Hellekant, Arendt et al. 2003, Arendt, Rugowski et al. 2011).

However, this report also showed that the tumors have a long latency (>1year) period, indicating that PRL has a weak oncogenic power (Rose-Hellekant, Arendt et al. 2003). In contrast, the disruption or loss of PRL or PRLR was shown to be associated with delays in the mammary gland tumor formation (Vomachka, Pratt et al. 2000, Oakes, Robertson et al. 2007). In addition, Jak2 ablation in PRL-induced mammary cancer model was sufficient to prevent the initiation of PRL-induced mammary tumorigenesis (Sakamoto, Triplett et al. 2010).

1.8.1.3 PRLR overexpression

Another, important aspect that highlights the possible role of PRL signaling pathway in breast tumorigenesis is the studies using breast cancer cell lines (Kavarthapu and Dufau 2016) and breast cancer samples (Ormandy, Hall et al. 1997, Touraine, Martini et al. 1998). Some studies using radiolabelled PRL highlight a PRLR expression in 20-60% in breast cancer samples (Holdaway and Friesen 1977, Rae-Venter, Nemoto et al. 1981, Turcot-Lemay and Kelly 1982). This level was increased to 95-100% mRNA PRLR detection using either PCR or in situ hybridization in breast carcinomas samples (Ormandy, Hall et al. 1997, Touraine, Martini et al. 1998). Moreover, some reports linked the increased in PRLR expression to specific pathological subtypes of breast cancer, including ER and PR positive breast cancer tumors (Ormandy, Hall et al. 1997) as well as lobular carcinomas (Tran-Thanh, Arneson et al. 2011).

Some other studies were able to detect PRLR protein expression in breast cancer samples (Mertani, Garcia-Caballero et al. 1998). However, most of the antibodies used were found to lack specificity for the PRLR (Galsgaard, Rasmussen et al. 2009). In addition, a group of recent reports using more specific antibodies to detect PRLR found PRLR to be expressed in low or undetectable levels in breast cancer samples (Galsgaard, Rasmussen et al. 2009, Hachim, Hachim et al. 2016). For that reason, the notion of up-regulation of PRLR in breast cancer should be evaluated carefully.

1.8.2. PRL and PRLR as a therapeutic target

Due to the tumorigenic role of PRL and its signaling pathway, several attempts emerged to target this pathway in breast cancer using diferent agents.

1.8.2.1 Dopamine agonists

As mentioned previously, the main source of circulating PRL is the pituitary gland, for that reason the first attempt to inhibit the PRL pathway was using dopamine agonists (Freeman, Kanyicska et al. 2000). The efficiency of dopamine agonist such as bromocriptin, in reducing circulating PRL had already been proved in patients with hyperprolactinemia (Gillam, Molitch et al. 2006). Interestingly, several clinical trails showed that while these medications were able to reduce circulating PRL levels in breast cancer patients, they have also shown disappointing results regarding their anti-tumor activity (Bonneterre, Mauriac et al. 1988, Anderson, Ferguson et al. 1993). The failure of these trials

meant that the attention had switched to new modalities of PRL pathway antagonists based on targeting PRL and PRLR activation.

1.8.2.2 Inhibition of PRLR activation

These treatment modalities are based on targeting the PRL-PRLR autocrine/paracrine loop and are divided into PRLR competitive antagonists and anti-PRLR antibodies (Figure 1.5).

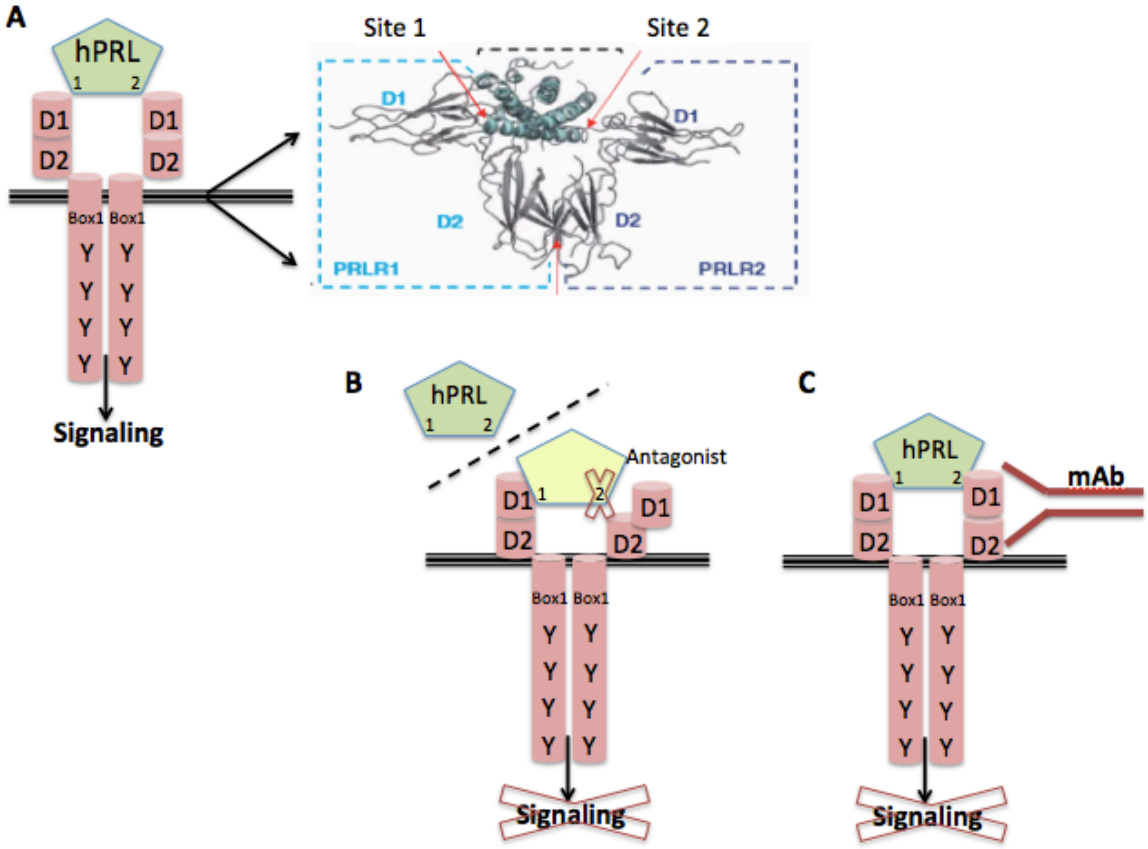

Figure 1.5 PRLR as a therapeutic target. A) The activation of PRLR induces the formation of a ternary complex involving one PRL moiety bound to a PRLR homodimers (PRLR1 and PRLR2). B) PRLR antagonist contains a mutation that avoids the proper interaction of binding site 2 with the PRLR. C) The PRLR neutralizing antibody binds to the PRLR in a non-competitive manner. Modified from Goffin and Touraine, (2015), Antagonistic properties of human prolactin analogs that show paradoxical agonistic activity in the Nb2 bioassay, Expert Opin Ther Targets.

PRLR competitive antagonists are mainly based on engineering human prolactin varients that will result in the formation of an incompetent ligand/receptor complex leading to an inhibiton of PRL signaling (Goffin, Kinet et al. 1996). Despite the fact that these compounds show some promising data, due to their short half life and decreased afinity attribited to mutations confering antagonism, they never reached to clinical trials (Goffin 2017). In contast, several PRLR neutralizing mAbs have been produced by different companies, and have been shown to be effective in inhibiting PRL signaling in preclinical models (Damiano, Rendahl et al. 2013, Otto, Sarnefalt et al. 2015). However, a recent clinical trial based on administration of (LFA102) mAb to patients with PRLR-positive advanced breast or prostatic cancer failed to provide any antitumor activity or clinical benefits (Agarwal, Machiels et al. 2016).

1.8.3 PRL as a tumor suppressor factor

As mentioned before, PRL hormone was found to play a vital role in inducing post-pubertal mammary gland development and terminal differentiation of the mammary epithelium (Horseman, Zhao et al. 1997, Lee and Ormandy 2012). For that reason, another point of veiw regarding the PRL role in breast cancer had also emerged, suggesting that PRLR signalling may act as a suppresor of breast cancer tumorigenesis. Recent studies have suggested that the PRL effects on cancer cells maybe similar to its physiological effects on mammary gland differentiation (Sultan, Xie et al. 2005, Nouhi, Chughtai et al. 2006).

Recently, using *in vitro* studies, PRL and its signaling pathway was found to be essential for inducing E-cadherin localization to the cell surface of luminal breast cancer cells (Sultan, Xie et al. 2005). This process is essential for increasing cell adhesion and is known to play an important role in suppressing tumor invasion. Also, reactivation of PRL/JAK2 signaling in the highly mesenchymal MDA-MB-231 breast cancer cell line was sufficient to reduce their mesenchymal and invasive properties. In comparison, suppression of this pathway in the epithelial-like T47D breast cancer cell line was sufficient to enhance their mesenchymal and invasive properties (Nouhi, Chughtai et al. 2006). Moreover, it was suggested that PRL can still play a pro-differentiation role in breast cancers cells through the suppression of stem CK5+ (basal-like) cells, believed to have stem cell-like properties and to be associated with chemotherapy resistance (Sato, Tran et al. 2014). This PRL effect was mediated though the suppression of the BCL6 oncogene (Sato, Tran et al. 2014).

In addition, the autocrine/paracrine loop which was proposed to be increased in breast cancer and used as an example of the tumor promotor role of PRL, was recently questioned and re-evaluated. Studies using cell lines and patient samples suggest that the PRL autocrine/paracrine loop is unlikely to be a cause of cell proliferation or tumorigenesis (Nitze, Galsgaard et al. 2013, Zhang, Cherifi et al. 2015).

More arguments supporting the protective role of PRL signalling comes from epidemiological studies that reported the loss of STAT5 activation during cancer progression (Nevalainen, Xie et al. 2004), Also the lower expression of

STAT5 in invasive carcinomas compared to well differentiated cancer was confirmed (Bratthauer, Strauss et al. 2006).

Taken together, these data suggest that the role of PRL pathway in breast tumorigenesis needs to be re evaluated.

1.8.4 PRL signaling pathway as a prognostic marker in breast cancer

Recent studies that examined the association between serum PRL levels and tumor characteristics, as well as patient outcomes, showed no clear association between patient outcome (Dowsett, McGarrick et al. 1983, Wang, Hampson et al. 1986, Wang, Stepniewska et al. 1995, Bhatavdekar, Patel et al. 2000) and tumor features like tumor size (Wang, Hampson et al. 1986, Arslan, Serdar et al. 2000), tumor stage (Mandala, Lissoni et al. 2002) , and lymph node involvement (Lissoni, Barni et al. 1995, Wang, Stepniewska et al. 1995). However, some reports showed an association between high prolactin serum levels, 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 overall survival (Wang, Stepniewska et al. 1995, Patel, Bhatavdekar et al. 1996, Bhatavdekar, Patel et al. 2000) as well as tumor recurrance (Wang, Stepniewska et al. 1995, Patel, Bhatavdekar et al. 1996). In contrast, other reports showed that PRL levels following surgical intervention were either non significant or to be associated with favorable patient outcome and prolonged survival (Lissoni, Barni et al. 1995, Mandala, Lissoni et al. 2002, Bignami, Lissoni et al. 2005).

The discovery of the proposed autocrine/paracrine PRL loop prompted investigation of the association between tissue expression of different PRL pathway members, tumor characteristics and its relation with patient outcomes.

In spite of the differernces in the expression levels of PRL and PRLR in these reports, many of them showed both PRL and PRLR expression to be associated with more favorable tumor characteristics, including well differentiated tumors, smaller tumor size and lymph node negative tumors. (Hachim, Hachim et al. 2016, Hachim, Shams et al. 2016) (Faupel-Badger, Duggan et al. 2014).

However, still other reports suggests that tumor expression of human PRL might be associated with worse patient outcome in both breast and endometrial carcinoma (Wu, Yang et al. 2011).

Moreover, it was also shown to be more highly expressed in the less aggressive non-TNBC subtypes compared with the TNBC breast cancer tumors (Hachim, Hachim et al. 2016). Indeed, loss of STAT5 activation or low expression of STAT5 was also shown to be associated with tumor progression as well as loss of differentiation and even antiestrogen therapy failure (Nevalainen, Xie et al. 2004, Bratthauer, Strauss et al. 2006, Peck, Witkiewicz et al. 2012). Off-note, more recent report showed that loss of the nuclear phosphorylated STAT5A and not STAT5B, to be associated with tumor progression and poor patient outcome (Peck, Witkiewicz et al. 2012). In addition, PRL , PRLR and STAT5 expression were shown to be associated with prolonged patient survival (Nevalainen, Xie et al. 2004, Hachim, Hachim et al. 2016, Hachim, Shams et al. 2016).

1.9 Breast cancer overview

Breast cancer is the most common cancer in females and the leading cause of their deaths worldwide (World Health Organization 2015). Breast cancer is a complex disease that comprises of various entities with different clinical, pathological and molecular profiles. Breast Cancer can be classified according to histopathological analysis or gene expression profiles. Histologically, tumours can be classified according to cancer cell growth patterns which undergo progressive development starting from hyperplasia to in situ, invasive and metastatic carcinoma (Simpson, Reis-Filho et al. 2005).

1.10 Histological classification of breast tumors

The tumors are classified as carcinoma in situ (CIS) or invasive/infiltrating breast carcinoma (IBC). CISs are considered to be pre-malignant lesions that remained in their normal location (ducts and lobes) in the mammary gland, in contrast to IBCs that have infiltrated outside of the ducts or lobes into the connective tissue with the potential to spread and cause metastasis (Cowell, Weigelt et al. 2013).

Ductal carcinoma in situ (DCIS) is considerably more common than lobular carcinoma in situ (LCIS) and comprises different histological types: comedo, cribiform, micro papillary, papillary and solid invasive carcinomas. On the other hand, Invasive breast carcinoma includes a group of several tumors, comprising invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC), tubular, apocrine, adenoid, mucinous (colloid), medullary, metaplastic and

papillary carcinomas. Invasive Ductal Carcinoma (IDC) is the most common histological subtype (75%), and can be sub-classified according to the levels of nuclear pleomorphism, glandular/tubule formation and mitotic index into well, moderately or poorly differentiated subtypes (Figure 1.6) (Li, Uribe et al. 2005, Malhotra, Zhao et al. 2010).

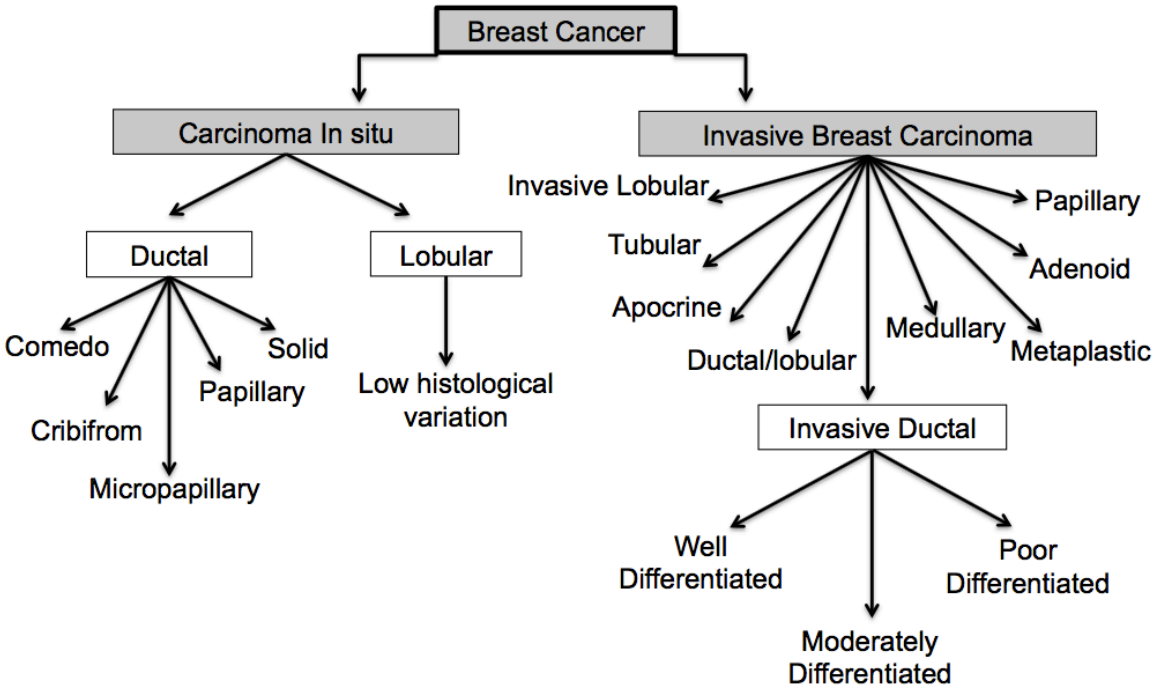

Figure 1.6 Histological types of Breast cancer. Breast cancer tumors are classified as carcinoma in situ or invasive breast carcinoma, each one with different intrinsic subtypes. Modified from Gautam K. Malhotra, et al., (2010), Histological, molecular and functional subtypes of breast cancers, Cancer Biology & Therapy*10: 10, 955-960*.

When dealing with invasive carcinomas, it is recommended to determine the status of classical immunohistochemistry (IHC) markers such as ER, PR, HER2 and the proliferation index (Ki67) (Harris, Fritsche et al. 2007), which helps in guiding clinical decisions. Together with the traditional clinico-pathological variables comprising of tumor size, tumor grade and lymph-node involvement, we can predict which patients are likely to respond to targeted therapies (Maughan, Lutterbie et al. 2010) (Payne, Bowen et al. 2008).

1.11 Molecular classification of breast tumors

Recent studies, used differential gene expression profiles from tumor samples using high-throughput microarray analysis (Perou, Sorlie et al. 2000) (Sorlie, Perou et al. 2001) to identify several molecular breast cancer subtypes. This was based on different clusters of intrinsic genes that reflected the phenotype of individual tumors and expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Notably, these molecular subtypes display the utility of predicting the patient clinical outcome and response to therapy (Parker, Mullins et al. 2009) (Hu, Fan et al. 2006).

These molecular subtypes, often referred as “intrinsic subtypes” include: Luminal A, Luminal B, HER2+, Normal-like and Basal-like (Dai, Li et al. 2015) Figure 1.7.

The Luminal A (50-60%) group is defined by positive ER, PRG expression. Usually, it contains low levels of proliferation related genes (ki67)

and represents low histological grades with a good outcome. The Luminal B (10-20%) group is ER, PRG positive and HER2 negative with high levels of proliferation related genes (ki67) or ER, PRG and HER2 positive expression. This group has a bad prognosis and a higher histological grade compared with the Luminal A group. HER2+ subgroup (15-20%) is characterized by overexpression of HER2 oncogene and is associated with high proliferation and aggressive behaviour (Sorlie, Perou et al. 2001) (Sorlie, Tibshirani et al. 2003) (Parker, Mullins et al. 2009). The normal breast-like group contains similar features to normal breast/adipose tissue and is enriched with genes of fibroadenoma. However, this group remains poorly described due to the possible contamination with normal tissue and its clinical significance has not yet being determined (Peppercorn, Perou et al. 2008).

The basal-like subtype (10-20%) is defined by the absence of ER, PRG and HER2 expression (triple negative phenotype) and the expression of basal cytokeratins CK5/6 and epidermal growth factor receptor (EGFR) (Rakha, Reis-Filho et al. 2008). Other genes usually found in normal breast basal/myoepithelial cells include basal cytokeratins CK14 and CK17; CD44 gene expression (Klingbeil, Natrajan et al. 2010), has also been associated to this group. The basal-like subtype is associated with high histological grade, and there is increasing evidence suggesting that the morphological features and molecular genetic profiles of this subtype are linked with tumors appearing in *BRCA1* germ-line mutation carriers (Turner and Reis-Filho 2006). Interestingly, BRCA1 gene silencing leads to down regulation of ER and up regulation of genes considered

markers of basal-like tumors, suggesting that BRCA1 dysfunction seems to be one of the drivers of basal-like tumors and of a subgroup of triple negative cancers (Gorski, James et al. 2010). In clinical practice basal-like and Triple Negative tumors are considered the same entity. However, they are not equivalent, despite the fact that 70% of basal-like tumors are Triple Negative tumors; there is 30% discordance between the two groups as previously described (Kreike, van Kouwenhove et al. 2007). Therefore, in order to identify both subtypes immunohistochemistry markers such as ER, PGR, HER2, EGFR and CK5/6 have been used as a gold standard for the identification of basal-like group tumors, while Triple Negative tumors will be negative for ER, PGR and HER2 (Nielsen, Hsu et al. 2004).

The Claudin-Low subgroup has recently been identified and represents 12-14% of all tumors. These tumors are associated with genes implicated with tight junctions and intracellular adhesion including Claudin 3, 4 and 7, cingulin, ocludin and E-cadherin. They are also enriched with cell proliferation and epithelial–mesenchymal transition genes and are associated with a cancer stem cell phenotype. This subgroup is closely related to basal-like tumors and is considered the breast cancer subgroup with the least favourable prognosis (Herschkowitz, Simin et al. 2007, Prat, Parker et al. 2010).

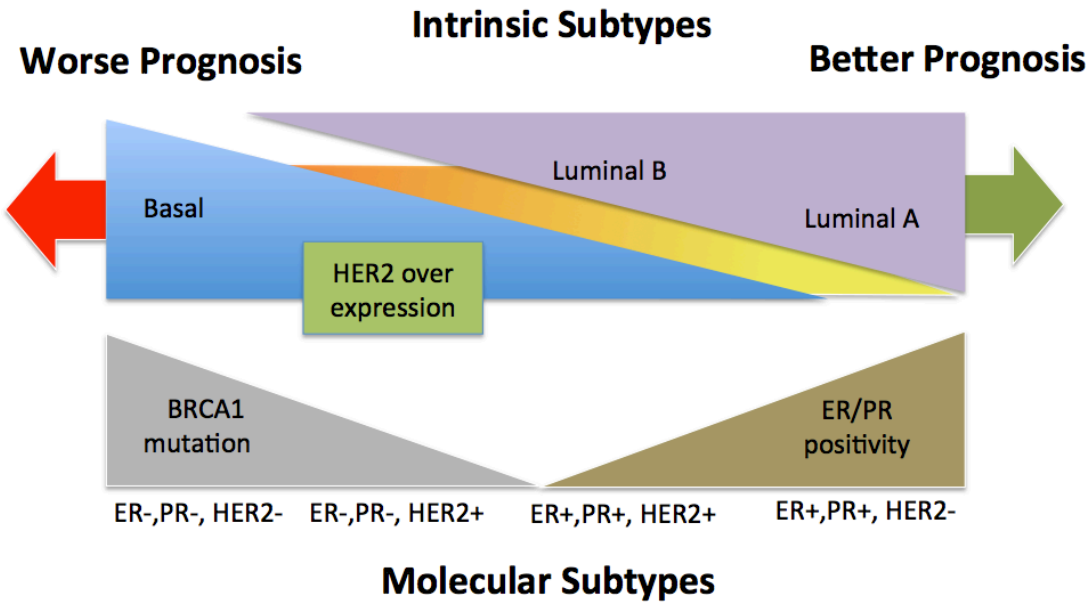

Figure 1.7 Patient outcomes based on breast tumor intrinsic subtypes. The intrinsic subtypes include: Luminal A, Luminal B, Her2 over expression and Basal, all display different clinical outcome and response to therapy. Modified from Xiaofeng Dai, et al. (2015), Breast cancer intrinsic subtype classification, clinical use and future trends, Am J Cancer Res 5(10): 2929-2943.

1.12 Gene profiling databases and breast cancer

In the past two decades, the discovery of microarray-based gene expression profiling has made the simultaneous analysis of thousands of genes in samples (Colombo, Milanezi et al. 2011). In breast cancer, this discovery has a wide group of applications. It has helped improve our understanding of cancer heterogeneity (Perou, Sorlie et al. 2000, Sorlie, Perou et al. 2001) and has lead to the discovery of more precise breast cancer molecular classifications (Curtis, Shah et al. 2012). In addition, it has led to the discovery of new markers and signatures that have either prognostic value or a potential therapeutic application (Perou, Sorlie et al. 2000, Sorlie, Perou et al. 2001, Wang, Klijn et al. 2005, Hess, Anderson et al. 2006, Saal, Johansson et al. 2007, Farmer, Bonnefoi et al. 2009).

The publically available databases allow researchers to investigate the expression levels of thousands of genes in large number of patient samples with sufficient clinical information (Colombo, Milanezi et al. 2011) to investigate the correlation between the gene expression levels and a wide variety of both clinical and pathological characteristics and patient outcome (Colombo, Milanezi et al. 2011, Ringner, Fredlund et al. 2011). The most commonly used data bases are the Gene Expression-Based Outcome for Breast Cancer Online and Breast Cancer Gene-Expression Miner v4.0.

1.12.1 GOBO: Gene Expression-Based Outcome for Breast Cancer Online: The GOBO database is a publically available and use a multifunctional user-friendly online tool that allows users to investigate the prognostic value of either a single gene or a group of genes in a dataset of 1881 breast cancer patients pooled from 11 public datasets (Ringner, Fredlund et al. 2011). In addition, the GSA-cell line application of this database allows the users to investigate the gene expression levels in 51 breast cancer cell lines, representing the different breast cancer subtypes (Neve, Chin et al. 2006). This database also allows rapid assessment between the gene expression levels and a panel of clinicopathological parameters including grade, molecular subtypes and ER status using the GSA- tumor application (Ringner, Fredlund et al. 2011).

Furthermore, using the same GSA-tumor application, the association between the gene expression levels and patient outcome using both distant metastasis free survival (DMFS) and relapse free survival (RFS) as endpoints are also available. Moerover, it allows investigation of the correlation with patient outcome in subgroups obtained from stratification of patients according to molecular subtypes, grade, ER status, LN involvement and tamoxifen treatment (Ringner, Fredlund et al. 2011).

1.12.2 Breast Cancer Gene-Expression Miner v4.0: This is another publicly available application that contains pooled data from more than 5000 breast cancer patients (Jezequel, Frenel et al. 2013). This application contains three modules: the expression, the prognostic and the correlation modules. The

expression modules allows the analysis of gene expression levels of the selected gene and different clincipathological parameters including age, grade and molecular subtypes. In comparison, the prognostic modules allows the investigator to assess the correlation between gene expression levels and patient outcome represented as metastatic free survial (MFS) or any event free survival (AEFS) (Jezequel, Frenel et al. 2013). In addition, the assessment of the correlation between gene expression levels and patient outcome in different breast cancer subgroups based on LN status, estrogen receptor status and molecular subtype is also available. A third module called the correlation module was added. This module gives the ability to investigate the correlation coefficient between two or more genes in all patient samples or in different molecular subtypes (Jezequel, Frenel et al. 2013).

1.13 Triple Negative Breast Cancer

Triple Negative Breast Cancer (TNBC) accounts for about 15% of all breast cancer cases and is defined using immunohistochemistry techniques by the lack of estrogen receptor (ER), progesterone receptor (PRG), and the human epidermal growth factor receptor 2 (HER2) (Carey, Perou et al. 2006).

The risk of developing TNBC varies with age, race, genetics, breastfeeding patterns, and parity. Several population-based studies have shown that TNBC has an onset at a young age (<50 years old), is frequently related to African–American ethnicity (Lund, Trivers et al. 2009) and has a well-established association to the hereditary breast cancer and BRCA-1 mutation status

(Foulkes, Stefansson et al. 2003). Interestingly, parity and young age at first full-term pregnancy increase the risk of developing TNBC compared with other breast cancer subtypes (Millikan, Newman et al. 2008). On the contrary, a longer duration of breastfeeding and an increasing number of children breastfed reduce the risk of developing TNBC (ElShamy 2016, Ma, Ursin et al. 2017). TNBC is a heterogeneous disease characterized by its distinctly aggressive biology and currently represents a challenge due to the lack of targeted therapies. From the clinico-pathological point of view TNBC represent the most aggressive tumors with the highest rates of recurrence and shorter overall survival in patients compared with other breast cancer subtypes. Cytotoxic chemotherapy still remains the mainstay in the treatment of TNBC patients, however these treatment approaches showed limited benefits, mostly due to toxic effects, resistance and tumor relapse (Foulkes, Smith et al. 2010).

1.14 Molecular stratification of TNBC

To better understand the complexity of the disease as well as the identification of molecular drivers that can be therapeutically targeted, TNBC has been classified into six different subgroups. Using gene expression profiling analysis, the two basal-like groups (BL1 and BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM) and luminal androgen receptor (LAR) were categorized (Lehmann, Bauer et al. 2011).

The Basal Like1 subgroup is enriched in cell proliferation, cell cycle components and DNA damage related genes, and the BRCA1 mutation has also

been associated with this group. The Basal Like 2 subtype is more associated with epidermal growth factor signaling (EGF) and exhibits mutations in PTEN gene. The Immunomodulatory subtype is related with lymphocytic infiltration, immune cell signaling, cytokine signaling, antigen processing and presentation, and signaling through core immune signal transduction pathways (NFKB, TNF, and JAK/STAT signaling). The Mesenchymal subgroup is associated with cell motility, cell differentiation pathways and epithelial-mesenchymal transition–associated genes, while the Mesenchymal stem-like subtype expresses low levels of proliferation genes and is highly enriched with stem cells. This subtype comprises of the highly aggressive Claudin-low subgroup. Finally the Luminal Androgen Receptor subgroup represents a different entity among TNBC subtypes. This subgroup represents 10% of total TNBCs. It displays luminal gene expression patterns with apocrine histology, is enriched in androgen receptor signaling and exhibits a high frequency of PI3K mutations.

This gene profiling analysis also describes that most of TNBCs are classified as basal-like tumors. The BL1 subtype correlates strongly with intrinsic molecular classification (85%). However, the BL2, IM, and M subtypes only moderately correlated to the basal-like molecular tumors by 31%, 58%, and 47%, respectively. The majority of LAR was grouped as either luminal A or luminal B (82%), confirming its luminal-like phenotype. Consequently, the majority of TNBCs exhibit a basal-like phenotype by IHC, while only around half correlate to the basal-like intrinsic gene set (Lehmann, Bauer et al. 2011) (Figure 1.8).

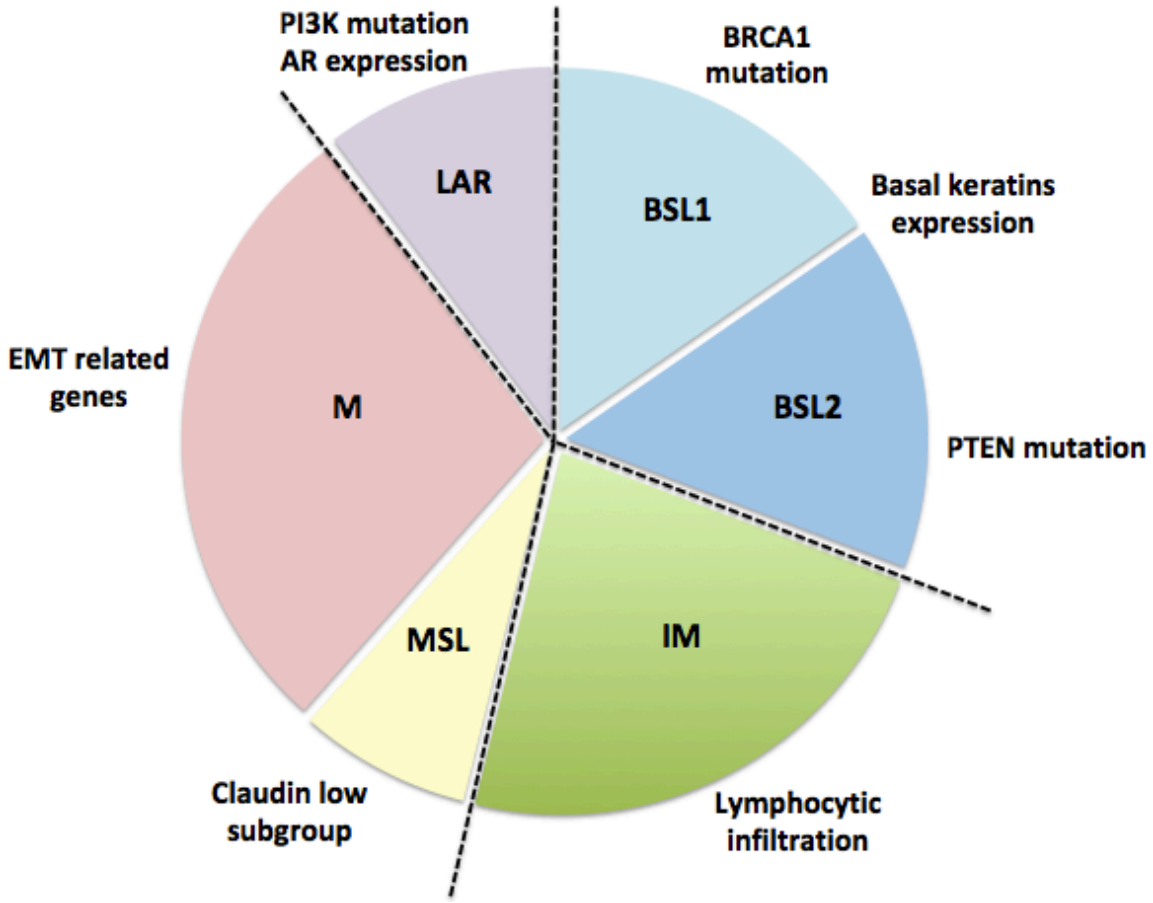

Figure 1.8 TNBC gene expression subtypes are associated with distinct molecular features. Graph depicting TNBC subtypes: BL1, basal-like 1; BL2, basal-like 2; IM, immunomodulatory; ML, mesenchymal-like; MSL, mesenchymal stem-like and LAR, luminal androgen receptor. Modified from Nicholas C. Turner, et al. (2013), Tackling the Diversity of Triple-Negative Breast Cancer, Clin Cancer Res; 19(23).

1.15 Genetics Drivers and clinical implications in TNBC

Many studies have addressed the genetics of TNBC in order to elucidate its aggressive biology. TP53 is the most common mutation occurring in 80% of these tumors. PTEN mutations and/or deletions are described in approximately 10% of TNBCs and are more predominant in the BSL2 subtype (Shah, Roth et al. 2012). PIK3CA mutations are found in 7-10% of TNBC tumors (Stemke-Hale, Gonzalez-Angulo et al. 2008) and are predominantly high in the LAR subgroup (Gonzalez-Angulo, Stemke-Hale et al. 2009). While the mutation or loss of RB1 is found in 20% of TNBC tumors, (Cancer Genome Atlas 2012) BRCA1 mutation is predominant in ~80% of the TNBC phenotype and is associated with the BSL1 subgroup (Lehmann, Bauer et al. 2011, Turner and Reis-Filho 2013).

The identification of these genetics drivers may help in finding molecular targets that will be critical to improve the survival in TNBC patients. Recent studies describe how the predominant gene expression signaling components per TNBC subgroup can be used to predict the sensitivity to targeted therapeutic agents using preclinical models (cell lines representatives of each subtype) as shown in Table 1.1.

TNBC subtype	Cell line Representative	Gene Category	Therapeutic targets/ Drugs
BSL1	HCC2157, HCC1599, HCC1937, HCC1143, HCC3153, HCC38, MDA-MB-468	DNA damage response and cell proliferation, BRCA1	Cisplatin, PARP inhibitors
BSL2	SUM149PT, CAL-851, HCC70, HCC1806, HDQ-P1	TP53, EGFR, MET signaling	mTOR, growth factor inhibitors
IM	HCC1187, DU4475	Immune Signaling	Cisplatin, PARP inhibitors
M	BT_549, CAL_51, CAL_120	EMT, Wnt, TGFβ, IG1FR, Notch, cell proliferation	mTOR, growth factor inhibitors, Src inhibitors
MSL	HS578T, MDA_MB_157, SUM159PT, MDA_MB_436, MDA_MB_231	EMT, Wnt, TGFβ, MAPK, Rac, PI3K, PDGF	mTOR, PI3K, MEK and growth factor inhibitors
LAR	MDA_MB_453, SUM185PE, HCC2185, CAL-148, MFM-223	AR signalling, FOXA1, PI3K	AR antagonist, PI3K inhibitors
UNCLASSIFIED	HCC1395	DNA damage response and cell proliferation	Cisplatin, PARP inhibitors

Table 1.1 Differential gene expression in TNBC subtypes with preclinical models and its pharmacological targetable drivers. Table describing the differential gene expression, the pharmacological agents and the cell lines that represent each TNBC subtype. Modified from Vandana G. Abramson et al., (2015) & Lehmann B, et al., (2011).

As TNBCs are clinically heterogeneous, different molecular subsets were proposed to categorize TNBC into more precise subgroups with different clinical outcome. This classification was based on a group of metagenes that not only reflect the distinct cancer cell origin but also reflect the non-neoplastic constituents of the tumor microenvironment. 16 metagenes were identified (Table 1.2), including a basal-like phenotype, apocrine/androgen receptor signaling signature, five signatures related to different types of immune cells (B-Cell, T-Cell, MHC class II, MHC class I and Interferon response), a stromal signature, the claudin-CD24 signature, markers of blood and adipocytes, an inflammatory signature (IL-8, CXCL1 and CXCL2) and an angiogenesis signature (associated with poor prognosis) (Rody, Karn et al. 2011). This study showed that the basal-like metagene had no significant effect on survival compared with the B-Cell and IL-8 metagenes. Patients with high expression of B-Cell and low expression of IL-8 metagenes have a significantly better prognosis (32%) and 5-year event-free survival compared with other TNBC patients, independent of histological grade. Decreased outcome was observed with high expression of angiogenesis (VEGF) and histone-related metagenes. The observations mentioned above are important due to the possible therapeutic interventions such as the inhibition of the IL-8 pathway or the activation of the immune system in the tumor microenvironment that could benefit patients with TNBC (Rody, Karn et al. 2011).

Biological component	Metagene name	Key markers
Basal-like phenotype	Basal-like	KRT-5,6, 14, 17, SOX10, SERP1, ELF5, EPHB3, GABRP
Apocrine/androgen receptor signalling	Apocrine	AR, FOXA1
Immune system		
• B-Cell	B-Cell	IgG
• T-Cell	T-Cell	TCR, LCK, ITK
• MHC class II	MHC-2	HLA-DR, -DM, -DP, -DQ
• MHC class I	MHC-1	HLA-A, -B, -C, -E, -F, -G
• Interferon response	IFN	OAS1, OAS2, OAS3, MX1
Stroma	Stroma	Decorin, Osteonectin, Fibronectin, COL5A1
Claudin-CD24 signature	Claudin-CD24	CLDN3, CLDN4, CD24, ELF3
Proliferation	Proliferation	BUB1, CDC2, STK6, BIRC5, TOP2A,
Blood	Haemoglobin	HBA1, HBA2, HBB
Adipocytes	Adipocyte	FABP4, PLIN, ADIPOQ, ADH1B
Angiogenesis	VEGF	VEGF, Adrenomedullin, ANGPTL4
Inflammation	IL-8	IL-8, CXCL1, CXCL2
HOXA gene cluster	HOXA	HOXA-4, -5, -7, -9, -10, -11
Histone gene cluster	Histone	Histones H2A, H2B

Table 1.2 Metagenes as representatives of each TNBC subtype and non-neoplastic constituents of the tumor microenvironment. Modified from Rody Kam, et al., 2011, PLoS One 6(12) e28403.

More recently, using mRNA expression and DNA profiling deep analysis from independent TNBC datasets, four TNBC stable subtypes with distinct clinical outcome were identified: luminal AR (LAR), Mesenchymal (MES), Basal-like immunosuppressed (BLIS), and Basal-like immune-activated (BLIA). These subtypes identify specific molecules per subgroup serving as biomarkers and potential targets (Burstein, Tsimelzon et al. 2015).

Subtype 1 designated as “LAR” in previous studies, is characterized by expression of AR, ER, prolactin, and ErbB4 signaling. Estrogen-regulated genes (PGR, FOXA, XBP1, GATA3) are also expressed in spite of its ER IHC negativity, due to the 1% ER protein levels. Also DHRS2, AGR2, FOXA1, CA12, AR, TOX3, KRT18, MUC1, PGR, ERBB3, RET, and ITGB5 are overexpressed. This finding suggests that this subtype may respond to anti-estrogen and anti-androgen treatment as well as MUC1 vaccines. Subtype 2 “MES” (designated as MSL/Claudin-low in previous studies) is characterized by up regulation of cell cycle, mismatch repair, DNA damage networks and IGF1 signalling. Genes including ADH1B, ADIPOQ, OGN, FABP4, CD36, NTRK2, EDNRB, GHR, ADRA2A, PLA2G2A, PPARG, ADRB2, PTGER3, IL1R1, and TEK are overexpressed. Therefore, β-blockers and IGF or PDGFR inhibitors, may be useful therapies for this subtype. Subtype 3 “BLIS”, comprise of the previously described subtypes BSL1 and BL2 and is enriched with SOX family transcription factors. All immune-regulated pathways are down regulated in this subgroup and on the other hand ELF5, HORMAD1, SOX10, SERPINB5, FOXC1, SOX8, TUBB2B, VTCN1, SOX6, KIT, and FGFR2 are up regulated. For that reason,

immune-based therapy can be useful. This subtype has the worst prognosis. Subtype 4 "BLIA" (previously described as the IM subtype) is characterized by up regulation of immune related genes and activation of STAT genes as well as CXCL9, IDO1, CXCL11, RARRES1, GBP5, CXCL10, CXCL13, LAMP3, STAT1, PSMB9, CD2, CTLA4, TOP2A and LCK. This suggests that STAT and cytokine inhibitors, cytokine receptor antibodies, or the CTLA4 inhibitor; ipilumimab can be useful in the treatment of this subtype. This subtype is considered to have best prognosis amongst all of them (Burstein, Tsimelzon et al. 2015).

Altogether, these studies highlight the importance of elucidating prognostic and therapeutic implications as well as more precise and clinically relevant TNBC subgroups. Therefore, development of specific prognostic biomarkers and new-targeted therapies are needed.

1.16 Key features of Triple Negative Breast Cancer

1.16.1 Cancer Stem cells (transcription factors and CSC markers)

TNBC represents poorly differentiated and high-grade tumors. This subtype is characterized by being highly enriched with cancer stem cells (CSCs) that confer the aggressive behavior and lead to chemo/radio therapy resistance, early relapse and metastasis (Anders and Carey 2009) (Phillips, McBride et al. 2006) (Kim, Joo et al. 2009).

CSC, also known as breast tumor-initiating cells, are a small subpopulation in the tumors with the ability of self-renew, differentiate and reconstitute a new tumor (Luo, Brooks et al. 2015). These CSCs are highly regulated by a complex

network of transcription factors in charge of maintaining its self-renewal capacity, pluripotent stage and cell fate. OCT4, NANOG and SOX2 are the most important transcription factors linked to these processes and have been related to several malignancies when they are deregulated or overexpressed (Ben-Porath, Thomson et al. 2008). OCT4 is critically involved in the self-renewal of undifferentiated embryonic stem cells (Niwa, Miyazaki et al. 2000). It forms a heterodimer with Sox2 and together they regulate Nanog by binding to its promoter (Rodda, Chew et al. 2005). Sox2 has also been shown to be a driver of the basal-like phenotype in sporadic breast cancers (Rodriguez-Pinilla, Sarrio et al. 2007). Nanog is the key factor in maintaining pluripotency and is normally expressed in embryonic stem cells and absent in adult tissues (Gawlik-Rzemieniewska and Bednarek 2016). Furthermore, recent studies suggest Nanog plays a role in cell cycle progression in breast cancer. Induction of Oct-4 and Nanog over-expression enhanced the invasiveness of CSCs, while knockdown of both Oct-4 and Nanog inhibited the migration of CSCs *in vitro* suggesting that Oct-4 and Nanog positively regulate the epithelial mesenchymal transition process (EMT), contributing to breast cancer metastasis (Wang, Lu et al. 2014). Recent findings have shown that Nanog is overexpressed in poorly differentiated breast cancers and correlate with poor prognosis in TNBC patients (Nagata, Shimada et al. 2014).

Another feature of CSCs is that they can be recognized by the expression of different markers such as CD44, CD24 and ALDH1 (Al-Hajj, Wicha et al. 2003, Ginestier, Hur et al. 2007). CD44 is a trans-membrane glycoprotein receptor for

hyaluronic acid and many other extracellular matrix components, as well as a cofactor for growth factors and cytokines. It is involved in adhesion and has been associated with tumor dissemination (Paulis, Huijbers et al. 2015). Recently a CD44-targeted monoclonal antibody photosensitizer conjugate for photo immunotherapy was developed against TNBC. This CD44-targeted conjugate demonstrated efficient elimination of CD44 positive cells following inhibition of tumor growth *in vivo* in TNBC xenograft (Jin, Krishnamachary et al. 2016).

CD24 is a surface glycoprotein that acts as a signal transducer. CD24 positive cells characterize more differentiated luminal epithelial cells. Its evaluation as a prognostic marker in breast cancer has always been in combination with CD44 expression referred as the $CD44^+/CD24^-$ phenotype (Kim, Kim et al. 2011). This highly tumorigenic $CD44^+/CD24^-$ subpopulation was shown to be able to form tumors when as few as 100 cells were injected into immune deficient mice (Al-Hajj, Wicha et al. 2003).

Gene profiling analysis of this subpopulation revealed that was associated with genes related with poor outcome, dissemination and resistance to therapy (enriched with cell cycle, calcium binding, chemotaxis, differentiation, ubiquitinization related genes and high levels of IL-1, IL-6, urokinase plasminogen activator was observed) (Paula A and Lopes 2017).

The expression of CD44 seems to be crucial among the $CD44^+/CD24^-$ subpopulation. There is evidence that knocking down its levels in combination with chemotherapy (doxorubicin) reveals promising results for eradicating its highly tumorigenic subpopulation via a decrease in cell proliferation and increase

in apoptosis (Van Phuc, Nhan et al. 2011). Likewise, knock out of CD44 was shown to cause differentiation into non-BCSCs with lower tumorigenic potential *in vivo* and a decrease of stem cell-related genes (Muc-1, MMP9, 7; Myc, cyclin D1, Bcl-2, LEF1, members of Wnt, hedgehog and PI3K signaling, TP53). The observations mentioned above open a new direction in treating breast cancer through 2 different approaches: gene therapy in combination with standard chemotherapy and the promising differentiation therapy (Pham, Phan et al. 2011).

The $CD44^+/CD24^-$ subpopulation was found to be highly expressed in TNBC (84%), driving its aggressive phenotype and poor prognosis (Honeth, Bendahl et al. 2008) (Buess, Rajski et al. 2009). The biological roles of $CD44^+/CD24^-$ cells in TNBC needs to be further clarified. However, sorting the $CD44^+/CD24^-$ population from TNBC cells revealed a higher capacity for proliferation, migration, invasion and tumorigenicity as well as lower adhesion ability than non $CD44^+/CD24^-$ cells (Ma, Li et al. 2014).

Furthermore, the $CD44^+/CD24^-$ subpopulation has shown to have a higher rate of proliferation and was found to be associated with a high risk of recurrence and mortality in TNBC patients(Wang, Wang et al. 2017). These observations also highlight that TNBC cells contain more cancer stem/progenitor cells with high Ki-67 proliferation index associated with poor outcome (Idowu, Kmieciak et al. 2012).

Aldehyde dehydrogenase (ALDH) is an enzyme critical for the detoxification of endogenous and exogenous aldehydes and converts retinol into retinoic acid. There are 19 ALDH genes. However, ALDH1 is considered a marker of cancer stem cells involved in their self-renewal and differentiation and is associated with poor prognosis. There are three main isotypes, ALDH1A1, ALDH1A2, and ALDH1A3 (Tomita, Tanaka et al. 2016). ALDH1A1 has been correlated with higher tumor grades, the development of metastasis and poorer patient outcomes (Ginestier, Hur et al. 2007) (Charafe-Jauffret, Ginestier et al. 2010). As few as 500-1000 ALDH+ cells have been shown to form tumors *in vivo* (Charafe-Jauffret, Ginestier et al. 2009).

Recently, the ALDH1A3 isotype was associated with higher grade/stage and breast metastatic disease (Marcato, Dean et al. 2011). Furthermore, $ALDH1^+$ phenotype was found to be associated with biological aggressiveness (tumor size/stage) and poor outcomes for TNBC patients (Ma, Li et al. 2017).

The combination of CD44, CD24 and ALDH1 is not commonly used as cancer stem cell marker in breast cancer. However it has been suggested that high ALDH activity and CSC marker expression ($ALDH^+/CD44^+/CD24^-$) enhanced malignant and metastatic properties of breast cancer stem cells as well as primary tumour growth. This stem cell subpopulation represents less than 1% of the total cancer cell population. However, its phenotype appears to be highly tumorigenic with the ability to generate tumors from as few as 20 cells (Croker, Goodale et al. 2009) (Ginestier, Hur et al. 2007).

1.17 Prognostic and Predictive Markers in Triple Negative Breast Cancer

TNBC is the subtype with the highest recurrence rate, frequency of metastasis and worst survival amongst breast cancer. Nowadays, the number of cancer-related parameters available to predict prognosis of TNBC patients has grown significantly. Clinico-pathological features have been used to estimate prognosis, including histological type and grade, lympho-vascular invasion, tumor size, lymph-node involvement and patient age (Zhou, Li et al. 2013).

Strong efforts have been made to develop biomarkers that provide not only prognostic but also predictive information in TNBC patients. Among them, some of the most promising markers are EGFR, Ki-67 expression, BRCA1 mutation, tumor-Infiltrating lymphocytes (TILs) Androgen receptor (AR) and PI3K pathway. Recent studies have suggested that Epidermal growth factor receptor (EGFR) is frequently overexpressed in TNBC tumors (50%). Moreover, patients with EGFR-positive TNBC had a significantly less favorable prognosis and a poorer response to neo-adjuvant chemotherapy than patients with EGFR- negative TNBC. These findings suggested a potential role of EGFR-targeted therapy in TNBC (Corkery, Crown et al. 2009, Nogi, Kobayashi et al. 2009).

The nuclear protein, Ki67 has been shown to be related to higher histologic grade, size, positive lymph-nodes status and short overall survival in breast cancer (Rakha, El-Sayed et al. 2007). Furthermore, several studies showed a positive correlation between Ki67 expression and pathologic tumor response to neoadjuvant chemotherapy in breast cancer (Jones, Salter et al. 2009) as well as in TNBC (Keam, Im et al. 2007). Ki67 has been demonstrated to be a predictor

for poor prognosis, and TNBC patients with high Ki67 expression seem to display resistance to anthracycline-based chemotherapy (Abubakar, Orr et al. 2016).

Previous studies showed the positive correlation between TNBC patients and BRCA1 mutation (~80%). Recently the positive correlation between BRCA1 mutation and decrease in the risk of distant metastasis and mortality in TNBC patients as demonstrated (Maksimenko, Irmejs et al. 2014). Moreover, this provide an opportunity to development of targeted therapies that has shown an improvement of outcome in TNBC- BRCA1 carriers (Anders, Winer et al. 2010).
The prognostic and predictive roles of tumor infiltrating lymphocytes (TILs) have been studied in BC. TILs were described to be predominant in basal-like tumors (Livasy, Karaca et al. 2006). Recently, the positive correlation between TILs and better outcome has been described in TNBC patients (Adams, Gray et al. 2014, Ibrahim, Al-Foheidi et al. 2014).

Moreover, some studies suggest that tumor infiltration by CD8 cytotoxic lymphocytes and absence of FOXP3 immunosuppressive regulatory cells could control tumor growth and carry a better prognosis (Miyashita, Sasano et al. 2015).

AR is a steroid hormone receptor, considered as prognostic/predictive marker. AR is expressed in 70% of all breast cancers subtypes (Gucalp and Traina 2010) including in ~30% of TNBCs. While, low expression of AR is related to metastasis in TNBCs (Sutton, Cao et al. 2012), AR-negativity correlates with poor patient outcomes (disease-free survival and overall survival) (Tang, Xu et al. 2012). Moreover, it is shown that inhibition of AR in TNBC patients could stabilize

the disease and offers an alternative route to improve patient survival (Gucalp, Tolaney et al. 2013). On the other hand, phosphorylation of AR at either ser-515 or ser-81 can serve as a surrogate for AR activation and potential targets for therapeutic intervention (Roseweir, McCall et al. 2017).

From the clinical point of view, the presence of PI3K mutations has a favorable prognostic value for hormone receptor-positive breast cancer patients (Kalinsky, Jacks et al. 2009, Pang, Cheng et al. 2014, Zardavas, Phillips et al. 2014). However, there have only been a few reports about the clinical implications of PI3K mutations in TNBC patients. For TNBC patients, AR and AR phosphorylation dependent on PI3K/Akt signaling pathway (pAR) are known as independent favorable prognostic markers (Takeshita, Omoto et al. 2013). Recently, it was shown that the presence of PIK3CA major mutations of cDNA could be a discriminatory predictor of relapse-free survival and breast cancer-specific survival. This is supported by the use of PI3K inhibitors as a combination therapy with AR inhibitors that are currently under investigation in TNBC patients (Takeshita, Yamamoto et al. 2015).

1.18 Current Therapies available for TNBC

Nowadays, no effective specific targeted therapy is readily available for TNBC. Patients do not benefit from hormonal or trastuzumab-based therapy because of the loss of target receptors (ER, PGR, and HER2). Therefore, surgery, radiotherapy and chemotherapy, individually or in combination, are the

only available modalities. However, some receptors/molecules have been identified as new therapeutic targets.

Chemotherapy remains the mainstay for TNBC patients in the neo-adjuvant, adjuvant or metastatic settings. Despite the aggressive clinical behavior of TNBC, ~30–40% of the patients that achieve pathological complete response (PCR) after neo-adjuvant chemotherapy will display high rates of survival. However, patients with residual disease after neo-adjuvant therapy are more likely to recur and die from metastatic disease (Liedtke, Mazouni et al. 2008, Masuda, Baggerly et al. 2013). This clinical behavior is due to the heterogeneity of the different TNBC subsets and the diversity of their biological responses to different treatments and targeted therapies.

1.18.1 Platinum agents: Carboplatin and cisplatin are the most common platinum salts used in the therapy. These salts bind directly to DNA, resulting in formation of DNA-platinum adducts that impede cell division and will lead to cell apoptosis. Interest in platinum-based chemotherapy in breast cancer has been renewed, based on the hypothesis that these agents are more effective in TNBC patients with BRCA-mutant (Tian, Zhong et al. 2015).

1.18.2 Non-platinum-based regimens: Anthracycline, Doxorubicin and taxanes are the most common agents in this category. While, anthracyclines are intercalating they are one of the most used drug classes in breast cancer. However, the use of these agents is often limited to the metastatic setting of the disease due to the cumulative dose levels from earlier adjuvant chemotherapy

that will increase the risk of toxicity. Therefore, its use is reserved for anthracycline-naive patients. In TNBC patients Anthracycline-containing regimens are effective in the neo-adjuvant setting (Liedtke, Mazouni et al. 2008). The studies performed with taxane-based chemotherapy proved their efficacy in TNBC treatment in any setting of the disease (neo-adjuvant, adjuvant, and metastatic). The use of taxanes is recommended as a first-line therapeutic option for TNBC (Mustacchi and De Laurentiis 2015). Recently, Nab-paclitaxel, an albumin-bound nanoparticle formulation of paclitaxel has enabled higher intra-tumoral concentrations and is demonstrated to be more effective and less toxic than conventional taxanes in metastatic TNBC (Schettini, Giuliano et al. 2016).

1.19 Targeted Therapies:

1.19.1 Poly(ADP-Ribose) polymerase inhibitors (PARP): PARP, a poly ADP-ribose polymerase, is a member of a family of enzymes that facilitates DNA repair by removing damaged and/or incorrect DNA sequences by different excision repair pathways. In patients with BRCA1/2 mutation, PARP inhibition induces a process referred to as “synthetic lethality” by accumulation of single strand breaks in BRCA-mutated cancers that lead to cell death. Currently, several PARP inhibitors such as olaparib, veliparib, rucaparib, niraparib, talazoparib (BMN673) and others are undergoing clinical development mainly in TNBC (Tutt, Robson et al. 2010, Dwadasi 2014).

1.19.2 EGFR inhibition: To date, 6 phase II clinical trials to investigate the efficacy and safety of anti-EGFR mAbs in TNBC patients have been reported.

Many studies using EGFR inhibitors have shown improvements in response rate as well progression-free survival (PFS) in metastatic TNBC patients using them in combination with other agents (~10%) (Carey, Rugo et al. 2012, Tredan, Campone et al. 2015).

1.19.3 AR inhibitors (Bicalutamide, Enzalutamide) / PI3K inhibitors: These agents are commonly used for LAR subtype cancers. The androgen-blocking agents were found to stabilize the disease in ~19% of TNBC patients (Gucalp, Tolaney et al. 2013). Moreover, the LAR subgroup is also characterized by expression of PI3K mutations, for that reason targeting PI3K and AR have also been also recently explored in TNBC patients (Gonzalez-Angulo, Stemke-Hale et al. 2009). In addition, pan-PI3K inhibitors elicited disease stabilization or partial response in TNBC (Bendell, Rodon et al. 2012).

1.19.4 Src Inhibitors (Desatinib): TNBC tumors display high sensitivity to multi-target kinase inhibitors compared with other tumors. In metastatic TNBC disease the use of src inhibitors in association with other chemotherapy agents showed a synergistic effect on decreasing tumor growth. Currently many trials are undergoing and reporting good tolerability and promising results as a single agent or in combination for metastatic TNBC patients (Tryfonopoulos 2009).

1.19.5 Cancer stem cell inhibitors: CSCs are resistant to many current cancer treatments and eliminating them will improve patient survival rates. Several anti-CSC strategies and associated targets have been proposed and validated for TNBC. Vitamin D3 in combination with androgen receptor agonist has shown to

inhibit CSC phenotype and induce differentiation. These agonists showed reduction of ALDH+ cells and up regulation of claudins and down regulation of vimentin and cytokeratin 5, consistent with more differentiated epithelial phenotype (Thakkar, Wang et al. 2016).

Also antibiotics like salinomycin as single agents or in combination with chemotherapy and/or deacetylatilase inhibitors have been explored with promising results (Gupta, Onder et al. 2009, Kai, Kanaya et al. 2015, Thakkar, Wang et al. 2016).

More recently, the CDK4 inhibitor Palbociclib was found to be preventing TNBC cancer stem cells and chemotherapy-resistant cancer cells from self-renewing and proliferating leading to their eradication (Dai, Zhang et al. 2016).

1.20 Emerging therapeutic modalities in TNBC

After 2 decades of great improvements in the management of TNBC, a number of important new trials and novel drugs have started to move the field forward. Currently, better understanding of the molecular basis of TNBC and the biological processes crucial for tumorigenicity has lead to the development of new therapeutic modalities. These modalities include targeting essential processes such as immunity modulation, modulation of epigenetics (induction of apoptosis and modulation of cell cycle), induction of cellular differentiation and senescence.

1.20.1 Immune-checkpoint Inhibitors: Immune-checkpoints refer to diverse molecules in charge of preventing excessive activity of the immune system under normal conditions. For that reason, inhibition of these checkpoints and enhancement of the T-cell response could be used as a different therapeutic approach. The higher levels of lymphocytic infiltration seen in TNBC compared with other breast cancer subtypes, and its favourable prognostic value, may indicate that immune-checkpoint inhibitors are an option to explore for the management of this disease.

Recently, anti-CTL4 antibodies (ipilimumab and tremelimumab) were evaluated in breast cancer, as well as PDL1/PD1 pathways that are overexpressed in 20% of TNBCs (Mittendorf, Philips et al. 2014). Early phase I and phase II trials with immune checkpoint inhibitors in TNBC as single agents or in combination with chemotherapy are being conducted (PD-1 inhibitors: Nivolumab and pembrolizumab / PD-L1 inhibitors: tezolizumab, avelumab, and durvalumab). These trials reported an overall response rate of up to 19% with clinical responses and a tolerable safety profile (Table 1.2). However this area needs to be further explored in TNBC (Nanda, Chow et al. 2016).

Trial	Phase	Drug	Subjects	Endpoints
KEYNOTE-012 NCTO1848834	Ib	Pembrolizumab	27 TNBC	ORR: 18.5% 1CR, 4PRs and 7SD, 6 month PFS: 24.4%
GO27831 NCTO1375842	I	Atezolizumab	21 TNBC	ORR: 19% 2CRs, 2PRs, 6 month PFS: 27%
JAVELIN NCTO1777200 4	Ib	Avelumab	168 58 TNBC	In TNBC: ORR: 8.6%, 5PR and 13 SD
GP28328 NCTO1633970	Ib	Atezolizumab Nab-Paclitaxel	32 TNBC	ORR: 38% 3% CR, 34% PR, 44% SD

CR complete response, ORR overall response rate, PFS progression-free survival, PR partial response, SD stable disease, TNBC triple negative breast cancer.

Table 1.3 Clinical Trial results using checkpoint inhibitors in metastatic TNBC. Phase I clinical trials results using checkpoint inhibitors in patients with metastatic TNBC. Modified from Isha Dua MD, et al., (2017), Immunotherapy for Triple-Negative Breast Cancer: A Focus on Immune Checkpoint Inhibitors, American Journal of Hematology / Oncology; 13(4): 20-27.

1.20.2 Modulating Epigenetics

Post-translational modifications including acetylation, phosphorylation, and methylation are primarily responsible for the regulation of gene expression. There is evidence that TNBC is characterized by having a DNA hypo methylation profile(Stirzaker, Zotenko et al. 2015) and that gene silencing in TNBC patients is performed by methylation and/or histone acetylation (Widschwendter and Jones 2002, Grushko 2010).

Some DNA methylation studies in breast cancer have focused on the methylation status of some tumor-related genes in invasive cancers. The DAPK and ID4 genes have gained importance in TNBC to understand the mechanism that can lead to therapeutic targets (Gheibi, Kazemi et al. 2012). While, DAPK is a gene associated with DNA apoptosis, it is known to be more hyper methylated in TNBC cases. There is strong association between DAPK hyper methylation and tumor grade and size in TNBC (Hafez, Al-Shabanah et al. 2015) and the inhibition of DAPK has been shown to suppress growth (Zhao, Zhao et al. 2015). ID4 are basic helix-loop-helix transcription factors that lack DNA binding activity and so its actions will depend on the protein-binding partner. These factors act as tumor suppressors repressed in ER positive tumors and pre-malignant lesions (de Candia, Akram et al. 2006). It was shown that ID4 hyper methylation is increased in TNBC and correlate positively with tumor size and number of lymph-nodes in TNBC, which suggest that ID4 could serve as a prognostic biomarker for predicting early metastasis and could explain the aggressiveness of TNBC (Hafez, Al-Shabanah et al. 2015).

1.20.3 Histone deacetylase inhibitors (HDI)

Post-translational modifications (PTMs) can induce an interaction between survival pathways and apoptosis by sensitizing cancer cells to therapeutic agents. These agents also could possibly reactivate genes that increase the sensitivity to different treatment agents available. Usually these drugs modulate gene expression through the induction of cell cycle arrest, apoptosis (induction of pro-apoptotic and inhibition of anti-apoptotic proteins), EMT regulation and differentiation upon gene transcriptional repression (Damaskos, Garmpis et al. 2017). Currently, four HDAC classes have been identified depending on their sequence homology to the yeast original enzymes, subcellular location, and enzymatic activity. While classes I, II, and IV are zinc-dependent enzymes, class III consists of a large family of silent information zinc-independent regulators. Furthermore, each class has a specific subcellular localization: class I is found primarily in the nucleus, class II is able to shuttle in and out of the nucleus, class IV has a cytoplasmic location, and the subcellular distribution of class III HDACs is unknown (Figure 1.9) (de Ruijter, van Gennip et al. 2003, Damaskos, Garmpis et al. 2017) (Rosato and Grant 2005).

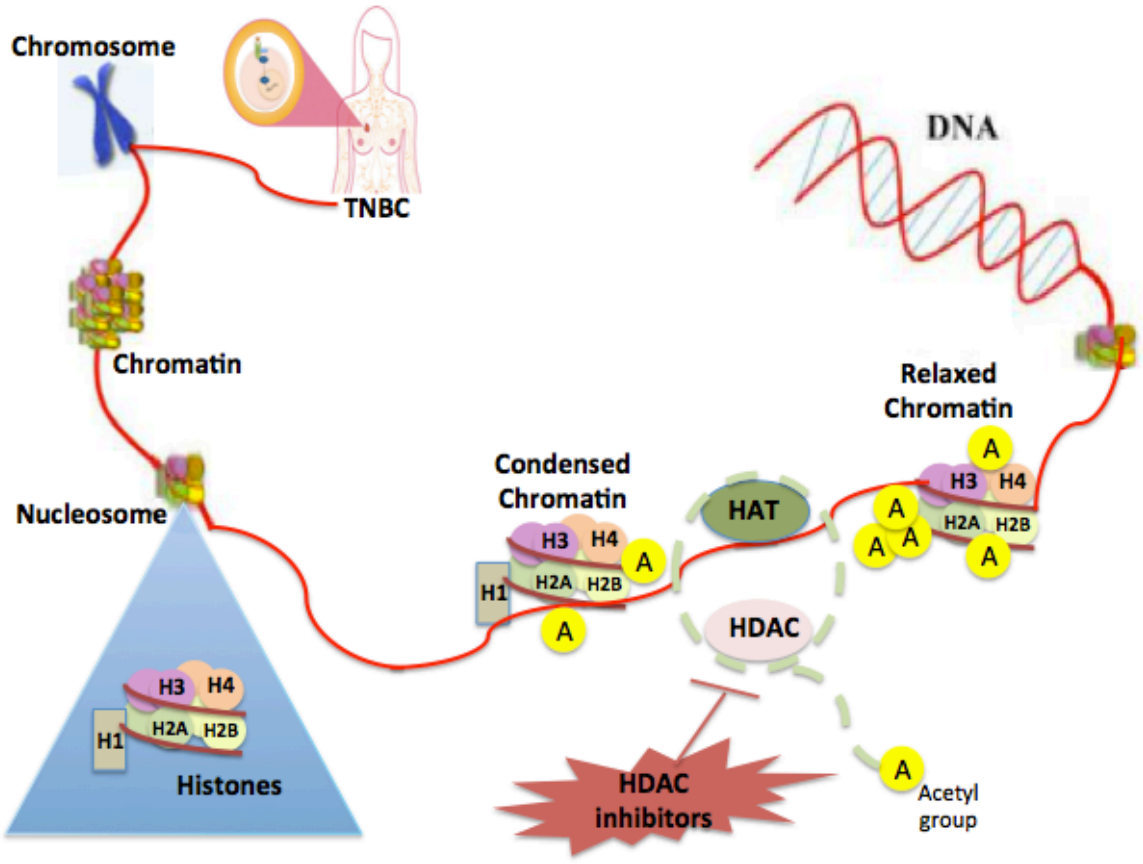

Figure 1.9 Therapeutic strategy targeting histone deacetylases (HDAC) against TNBC. Several HDAC inhibitors have been classified according to their specificity for HDAC subtypes and reported to enhance the acetylation of histones in tumor cells by the histone acetyl transferases (HATs). Modified from Garmpis N *et al*., (2017), Histone Deacetylase Inhibitors in Triple Negative Breast Cancer: progress and promises, Cancer genomics & proteomics 14: 299-313.

Moreover, agents acting on PTMs can be used to primarily treat some resistant cancers (Singh, Zhang et al. 2010, Khan and La Thangue 2012, Bolden, Shi et al. 2013). Acetylation is a major PTM, which is critical for cycle arrest and apoptosis. Therefore, histone deacetylase agents have shown their anti-tumor activity as useful cancer therapeutics (Khan and La Thangue 2012, Bolden, Shi et al. 2013). Vorinostat is one of the most advanced pan-HDAC inhibitors. Its main mechanism of action is the induction of changes in acetylating motif and downstream effects on apoptotic pathways. Breast cancer preclinical studies showed growth inhibition caused by G_1 and G_2-M cycle phase arrest and apoptosis. In clinical trials it showed modest clinical benefit and high toxic effects (Luu, Morgan et al. 2008). However, preclinical studies have demonstrated that its use can reactivate estrogen receptors and inhibit metastasis in TNBC cells (Palmieri, Lockman et al. 2009, Stark, Burger et al. 2013). Clinical trials have reported a complete pathological response in TNBC patients (Tu, Hershman et al. 2014).

Panobinostat as either single or combination agent showed decreased tumor growth through inhibition of the cell cycle and an increase in apoptosis, as well as cell morphology changes and reversal of EMT in *in vitro* and *in vivo* TNBC preclinical models (Rao, Balusu et al. 2012, Tate, Rhodes et al. 2012, Rhodes, Tate et al. 2014).

1.20.4 Induction of Cellular Differentiation

The aim of the differentiation therapy is the reactivation of cellular differentiation programs in cancer cells in order to induce a more differentiated/mature phenotype that resembles the “normal/benign” potentially causing the loss of tumorigenic potential and further proliferation (Figure 1.10) (Cruz and Matushansky 2012). Although differentiation therapy does not eliminate the cancer cells, it stops their growth and allows standard therapies (such as chemotherapy/radiotherapy) to eradicate the malignant cells.

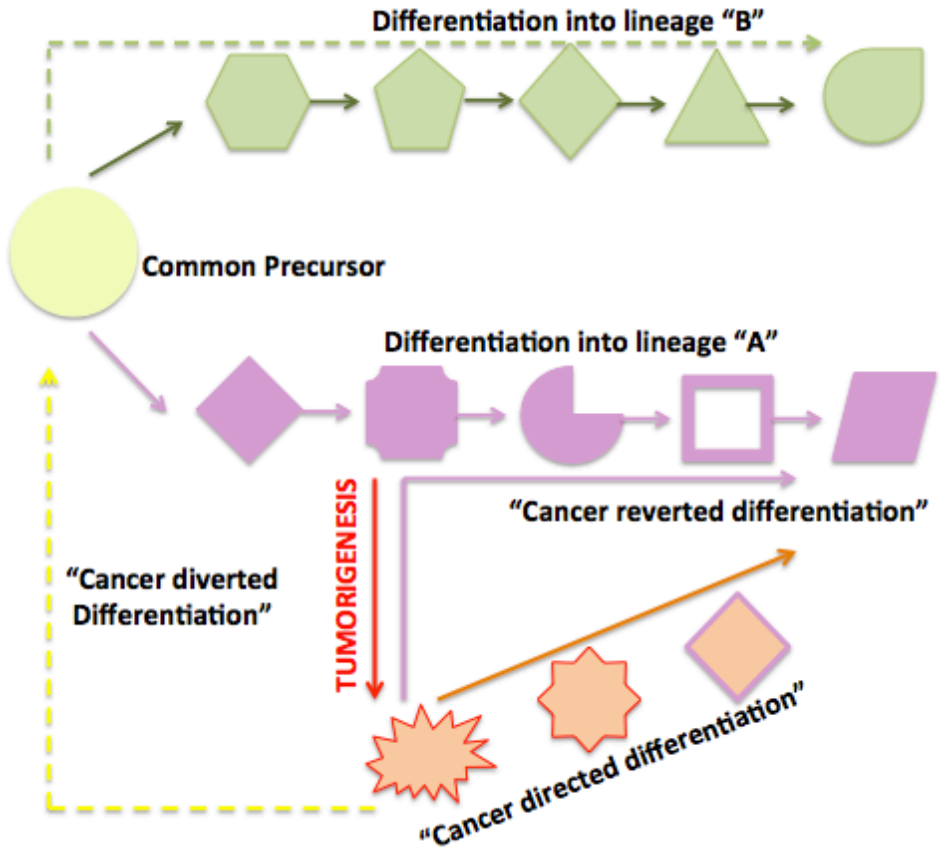

Figure 1.10 Schematic representation of multiple ways in which cancer maybe differentiated. Cancer differentiation therapy can occur in three different ways: 1) cancer directed differentiation (without correcting the underlying oncogenic mechanisms that have resulted in the initial differentiation block); 2) cancer reverted differentiation (correction of the underlying oncogenic mechanism results in natural restoration of endogenous differentiation pathways); and 3) cancer diverted differentiation (cancer cell is redirected to an earlier stage of differentiation where access to alternative differentiation routes may be feasible). Modified from Filemon de la Cruz et al., (2012), Solid Tumor Differentiation Therapy – Is It Possible?, Oncotarget; 3: 559-567

Recently, the cyclin-dependent kinase (CDK4) was found to act as cancer stem cell regulator and novel prognostic marker in TNBCs. Furthermore, it was found that CDK4 inhibition using palbociclib prevents CSC renewal and is able to reverse the mesenchymal-like phenotype observed in TNBC back to a luminal/epithelial-like phenotype with a better prognosis, highlighting CDK4 as promising differentiation treatment in TNBC.

Another differentiation agent is all-trans-retinoic acid (ATRA). This agent is well studied in the treatment of acute promyelocytic leukemia (APL); however, its use in solid tumors needs to be further explored. Retinoic acid (RA) transduces its signals by binding to specific nuclear retinoic acid receptors (RAR), which include RAR α, β, and γ (Samarut and Rochette-Egly 2012). While studies demonstrate that retinoic acid receptor (RARα) expression is an estrogen-induced gene (Roman, Ormandy et al. 1993). Its expression in TNBC is epigenetic-silenced (Tang and Gudas 2011). As TNBC, lacks expression of hormone receptors including ER, it was recently suggested that inducing ER expression is a potential strategy for sensitization of triple-negative breast cancers to adjuvant endocrine therapies. Recent studies showed that RA and estradiol induce the increase of expression of RA-inducible genes and lead to increase of cell growth. However, when a combination of estradiol, RA and tamoxifen is used, TNBC cell proliferation was significantly decreased *in vitro (Coyle, Dean et al. 2014).*

RAR-β has also been observed to be epigenetic-silenced in breast cancer stem cells. A recent preclinical study using a combined therapy with entinostat,

ATRA and doxorubicin (designated as EAD) observed a significant TNBC tumor regression and restoration of epigenetically silenced RAR-b expression *in vivo* (patient-derived metastatic cells). Moreover, gene analysis revealed that ELF3 improved cell differentiation in response to EAD therapy. These findings demonstrate that EAD therapy was successful in decrease TNBC tumor growth and progression.

Another important point regarding differentiation is its relation with other tumor suppression mechanisms (Merino, Nguyen et al. 2016). It is still an open question whether retinoids can induce cell cycle arrest and consequently leads to senescence or whether independent mechanisms are involved (Shilkaitis, Green et al. 2015). For that reason, many challenges remain for cancer differentiation therapy particularly in TNBC. An in depth exploration of the molecular mechanisms of epigenetic networks and senescence may provide new insights for treatment with differentiation therapy.

1.20.5 Cellular Senescence

Cellular senescence is defined by a state of irreversible growth arrest in which cells stop dividing but remain metabolically active. This process can be triggered by multiple stimuli including telomere shortening, DNA damage, increase of mitogens or oncogenic proteins, etc. Together these mechanisms limit the abnormal replication or removal of damaged cells and protect against cancer development. In normal physiology, senescence is a developmental process that play a role in organogenesis, aging and nowadays is considered a

tumor suppressor mechanism. This process is controlled by the telomere length and is called replicative senescence. Senescence can be induced independently of telomere length by stress-induced senescence and oncogene-induced senescence (Munoz-Espin and Serrano 2014).

While, replicative senescence is produced when telomere length is critically short and is sensed as DNA damage, stress-induced senescence is produced by reactive oxygen species. On the other hand oncogene-induced senescence is activated by several oncogenes caused by aberrant activation of oncoproteins in normal cells or by constitutively activation of the enzyme telomerase (Rodier and Campisi 2011).

1.20.5.1 Principal features of senescent cells

Senescent cells differ from non-dividing cells such as quiescent and terminally differentiated cells because they display permanent growth arrest, remaining metabolically active and resistant to apoptotic signals. Senescent cells are mostly arrested in the G1 phase of the cell cycle. This is mainly controlled by the RB family of tumor suppressors, which blocks the activity of transcription factors of the E2F family (Takahashi, Ohtani et al. 2007).

Moreover, senescent cells are characterized by the expression of numerous markers and morphological changes (Childs, Gluscevic et al. 2017). The first marker to be used for the specific identification of senescent cells was the senescence-associated β-galactosidase (SA-βgal), an enzyme that reflects lysosomes biogenesis and is considered the gold standard in detecting

senescent cells (Dimri, Lee et al. 1995). Furthermore, the absence/down regulation of proliferative markers (p16INK4, ARF, p53, p21, p15, p27 and hypo phosphorylated RB) and chromatin alterations are features of senescent cells.

Senescence-associated heterochromatin foci (SAHF) are found in the nucleus of senescent cells, and are a transcriptionally repressive heterochromatin structure enriched with histone modifications including lysine9-trimethylated histone H3 (H3K9me3), heterochromatin-associated protein 1 homologue-γ (HP1γ), the histone variant macroH2A and p53 binding protein 53BP1. SAHFs are believed to repress the expression of proliferation-promotion genes (including cyclin A and E) thus contributing to cell cycle arrest (Zhang, Poustovoitov et al. 2005, Kosar, Bartkova et al. 2011). This process is believed to be mainly under the control of the p16/RB pathway.

Retinoblastoma protein (RB) is a potent tumor suppressor and cell cycle regulator in its non-phosphorylated state. After phosphorylation by the CDK4/6 kinases, RB loses its repressive function, releasing E2F transcription factors and consequently allowing them to activate growth-promoting genes. Another important function is that RB interacts with the histone methyltransferase SUV39H1 and the HP1 protein, required for cell cycle regulated H3K9me3 at the cylcin E promoter (Nielsen, Schneider et al. 2001). H3K9me3 is an important histone modification that is associated with heterochromatin-gene transcription inhibition and controls reprograming-cell identity (Becker, Nicetto et al. 2016).

An important feature of senescent cells is that they can no longer replicate but remain metabolically active. However, they can adopt an immunogenic phenotype called senescent associated secretory phenotype (SASP) that creates an inflammatory microenvironment that may lead to the eradication of senescent cells. SASP involve several cytokines (IL-1, IL-6, IL8, etc.), chemokines (MCP-1, CXCR1,2, CXCL etc.), growth factors (IGFBPs, VEGF, PAI-1, etc.) and proteases (metalloproteinases) (Campisi 2013) and is still under investigation into its clinical significance.

1.20.5.2 Cellular senescence and cancer

Cellular senescence is recognized as a potent tumor-suppressive mechanism, that produces several changes in gene expression and in extracellular matrix composition (Campisi 2005). Furthermore, the increase in the secretion of numerous cytokines, chemokines, growth factors and proteases known, as SASP will induce an environment that on the one hand is protective and will induce protective senescent-cell clearance and on the other hand will provide favorable conditions for tumorigenesis (Figure 1.11) (Krtolica, Parrinello et al. 2001, Parrinello, Coppe et al. 2005, Coppe, Kauser et al. 2006, Laberge, Awad et al. 2012, Lee and Lee 2014).

Controversially, as a tumor promoter it is suggested that SASP will induce an immune response that can lead to chronic inflammation stimulating tumor growth and angiogenesis and ultimately producing tumor spread. Therefore, more studies are required to elucidate the mechanisms causing the SASP, explore

differences in the SASP among cell types, and investigate the nature of the SASP in tumor progression.

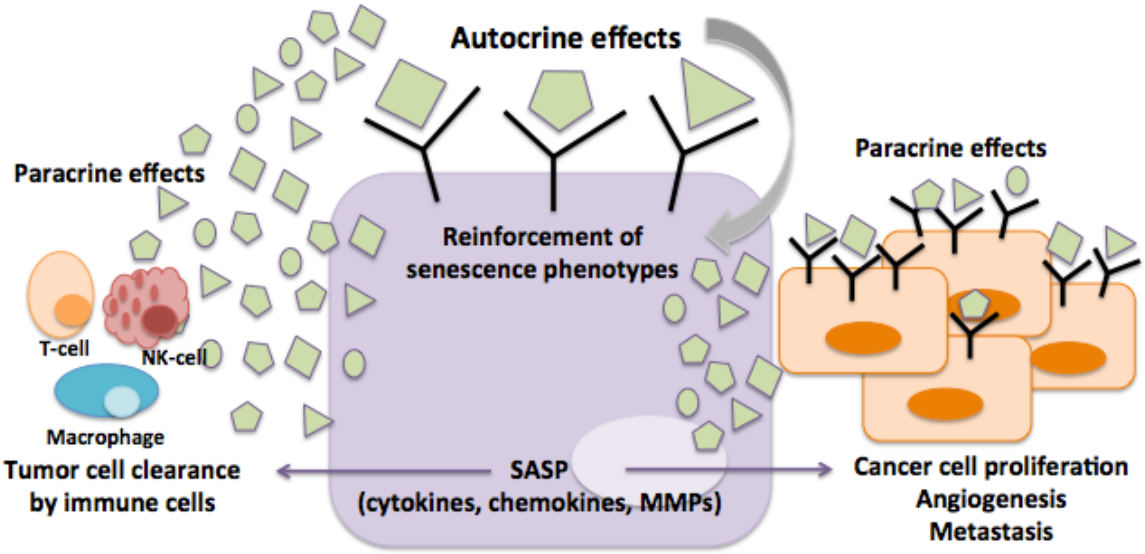

Figure 1.11 Pleiotropic nature of senescent cells. Senescence associated secretory phenotype (SASP) contributes to the maintenance of the senescent phenotype and secretion of pleiotropic factors able to induce immune regulation and affect tumor microenvironment. Modified from Lee M, et al., (2014), Exploiting tumor cell senescence in anticancer therapy, BMB Reports 47(2): 51-59.

Chapter 2: Prolactin pro-differentiation pathway in triple negative breast cancer: impact on prognosis and potential therapy

Chapter 2:

Prolactin pro-differentiation pathway in triple negative breast cancer: impact on prognosis and potential therapy

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2.1 Preface

Prolactin hormone (PRL) and its downstream Jak2/Stat5 signalling pathway are critical for mammary gland development and promotion of terminal differentiation of the mammary epithelial cells. However, the role of PRL in breast cancer development/progression still needs to be fully evaluated. Previous work has implicated an anti-tumorigenic role for PRL in breast carcinogenesis. However, the detailed mechanism(s) by which PRL induces these effects needs to be further investigated. TNBC is a heterogeneous disease, characterized by aggressive phenotype, poor overall survival and lack of targeted therapy. In this chapter, we thus examined the role of PRL and its downstream signalling pathway Jak2/Stat5 in regulating TNBC tumorigenicity.

Here the role of prolactin-mediated differentiation pathway in prognosis, sub-classification and potential therapeutic option in TNBC were evaluated. Using immunohistochemistry analysis we were able to detect PRLR in ~3% of TNBC cases. Moreover, using gene profiling bioinformatics data of 580 TNBC's indicated that PRLR gene expression can be detected in ~30% of the cases. Most importantly, these analyses also indicated that gene expression of PRL signaling pathway components individually (PRL, PRLR, Jak2 and Stat5a) or as a gene signature were able to detect TNBC patients with better outcomes. In addition, examining the correlation of gene expression of PRLR with established molecular subtypes of TNBC it showed that PRLR expression could sub-classify TNBC patients into a new subgroup (TNBC-PRLR) characterized by epithelial-

luminal differentiation. To evaluate the potential therapeutic value of PRL in TNBC we showed that restoration/activation of PRL pathway in TNBC cells representative of mesenchymal or TNBC-PRLR subgroups led to induction of epithelial phenotype and suppression of tumorigenesis *in vitro* and *in vivo*. Those results together indicate for the first time that PRLR is a marker of favorable prognosis in TNBC patients and the activation of its signaling pathway decreases the aggressive and invasive phenotype of TNBC cells.

2.2 Abstract

Triple negative breast cancer (TNBC) is a heterogeneous disease associated with poor clinical outcome and lack of targeted therapy. Here we show that prolactin (PRL) and its signalling pathway serve as a sub-classifier and predictor of pro-differentiation therapy in TNBC. Using immunohistochemistry and various gene expression in silica analyses we observed that prolactin receptor (PRLR) protein and mRNA levels are down regulated in TNBC cases. In addition, examining correlation of PRLR gene expression with metagenes of TNBC subtypes (580 cases), we found that PRLR gene expression sub-classifies TNBC patients into a new subgroup (TNBC-PRLR) characterized by epithelial-luminal differentiation. Importantly, gene expression of PRL signalling pathway components individually (PRL, PRLR, Jak2 and Stat5a), or as a gene signature is able to predict TNBC patients with significantly better survival outcomes. As PRL hormone is a druggable target we determined the biological role of PRL in TNBC biology. Significantly, restoration/activation of PRL pathway in TNBC cells representative of mesenchymal or TNBC-PRLR subgroups led to induction of epithelial phenotype and suppression of tumorigenesis. Altogether, these results offer potential new modalities for TNBC stratification and development of personalized therapy based on PRL pathway activation.

2.3 Introduction

Triple negative breast cancer (TNBC) is typified by lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2). This subtype of breast cancer is characterized by poor histological characteristics, high rate of recurrence, poor patient outcome and lack of targeted therapy (Sorlie, Perou et al. 2001, Metzger-Filho, Tutt et al. 2012). Loss of cellular differentiation is a key feature of TNBC tumors that may contribute to its unfavourable/aggressive phenotype (Nielsen, Hsu et al. 2004, Onitilo, Engel et al. 2009). Therefore, better understanding of the molecular pathways involved in cellular differentiation may provide new opportunities for better patient's stratification, prognosis and personalized therapy in this breast cancer subtype (Perou, Sorlie et al. 2000, Foulkes, Smith et al. 2010).

Studies performed to understand the biology of TNBC revealed that it is a heterogeneous disease(Abramson, Lehmann et al. 2015). Based on gene expression analyses, TNBC has been categorised into six subgroups including basal-like (BL1 and BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM) and luminal androgen receptor (LAR)(Lehmann, Bauer et al. 2011). The basal-like (BL1 and BL2) subtypes are highly enriched in gene expression patterns associated with proliferation-related genes as well as genes involved in DNA damage response. The mesenchymal (M and MSL) subtypes are enriched in gene expression patterns associated with epithelial-to-mesenchymal transition process(Lehmann, Bauer et al. 2011). The immunomodulatory subtype is characterized for gene ontologies of immune cell

processes that include immune cell and cytokine signalling, antigen processing and presentation(Denkert, Loibl et al. 2010, Loi, Sirtaine et al. 2013). The LAR subgroup is typified for being enriched in genes related with androgen receptor (AR) signalling and has been associated with good prognosis within TNBC(He, Peng et al. 2012, Tang, Xu et al. 2012). In addition to this classification of TNBC other studies have generated various gene signatures distinguishing molecular subsets (basal-like, mesenchymal-like (Claudin-low) and luminal androgen receptor) as well as non-neoplastic cell populations (epithelial claudin-CD24 signature, stromal signature, markers of blood, adipocytes, angiogenesis and inflammatory signature)(Rody, Karn et al. 2011). Due to this diversity in the histological and molecular features as well as limited availability of well-defined molecular targets, developing treatments against TNBC remains challenging.

Extensive studies both in vitro and in vivo highlighted PRL and its downstream Jak2/Stat5 signalling pathway as central to mammary gland development and terminal differentiation of the mammary epithelial cells(Ormandy, Binart et al. 1997, Hennighausen and Robinson 2005). On the other hand, the role of PRL in breast cancer development/progression is not fully elucidated. Previous studies suggested that PRL could lead to breast cancer development by functioning as a local growth factor through a PRL/PRLR autocrine loop (Chen, Ramamoorthy et al. 1999, Vonderhaar 1999, Chen, Holle et al. 2002, Clevenger, Furth et al. 2003). Furthermore, studies using transgenic mice designed to overexpress PRL in mammary epithelial cells resulted in the development of mammary tumors(Wennbo, Gebre-Medhin et al. 1997, Rose-

Hellekant, Arendt et al. 2003). As well, PRL and PRLR were found to play a permissive role in oncogene-induced mammary tumors(Oakes, Robertson et al. 2007). PRL was also found to cooperate with loss of p53 to induce Claudin-low mammary carcinomas(O'Leary, Rugowski et al. 2014) and was associated with interfering with BRCA1 regulation of expression of the cell cycle inhibitor p21(O'Leary, Rugowski et al. 2014). In addition, PRL and PRLR were recently implicated in breast cancer metastatic spread (Yonezawa, Chen et al. 2015, Sutherland, Forsyth et al. 2016). While the above studies highlight a role for PRL in promoting tumorigenesis, many recent studies, including ours, suggested a different role as a potential suppressor of breast carcinogenesis. Indeed, we have previously shown that PRL, through PRLR/Jak2 signalling suppresses epithelial-mesenchymal-transition (EMT) and reduces the invasive properties of breast cancer cells(Nouhi, Chughtai et al. 2006). Furthermore, using both mammary epithelial cells and human breast cancer cells we showed that PRL blocks growth factor-induced mammary cell proliferation and viability of breast cancer cells(Haines, Minoo et al. 2009). More recently we also found that expression of PRLR and PRL in human breast cancer correlate with favourable prognosis and better patient outcome (Hachim, Hachim et al. 2016, Hachim, Shams et al. 2016). In support of these findings, PRL and PRLR expression were found to be down regulated in breast cancer patients and breast cancer cell lines(Galsgaard, Rasmussen et al. 2009, Nitze, Galsgaard et al. 2013). Moreover, expression/activation of the PRL effector molecule Stat5a was found to associate positively with increased levels of histologic differentiation of breast

cancer tissues and to distinguish breast cancer patients with favourable prognosis and response to endocrine therapy (Yamashita, Nishio et al. 2006). Stat5a loss of expression was also found to be associated with tumor progression and unfavourable clinical outcomes (Peck, Witkiewicz et al. 2012). As well, the PRL-responsive milk proteins whey acidic protein (WAP) and α-casein were also shown to inhibit tumorigenesis and breast cancer cell invasion (Ikeda, Nukumi et al. 2004, Nukumi, Iwamori et al. 2007, Bonuccelli, Castello-Cros et al. 2012). Together these findings provide compelling evidence regarding the role of PRL pathway in maintaining tissue differentiation and as a suppressor of breast carcinogenesis. This unexpected suppressive role of PRL in breast cancer is still emerging and needs to be further elaborated. In addition, the role of PRL in TNBC has not yet been investigated.

In this study, we evaluated the role PRL differentiation pathway in prognosis and suppression of tumorigenesis in TNBC. Using tissue microarrays and gene profiling databases of breast cancer patients, our results identified a novel and relevant subgroup within TNBC characterized by PRLR expression and luminal-epithelial characteristics. This TNBC-PRLR subgroup showed better prognosis represented as prolonged disease free survival. Furthermore, functional studies using TNBC cell lines showed that activation of PRL signalling pathway suppresses the aggressive nature of TNBC cells in vitro and tumorigenesis in vivo. Overall, these findings propose a new management strategy for TNBC patients. This approach is based on screening for PRLR

expression in patients that may benefit from the use of PRL hormone as a novel pro-differentiation therapy.

2.4 Results

2.4.1 PRLR expression is down regulated in TNBC

To decipher the role of PRL in TNBC, we first examined PRLR protein expression in TMA composed of 43 TNBC cases representing different grades, stages and histological types (Figure S1A). Interestingly, our analysis revealed that PRLR protein is expressed in only ~2% (1 case) of the cases examined (Figures 1A & 1B and Figure S1A). This down regulation of PRLR in TNBC cases was irrespective of grade, stage and histological type (Figures S1B, S1C & S1D). We next analyzed PRLR gene expression levels in different breast cancer molecular subtypes including TNBC (660 patients), Her-2 (170 patients), luminal A (703 patients) and luminal B (170 patients) using robust single sample predictor classification (RSSPC) in bc-GenExMiner 3.0 database. The Molecular subtype prognostic analysis tool of this program allows automatic beforehand classification of PRLR gene expression levels into three equal quartiles (low, intermediate and high). Our analyses showed that intermediate/high PRLR gene expression levels are least in TNBC (29%) compared to Her-2 (68%), luminal A (84%) and luminal B (78%) molecular subtypes (Figure 1C). Together, these results indicate that while PRLR protein expression is down regulated in TNBC its gene expression is still preserved in 29% of cases.

2.4.2 The prognostic relevance of PRL differentiation pathway in TNBC

Next we investigated whether PRL pathway expression could impact the prognosis of TNBC patients. To assess this point, we analyzed the association between PRLR gene expression and patient outcome, any event free survival (AEFS) using bc-GenExMiner 3.0 database of basal-like intrinsic breast cancer subgroup (representing TNBC) in two sub-classification methods (Hu and Sorlie) (Sorlie, Perou et al. 2001, Hu, Fan et al. 2006). Interestingly, we observed a significant association between PRLR gene expression and prolonged AEFS (Hu 1,072 patients and Sorlie 724 patients) (Figures 2A & 2B). Next we investigated the prognostic value of PRL signalling components Jak2 and Stat5a using the same methods of classification mentioned above. Interestingly, we also observed a significant correlation between Jak2 (Hu 1,122 patients and Sorlie 778 patients) (Figures 2C & 2D) and Stat5a (Hu 1,124 patients and Sorlie 770 patients) (Figures 2E & 2F) gene expression and prolonged AEFS.

Moreover, we investigated PRLR, Jak2 and Stat5a gene expression individually in relation to relapse free survival (RFS) using KM plotter database of TNBC patients(Zsuzsanna Mihály 2013). Our results showed the same trend of significant association between PRLR, Jak2 and Stat5a gene expression and prolonged RFS (Figure S2) in 580 TNBC patients. We next investigated the prognostic power of PRL hormone gene expression in TNBC. Indeed, while PRL gene expression showed only marginal significance with better AEFS using Hu (1,139 patients) and Sorlie (783 patients) methods of classification (bc-GenExMiner 3.0), it was significantly associated with prolonged RFS using KM

plotter database (580 patients) (Figure S3). For better evaluation of the prognostic role of PRL and its signalling pathway in determining TNBC patient outcome, we next generated a gene signature representing PRL pathway including PRL, PRLR, Jak2 and Stat5a. Gene expression levels were classified into high or low according to the mean expression levels for each gene grouped together. This was achieved using the multi-gene classifier tool of KM plotter database (Materials and Methods) in 580 TNBC patients. Importantly, our results showed a significant association between high PRL pathway based gene signature and prolonged RFS (P=6.5e−09) in TNBC patients (Figure 2G). Together, these findings indicate that PRL pathway expressers constitute a subgroup within TNBC patients displaying favourable outcome and prolonged survival.

2.4.3 PRLR is a novel sub-classifier of TNBC patients

Recent studies have indicated the heterogenic nature of TNBC that impacts treatment options and patient outcome(Lehmann, Bauer et al. 2011, Rody, Karn et al. 2011). Therefore, here we examined PRLR gene expression in relation to metagenes representative of the molecular heterogeneity of TNBC using robust molecular subtype predictor classification (RMSPC) in bc-GenExMiner 3.0 that includes a cohort of 580 TNBC patients.

Interestingly, as shown in Figure 3, PRLR gene expression was inversely correlated with genes related to basal-like subtype (basal keratins KRT14, KRT14, KRT5 and KRT6a), mesenchymal-like (Claudin low) and genes

representative of the non-neoplastic cell populations. However, PRLR gene expression showed a significant positive correlation with members of two metagene clusters. The first metagene represents luminal-like genes associated with LAR signalling (FOXA1 and AR) and the second metagene represents epithelial cell-cell adhesion and luminal differentiation (Claudin-CD24). These data suggest that PRLR identifies a novel and distinct subgroup of TNBC with luminal-epithelial differentiation. To validate and gain further insights into this new TNBC-PRLR subgroup, we analyzed the positive association between PRLR and AR protein expression using immunohistochemistry in the TMA of human TNBC cores used above(Harvey, Clark et al. 1999). As expected, AR protein expression was positive in ~29% (12 cases) of TNBC cases. Interestingly, only ~10% (1 case) of these cases showed positive association with PRLR protein expression (Figures 4A & 4B and Figure S4) confirming the metagenes association findings described above and highlighting that TNBC-PRLR is an independent subgroup. Previous reports have suggested that AR expression is a marker of favourable prognosis in TNBC(Luo, Shi et al. 2010, Tang, Xu et al. 2012). Therefore, using the prognostic gene expression analysis tool of bc-GenExMiner 3.0 database, we then analyzed the prognostic value of AR gene expression in comparison to that of PRLR gene expression using the same methods of classification (Hu and Sorlie) as indicated in Figure 2. In contrast to PRLR, AR gene expression showed no significant association with better patient outcome (Hu 1,072 patients and Sorlie 724 patients) (Figures 4C & 4D). Together, these results indicate that PRLR expression is an independent

biomarker of favourable patient outcome in TNBC and defines a novel TNBC subgroup.

2.4.4 Restoring PRL signalling pathway in TNBC cells reduced cell viability, invasion capacity, mesenchymal properties and tumorigenesis

Previous studies have shown that the highly aggressive mesenchymal-like TNBC cell line MDA-MB-231 lacks expression of the PRLR (Ballestar, Paz et al. 2003). To investigate the role of PRL and its signalling pathway in regulating TNBC biology we restored PRLR expression in MDA-MB-231 cell line using a dox-dependent lentiviral transduction method (Materials and Methods). As shown in Figure 5A, significant PRLR protein expression was induced following dox treatment in MDA-MB-231/PRLR cells in comparison to MDA-MB-231/vector cells. In contrast to MDA-MB-231/vector cells, treatment of MDA-MB-231/PRLR cells with rhPRL led to the activation of PRL signalling molecule Stat5, indicative of a successful restoration of the PRL pathway in MDA-MB-231/PRLR cells (Figure 5A). We next investigated the biological effects of restoring PRL signalling in regulating cell viability of MDA-MB-231 cells. Interestingly, our results showed that PRL treatment induced a significant decrease in cell viability (34%-40%) of MDA-MB-231/PRLR cells in comparison to MDA-MB-231/Vector cells (P=2.209e-006) (Figure 5B). We next performed trans well invasion assays to gain further insights on the role of PRL pathway restoration in modifying the high invasive capacity of these TNBC cells. As shown in Figure 5C, activation of PRL signalling pathway dramatically decreased the invasive capacity (78.5%) of

MDA-MB-231/PRLR cells. As shown in Figure S5, this loss of invasive activity of MDA-MB-231/PRLR is not due to loss of cell viability. Next we examined the ability of PRL in regulating the expression of EMT markers including transcription factors (slug, snail, twist and zeb1) as well as E-cadherin, vimentin and fibronectin. As shown in Figure 5D, we observed down regulation of all mesenchymal markers examined in MDA-MB-231/PRLR cells when compared to MDA-MB-231/vector cells following hPRL treatment. On the other hand, the epithelial marker E-cadherin was significantly up regulated by hPRL in MDA-MB-231/PRLR cells when compared to control MDA-MB-231/vector cells. Together these results indicate that restoring PRL pathway in TNBC suppress their aggressive behaviour and mesenchymal phenotype.

Finally, we analyzed the role of PRL pathway restoration in regulating tumorigenesis of MDA-MB-231 cells using NOD/SCID mouse xenograft animal model. Animals were inoculated with either MDA-MB-231/vector or MDA-MB-231/PRLR cells subcutaneously into the right flank of each mouse. Animals were randomly assigned into three groups: MDA-MB-231/vector and MDA-MB-231/PRLR treated with dox and hPRL and MDA-MB-231/PRLR treated with dox only. Animals were treated intra-peritoneal from day 1 following cell implantation and tumor growth was monitored for 8 weeks (Supplementary Materials and Methods). As shown in Figure 5E, MDA-MB-231/vector xenografts showed considerable tumor growth reaching a volume of 200.81 mm3 at the time of sacrifice. Moreover, within the MDA-MB-231/PRLR untreated group only one mouse showed tumor growth that reached a maximum volume of 0.875mm3

suggesting that mouse PRL while it is described as a weak agonist of the hPRLR, it is sufficient to induce activation of the overexpressed hPRLR. Importantly, all mice within the MDA-MB-231/PRLR treated group failed to develop any detectable tumors throughout the period examined (Figure 5E) suggesting that PRL abrogates tumor formation in vivo. Altogether, these results indicate that restoration of the PRL pathway in TNBC results in suppression of cell viability, invasion capacity and tumorigenesis.

2.4.5 PRL supresses cell viability and tumor growth of TNBC-PRLR subgroup

Our previous results showed that PRLR gene expression sub-classifies a distinct population of TNBC tumors enriched with luminal and epithelial differentiation gene signatures associated with favourable outcome. To investigate the role of PRL and its signalling pathway in this TNBC subtype we tested PRL pathway activation in a representative cell line (MDA-MB-453). As shown in Figure 6A, MDA-MB-453 cells express endogenous levels of PRLR (Ormandy, Clarke et al. 1992, Lehmann, Bauer et al. 2011). PRL stimulation of these cells also resulted in Stat5 activation suggesting the presence of a functional PRL pathway in this cellular model of TNBC-PRLR subgroup. Next we investigated the role of PRL in regulating cellular viability in this model. Interestingly, we found that PRL caused a significant reduction in cell viability after 72hrs of treatment (~15%) (P=0.0001) (Figure 6B). To further characterize the role of PRL in TNBC-PRLR subgroup, we analyzed the effects of PRL in

regulating tumorigenesis using NOD/SCID/MDA-MB-453 animal xenograft model. Animals were inoculated with MDA-MB-453 cells subcutaneously into the right flank of each mouse. The mice were randomly assigned into two groups according to PRL treatment into MDA-MB-453 untreated and MDA-MB-453 treated (Supplementary Materials and Methods). Tumor growth and/or progression of the disease were monitored up to 8 weeks after cell inoculation. Notably, our results revealed that in the absence of PRL, mice showed signs of dissemination of the disease as well as a high incidence of morbidity (50%). These were assessed and measured by the appearance of paraneoplastic conditions (cachexia, anorexia, dehydration, respiratory difficulties, loss of weight, changes in the texture and coloration of the fur, skin dryness and loss of vibrissae), predominance of lethargic behaviour as well as the presence of palpable tumors (Figure 6C). In contrast, none of the above mentioned features were seen in the PRL treated group. PET/SPECT/CT studies were next performed to further evaluate disease progression. PET/SPECT/CT fusion of coronal and axial views of untreated group revealed the presence of high fludeoxyglucose (FDG) uptake area in the flanks, thymus and liver in addition to the expected normal tissues (brain, heart, and bladder) that normally exhibit a high rate of FDG uptake. Importantly, in the PRL treated group no FDG uptake was observed except for brain, heart, and bladder suggesting absence of tumor formation in this group of mice (Figure 6D and Figure S6). All animals were subjected to necropsy to confirm the presence or the absence of secondary tumors by gross examination after PET/SPECT/CT analysis. The lack of tumor

growth and/or dissemination in the PRL treated group was also confirmed by histological examination of lung and liver tissues (Figure S7). These findings further demonstrate the growth inhibitory effects of PRL in vitro and in vivo in TNBC-PRLR subgroup and highlight the possible use of PRL as a novel therapeutic strategy in TNBC. Altogether, these results emphasize that PRLR expression can categorize a specific TNBC subgroup with epithelial-luminal differentiation and favourable prognosis. Furthermore, PRLR can be used as a predictive marker for the possible use of PRL as a pro-differentiation therapy in breast cancer.

2.5 Discussion

TNBC represents an enormous clinical challenge due to its aggressive nature, heterogeneity and lack of targeted therapy (Dent, Hanna et al. 2009, Foulkes, Smith et al. 2010). Histologically, the majority of TNBC tumors are poorly differentiated and show high grade, a characteristic that promotes the aggressive phenotype resulting in poor overall survival (Brouckaert, Wildiers et al. 2012). Recent advances in the field have helped in characterizing 6 different TNBC intrinsic subgroups (Lehmann, Bauer et al. 2011). Still, identification of novel biomarkers in this breast cancer subtype is critically needed to help understand the biology of TNBC and the development of new tools for prognosis and therapy.

Loss of cellular differentiation is a common feature of TNBC tumors. In addition, TNBC tumor cells are thought to originate from a progenitor mammary stem cell population. Therefore, elucidating the role of mammary differentiation pathways in TNBC biology might provide novel approach in advancing classification, prognosis and treatment. PRL hormone is known to play an important role in mammary gland development and terminal differentiation of mammary epithelial cells (Hennighausen and Robinson 2005). The role of PRL in breast cancer development/progression is not fully elucidated and further studies are clearly required to clarify its role. Previous work described PRL and its receptor to play a permissive role in the development of mammary tumors and metastasis(Wennbo, Gebre-Medhin et al. 1997, Chen, Ramamoorthy et al. 1999, Vonderhaar 1999, Chen, Holle et al. 2002, Clevenger, Furth et al. 2003, Rose-

Hellekant, Arendt et al. 2003, Oakes, Robertson et al. 2007, O'Leary, Rugowski et al. 2014, Yonezawa, Chen et al. 2015, Sutherland, Forsyth et al. 2016). However recent studies have not only questioned this role of PRL but highlighted that it can act as a suppressor of breast tumorigenesis (Nouhi, Chughtai et al. 2006, Haines, Minoo et al. 2009). In addition PRL and PRLR were found to be down-regulated in breast cancer and their expression correlate with good prognostic and better patient outcome (Galsgaard, Rasmussen et al. 2009, Nitze, Galsgaard et al. 2013, Hachim, Hachim et al. 2016, Hachim, Shams et al. 2016). This is consistent with recent evidence showing that PRLR receptor antagonists did not show any anti-tumorigenic effects and therapeutic benefits in clinical trials(Agarwal, Machiels et al. 2016).

Here we examined the prognostic and therapeutic role of PRL and its signalling pathway in TNBC. Our results indicate that while PRLR expression is down regulated in TNBC in comparison to other breast cancer molecular subtypes, intermediate/high PRLR mRNA levels are still preserved in ~30% of TNBC cases. Based on metagene cluster analyses we identified a TNBC-PRLR subgroup. This subgroup represents a distinct TNBC subgroup characterized by luminal-like differentiation (FOXA1 and AR) and epithelial (claudin-CD24) gene signatures. Moreover, our gene expression/prognosis analyses revealed that TNBC-PRLR subgroup has better patient overall survival outcomes in comparison to all subgroups of TNBC. Interestingly, these results are in agreement with a recent study describing PRL pathway to be enriched in the TNBC LAR subtype (Yu, Zhu et al. 2013, Burstein, Tsimelzon et al. 2015).

Altogether these results indicate that PRLR represents an independent and precise marker to distinguish a unique TNBC subgroup with specific molecular and prognostic features.

Nowadays, cytotoxic chemotherapy remains the mainstay of treatment for patients with TNBC in spite of the increasing number of targeted therapies. Targets such as epidermal growth factor receptor (EGFR) (Baselga, Gomez et al. 2013), vascular endothelial growth factor receptor (VEGFR)(von Minckwitz, Eidtmann et al. 2012), DNA repair molecules(Farmer, McCabe et al. 2005), cell-cycle control and cell survival genes(Lehmann and Pietenpol 2012, Lin, Sampath et al. 2013) as well as inhibitors of AR(Gucalp, Tolaney et al. 2013) have been utilized to develop treatment modalities against TNBC. Still these treatment approaches show limited benefits, mostly due to toxic effects, resistance and tumor relapse. Pro-differentiation based therapies have been recently proposed in the hope of developing less aggressive treatments against cancer based on the reprograming/reversing cancer cells into less aggressive benign phenotype (Fenaux, Chevret et al. 2000, Yan and Liu 2016). Our present study supports the pro-differentiation concept as a mean to revert/suppress tumorigenesis. Indeed, we provide in vitro and in vivo evidence indicating that restoration and activation of the PRL differentiation program in TNBC results in reversal of the highly proliferative, invasive, mesenchymal and tumorigenic phenotype through induction of cell differentiation. This reprograming into more epithelial and non-invasive features may explain the better overall survival seen in TNBC-PRLR subgroup.

Together our findings highlight the relevance of using differentiation pathways in suppressing tumorigenesis. Indeed, cancer cells are known to be plastic in nature and can be reverted to a less aggressive phenotype under favourable stimulus. Therefore exploiting pro-differentiation pathways in cancer should be considered as a viable avenue for developing novel prognostic and therapeutic approaches.

2.6 Material and Methods

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

2.6.1 Generation of stable cell lines: MDA-MB-231 parental cells were used to generate stable cell lines (MDA-MB-231/vector and MDA-MB-231/PRLR) overexpressing the human long from PRLR cDNA using doxycycline (dox)–dependent lentiviral system.

2.6.2 Tissue microarray: TNBC tissue microarray (43 cases with clinico pathological data) was purchased from US Biomax.

2.6.3 Immunohistochemistry: Slides were incubated with a rabbit polyclonal antibody to PRLR-L (Santa Cruz #sc-20992) as describe previously (Hachim, Hachim et al. 2016) and with a rabbit polyclonal antibody to AR (Santa Cruz #CO215), using positive and negative controls for both (Figure S1A and Figure S4 respectively).

2.6.4 Gene expression analysis: Breast Cancer Gene-Expression Miner Version 3.0 (bc-GenExMiner 3.0) database was used to evaluate the mRNA levels of PRLR in different molecular subtypes (Jezequel, Campone et al. 2012). As indicated in bc-GenExMiner 3.0 the biological validation of this tool was extensively tested and approved for different genes as indicated "http://bcgenex.centregauducheau.fr/BC-GEM/GEM_Aide.php#Data_Validation". The prognosis gene expression analysis tool of bc-GenExMiner 3.0 was used to assess the association between PRL, PRLR, Jak2 and Stat5a mRNA levels and patient outcome, using gene symbol. The gene expression correlation analysis

tool of bc-GenExMiner 3.0 was used to study the correlation between PRLR mRNA levels and members of the different metagenes within TNBC using robust single sample predictor classification (RSSPC)(Rody, Karn et al. 2011). Kaplan-Meier plotter database was used to evaluate PRL signaling pathway components individually or as a single gene signature, using the following probeset ID (Affimetrix): PRL (205445_at), PRLR (206346_at), Jak2 (205841_at) and Stat5a (203010_at) in relation to patient outcome(Gyorffy, Lanczky et al. 2010).

2.6.5 Cell lysis, immunoprecipitations and western blotting: For whole cell lysates and immunoprecipitations, cells were lysed in lysis buffer as described previously (Ali and Ali 1998).

2.6.6 Invasion assay: 80 x10^3 cells were seeded in 24-well plates HTS multi-well insert system coated with Matrigel. Invasion assays were performed for 24 hours as described previously (Nouhi, Chughtai et al. 2006).

2.6.7 RNA extraction and qRT-PCR: Total RNA from MDA-MB-231/PRLR and MDA-MB-231/vector cells treated with hPRL for 72hrs was isolated, reverse transcribed and used for PCR amplification. RT-qPCR of EMT markers (slug, snail, twist, FN1, vimentin, E-cadherin, zeb1) was performed.

2.6.8 MTT assay: MTT assays were performed as previously described (Cocolakis, Lemay et al. 2001).

2.6.9 Animal models: All experimental animal work was performed in a specific-pathogen-free animal facility according to the guidelines and ethical regulations of the Research Institute McGill University Health Centre approved animal used

protocol (#2014-7492) in accordance with Canadian Council of animal care guidelines.

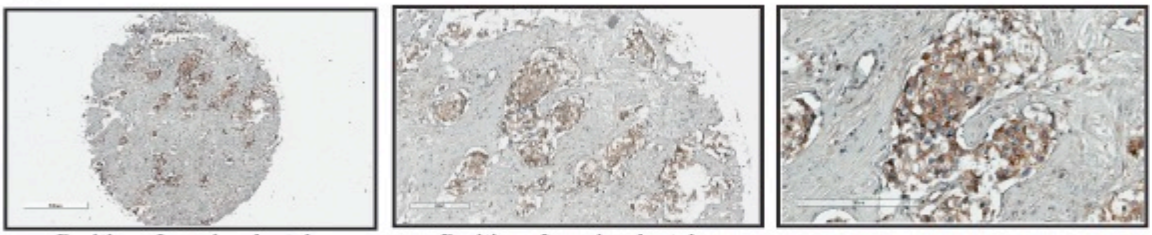
2.6.10 MDA-MB-231 xenograft: 18 Female NOD/SCID mice were purchased from Charles River Laboratories (Saint-Constant, QC, Canada) and randomly assigned into three groups (n=6 mice/group) according to PRL treatment: MDA-MB-231/vector, MDA-MB-231/PRLR untreated and MDA-MB-231/PRLR treated. The mice were injected intra-peritoneal with doxycycline (20 mg/kg) daily. Treated group was injected intra-peritoneal every second day with rhPRL (0.1mg/g). Tumor growth was monitored up to 8 weeks after implantation.

2.6.11 MDA-MB-453 xenograft: 12 Female NOD/SCID mice were purchased from Charles River Laboratories (Saint-Constant, QC, Canada) and randomly assigned into two groups (n=6 mice/group) according to rhPRL treatment: MDA-MB-453 untreated or MDA-MB-453 treated. The mice were treated intra-peritoneal with either vehicle or rhPRL (0.1mg/g) each second day. Tumor growth was monitored up to 8 weeks after implantation.

2.6.12 Whole-body imaging of NOD/SCID/xenograft mice using PET/SPECT/CT scans: PET/SPECT/CT scan was performed on three mice from each group of MDA-MB-453 animal xenograft. At the end of the experiment mice were sacrificed by CO2 asphyxiation and subjected to necropsy.

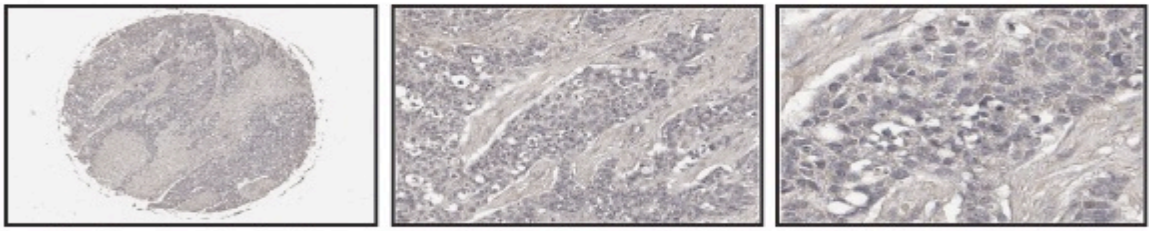
Further detailed information can be found in the Appendix: Extended Materials and Methods.

A)



Positive - Invasive ductal carcinoma- 10X | Positive - Invasive ductal carcinoma- 40X | Positive - Invasive ductal carcinoma- 100X

B)



Negative - Invasive ductal carcinoma- 10X | Negative - Invasive ductal carcinoma- 40X | Negative - Invasive ductal carcinoma- 100X

C)

Molecular Subtype	Basal Like			HER-2			Luminal A			Luminal B			Normal -like		
No. patients	660			170			703			170			301		
PRLR expression	Low	Int.	High	Low	Int.	High	Low	Int.	High	Low	Int.	High	Low	Int.	High
	71%	21%	8%	32%	40%	28%	15%	32%	52%	22%	40%	38%	40%	41%	19%

Figure 2.1: PRLR protein and gene expression in TNBC cases.

A) Positive immunohistochemical staining of PRLR in a case of invasive ductal carcinoma (10X, 40X and100X). B) Negative immunohistochemical staining of PRLR in a case of invasive ductal carcinoma (10X, 40X and100X). C) Table represents PRLR gene expression levels in different breast cancer molecular subtypes stratified according to robust single sample predictor classification (RSSPC) method using breast cancer gene-expression miner v3.0 database. PRLR gene expression levels were stratified into high, intermediate and low levels (Materials and Methods).

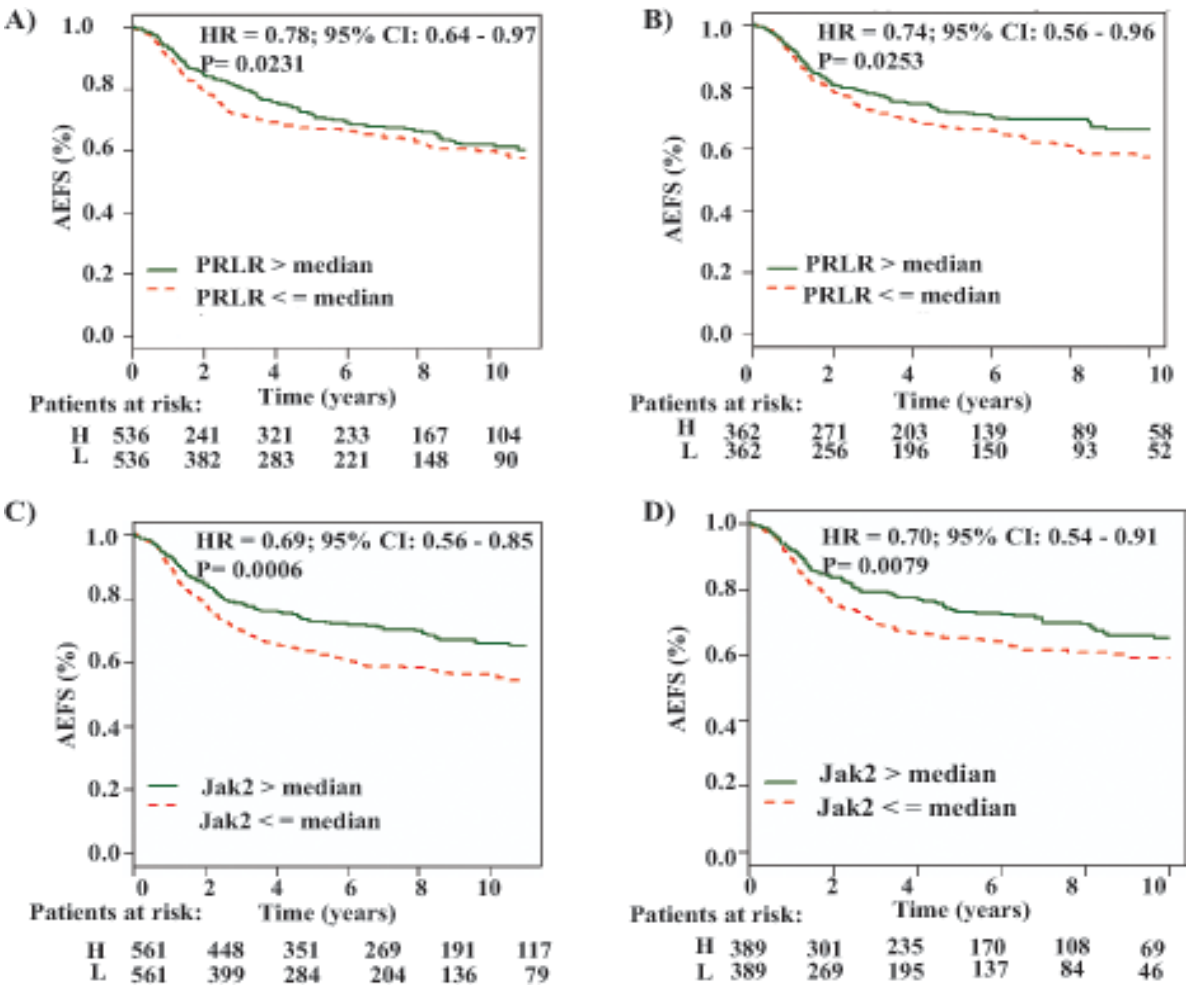

A)
HR = 0.78; 95% CI: 0.64 - 0.97
P= 0.0231
PRLR > median
PRLR <= median
AEFS (%)
Time (years)
Patients at risk:
H 536 241 321 233 167 104
L 536 382 283 221 148 90
B)
HR = 0.74; 95% CI: 0.56 - 0.96
P= 0.0253
PRLR > median
PRLR <= median
AEFS (%)
Time (years)
Patients at risk:
H 362 271 203 139 89 58
L 362 256 196 150 93 52
C)
HR = 0.69; 95% CI: 0.56 - 0.85
P= 0.0006
Jak2 > median
Jak2 <= median
AEFS (%)
Time (years)
Patients at risk:
H 561 448 351 269 191 117
L 561 399 284 204 136 79
D)
HR = 0.70; 95% CI: 0.54 - 0.91
P= 0.0079
Jak2 > median
Jak2 <= median
AEFS (%)
Time (years)
Patients at risk:
H 389 301 235 170 108 69
L 389 269 195 137 84 46

Figure 2.2

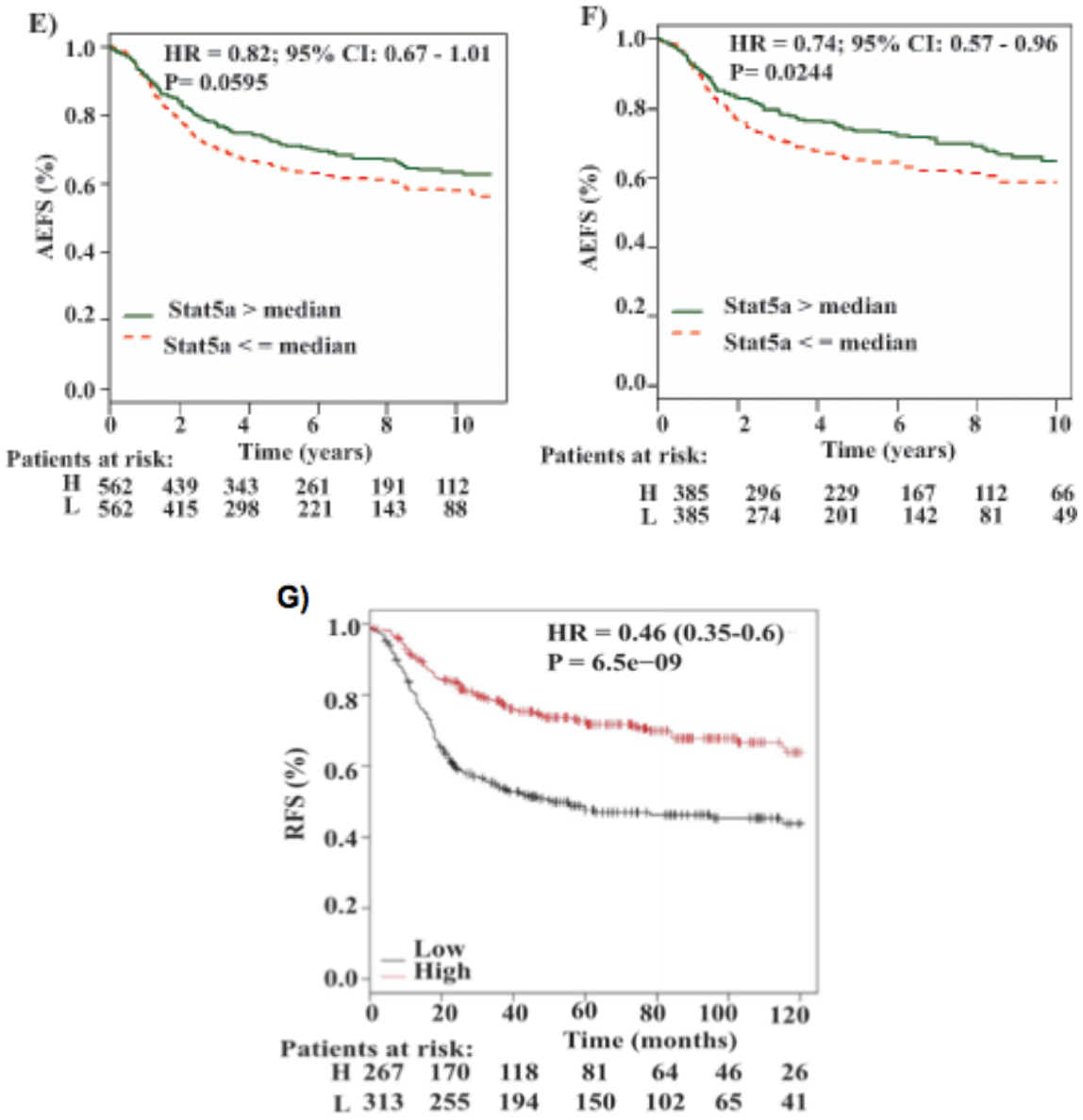

E)
HR = 0.82; 95% CI: 0.67 - 1.01
P= 0.0595
AEFS (%)
Stat5a > median
Stat5a < = median
Time (years)
Patients at risk:
H 562 439 343 261 191 112
L 562 415 298 221 143 88
F)
HR = 0.74; 95% CI: 0.57 - 0.96
P= 0.0244
AEFS (%)
Stat5a > median
Stat5a < = median
Time (years)
Patients at risk:
H 385 296 229 167 112 66
L 385 274 201 142 81 49
G)
HR = 0.46 (0.35-0.6)
P = 6.5e−09
RFS (%)
Low
High
Time (months)
Patients at risk:
H 267 170 118 81 64 46 26
L 313 255 194 150 102 65 41

Figure 2.2

Figure 2.2: Expression of PRL signalling pathway components correlates with favourable patient's outcome in TNBC.

Kaplan-Meier survival curves for PRLR (A & B), Jak2 (C & D) and Stat5a (E & F) gene expression levels according to Hu et al and Sorlie methods respectively using AEFS as an endpoint. Gene expression is stratified by median into high (green line) and low (red line) expression levels using breast cancer gene-expression miner v3.0. G) Kaplan-Meier survival curves for PRL pathway (PRL, PRLR, Jak2 and Stat5) based gene signature using RFS as an endpoint in basal breast cancer subtype using the KM plotter database.

Biological component	Metagene name	Key markers	PRLR expression in basal only

Basal-like phenotype — Basal-like — KRT5; KRT6a; KRT14; KRT17; SOX10; SFRP1; ELF5; EPHB3; GABRP

Correlation	Parameters	ELF5	EPHB3	KRT14	KRT17	KRT5	KRT6A
PRLR	R	0.17	0.09	-0.13	-0.2	-0.1	-0.16
PRLR	P	< 0.0001	0.0319	0.003	0.0516	0.0226	0.0002
PRLR	No	554	554	554	96	554	554

Apocrine/ Androgen receptor signalling — Apocrine — AR; FOXA1

Correlation table	Parameters	FOXA1	AR
PRLR	R	0.2	0.22
PRLR	P	< 0.0001	< 0.0001
PRLR	No	554	554

Immune system:

B-Cell — B-Cell — IgG FCGR1AFCGR 2A; FCGR 3A; FCGR 1B; FCGR 2B; FCGR 3B; FCGR 1C; FCGRT;IGHE

Correlation	Parameters	MX1	FCGR1A	FCGR2A	FCGR1B	FCGR2B	FCGRT
PRLR	R	-0.08	-0.12	-0.16	-0.1	-0.22	-0.1
PRLR	P	0.0588	0.0061	0.0002	0.0361	< 0.0001	0.015
PRLR	No	554	554	554	458	554	554

T-Cell — T-Cell — TCR=TRBTRA; TRG; LCK; ITK

Correlation	Parameters	ITK	LCK	HLA-RB1	HLA-RB4	HLA-DMB	HLA-DPA1	HLA-DPB1	HLA-DQB1
PRLR	R	-0.12	-0.19	-0.1	-0.08	-0.1	-0.07	-0.09	-0.1
PRLR	P	0.0055	< 0.001	0.015	0.0573	< 0.001	< 0.0001	< 0.0001	< 0.0001
PRLR	No	554	553	554	554	5300	5436	5607	5436

Correlation	Parameters	HLA-A	HLA-B	HLA-C	HLA-E	HLA-F	HLA-G
PRLR	R	-0.17	-0.13	-0.15	-0.21	-0.19	-0.11
PRLR	P	0.0001	0.0024	0.0005	< 0.0001	< 0.0001	0.007
PRLR	No	554	554	554	554	554	554

Stroma — Stroma — Decorin; DCN; Osteonectin SPARC; Fibronectin FN1; COL5A1

Correlation	Parameters	DCN	COL5A1
PRLR	R	-0.09	-0.09
PRLR	P	0.0412	0.0426
PRLR	No	554	554

Claudin-CD24 — 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	0.0106	0.0033	0.0378
PRLR	No	554	554	554	554

Proliferation — Proliferation — BUB1; CDC2 (CCND2); STK6 (AURKA); BIRC5; TOP2A;

Correlation	Parameters	CCND2	BIRC5
PRLR	R	-0.13	0.09
PRLR	P	0.0015	0.026
PRLR	No	554	554

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<tr><th>Biological component</th><th>Metagene name</th><th>Key markers</th><th>PRLR expression in basal only</th></tr>
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<tr><td>PRLR</td><td>r</td><td>-0.15</td><td>-0.22</td><td>-0.14</td></tr>
<tr><td>PRLR</td><td>p</td><td>0.0003</td><td>0.0313</td><td>0.001</td></tr>
<tr><td>PRLR</td><td>No</td><td>554</td><td>96</td><td>554</td></tr>
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</td></tr>
<tr><td>Adipocytes</td><td>Adipocyte</td><td>FABP4; PLIN1; ADIPOQ; ADH1B</td><td>NOTHING</td></tr>
<tr><td>Angiogenesis</td><td>VEGF</td><td>VEGF; adrenomedullin (ADM); ANGPTL4</td><td>
<table>
<tr><td>Correlation</td><td>Parameters</td><td>ADM</td><td>ANGPTL4</td><td>VEGFC</td></tr>
<tr><td>PRLR</td><td>r</td><td>-0.09</td><td>-0.1</td><td>-0.07</td></tr>
<tr><td>PRLR</td><td>p</td><td>0.0319</td><td>0.0251</td><td>0.0796</td></tr>
<tr><td>PRLR</td><td>No</td><td>554</td><td>511</td><td>554</td></tr>
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<tr><td>Inflammation</td><td>IL-8</td><td>IL-8; CXCL1; CXCL2</td><td>
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<tr><td>PRLR</td><td>r</td><td>-0.08</td><td>-0.13</td></tr>
<tr><td>PRLR</td><td>p</td><td>0.0469</td><td>0.0015</td></tr>
<tr><td>PRLR</td><td>No</td><td>554</td><td>554</td></tr>
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</td></tr>
<tr><td>HOXA gene cluster</td><td>HOXA</td><td>HOXA-4; 5; 7; 9; 10; 11</td><td>NOTHING</td></tr>
<tr><td>Histone gene cluster</td><td>Histone</td><td>Histones H2A; H2B</td><td>NOTHING</td></tr>
</table>

Figure 2.3

Figure 2.3: PRLR gene expression and its association with different TNBC gene signatures representative of molecular subtypes

Analysis of PRLR gene expression levels in association with different clusters of correlated genes (metagenes) used to distinguish molecular heterogeneity of TNBC. PRLR gene expression inversely correlates with genes related to basal-like subtype (basal keratins KRT14, KRT14, KRT5 and KRT6a), mesenchymal-like (Claudin low) and genes representative of the non-neoplastic cell populations (red). PRLR expression shows a significant positive correlation with members of two metagene clusters (green) apocrine/androgen receptor signalling (FOXA1 and AR) and claudin-CD24 (CLDN3, CLDN4 and CD24) representing luminal/epithelial differentiation.

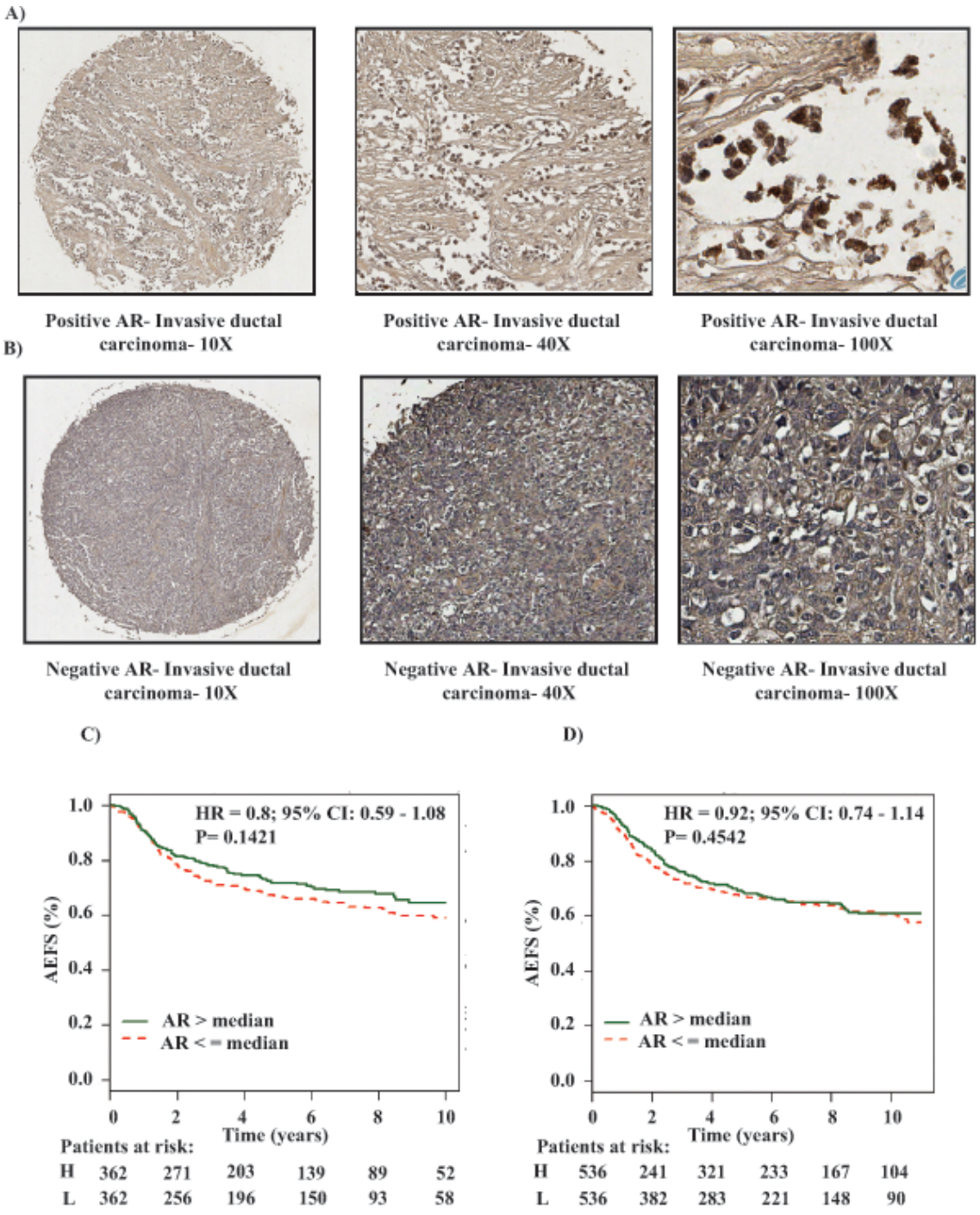
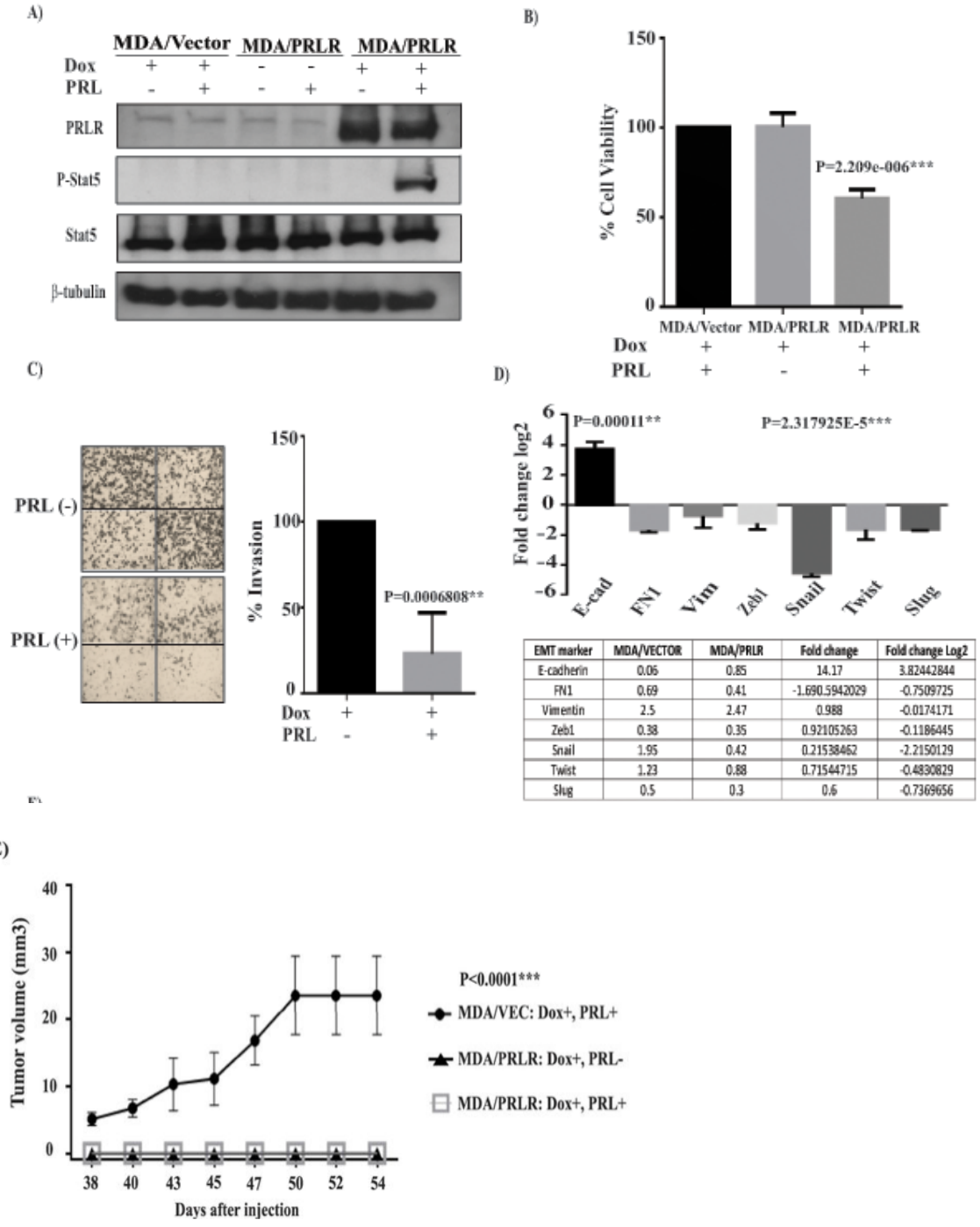

A)
Positive AR- Invasive ductal carcinoma- 10X
Positive AR- Invasive ductal carcinoma- 40X
Positive AR- Invasive ductal carcinoma- 100X
B)
Negative AR- Invasive ductal carcinoma- 10X
Negative AR- Invasive ductal carcinoma- 40X
Negative AR- Invasive ductal carcinoma- 100X
C)
HR = 0.8; 95% CI: 0.59 - 1.08
P= 0.1421
AEFS (%)
AR > median
AR <= median
Time (years)
Patients at risk:
H 362 271 203 139 89 52
L 362 256 196 150 93 58
D)
HR = 0.92; 95% CI: 0.74 - 1.14
P= 0.4542
AEFS (%)
AR > median
AR <= median
Time (years)
Patients at risk:
H 536 241 321 233 167 104
L 536 382 283 221 148 90

Figure 2.4

Figure 2.4: AR protein and gene expression in TNBC cases

A) Positive immunohistochemical nuclear staining of AR in PRLR positive breast cancer case (4X, 40X and100X). B) Negative immunohistochemical staining of AR in a TNBC case (4X, 40X and 100X). C & D) Kaplan-Meier survival curves for AR m-RNA levels in basal-like subtype stratified according to Sorlie's and Hu's classifications respectively, using AEFS as an endpoint using the breast cancer gene-expression miner v3.0. Gene expression is stratified according to median into high (green line) and low (red line) expression levels.



EMT marker	MDA/VECTOR	MDA/PRLR	Fold change	Fold change Log2
E-cadherin	0.06	0.85	14.17	3.82442844
FN1	0.69	0.41	-1.690.5942029	-0.7509725
Vimentin	2.5	2.47	0.988	-0.0174171
Zeb1	0.38	0.35	0.92105263	-0.1186445
Snail	1.95	0.42	0.21538462	-2.2150129
Twist	1.23	0.88	0.71544715	-0.4830829
Slug	0.5	0.3	0.6	-0.7369656

Figure 2.5

Figure 2.5: Restoring PRL-differentiation pathway in TNBC suppresses tumorigenesis.

A) Cells (MDA-MB-231/vector and MDA-MB-231/PRLR) were incubated in DMEM (2% FBS) and stimulated or not with dox (100 ng/ml) O/N and stimulated or not with hPRL 250 ng/ml for 15 minutes. Cell lysates were immune detected using antibodies to PRLR, p-Stat5, Stat5 and β-tubulin. B) Control MDA-MB-231/vector and MDA-MB-231/PRLR cells were plated in starvation media and treated or not with dox (100 ng/ml) and hPRL (250 ng/ml) for 72hrs. MTT assays were performed and the results are presented as means ± SEM for triplicates of five independent experiments (p=2.209e-006). C) MDA-MB-231/PRLR cells were stimulated with dox (100 ng/ml) and stimulated or not with hPRL (250 ng/ml) for 72hrs. Then equal number of cells was plated on Matrigel for invasion for 24hrs. Columns represent means of triplicates of three independent experiments (P=0.00068). Microscope images of invaded cells taken from 4 fields of a representative well (left). D) MDA-MB-231/vector and MDA-MB-231/PRLR cells were treated with dox (100ng/ml) and hPRL (250 ng/ml) for 72hrs and the expression of EMT markers (as indicated) was examined using q-RT-PCR. Results are expressed as log2 fold change of triplicates of three independent experiments. E) Graph depicting tumor volume of MDA-MB-231/Vector or MDA-MB-231/PRLR xenografts after treatment for a period of 8 weeks as indicated.

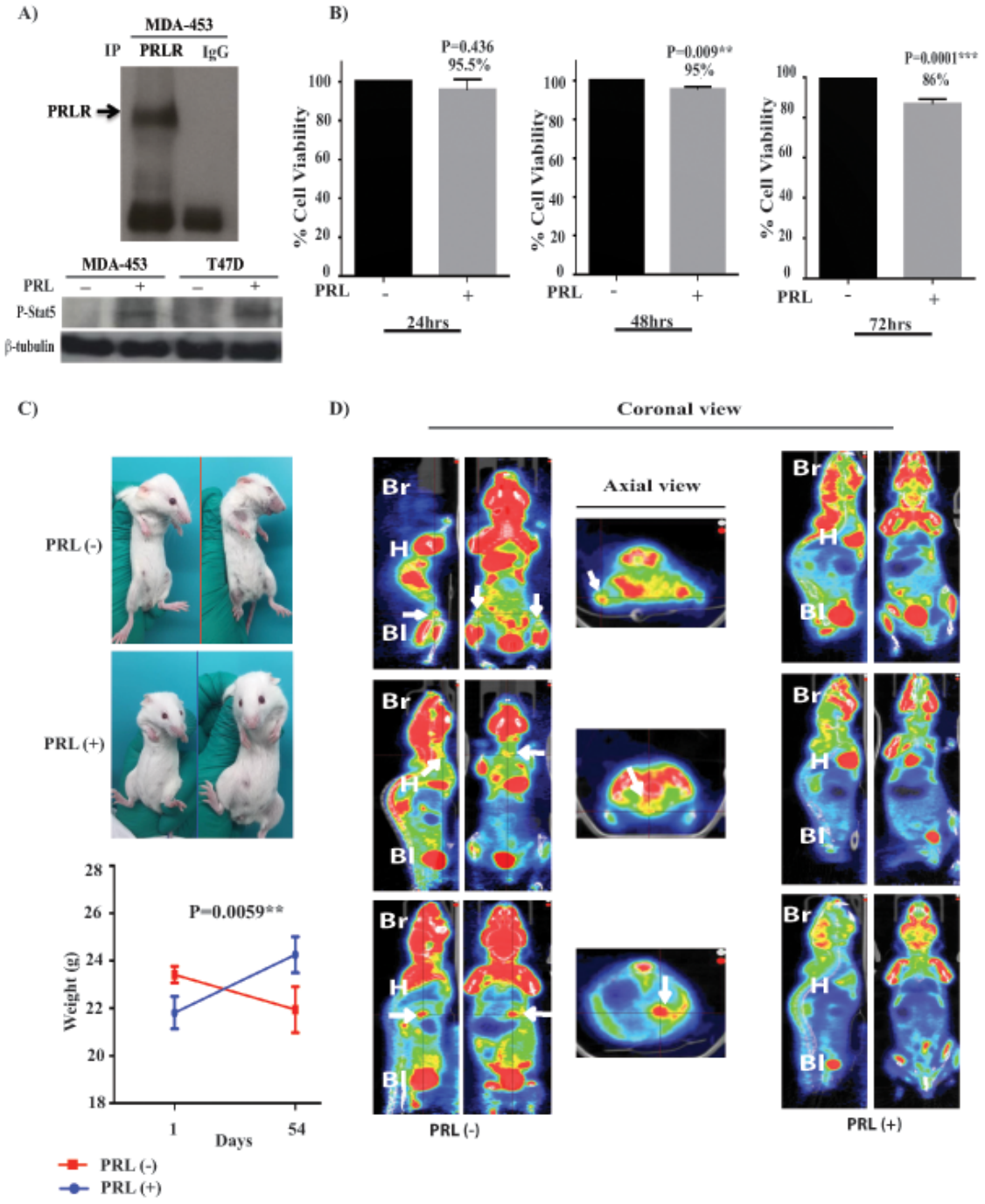

A)
MDA-453
IP PRLR IgG
PRLR
MDA-453 T47D
PRL – + – +
P-Stat5
β-tubulin
B)
P=0.436
95.5%
P=0.009**
95%
P=0.0001***
86%
% Cell Viability
PRL - + - + - +
24hrs
48hrs
72hrs
C)
PRL (-)
PRL (+)
P=0.0059**
Weight (g)
Days
PRL (-)
PRL (+)
D)
Coronal view
Axial view
Br
H
Bl
PRL (-)
PRL (+)

Figure 2.6

Figure 2.6: PRL suppresses viability and tumorigenesis of MDA-MB-453 cells representative of TNBC-PRLR subgroup

A) MDA-MB-453 cells were lysed and immune precipitated using antibody to PRLR or control IgG and immune detected using antibody to PRLR (Materials and Methods). MDA-MB-453 cells were incubated in L-15 (2% FBS) for an O/N period. Cells were then stimulated or not with hPRL (250ng/ml) for 15 minutes. Cell lysates were immune detected using antibodies to p-Stat5 and β-tubulin. B) MDA-MB-453 cells ($5x10^3$ cells) were plated in L-15 (2% FBS) and treated or not treated with hPRL (250ng/ml) for 24-72 hrs as indicated. MTT assays were performed and results are presented as the mean ± SEM of triplicates of five independent experiments. C) Representative pictures of NOD/SCID mice untreated or treated with r hPRL for 8 weeks. Graph depicting measurements of body weight in untreated or treated MDA-MB-453 xenograft mice. D) Whole-body imaging of MDA-MB-453 xenograft using PET/SPECT/CT scan. FDG uptake is observed in brain (Br), heart (H) and bladder (Bl) as well as in xenograft tumors (white arrows).

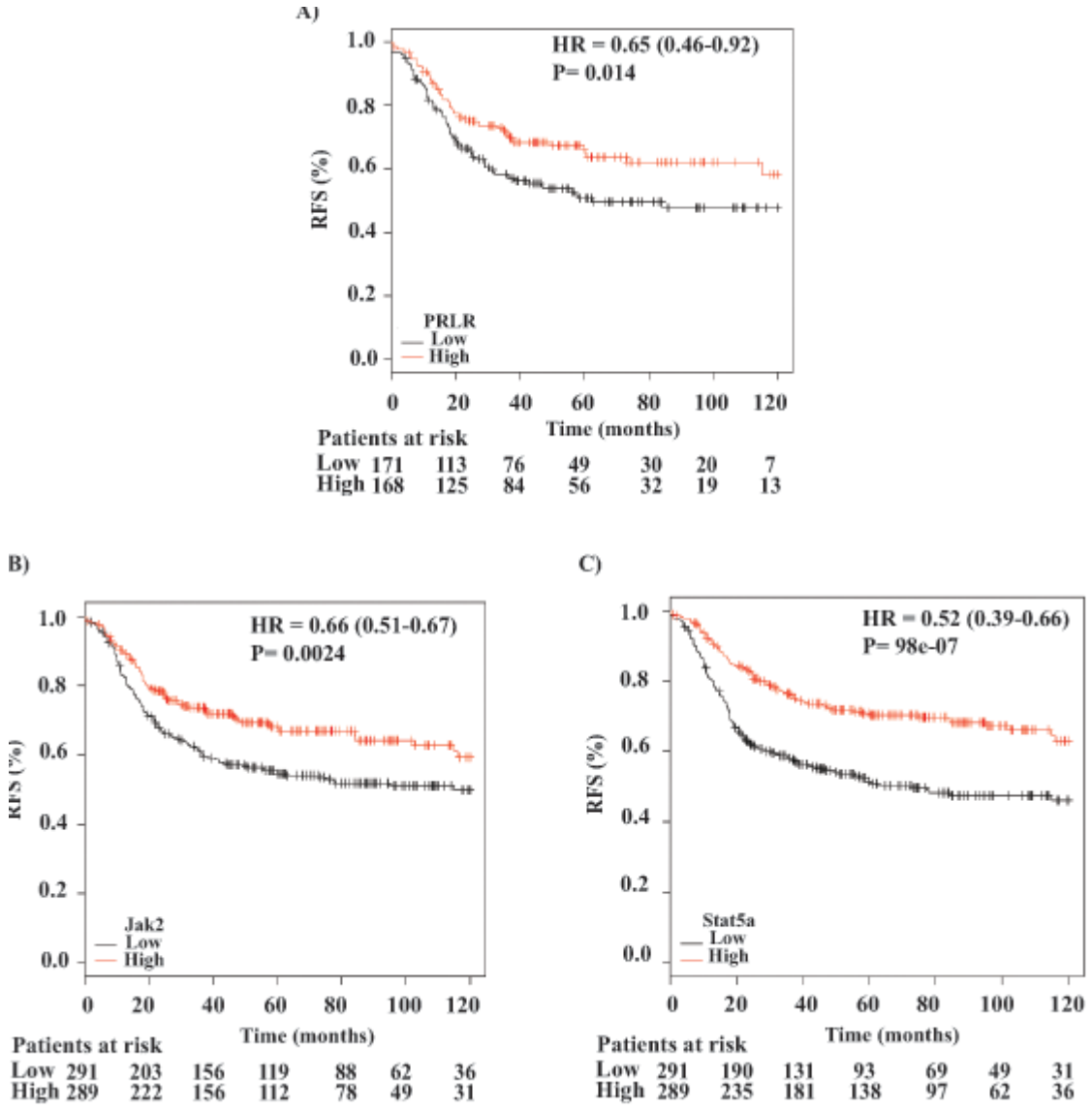


Figure S1: A & B) Kaplan-Meier survival curves for PRL gene expression levels in basal-like subtype according to Hu et al and Sorlie methods respectively using AEFS as an endpoint stratified by median into high (green line) and low (red line) expression levels using breast cancer gene- expression miner v3.0. In each graph HR and P values are indicated.

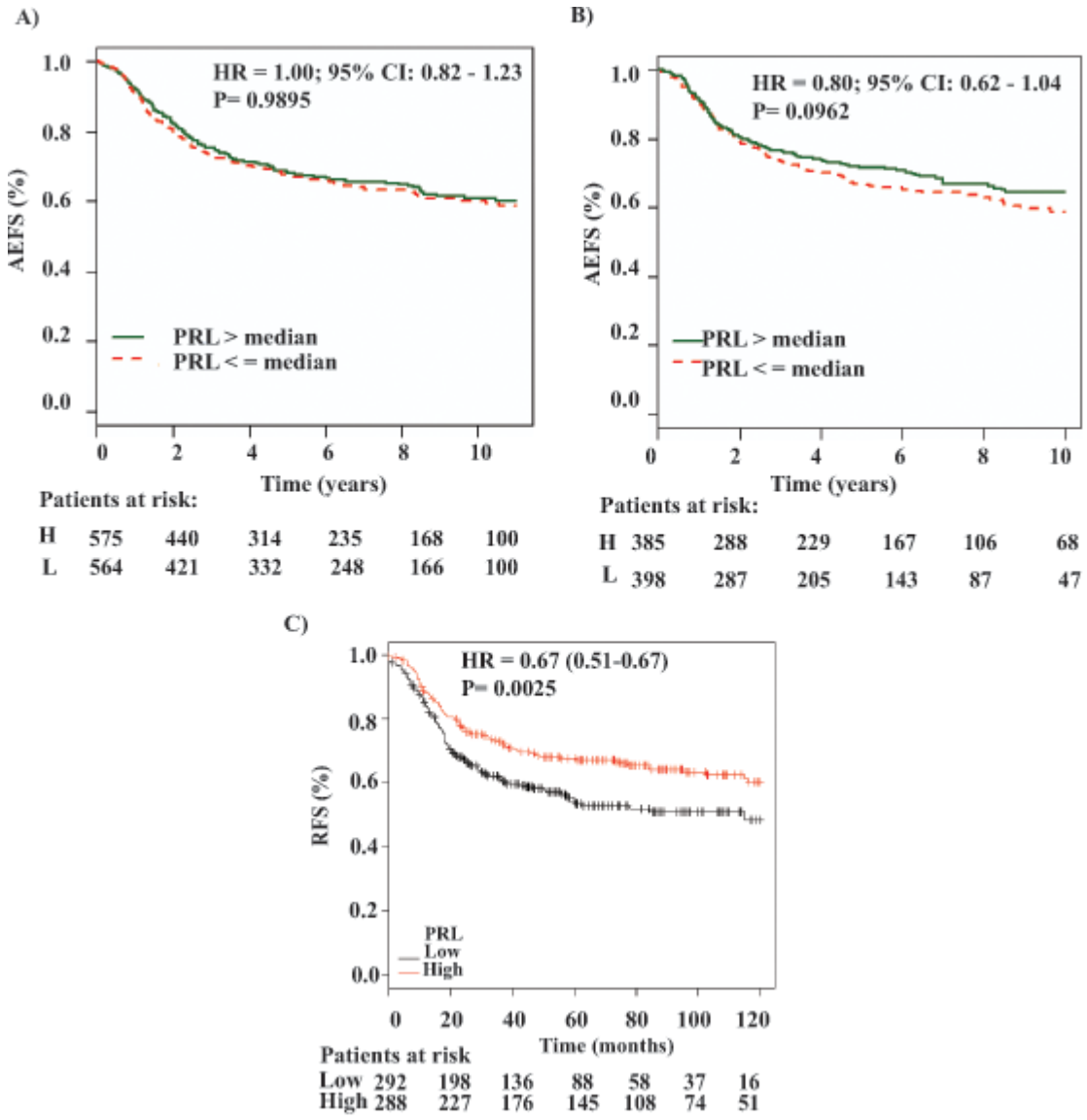
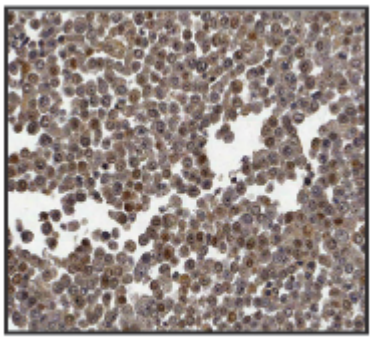


Figure S2: Kaplan-Meier survival curves for PRL (A), PRLR (B), Jak2 (C), Stat5a (D) gene expression levels in basal-like subtype, using DMFS as an endpoint using GOBO database stratified with median into high (black line) and low (red line) expression levels



AR expression in T47D cells
(40X)

Figure S3: Positive immunohistochemical staining of AR protein showing nuclear stain in T47D cells

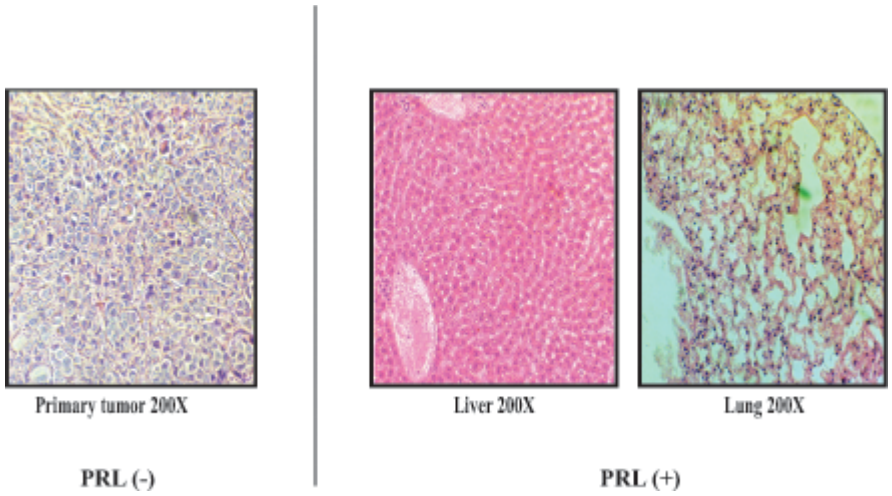


Figure S4: Histopathological analysis of primary tumors and organs of MDA-MB-453 xenograft animal models. H&E staining of primary breast cancer tumor obtained from the flanks of MDA-MB-453 untreated mice (left panel), liver (middle panel) and lungs (right panel) tissues of hPRL treated xenograft mice.

PRL (-)	Time after injection (hours)	Prolactin levels (ug/L)
1	2	<0.3
	4	<0.3
2	2	<0.3
	4	<0.3
3	2	<0.3
	4	<0.3
PRL (+)	**Time after injection (hours)**	**Prolactin levels (ug/L)**
1	2	53.2
	4	19.8
2	2	24.3
	4	22.7
3	2	0.9
	4	0.5

Table S1: Serum levels of hPRL in MDA-MB-231/PRLR xenograft animal mouse model. Serum levels hPRL detected by radioimmunoassay at 2hrs and 4hrs post intra-peritoneal injection of recombinant hPRL (0.1ug/g of body weight) in untreated and treated mice.

Chapter 3: Prolactin pro-differentiation pathway activation depletes cancer stem cells and inhibits tumorigenesis through the induction of cellular senescence in triple negative breast cancer

Chapter 3:

Prolactin pro-differentiation pathway activation depletes cancer stem cells and inhibits tumorigenesis through the induction of cellular senescence in triple negative breast cancer

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3.1 Preface

In our previous study, we have determined the biological role of PRL in TNBC. Significantly, we demonstrated that pro-differentiation pathways as a mean to revert/suppress tumorigenesis. We determined that activation of PRL signalling pathway in TNBC results in reversal of the mesenchymal highly aggressive phenotype of TNBC through induction of cell differentiation to epithelial less aggressive phenotype. Furthermore, our results identified PRLR as a novel and relevant sub-classifier, able to detect a new subgroup (TNBC-PRLR) with better overall survival. Altogether, these findings led us to propose a new management strategy for TNBC patients. This approach is based on screening for PRLR expression in patients that may benefit from the use of PRL hormone as potential therapy. This prompted us to investigate what are the mechanisms through which PRL induces its anti-tumorigenic and anti-tumor progression effects in TNBC. Different mechanisms by which PRL regulates TNBC tumorigenicity were studied, as well as the role of PRL pathway in regulating TNBC stemness, tumor initiation capacity and cell fate.

Our results indicate that PRL treatment is able to inhibit tumor initiation capacity of TNBC cancer stem cells by reduction of its tumor-sphere formation ability. Importantly, PRL treatment is able to change the CSC phenotype from $CD44^{hi}/CD24^{low}$ and ALDH+ sub-populations (highly tumorigenic) to $CD44^{low}/CD24^{low}$ and ALDH- (non-tumorigenic) sub-populations and decrease stem cell markers (OCT4, SOX2 and Nanog) implicated in self-renewal.

Moreover, we showed that PRL treatment is able to induce cellular senescence through regulation of cell cycle arrest and heterochromatin formation. PRL treatment was also able to induce SAβ-galactosidase, to induce the formation of senescence associated heterochromatin foci (SAHF) and to inhibit the senescence-associated secretory phenotype (SASP). Furthermore, we demonstrated that PRL inhibits tumour progression *in vivo.*

3.2 Abstract

Triple negative tumors account for ~20% breast cancer. The management of these tumors represents a challenge due to its aggressive phenotype, heterogeneity and lack of targeted therapy. Loss of differentiation as well as enrichment with stem cell populations is believed to play an essential role in cancer invasion, metastasis and chemo and radiotherapy resistance. in this breast cancer subtype. Previously, we showed that PRL pathway in TNBC tumors was associated with a decrease in aggressive phenotype, favourable prognosis and inhibits tumorigenesis. However, the mechanisms through which PRL produce its effect was not fully elucidated. Here, we investigated different potential mechanisms though which PRL might produce its anti-tumorigenic effect. Interestingly, we found that PRL induces cellular differentiation of cancer stem cells (CSC) by reducing their tumor initiation, self-renewal capacity and their transcriptional network. Furthermore, PRL treatment is able to change CSC highly tumorigenic sub-populations ($CD44^+/CD24^-$ and $ALDH^+$) into non-tumorigenic sub-populations ($CD44^-/CD24^-$ and $ALDH^-$). In addition, PRL was found to induce cellular senescence program through epigenetic regulation of heterochromatin. PRL treatment was also able to induce SAβ-galactosidase, regulate different genes/proteins expressed in cellular senescence and formation of SAHFs. Furthermore, we found PRL to inhibit senescence-associated secretory phenotype and inhibit tumour progression *in vivo.* All this findings highlight the use of PRL as potential new approach for TNBC personalized therapy.

3.3 Introduction

Triple Negative Breast Cancer (TNBC) account for about 10-20% of all breast cancer cases and is characterized by the lack expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)(Rakha, El-Sayed et al. 2007, Foulkes, Smith et al. 2010). TNBC represents high-grade tumors with aggressive phenotype. This subtype represents the worse prognosis and patients are at high risk of early relapse and metastasis(Anders and Carey 2009) due to the presence of high levels of cancer stem cells (CSCs). These CSCs have been shown to be responsible for resistance to chemo- and radio-therapies(Kim, Joo et al. 2009) (Phillips, McBride et al. 2006, Wicha, Liu et al. 2006, Li, Lewis et al. 2008, Crown, O'Shaughnessy et al. 2012). For that reason, no targeted therapy is readily available, and more efforts are needed to provide new avenues for developing novel therapies against this breast cancer subtype.

CSCs are a small population of cells within the tumors, capable of self-renew, differentiate into multiple lineages, being responsible for tumor initiation, metastasis and recurrence(Dalerba, Cho et al. 2007) (Charafe-Jauffret, Ginestier et al. 2009). These properties are under control of molecular mechanism in a highly regulated manner by several transcription factors such as Oct4, Sox2 and Nanog (Rodriguez-Pinilla, Sarrio et al. 2007, Kim and Nam 2011, Lu, Mazur et al. 2014). These transcription factors significantly increase the potential of CSCs to form tumorspheres and promote metastatic invasion when they are deregulated or overexpressed (Simoes, Piva et al. 2011). Moreover, also they also act as

molecular switches that control the CSC fate and differentiation during cancer development (Takahashi, Tanabe et al. 2007). Additionally, a Nanog/Oct4/Sox2 expression signature was directly associated with high-grade TNBC basal-like subtype and with poor clinical outcome (Ben-Porath, Thomson et al. 2008).

Other feature of CSCs is that they can be recognized by their expression of different markers. In TNBC CSCs are recognized by surface markers such as CD44, CD24 and the enzyme aldehyde dehydrogenase 1 (ALDH1) (Al-Hajj, Wicha et al. 2003, Ginestier, Hur et al. 2007). The adhesion molecule CD44 together with no or very low levels of CD24, referred to as CD44+/CD24- phenotype(Al-Hajj, Wicha et al. 2003), was found to be the highest population expressed in TNBC and associated with poor prognosis (Honeth, Bendahl et al. 2008, Buess, Rajski et al. 2009). Furthermore, ALDH1, a detoxifying enzyme involved in catalyzing the oxidation of intracellular aldehydes, has been suggested as another putative BCSC marker (Ginestier, Hur et al. 2007, Charafe-Jauffret, Ginestier et al. 2009). ALDH1+ phenotype was found to be associated with biological aggressiveness (tumor size/stage) and poor outcomes of TNBC patients (Ma, Li et al. 2017). Therefore, cancer stem cells are an interesting target in TNBC. For that reason, the identification of the possible mechanism that contributes to differentiation/eradication of these two TNBC CSC populations will provide an approach to prevent relapse, drug resistance and metastasis.

On the other hand, many emerging approaches for treating breast cancer patients was based on activation of apoptotic signals leading to cell death

promotion (Perez-Mancera, Young et al. 2014). However, recently, cellular senescence was proposed as an intrinsic tumor suppressor mechanism (d'Adda di Fagagna 2008, Kuilman, Michaloglou et al. 2010). Senescence is a non-reversible state characterized by distinct cellular morphology, formation of senescence-associated heterochromatin foci and expression of senescence-associated genes that are also linked to differentiation. For that reason, senescence as a tumor suppressor mechanism and its relation with differentiation in TNBC is also important to be investigated.

Prolactin hormone (PRL) is one of the major regulators of mammary gland development and terminal differentiation of the mammary epithelial cells (Ormandy, Binart et al. 1997, Hennighausen and Robinson 2005). However, its role in breast carcinogenesis is not fully elucidated. Many studies have implicated PRL in promoting tumorigenesis through a PRL/PRLR autocrine loop (Chen, Ramamoorthy et al. 1999, Vonderhaar 1999, Chen, Holle et al. 2002, Clevenger, Furth et al. 2003) or cooperating with p53 loss to induce Claudin low mammary carcinomas(O'Leary, Rugowski et al. 2014). Also was suggested to interfere in BRCA1-p21 regulation (O'Leary, Rugowski et al. 2014). In addition, PRL and it signalling was recently implicated with breast cancer metastatic spread (Yonezawa, Chen et al. 2015, Sutherland, Forsyth et al. 2016). While the above studies highlight PRL as promoter of carcinogenesis, other studies, including ours, suggested a different role as a potential suppressor of breast tumorigenesis. Indeed, we have previously shown that PRL, through PRLR/Jak2 signalling suppresses epithelial-mesenchymal-transition (EMT) and reduces the

invasive properties of breast cancer cells (Nouhi, Chughtai et al. 2006). Moreover, PRLR and PRL in human breast cancer were found to correlate with favourable patient outcome (Hachim, Hachim et al. 2016, Hachim, Shams et al. 2016). In support to these findings, PRL and PRLR expression were found down regulated in breast cancer patients and breast cancer cell lines (Nitze, Galsgaard et al. 2013). Furthermore PRL effector molecule Stat5a was found to be associated also with favourable prognosis(Yamashita, Nishio et al. 2006) and its loss of expression to be associated with tumor progression and unfavourable clinical outcomes(Peck, Witkiewicz et al. 2012) Importantly, was recently demonstrated that PRL differentiation pathway is also able to identify the novel TNBC-PRLR subgroup characterized by luminal-epithelial phenotype and favourable outcome. Furthermore, functional studies demonstrated that activation of PRL signalling pathway suppresses the aggressive nature of TNBC cells in vitro and tumorigenesis in vivo (Lopez-Ozuna, Hachim et al. 2016).

Together these findings provide evidence regarding the role of PRL pathway in maintaining tissue differentiation as a suppressor of breast carcinogenesis. As CSCs are believed to be responsible of relapse and metastasis in TNBC, PRL differentiation pathway effects in TNBC CSCs should be elucidated. In addition, there are no studies that evaluate the cell fate after PRL treatment. For that reason, cellular senescence and its possible tumor suppression role in TNBC has also to be examined. In this study, we evaluated the effects of PRL pro-differentiation signalling in TNBC CSCs differentiation and cellular senescence. Using tumor formation assays and FACS analysis we were

able to demonstrate that PRL was able to inhibit tumor formation capacity and self-renewal of $CD44^{+}/CD24^{-}$ and $ALDH^{+}$ TNBC CSC subpopulations. In addition, using qPCR analysis, we also find that PRL was able to inhibit the mRNA levels of group of CSC transcriptional markers including OCT4, SOX2 and NANOG. Moreover, we also demonstrated that PRL treatment was also able to induce SAβ-galactosidase and different regulatory genes/proteins expressed in cellular senescence. This study provide for the first time more detailed information about the mechanism through which PRL induces its anti-tumorigenic and anti-tumor progression effects in vitro and in vivo, highlighting its possible use as a novel TNBC therapeutic approach.

3.4 Results

3.4.1 Breast cancer stem cell populations in human breast cancer cell lines representative of basal-TNBC and TNBC-PRLR sub-groups

TNBC tumors are highly enriched with $CD44^+/CD24^-$ and $ALDH^+$ CSC populations; considered as the most tumorigenic within TNBC. For that reason, we first evaluated the levels of these CSC sub-populations present in TNBC cells representative of the basal subtype in comparison to the sub-populations found in the TNBC-PRLR subtype. We used the highly aggressive mesenchymal-like TNBC cell line MDA-MB-231, the MDA-MB-231/PRLR in which PRLR expression was restored, MDA-MB-231/vector as a control and the MDA-MB-453 cell line as representative of the TNBC-PRLR subgroup that expresses the PRLR endogenously (Lopez-Ozuna, Hachim et al. 2016). CD44 and CD24 staining was performed in both cellular models using flow cytometry analysis. Interestingly, as can be seen in Figure S1, we found that $CD44^+/CD24^-$ mesenchymal-like stem cell population is highly enriched in MDA-MB-231WT, MDA-MB-231/vector and MDA-MB-231/PRLR cells (~80%) and least present in MDA-MB-453 cells (8%). We next examined the proportion of $ALDH^+$ breast cancer stem cell population in the two model cell systems. $ALDH^+$ epithelial-like CSCs population was found to represent ~2% in MDA-MB-231 WT, MDA-MB-231/PRLR and MDA-MB-231/vector cells and ~3% in the MDA-MB-453 cells. Together these results suggest that TNBC-PRLR subgroup have reduced levels of the aggressive

mesenchymal breast cancer stem cell sub-population in comparison to the basal subtype.

3.4.2 PRL signaling pathway activation in TNBC is able to induce differentiation of $CD44^+/CD24^-$ and $ALDH^+$ CSC into non-tumorigenic populations

The $CD44^+/CD24^-$ and $ALDH^+$ CSCs populations are considered to have the highest tumorigenic potential. For that reason, we investigated the role of PRL in regulating these populations. $CD44^+/CD24^-$ sub-population was examined using flow cytometry analysis (FACS) in MDA-MB-231/PRLR treated or not with hPRL for 72h. As shown in Figure 3.1A (right and left panels), as expected control MDA-MB-231/PRLR cells contain ~77% of the tumorigenic CD44+/CD24- sub-population. Importantly, upon PRL treatment of MDA-MB-231/PRLR cells we observed a significant decrease in this tumorigenic population by 80% (~15%, P=0.0001), and the generation of $CD44^-/CD24^-$ in comparison with untreated cells. This suppression of CD44+/CD24- population upon PRL treatment was also confirmed by examining the role of PRL in suppressing expression of each stem cell markers CD44 and CD24 individually. These results implicate PRL as an important suppressor of the mesenchymal breast cancer stem cell sub-population. As shown above MDA-MB-453 cells representative of TNBC-PRLR subgroup did not show enrichment in the tumorigenic $CD44^+/CD24^-$ population but did show the presence of $ALDH^+$ cancer stem cells (~3%), therefore we next examined using FACS whether PRL treatment is also able to suppress the ALDH+ stem cell population. Interestingly, PRL treatment of MDA-MB-453 cells

caused a significant reduction in $ALDH^+$ population by 80.3% (P=0.0003) in comparison with untreated cells (Figure 3.1B). Together, these results emphasize for the first time the important pro-differentiation role of PRL in suppressing TNBC CSCs and differentiating them into non-tumorigenic populations.

3.4.3 PRL down-regulates transcription factors implicated in stemness and self-renewal of breast cancer stem cells (BCSCs) in TNBC

CSCs possess high tumor initiation and self-renewal capacity. To better evaluate the role of PRL in modulating BCSCs behavior, we next examined using qPCR analysis, the role of PRL in regulating the m-RNA expression levels of transcription factors important for self-renewal and pluripotency including OCT4, SOX2 and NANOG. Importantly, PRL treatment of MDA-MB-231/PRLR cells resulted in a significant down-regulation of gene expression of each transcription factor OCT4 (5 folds), SOX2 (6.6 folds) and NANOG (6.9 folds) (Figure 3.2A). In accordance with this data we also observed a significant reduction in CD44 mRNA levels (2.3 folds) and a modest but significant increase in CD24 levels (1.3 folds) in MDA-MB-231/PRLR following PRL treatment (Figure 3.2A). Similarly, PRL stimulation of MDA-MB-453 cells also led to a significant reduction in gene expression of OCT4 (1.74 folds), SOX2 (1.8 folds) NANOG (2.39 folds) as well as CD44 (2.62 folds) mRNA levels following PRL treatment (Figure 3.2B). Together, these findings indicate that PRL may suppress TNBC stemness by

suppressing the expression of the self-renewal transcriptional network following PRL treatment in both MDA/PRLR and MDA-MB453 cells.

3.4.4 PRL suppresses *in vitro* tumor formation capacity and cell viability of the most tumorigenic TNBC BCSC sub-populations $CD44^{+}/CD24^{-}$ and $ALDH^{+}$

The ability of generating new tumors represents one of the cardinal features of CSCs. Therefore; we next examined the role of PRL in regulating the tumor initiation capacity of TNBC CSCs. Using *in vitro* tumorsphere formation assay, MDA-MB-231/Vector and MDA-MB-231/PRLR cells were either untreated or treated with hPRL for 72hrs. Cells were then seeded under low-attachment conditions in the presence or the absence of hPRL for a period of 7 days. As shown in Figure 3.2C PRL treatment was able to reduce primary tumor spheres formation (40.81%) (P=0.0006) in MDA-MB-231/PRLR in comparison with control vector cells or untreated MDA-MB-231/PRLR cells. Primary tumorspheres were dissociated and re-plated for secondary tumorsphere formation under PRL stimulation for additional 7 days. Importantly, we also observed a decrease of secondary tumor spheres formation (74.2%) (P=0.0001) in MDA-MB-231/PRLR treated cells in comparison to control and untreated cells. Moreover, as shown in Figure 3.2D PRL was also able to significantly suppress primary (36.6%) (P=0.0001) and secondary (32.1%) (P=0.0001) tumor-sphere formation in MDA-MB-453 cells in comparison with non-treated cells. These results together

highlight the critical inhibitory effect of PRL in tumor formation capacity and self-renewal of $CD44^+/CD24^-$ and $ALDH^+$ TNBC BCSC subpopulations.

We next investigated the effects of PRL treatment in regulating *in vitro* tumor formation capacity of the various TNBC stem cells subpopulations. For this reason, we sorted the BCSC sub-populations present in MDA-MB-231/PRLR: $CD44^+/CD24^-$, $CD44^+/CD24^+$ and $CD44^-/CD24^-$ and we plated them for tumorsphere formation assay. We found that PRL treatment was able to decrease significantly the tumor formation capacity of BCSCs present in all TNBC sub-populations (~40%, 62% and 59% respectively) (P=0.0001, P=0.0045, P=0.0166, respectively) (Figure 3.2E). In the same manner, PRL treatment was also sufficient to cause a significant reduction (70%) ((P=0.0001) in the tumor formation capacity of $ALDH^+$ cells sorted from MDA-MB-453 that express PRLR endogenously (Figure 3.2F).

Next, we evaluated the role of PRL in regulating cell viability of the most tumorigenic BCSC sub-populations. The $CD44^+/CD24^-$ and $ALDH^+$ stem cell populations were sorted from MDA-MB-231/PRLR and MDA-MB-453 cells, respectively, using flow cytometry and subjected to a cell viability assay. Interestingly, our results showed that PRL treatment induced a significant decrease in cell viability of $CD44^+/CD24^-$ populations at 24h, 48h and 72h (32%, 21%, 23% respectively) (P=0.0006, P=0.0087, P=0.0031, respectively) in comparison with untreated cells. The same significant reduction in viability of $ALDH^+$ was observed following PRL treatment (23%) (P=0.031) (Figure 3.2G).

Altogether, these results demonstrate that PRL is able to inhibit the tumorsphere formation capacity of TNBC BCSC sub-populations as well as decrease their cell viability.

3.4.5 $CD44^+$ stem cell marker is highly expressed in TNBC patients and correlates with poor survival outcomes

Next, to evaluate the clinical significance of PRL ability to reduce $CD44^+$/CD24- BCSC population, we analyzed the expression levels of CD44 stem cell marker m-RNA levels in TNBC clinical cases and its association with patient's outcome using publicly available databases of breast cancer patients. First, we examined the gene expression levels of CD44 in TNBC patients in comparison with non-TNBC patients in a large cohort of 4703 patients using the Customize Expression Analysis tool of bc-GenExMiner4.0 database. Our analysis showed that CD44 gene expression levels are higher in TNBC patients (417 patients) in comparison with the non-TNBC patients (4286 patients) (P=0.0001) (Figure 3.3A). To start investigating the clinical relevance of the expression of CD44 and its association with patient outcome, we further analyzed the association between CD44 gene expression and patient outcomes including; any event free survival (AEFS), metastasis-free survival (MFS) and relapse-free survival (RFS) using the Prognostic Analysis Tool of bc-GenExMiner4.0 database in basal-like intrinsic breast cancer subgroup (representing TNBC). Also, KM plotter database was used in this analysis. Interestingly, we found a

significant association between CD44 gene expression levels and decrease of AEFS (242 patients P=0.0462) (Figure 3.3B), MFS (169 TNBC patients, P=0.0211) (Figure 3.3C) and RFS (patients 618 P=0.059) (Figure 3.3D). Together, these findings indicate that expression of CD44 stem cell marker in TNBC patients displays an unfavorable outcome and decrease survival.

3.4.6 PRL reduces cell proliferation in TNBC cells though induction of cell cycle arrest independent of apoptosis

To decipher the mechanisms that might explain the ability of PRL to suppress cell viability of TNBC cells, we evaluated the effect of PRL in regulating apoptosis and cell cycle. Initially, the effects of PRL treatment on apoptosis were assessed using caspase 3/7 assays. Our results showed no significant change in caspase 3/7 activity in MDA-MB-231/PRLR cells treated with hPRL for 24h, 48h and 72h in comparison with untreated cells (Figure S2A). The same pattern of behavior was also observed in MDA-MB-453 cells (Figure S2C). To further support our findings we quantified the apoptotic cells using Annexin V/PI double staining assay. As presented in Figures S2B and S2D respectively, neither MDA-MB-231/PRLR cells nor MDA-MB-453 cells showed any significant increase in Annexin V staining in comparison to control cells following 72h treatment with hPRL.

Next, we analyzed the effect of PRL treatment on cell cycle progression, which might represent another mechanism through which PRL affect breast

cancer cell growth. Therefore, we examined the effects of PRL treatment on cell cycle progression using flow cytometry analysis. MDA-MB-231/PRLR and MDA-MB-453 cells were synchronized and treated with hPRL for 72h. Our results reveled that PRL treatment is able to induce ~5% increase in G1 arrest in both cellular models in comparison with its controls (untreated cells) (Figure S2E and S2F) (P=0.0001). All together, these results highlighted the inhibitory effects of PRL on cell proliferation and demonstrate that PRL suppression in cell viability can be attributed to cell cycle arrest independent of apoptosis.

3.4.7 PRL induces cellular senescence in TNBC cells

To further elucidate the role of PRL in modulating TNBC cell behavior, we investigated the effects of PRL on cellular senescence. For that reason, we analyzed the expression of senescence-associated β-galactosidase (SA-β-gal) activity in TNBC cells after PRL treatment. As shown in Figure 3.4A, we found a significant increase in SA-β-gal activity in MDA-MB-231/PRLR in comparison with untreated cells after 72h treatment (~32%) (P=0.0001). The same pattern of SA-β-gal increment was observed in MDA-MB-453 cells after 5 days treatment in comparison with untreated cells (~36%) (Figure 3.4B) (P=0.0001). No significant increase in SA-β-gal activity was found when cells were treated for 24h and 48h with hPRL in both cellular models (Figure S3A and S3B). Next, using RT-qPCR we examined the cell cycle regulatory genes associated with senescence phenotype. We found a significant up regulation of m-RNA expression of RB

(1.52 folds) p21 (1.69 folds), p15 (2.49 folds), and INKC4 (2.38 folds) in MDA-MB-231/PRLR following 72h PRL treatment. In contrast, we observed a significant down regulation of cyclin E m-RNA (-1.5 folds) involved in cell cycle progression with no significant change in m-RNA levels of p16 gene (Figure 3.4C). PRL treatment of MDA-MB-453 cells also showed the same significant trend of up regulation of RB (1.53 folds) and down regulation of cyclin E (-1.5 folds) m-RNA levels in comparison with untreated cells (Figure 3.4D). To further evaluate PRL role in negative regulation of cyclin E we examined the association between PRLR mRNA expression levels and cyclin E mRNA levels in clinical samples of 362 TNBC patients using the correlation analysis tool of bc-GenExMiner4.0 database. Interestingly and in support of our data PRLR expression showed a negative association with cyclin E mRNA levels (P=0.0054) that further confirm the ability of PRL and its signalling pathway in repressing cyclin E expression (Figure S3C).

To further examine the role of PRL in inducing an irreversible non-proliferative state of cellular senescence phenotype in TNBC cells, MDA-MB-231/PRLR cells were treated for 72h with hPRL and cells were then plated for a week in growth factors enriched media. Interestingly, as shown in Figure 3.4E we found that PRL pre-treated cells have reduced cell viability (25.3%) (P=0.0001) compared with untreated cells. Moreover, we also found a decrease in cell viability (24.8%) (P=0.0001) in MDA-MB-453 cells pre-treated for 5 days with PRL compared with non-treated group (Figure 3.4F). These results showed that

PRL treatment induced a long-term resistance to growth signals, a characteristic of senescent cells.

3.4.8 PRL induces chromatin modifications and formation of SAHFs in TNBC cells

Senescence is typically accompanied by alterations in the components of the nuclear envelope and in the organization of the chromatin. The formation of senescence-associated heterochromatic foci (SAHF) is a key feature of senescence phenotype. For that reason, we first attempted to evaluate the role of PRL in regulating the expression of genes involved in the nuclear membrane structure and chromatin arrangement in TNBC cells. Using RT-qPCR we analyzed the m-RNA levels of histone H2AX, 53 binding protein 1 (53BP1), heterochromatin protein 1 (HP1) and Lamin B1 following PRL treatment of MDA-MB-231/PRLR cells for 72h. We found a significant up regulation of H2AX (2.9 folds), HP1 (1.5 folds) and 53BP1 (2 folds), and down regulation of Lamin B1 (-2.23 folds) (Figure 3.4G). The same behavior was also observed in MDA-MB-453 cells upon PRL treatment. We found PRL to induce up-regulation of m-RNA levels of H2AX (2.5 folds), as well as HP1 (1.5 folds), whereas, it caused down regulation of Lamin B1 gene expression (1.89 folds) and no significant difference was observed in 53BP1 (Figure 3.4H).

As part of the SAHF, we also measured the hallmark of heterochromatin formation such as trimethylation at Lys9 of histone 3 (H3K9me3). As shown in Figure 3.4I, a significant H3K9me3 protein expression was induced (~35%)

following 72h PRL treatment in MDA-MB-231/PRLR cells and after 5 days PRL treatment in MDA-MB-453 cells in comparison with untreated cells. In addition, and to confirm the up-regulation of H3K9me3, usually present in SAHFs, we performed confocal fluorescence microscopy of H3K9me3 in MDA-MB-453 cells following 5 days of PRL treatment. As expected, we found that PRL treatment was sufficient to induce a significant increase in H3K9me3-DAPI colocalization in MDA-MB-453 treated cells (P=0.0001) in comparison with un-treated cells (Figure 3.4J). These results together highlight the possibility of PRL-induces cellular senescence program through epigenetic regulation of heterochromatin in TNBC cells.

3.4.9 PRL-treated MDA-MB-453 cells display senescence phenotype associated morphological changes

Senescence is characterized by distinct morphological changes. For that reason, ultra-structural changes in MDA-MB-453 cells were examined by electron microscopy (EM). Cells were treated with hPRL for 5 days and processed for EM analysis. As can be seen in Figure 3.5A control untreated MDA-MB-453 cells display normal cell morphology at the nuclear level, which displays a nucleolus (nl) surrounded by homogenous chromatin, and is enveloped by a regular membrane (nm). The cytoplasm contains highly dense mitochondria (mt) with classical divisions of internal cristea (crests), regular lysosomes (ly) containing granules and complete rough reticulum (rr) seen in the periphery. Golgi

apparatus (go) also is displayed as sacks in the periphery with a normal shape. On the other hand MDA-MB-453 cells treated with hPRL for a period of 5 days display a different morphology that correlates with senescence phenotype. As shown in Figure 3.5B cells become more heterogeneous, displaying enlarged cytoplasm with ultra-structural alterations. Nuclei exhibit irregular shapes (deeply multi-lobed) with high chromatin density indicative of heterochromatin formation in the periphery and loss of the nucleolus. Mitochondrial density decreases, and structural differences are clearly visible. In PRL treated cells the mitochondria appear to have lost their normal transverse cristae seen in control cells. We can observe also disturbances in rough reticulum that is present in the peripheries in small pieces as well as Golgi apparatus. Altogether these findings supported features present in senescence phenotype.

3.4.10 Activation of PRL pathway turns off senescence-associated secretory phenotype essential for tumour progression

Associated with cellular senescence is the senescence-associated secretory phenotype (SASP), characterized by the ability of the cells to secrete highly pleiotropic factors including growth factors and cytokines. These factors are found to be important for recruitment of inflammatory cells, alterations of tissue microenvironment and have been proposed to play a role in tumor progression. For that reason, we aimed to examine the effects of PRL in regulating SASP. Using RT-qPCR, we evaluated the mRNA levels of pro and anti-inflammatory

cytokines, factors involved in tissue remodeling, angiogenesis and hemostasis. These include: IL-1, IL-6, IL-8, IL-2, IL-4, IL-10, IGFBP7, CXCR1, CXCR2 and MCP-1; matrix metalloproteinases (MMPs) 2, 3 and 9 and its inhibitors (TIMP-1, 2), vascular endothelial growth factor A (VEGFA) and plasminogen activator inhibitor-1 (PAI-1). We first evaluated these SASP-associated genes in MDA-MB-231/PRLR cells after 12h PRL treatment. Our results showed an up regulation of all the factors between 3-10 folds. However, after 24h PRL treatment, we found a decrement in expression of these factors that finally become down regulated after 72h of PRL treatment (Figure 3.6 A). In the same manner, we evaluated SASP-associated genes in MDA-MB-453 cells after 12h of PRL treatment. Interestingly, we found a strong down regulation of all SASP-associated genes (between 3-9 folds). This decrement continues following 24h and 5 days treatment with PRL (Figure 3.6 B). Together, our results suggest that PRL is able to down regulate SASP associated with tumor progression in the mesenchymal MDA-MB-231/PRLR cells and the MDA-MB-453 cells representative of TNBC-PRLR subtype.

To further evaluate the role of PRL in suppressing SASP, we examined the association between PRLR m-RNA expression and m-RNA expression of genes involved in SASP, using publically available data of 374 TNBC patients in bc-GenExMiner4.0 database. Interestingly, PRLR gene expression, showed a significant inverse correlation with most of the genes involved in SASP mentioned above (Figure 3.6C). Altogether, this data highlights that PRL is able to induce cellular senescence without the activation of SASP.

3.4.11 PRL is able to inhibit tumor progression in TNBC NOD/SCID/MDA-MB-231 animal xenograft model

Next, we analyzed the effects of PRL in regulating stemness and tumor progression in TNBC using MDA-MB-231 cells, which contains ~80% of $CD44^{+}/CD24^{-}$ stem cell population *in vivo*. NOD/SCID mice were inoculated with either MDA-MB-231/vector or MDA-MB-231/PRLR cells into the right mammary fat pad of each mouse. After 8 weeks of cell implantation 10/10 mice from MDA-MB-231/vector group developed tumors compared with MDA-MB-231/PRLR group that only 15/20 mice developed tumors. Animals were randomly assigned into three groups: MDA-MB-231/vector treated with dox and hPRL (10 mice), MDA-MB-231/PRLR treated with dox and hPRL (8 mice) and MDA-MB-231/PRLR treated with only dox (7 mice). Animals were treated intra-peritoneal after tumor size reach ~0.3cm. Tumor growth was monitored for 3 weeks during the treatment (Materials and Methods). As shown in Figure 3.7A, a significant retardation in tumor growth was observed in MDA-MB-231/PRLR treated group, which tumors reached a maximum volume of 348.52 mm3 in comparison with untreated group (579.27 mm3) and MDA-MB-231/vector group (833.73 mm3) at the time of sacrifice (P=0.0001). These results suggest that PRL abrogates tumor progression *in vivo*. Figure 3.7B depicted the tumors collected from mice at the time of the necropsy. Altogether, these results indicate that PRL pathway plays a role in inhibition of tumor growth and progression *in vivo*.

3.4.12 PRL reduces cell proliferation; stem cell markers expression while induces histone hyper-methylation in TNBC animal xenograft

We next evaluated whether PRL-induced growth inhibition observed in the tumors obtained from the animal xenograft model was also associated with changes in cell proliferation and stem cell markers. Initially, we evaluated the PRLR protein expression in MDA-MB-231/vector or MDA-MB-231/PRLR xenografts. As expected, while around 90% of malignant cells showed a detectable PRLR expression in MDA-MB-231/PRLR tumors, most of MDA-MB-231/vector showed no detectable PRLR protein expression (Figure 3.7C). Next, we evaluated if the reduction in tumor growth observed in treated and untreated MDA-MB-231/PRLR compared to MDA-MB-231/vector was also associated with changes in the levels of expression of Ki-67, the most important proliferation marker expressed in the active phase of the cell cycle. Interestingly, IHC analysis of KI67 of MDA-MB-231/PRLR xenografts showed a significant reduction in Ki-67 levels (P=0.0026) when compared to MDA-MB-231/Vector xenografts. Importantly, this reduction in Ki67 expression was doubled after PRL treatment (P=0.0002) compared to the vector group (Figure 3.7D).

Next, we evaluated whether restoration and activation of PRL pathway in TNBC tumors can result in reduction of CD44 expression *in vivo*. Indeed, as shown in figure 3.7E, PRLR restoration was sufficient to significantly reduce CD44 levels by around 30% (P=0.0004) in comparison to vector xenografts.

Moreover, tumor sections from the MDA-MB-231/PRLR xenografts treated with PRL showed additional reduction of nearly 60% of CD44 levels compared to the vector group ($P<0.0001$) (Figure 3.7E). This provides strong pre-clinical evidence about the ability of PRL hormone in inducing differentiation of MDA-MB-231 by eradicating the CD44 positive stem cell population and reducing their proliferative capacity

Finally, we evaluated the methylation pattern of H3 (H3K9me3), as a marker of SAHFs formation in samples of tumors obtained from our animal xenograft models. Interestingly, we corroborate that xenograft tumors from MDA-MB-231/PRLR treated group showed significantly higher levels of methylation compared to MDA-MB-231/PRLR untreated and MDA-MB-231/vector xenografts ($P=0.0002$), emphasizing the ability of PRL to induce heterochromatin formation *in vivo* in TNBC tumors (Figure 3.7F). Altogether, these results highlight the strong growth inhibitory effects of the PRL pathway in TNBC and emphasize the possible clinical benefits of the PRL hormone as a differentiation therapy that might help in converting the highly aggressive TNBC cells enriched with stem cells into more differentiated and non-tumorigenic cells.

3.5 Discussion

TNBC accounts for only 15% of all breast cancer cases, is associated with the worst patient outcome, higher risk of recurrence and drug resistance. This aggressive behaviour is attributed to different histological and molecular features including loss of differentiation and high level of proliferative genes (Podo, Buydens et al. 2010, Brouckaert, Wildiers et al. 2012). For that reason, understanding molecular pathways involved in cellular differentiation and cell fate determination is critical. In addition, TNBC is well known to be highly enriched with stem cell populations able to self-renew, invade and cause metastasis (Sheridan, Kishimoto et al. 2006, Dalerba, Cho et al. 2007, Charafe-Jauffret, Ginestier et al. 2009). Therefore, regulation of stem cell transition between un-differentiated (self-renewal) to differentiated status might help in providing new approaches to treat the aggressive TNBC. These approaches will allow reprograming TNBC aggressive cells into more differentiated with less replicative capacity and more sensitive for conventional therapies (Agur, Kirnasovsky et al. 2011).

Prolactin hormone (PRL) is one of the major regulators of terminal differentiation of the mammary epithelial cells. In addition, it is an important factor in the determination of mammary epithelial cell fate though the induction of luminal progenitor cells commitment into alveolar cells essential for lactation (Sackmann-Sala, Guidotti et al. 2015). Being lactation proved to have a protective effect against pregnancy-associated TNBC (ElShamy 2016).

Previously, many studies have implicated PRL in promoting breast carcinogenesis (Ginsburg and Vonderhaar 1995, Brockman, Schroeder et al. 2002, Clevenger, Furth et al. 2003, Lee and Ormandy 2012, O'Leary, Rugowski et al. 2014, Sutherland, Forsyth et al. 2016). However, recent study highlighted a different role as a potential suppressor of breast tumorigenesis *in vitro* and *in vivo* and as biomarker of favourable prognosis (Lopez-Ozuna, Hachim et al. 2016). However, the mechanisms though which PRL produce its effect still needs to be deeply investigated. Here, we showed that PRL pathway produce its anti-tumorigenic effect in TNBC through induction of two highly related cell fate mechanisms: cellular differentiation and senescence.

Our results showed that PRL treatment was able to reduce $CD44^{+}/CD24^{-}$ (express in MDA-MB-231) and $ALDH^{+}$ (express in MDA-MB-453 cells) CSC populations and induce its differentiation into non-tumorigenic phenotype. In support to these findings, PRL also showed the capacity to down regulate Oct4, Sox2 and Nanog transcription factors, essential for self-renewal and pluripotency (Hadjimichael, Chanoumidou et al. 2015). This highlights a new possible approach to reprogram TNBC CSCs populations and inhibit their replicative capacity through the induction of differentiation. This *in vitro* finding was corroborated *in vivo* through a mammary fat pad animal xenograft, which demonstrated the growth inhibitory and anti tumor progression effects of PRL treatment. We found in the tumors obtained from this animal xenograft that PRL treatment is able to decrease cell proliferation confirmed by decrease of ki67 and CD44 stem cell marker.

The other mechanism though which PRL produce its anti-tumorigenic effect is cellular senescence, which is associated to irreversible cell proliferation arrest (Sledge and Pegram 2015) (Prieur and Peeper 2008, Campisi 2013). The ability of PRL to induce the SA-β-gal observed in our findings was reported previously as unexpected and non-conclusive finding (Nitze, Galsgaard et al. 2013). Our study showed that PRL treatment was able to induce SA-β-gal and regulate senescence associated cell cycle regulatory genes including RB, p21, p15, INKC4 and cyclin E to maintain irreversible cell cycle exit. Likewise PRL treatment was sufficient to induce irreversible growth arrest even after stimulation with growth factors enriched media, confirming the long-term cell cycle exit that differs from quiescence. In addition, we observed key morphological changes and chromatin rearrangement by EM, as well as depletion of nuclear proteins as Lamin B1 (Sadaie, Salama et al. 2013).

Interestingly, our results revealed that, while PRL treatment was able to induce multiple senescence-associated features also down regulates many immune response and microenvironment regulators (SASP) that plays an important role in tumor progression such as IL1, IL-6, IL8 and VEGF (Liu, Uppal et al. 2015) (Coppe, Desprez et al. 2010). These findings are consistent with our previous reports showing PRLR to be negatively correlated with two gene signatures associated with angiogenesis (VEGF) and with inflammatory genes and chemokines including IL-8 and CXCL1 in clinical patient samples (Lopez-Ozuna, Hachim et al. 2016). These immune regulators was suggested to play an important role in promoting tumorigenesis and cancer progression in

premalignant cells but not in normal cells (Perez-Mancera, Young et al. 2014). Furthermore, continuous expression of theses cytokines was found to be associated with worse outcome and might confer resistance to chemotherapy (Jackson, Pant et al. 2012, Sun, Campisi et al. 2012). For that reason, PRL induce senescence without propagation of the SASP highlight its possible use as a possible therapeutic agent. Another interesting finding is the link between induction of differentiation and senescence, through stimulation of heterochromatin formation and growth control (Leszczyniecka, Kang et al. 2002). One of the genes that showed to play a role in both processes is RB, which is important for chromatin cohesion & structure in differentiation and senescence independent of E2F activity.

Based on our observations, PRL has the ability to regulate differentiation and senescence and let us to propose that PRL might induce these processes through the modulation of RB protein leading to induction of methylation of histone H3, which induces arrest through HP1 stimulation and cyclin E inhibition (Figure S4). This model is supported by the fact that this axis was found to be important in differentiation and senescence (Stewart, Li et al. 2005, Bandyopadhyay, Curry et al. 2007), (Panteleeva, Boutillier et al. 2007). Our finding was in accordance with reports that proposed that knockdown of HP1 was sufficient to reduce cell cycle arrest and terminal differentiation in a neural model (Panteleeva, Boutillier et al. 2007). Additionally, two recent reports also showed that H3K9me3-decorated heterochromatin is important for controlling terminal differentiation, determination cell fate through epigenetic regulation and silencing

of genes (Allan, Zueva et al. 2012, Liu, Magri et al. 2015). Together, our data indicate induction of both processes senescence and differentiation by PRL might be parallel or overlapping.

In addition, the fact that terminal differentiation and senescence are both characterized by irreversible growth arrest (Leszczyniecka, Kang et al. 2002), and that cellular senescence is an irreversible condition compared to terminal differentiation (Narita and Lowe 2005, Campisi 2013) led us to hypothesize a sequence of events initiated by PRL treatment. These events include the induction of TNBC cancer cell differentiation into non-tumorigenic phenotype and activation of tumor suppressor mechanisms such as the activation of the RB pathway. Furthermore, these cells will become susceptible to undergo cellular senescence leading to permanent growth arrest and resistant to oncogenic signals (Becker, Nicetto et al. 2016). Altogether, highlight the ability of PRL treatment to induce reprograming of the TNBC cells shifting from highly proliferative and stem cell-like phenotype into more differentiated, less aggressive and non-proliferative phenotype. The ability of PRL to induce these changes emphasizes the potential use of PRL hormone as a novel therapy for TNBC patients able to inhibit tumor progression and recurrence.

3.6 Material and Methods

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

3.6.1 Cell culture: Human breast cancer cell lines MDA-MB-231/PRLR, MDA-MB-231/vector and MDA-MB-453 were cultured in DMEM containing 10% FBS. All cell lines were grown at 37 °C in 5% CO2.

3.6.2 Cell lysis and western blotting: For whole cell lysates and western blots, cells were lysed in SDS lysis buffer as described previously (Ali and Ali 1998).

3.6.3 Tumorsphere formation assay: MDA-MB-231/PRLR and MDA-MB-453 cells were treated for 72h and 5 days with or without PRL respectively. Cells were trypsinized and 1000 cells per well were plated in a 12-well low-attachment plate (Corning). Cells were grown for 7 days in DMEM-free serum supplemented with B27 (Invitrogen) and hPRL (250ng/ml).

3.6.4 Flow cytometry analysis: Adherent cells were dissociated into single cells by trypsin-EDTA and filtered through a 40µm nylon mesh (BD Biosciences, San Diego, CA). 1×10^6 cells were washed with PBS containing 0.5% FBS, incubated with anti-CD44 conjugated to APC-cy7, anti-CD24 conjugated to APC (BD Biosciences). Cells were then washed with 0.5%PBS-FBS for 3 times. After washing, cells were analyzed with Accuri C6 flow cytometer (BD Biosciences) and Flowjo software (Tree Star Inc). Single $CD44^+/CD24^-$, $CD44^+/CD24^+$, $CD44^-/CD24^-$ and $CD44^-/CD24^+$ cell was sorted using FACSAria into either 12-well low-attachment plate to perform tumorsphere analysis or to 96-well low attachment plate to perform MTT assay.

3.6.5 ALDEFLUOR assay: The assay was performed as manufacturer's protocol. 1 × 10^6 MDA-MB-453 cells were centrifuged and resuspended in 1 ml ALDH assay buffer. 5µl substrate was added into the cell suspension. DEAB was used as negative control. Cells were then incubated for 40 min at 37 °C. Percentage of $ALDH^+$ cells were analyzed with Accuri C6 flow cytometer and Flowjo software.

3.6.6 RNA extraction and qRT-PCR: Total RNA from MDA-MB-231/PRLR, MDA-MB-231/vector and MDA-MB-453 cells treated with hPRL for 72hrs and 5 days respectively, were isolated, reverse transcribed and used for PCR amplification. RT-qPCR of CSC transcription factors (Oct4, Sox2 and Nanog), senescent-associated genes (RB, p21, p16, p15, INKC4, Cyclin E, H2AX, 53BP1, HP1 protein and Laminin B1), senescence-associated secretory phenotype genes (IL-1, IL-6, IL-8, IL-2, IL-4, IL-10, IGFBP7, CXCR1, CXCR2 and MCP-1; matrix metalloproteinases (MMPs) 2, 3 and 9 and its inhibitors (TIM-1, 2), VEGFA and PAI-1) was performed.

3.6.7 MTT assay: MTT assays were performed as previously described (Cocolakis, Lemay et al. 2001).

3.6.8 Caspase Glo 3/7 Assay: Cells were plated in 96-well plates at 5000 cells/100µL in medium supplemented with 2% FBS. Caspase 3/7 activity was measured using the Caspase Glo 3/7 Assay (Promega) according to manufacturer's instructions. Cells were incubated with equal volumes of medium and Caspase Glo reagent for 30 minutes at room temperature, and luminescence was measured using the EG & G Berthold luminometer (Bad Wildbad, Germany).

3.6.9 Annexin V Apoptosis Detection assay: The assay was performed as manufacturer's protocol. $1X10^6$ cells were washed twice and resuspended with PBS-2%FBS (Assay buffer). Cells were then double-stained with Annexin V conjugated to FITC and PI for 15 minutes in the dark at room temperature, and then analyzed by flow cytometry.

3.6.10 Cell cycle analysis: Cells were harvested and washed with cold PBS, and then fixed with 70% ethanol for 30 min at RT. The fixed cells were washed with cold PBS twice, added 500µL DNA staining solution (including 200 µg/mL RNase A and 20 µg/mL propidium iodide staining solution) and incubated for 30 minutes. Finally, cells were analyzed by flow cytometry in the presence of the dye.

3.6.11 Senescence-associated β-galactosidase assay: The assay was performed as manufacturer's protocol. SA-β-gal staining was performed using SA-β-gal staining kit (Cell signalling). MDA-MB-231/PRLR (treated and untreated with hPRL for 72h) and MDA-MB-453 (treated and untreated with hPRL for 5 days) cells were fixed by Fixative Solution for 15 min, followed by β-gal solution incubation overnight at 37°C. The staining was checked under microscope for the development of green color.

3.6.12 Immunofluorescence microscopy: MDA-MB-453 was treated for 5 days with or without hPRL. Cells were trypsinized and grown on coverslips for 1 day, next, were fixed with 3.7% formaldehyde for 15 minutes and permeabilized in 0.1% Triton X-100 for 5 minutes. Cell were then washed with PBS and blocked for 1h in 2% normal donkey serum. Cells were incubated with an anti-H3K9me3

specific antibody for 1h, washed with PBS and incubated with Alexa Fluor®456 goat anti-rabbit antibody and DAPI for 1h. Stained coverslips were mounted with SlowFade® Gold antifade reagent. Confocal analysis was performed using a Zeiss LSM 510 Meta Axiovert confocal microscope using 63× objectives.

3.6.13 Electron microscopy: Cells of each group were harvested and fixed at 1×10^6 with 2.5% glutaraldehyde for 6h at 4°C. Then the cells were processed for ultra-structure analysis in the Facility for Electron Microscopy Research (FEMR), McGill University.

3.6.14 Immunohistochemistry: Slides were incubated with a rabbit polyclonal antibody to PRLR-L (Santa Cruz #sc-20992) as describe previously (Hachim, Hachim et al. 2016) and with a rabbit polyclonal antibody to Ki67 (Abcam #16667), CD44 (Abcam #ab51037) and H3K9me3 (Abcam #176916).

3.6.15 Gene expression analysis: Breast Cancer Gene-Expression Miner database Version 4.0 (bc-GenExMiner) was used to evaluate the mRNA levels of CD44 in TNBC patients (Jezequel, Campone et al. 2012). The prognosis gene expression analysis tool of bc-GenExMiner 4.0 was used to assess the association between CD44 mRNA levels and patient outcome (AEFS and MSF), using gene symbol. The KM plotter database was also used to investigate the association between the expression levels of CD44 and patient outcome (RFS). The correlation module of the Breast Cancer Gene-Expression Miner v4.0 was used to evaluate the association between PRLR and different genes including cyclin E1, self-regulatory factor, immune check points genes, microenvironment regulator genes and different interleukins and signalling molecules.

3.6.16 Animal models: All experimental animal work was performed in a specific-pathogen-free animal facility according to the guidelines and ethical regulations of the Research Institute McGill University Health Centre approved animal used protocol (#2014-7492) in accordance with Canadian Council of animal care guidelines.

3.6.17 MDA-MB-231 xenograft: 30 Female NOD/SCID mice were purchased from Charles River Laboratories (Saint-Constant, QC, Canada) and randomly assigned into three groups according to PRL treatment: MDA-MB-231/vector, MDA-MB-231/PRLR untreated and MDA-MB-231/PRLR treated. The mice were injected in the mammary fad pat. After the tumors reach a minimum volume of 0.5cm (after 8 weeks of cell implantation), animals were treated with doxycycline (20 mg/kg) daily. MDA-MB-231/vector, MDA-MB-231/PRLR treated group were injected intra-peritoneal every second day with hPRL (0.1mg/g). Tumor growth was monitored for 3 weeks during the treatment**.** At the end of the experiment mice were sacrificed by CO2 asphyxiation and subjected to necropsy. Tumors were collected from mice at the time of the necropsy and fixed with 4% paraformaldehyde.

3.6.18 Statistical analysis: All results are presented as the mean ± SEM for at least three individual experiments. The difference between groups was analyzed using Student's *t*-test, and $*P < 0.05$ was considered statistically significant.

Further detailed information can be found in the Appendix: Extended Materials and Methods.

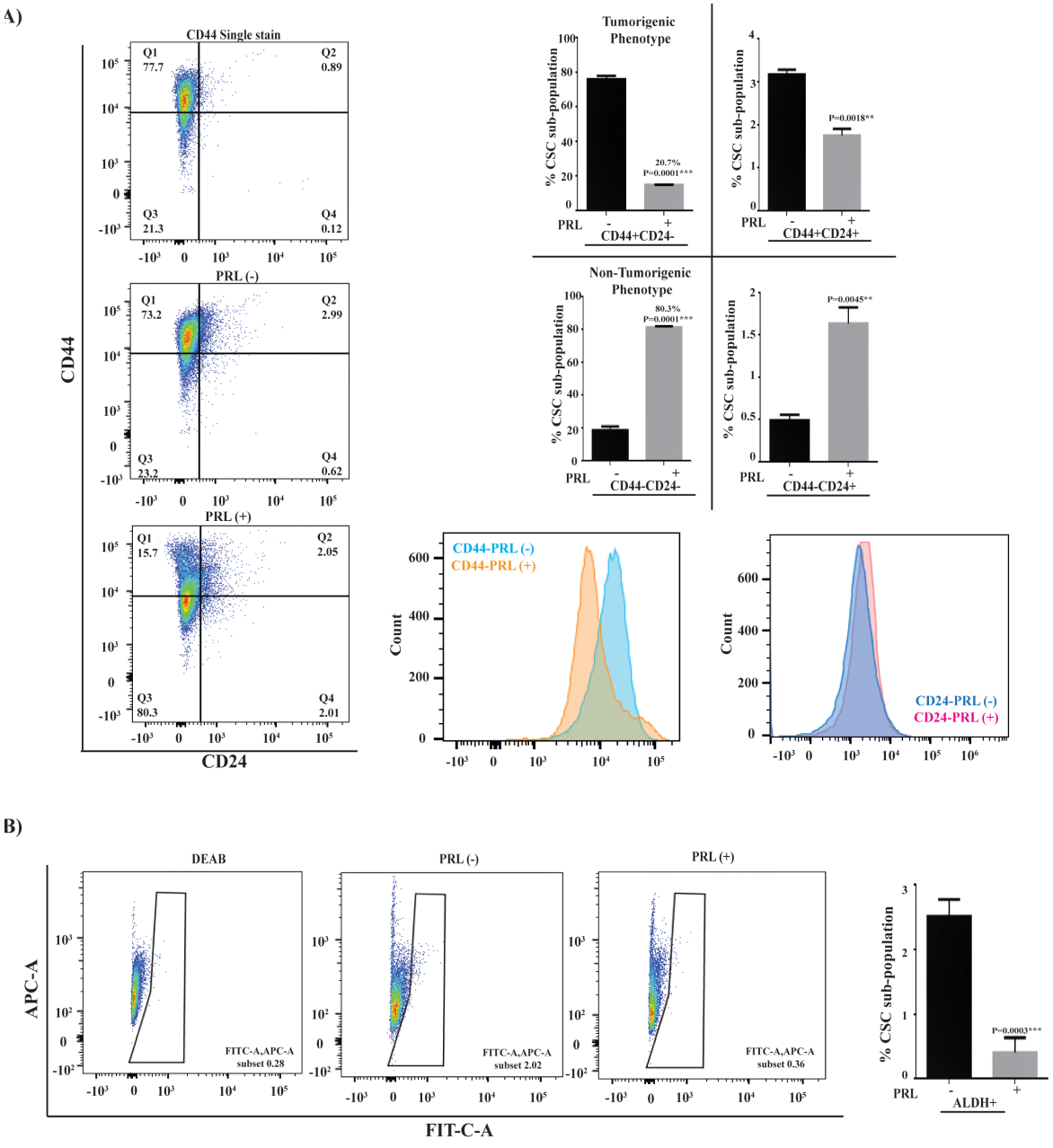

A)
CD44 Single stain
Q1 77.7
Q2 0.89
Q3 21.3
Q4 0.12
PRL (-)
Q1 73.2
Q2 2.99
Q3 23.2
Q4 0.62
PRL (+)
Q1 15.7
Q2 2.05
Q3 80.3
Q4 2.01
CD44
CD24
Tumorigenic Phenotype
% CSC sub-population
20.7%
P=0.0001***
PRL
CD44+CD24-
P=0.0018**
CD44+CD24+
Non-Tumorigenic Phenotype
80.3%
P=0.0001***
CD44-CD24-
P=0.0045**
CD44-CD24+
CD44-PRL (-)
CD44-PRL (+)
Count
CD24-PRL (-)
CD24-PRL (+)
B)
DEAB
PRL (-)
PRL (+)
APC-A
FITC-A,APC-A subset 0.28
FITC-A,APC-A subset 2.02
FITC-A,APC-A subset 0.36
FIT-C-A
% CSC sub-population
P=0.0003***
PRL
ALDH+

Figure 3.1

Figure 3.1: PRL treatment is able to induce phenotypical changes in TNBC stem cells.

A) MDA-MB-231/PRLR cells were treated or not with dox (100ng/ml) and hPRL (250ng/ml) for 72h and cells were stained with anti-CD24-FITC and anti-CD44-PE followed by flow cytometry analysis. Left panels represent a dot plot of MDA-MB-231/PRLR cells treated or not with hPRL for 72h. The percentage of CD44 and CD24 cells in each population is indicated in the following panel (right panel). Representative histogram of CD44 and CD24 distribution levels after PRL treatment are shown (right lower panels). B) MDA-MB-453 cells were treated or not with hPRL (250ng/ml) for 5 days and $ALDH^+$ were assessed by aldeflour assay followed by flow cytometry analysis. Lower panel is represented by a dot plot of aldeflour activity in MDA-MB-453 cells in the presence of DEAB with and without hPRL treatment for 5 days. The percentage of $ALDH^+$ population is indicated in the lower right panel. Data represent mean ± SEM of three independent experiments.

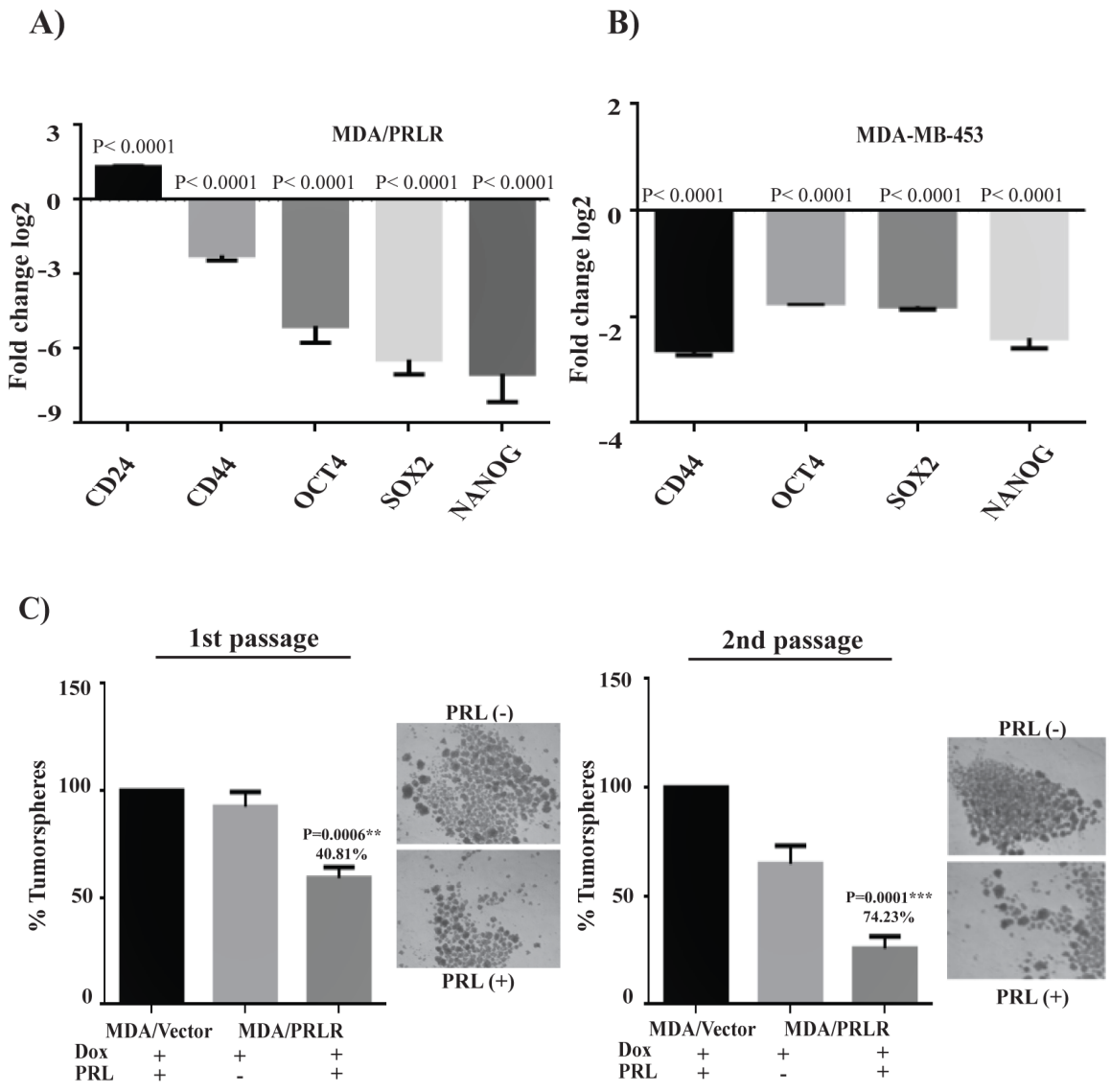

A)
MDA/PRLR
Fold change log2
P< 0.0001
P< 0.0001
P< 0.0001
P< 0.0001
P< 0.0001
CD24
CD44
OCT4
SOX2
NANOG
B)
MDA-MB-453
Fold change log2
P< 0.0001
P< 0.0001
P< 0.0001
P< 0.0001
CD44
OCT4
SOX2
NANOG
C)
1st passage
% Tumorspheres
P=0.0006**
40.81%
PRL (-)
PRL (+)
MDA/Vector
MDA/PRLR
Dox
PRL
2nd passage
% Tumorspheres
P=0.0001***
74.23%
PRL (-)
PRL (+)
MDA/Vector
MDA/PRLR
Dox
PRL

Figure 3.2

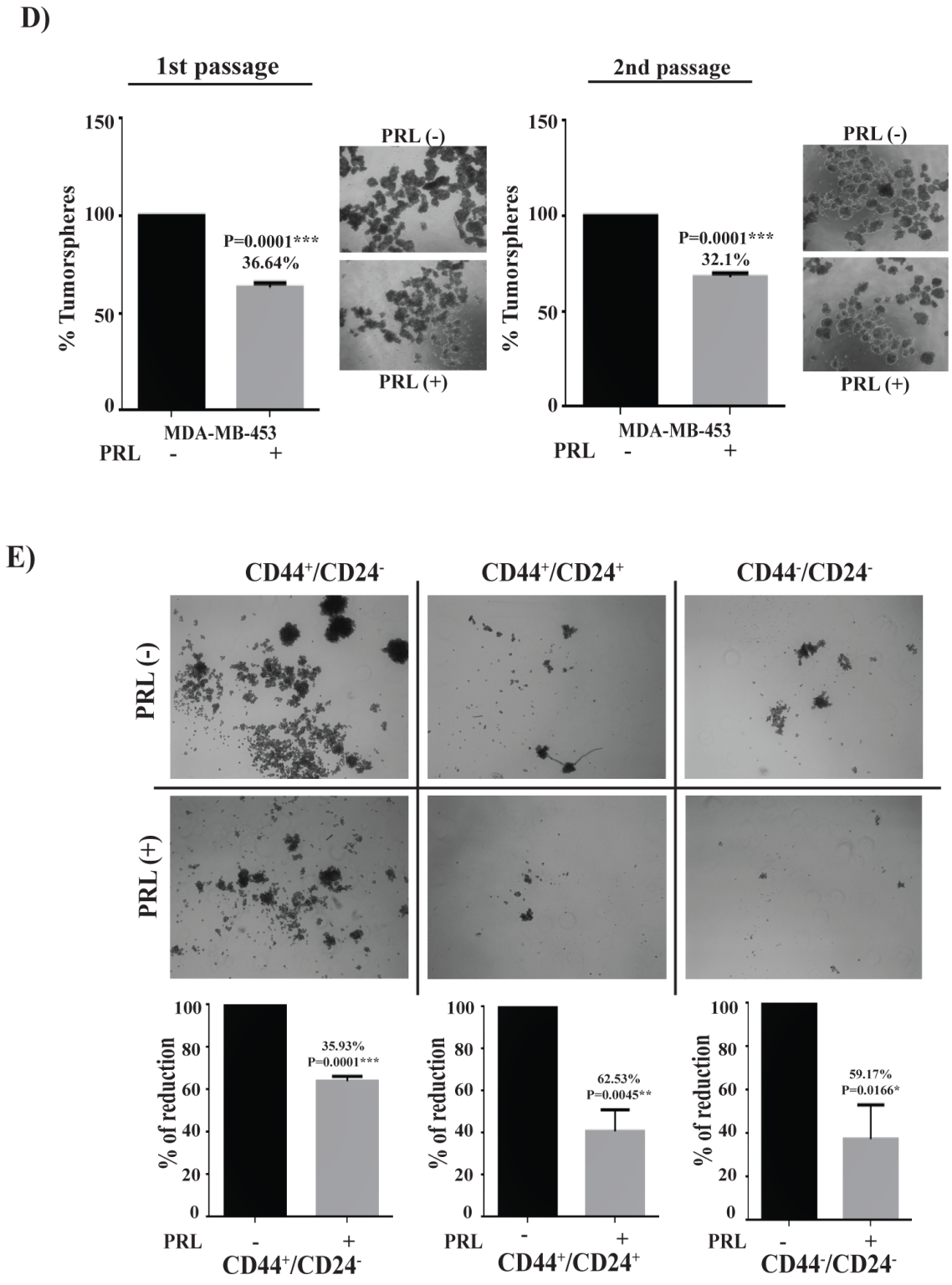

D)
1st passage
2nd passage
% Tumorspheres
150
100
50
0
P=0.0001***
36.64%
32.1%
PRL (-)
PRL (+)
MDA-MB-453
PRL - +
E)
CD44+/CD24-
CD44+/CD24+
CD44-/CD24-
PRL (-)
PRL (+)
% of reduction
100
80
60
40
20
0
35.93%
P=0.0001***
62.53%
P=0.0045**
59.17%
P=0.0166*
PRL - +

Figure 3.2

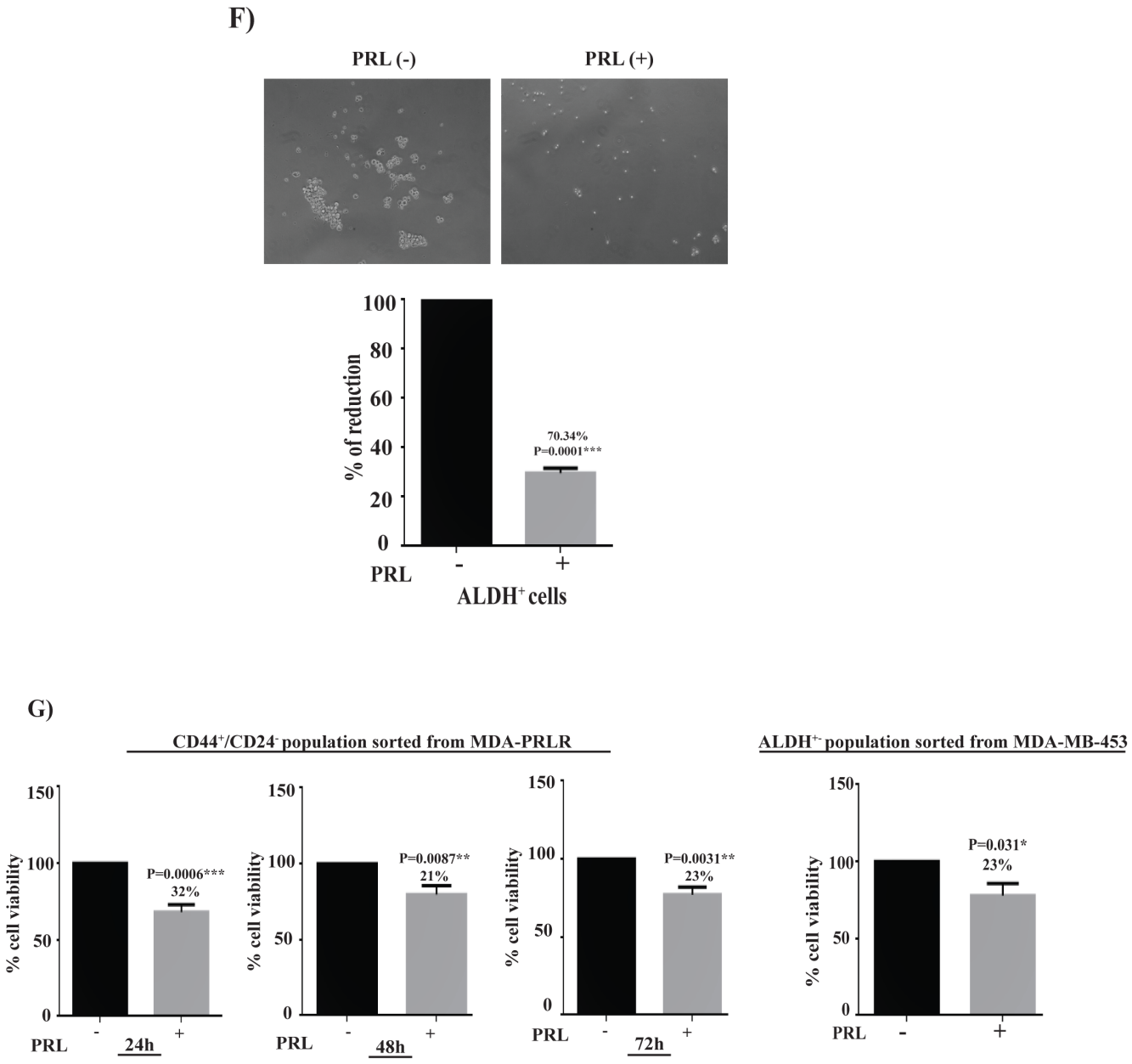

F)
PRL (-)
PRL (+)
100
80
60
40
20
0
% of reduction
70.34%
P=0.0001***
PRL
-
+
ALDH+ cells
G)
CD44+/CD24- population sorted from MDA-PRLR
ALDH+- population sorted from MDA-MB-453
150
100
50
0
% cell viability
P=0.0006***
32%
P=0.0087**
21%
P=0.0031**
23%
P=0.031*
23%
PRL
24h
48h
72h

Figure 3.2

Figure 3.2: PRL treatment induces stem cell differentiation and inhibits TNBC BCSC viability

A) MDA-MB-231/PRLR cells were treated or not with dox (100ng/ml) and hPRL (250ng/ml) for 72h and the expression of stem cell markers and transcription factors (CD24, CD44, OCT4, SOX2 and Nanog) was examined using qRT-PCR ($P < 0.05$). Results are expressed as log2 fold change of triplicates of three independent experiments. B) MDA-MB-453 cells were treated or not with hPRL (250ng/ml) for 5 days and the expression of stem cell markers and transcription factors (CD24, CD44, OCT4, SOX2 and Nanog) was examined using qRT-PCR ($P < 0.05$). Results are expressed as log2 fold change of triplicates of three independent experiments. C) MDA-MB-231/vector and MDA-MB-231/PRLR were treated or not with dox (100ng/ml) and hPRL (250ng/ml) for 72h. Left and right panel respectively, depict primary and secondary tumorsphere formation of MDA-MB-231/vector cells compared with MDA-MB-231/PRLR treated and un-treated cells. Representative microphotographs of primary and secondary tumorspheres respectively, following a 7-day treatment period is shown. D) MDA-MB-453 were treated or not with hPRL (250ng/ml) for 5 days. Left and right panel respectively, depict primary and secondary tumorsphere formation of MDA-MB-453 treated and un-treated cells. Representative microphotographs of primary and secondary tumorspheres respectively, following a 7-day treatment period are shown. E) The $CD44^+/CD24^-$, $CD44^+/CD24^+$ and $CD44^-/CD24^-$ cell populations were sorted from MDA-MB-231/PRLR and subjected to tumorsphere formation assay under hPRL stimulation. Representative microphotographs of primary tumorspheres after 7-

day incubation are shown. F) $ALDH^+$ cell population isolated from MDA-MB-453 was subjected to tumorsphere formation assay under hPRL stimulation. Representative microphotographs of primary tumorspheres after 7-day incubation are shown. G) The $CD44^+/CD24^-$ cell population sorted from MDA-MB-231/PRLR were plated and treated or not with hPRL (250 ng/ml) for 24h, 48h and 72h. MTT assays were performed and the results are presented as means ± SEM for triplicates of three independent experiments. $ALDH^+$ cell population was isolated from MDA-MB-453 and treated or not with hPRL (250 ng/ml) for 5 days. MTT assays were performed and the results are presented as means ± SEM for triplicates of three independent experiments.

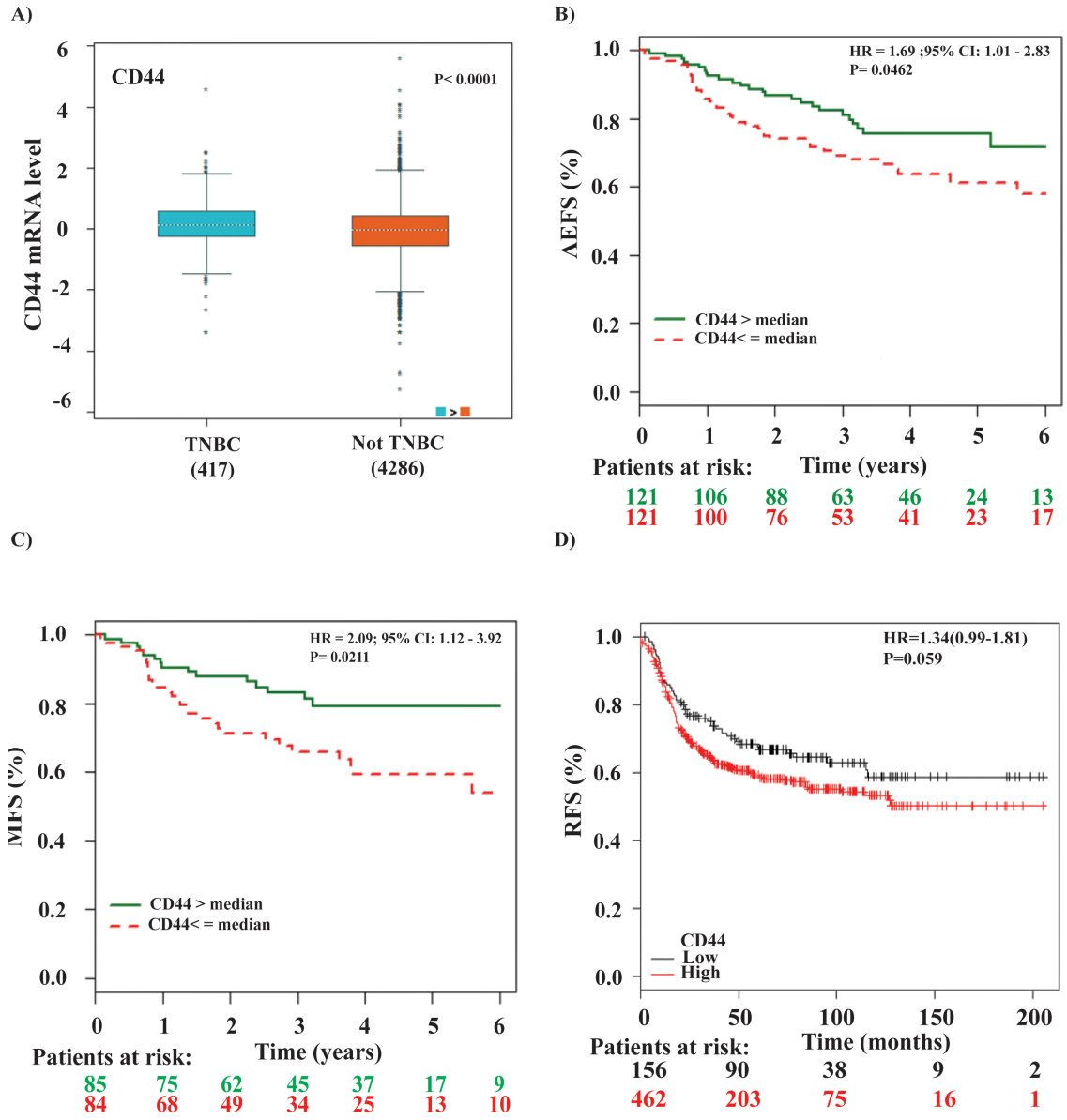

A)
CD44
P< 0.0001
CD44 mRNA level
6
4
2
0
-2
-4
-6
TNBC
(417)
Not TNBC
(4286)
B)
HR = 1.69 ;95% CI: 1.01 - 2.83
P= 0.0462
AEFS (%)
1.0
0.8
0.6
0.4
0.2
0.0
CD44 > median
CD44< = median
0 1 2 3 4 5 6
Time (years)
Patients at risk:
121 106 88 63 46 24 13
121 100 76 53 41 23 17
C)
HR = 2.09; 95% CI: 1.12 - 3.92
P= 0.0211
MFS (%)
1.0
0.8
0.6
0.4
0.2
0.0
CD44 > median
CD44< = median
0 1 2 3 4 5 6
Time (years)
Patients at risk:
85 75 62 45 37 17 9
84 68 49 34 25 13 10
D)
HR=1.34(0.99-1.81)
P=0.059
RFS (%)
1.0
0.8
0.6
0.4
0.2
0.0
CD44
Low
High
0 50 100 150 200
Time (months)
Patients at risk:
156 90 38 9 2
462 203 75 16 1

Figure 3.3

Figure 3.3: CD44 expression in TNBC as marker of poor patient outcome.

A) Kaplan-Meier curves of CD44 gene expression levels in TNBC patients in comparison with non-TNBC patients using the customize expression analysis tool of bc-GenExMiner4.0 database. B) Kaplan-Meier survival curves of CD44 gene expression levels and its association with patient outcome using AEFS as an endpoint. Gene expression is stratified by median into high (green line) and low (red line) using bc-GenExMiner4.0 database. C) Kaplan-Meier survival curves of CD44 gene expression levels and its association with patient outcome using metastasis-free survival (MFS) as an endpoint. Gene expression is stratified by median into high (green line) and low (red line) using bc-GenExMiner4.0 database. D) Kaplan-Meier survival curves of CD44 gene expression levels and its association with patient outcome using relapse-free survival (RFS) as an endpoint. Gene expression is stratified by median using KM plotter database.

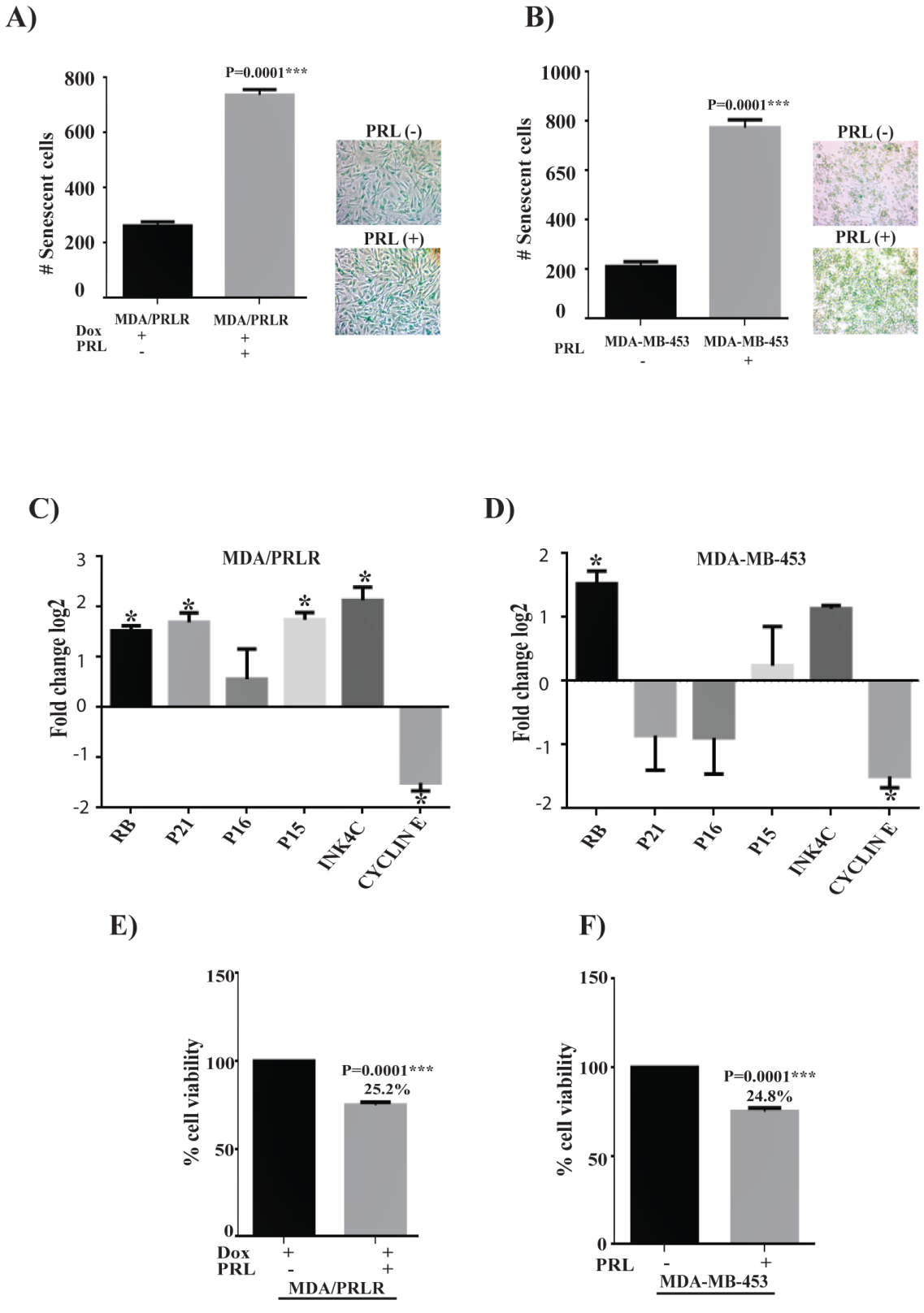

A)
800
600
400
200
0
# Senescent cells
P=0.0001***
PRL (-)
PRL (+)
MDA/PRLR
MDA/PRLR
Dox
PRL
+
-
+
+
B)
1000
800
650
400
200
0
# Senescent cells
P=0.0001***
PRL (-)
PRL (+)
PRL
MDA-MB-453
MDA-MB-453
-
+
C)
MDA/PRLR
Fold change log2
3
2
1
0
-1
-2
RB
P21
P16
P15
INK4C
CYCLIN E
D)
MDA-MB-453
Fold change log2
2
1
0
-1
-2
RB
P21
P16
P15
INK4C
CYCLIN E
E)
150
100
50
0
% cell viability
P=0.0001***
25.2%
Dox
PRL
+
-
+
+
MDA/PRLR
F)
150
100
50
0
% cell viability
P=0.0001***
24.8%
PRL
-
+
MDA-MB-453

Figure 3.4

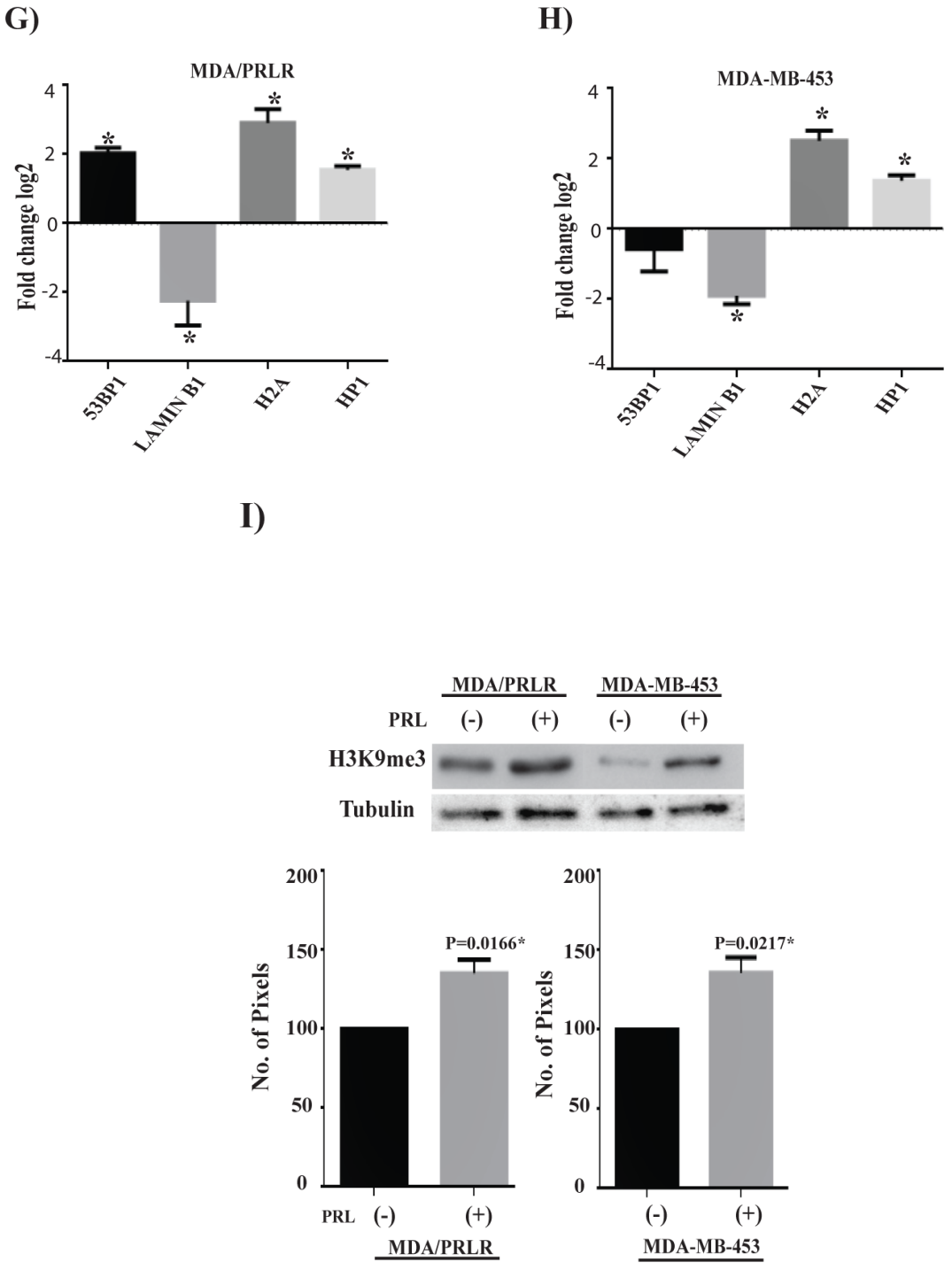

G)
MDA/PRLR
Fold change log2
4
2
0
-2
-4
53BP1
LAMIN B1
H2A
HP1
H)
MDA-MB-453
Fold change log2
53BP1
LAMIN B1
H2A
HP1
I)
MDA/PRLR
MDA-MB-453
PRL
(-)
(+)
(-)
(+)
H3K9me3
Tubulin
No. of Pixels
200
150
100
50
0
P=0.0166*
P=0.0217*
PRL
(-)
(+)
MDA/PRLR
MDA-MB-453

Figure 3.4

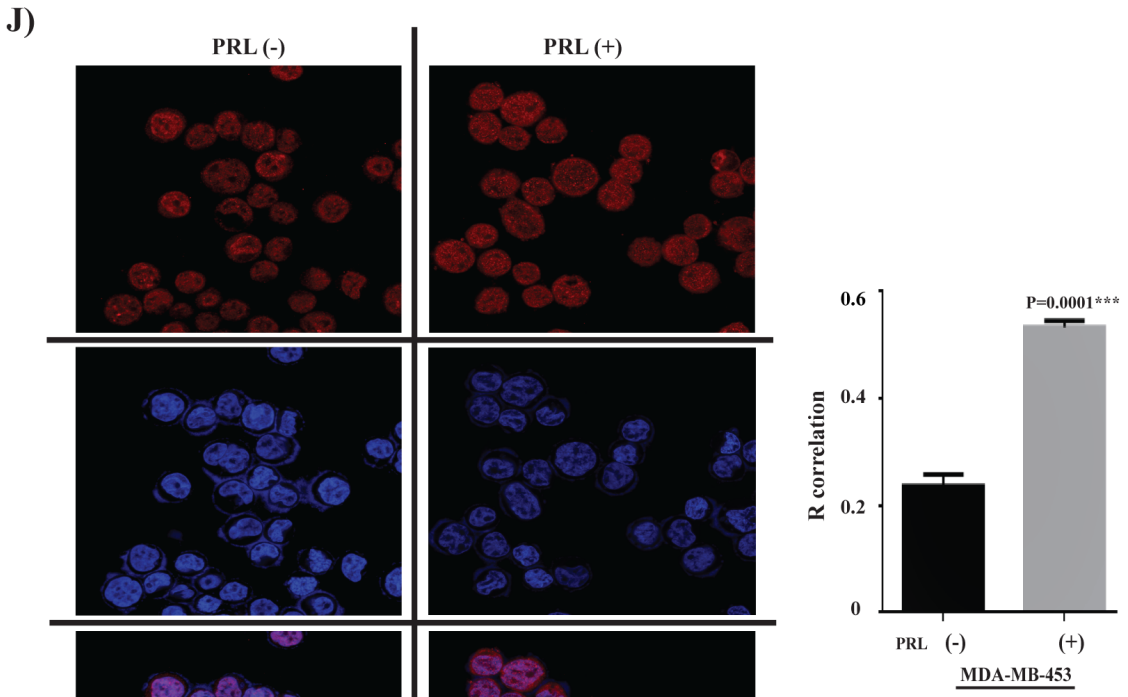


Figure 3.4

Figure 3.4: PRL treatment increases SAβ-gal staining and affects regulatory genes/proteins expressed in cellular senescence

A) MDA-MB-231/PRLR cells were treated or not with dox (100ng/ml) and hPRL (250ng/ml) for 72h following assessment of positive SAβ-gal staining. Representative microphotographs of positive SAβ-gal staining after 72h PRL treatment are shown (P=0.0001). B) MDA-MB-453 cells were treated or not with hPRL (250ng/ml) for 5 days following assessment of positive SAβ-gal staining Representative microphotographs of positive SAβ-gal staining after 5 days PRL treatment are shown (P=0.0001). C) MDA-MB-231/PRLR cells were treated or not with dox (100ng/ml) and hPRL (250ng/ml) for 72h and the expression of cell cycle senescence-associated genes (RB, p21, p16, p15, INK4C and cyclin E) were examined using qRT-PCR ($P < 0.05$). Results are presented as means ± SEM for triplicates of three independent experiments. D) MDA-MB-453 cells were treated or not with hPRL (250ng/ml) for 5 days and the expression of cell cycle senescence-associated genes (RB, p21, p16, p15, INK4C and cyclin E) were examined using qRT-PCR ($P < 0.05$). Results are presented as means ± SEM for triplicates of three independent experiments. E) MDA-MB-231/PRLR cells were plated and treated or not treated with dox (100ng/ml) and hPRL (250 ng/ml) for 72h. Next, cells were re-plated in growth media (DMEM-10% FBS) for one week. MTT assay was performed and the results are presented as means ± SEM for triplicates of three independent experiments (P=0.0001). F) MDA-MB-453 cells were plated and treated or not treated with hPRL (250 ng/ml) for 5 days. Next, cells were re-plated in growth media (DMEM-10% FBS) for one

week. MTT assay was performed and the results are presented as means ± SEM for triplicates of three independent experiments (P=0.0001). G) MDA-MB-231/PRLR cells were treated or not with dox (100ng/ml) and hPRL (250ng/ml) for 72h and the effect on nuclear membrane and chromatin modifications related-genes expressed in senescence phenotype (53BP1, Lamin B1, H2A, HP1) were examined using qRT-PCR ($P < 0.05$). Results are presented as means ± SEM for triplicates of three independent experiments. H) MDA-MB-453 cells were treated or not with hPRL (250ng/ml) for 5 days and the effect on nuclear membrane and chromatin modifications related-genes expressed in senescence phenotype (53BP1, Lamin B1, H2A, HP1) were examined using qRT-PCR ($P < 0.05$). Data is presented as mean ± SEM of three independent experiments. I) MDA-MB-231/PRLR and MDA-MB-453 were stimulated or not with hPRL (250ng/ml) for 72hrs and 5 days respectively. Cell lysates were immune detected using antibodies to H3K9me3 and β-tubulin. J) MDA-MB-453 was stimulated or not with hPRL (250ng/ml) for 5 days. H3K9me3 staining was assessed using confocal microscopy. Right panel depict H3K9me3-DAPI colocalization. Data is presented as mean ± SEM of three independent experiments ($P < 0.05$).

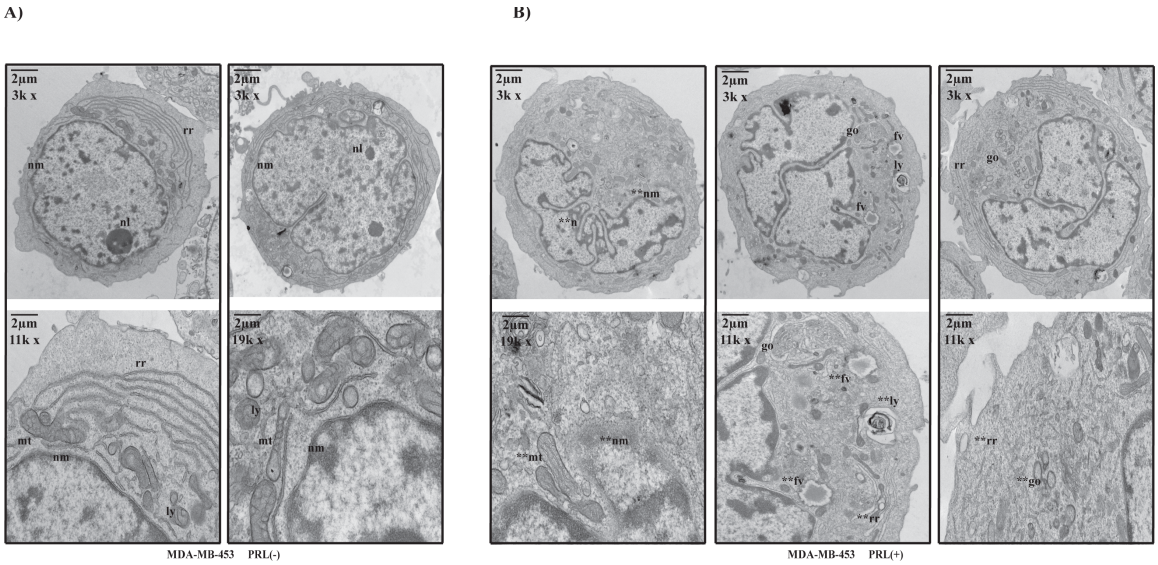


Figure 3.5: PRL induces senescence-associated ultra-structural phenotype in TNBC cells

A) MDA-MB-453 cells were treated or not with hPRL (250ng/ml) for 5 days and processed for electron microscopy (EM). EM views of MDA-MB-453 un-treated cells (2 different cells) at 11000x and 19000x magnification respectively; nuclear membrane (nm), nucleolus (nl), mitochondria (mt), lysosome (ly) and rough endoplasmic reticulum (rr) can be appreciated. B) Ultra structural changes in cellular senescence. EM views of MDA-MB-453 treated cells with PRL for 5 days (3 different cells) at 11000x and 19000x magnification respectively; multi-lobulated nucleus (**n), loss on continuity in the nuclear membrane (**nm), irregular mitochondria, Golgi apparatus and rough endoplasmic reticulum patterns (**mt, **go, **rr), presence of fatty vesicles (**fv).

A)

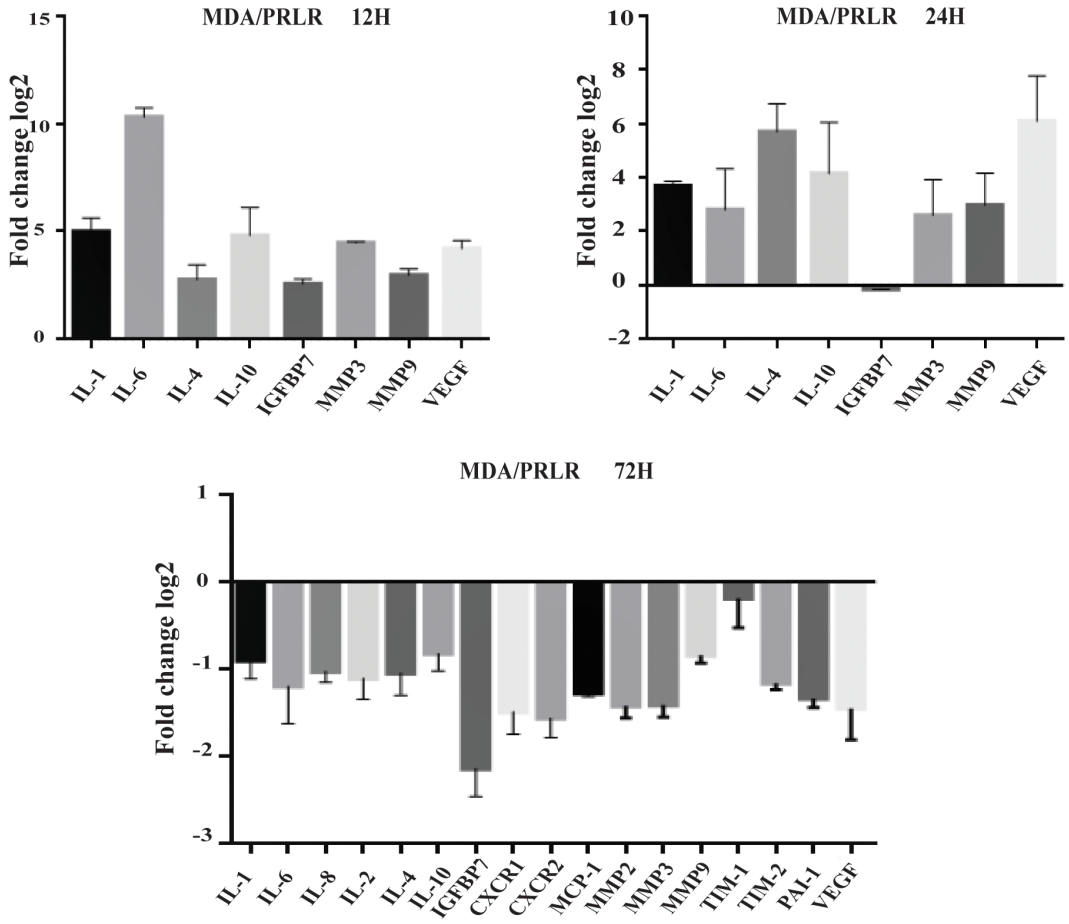
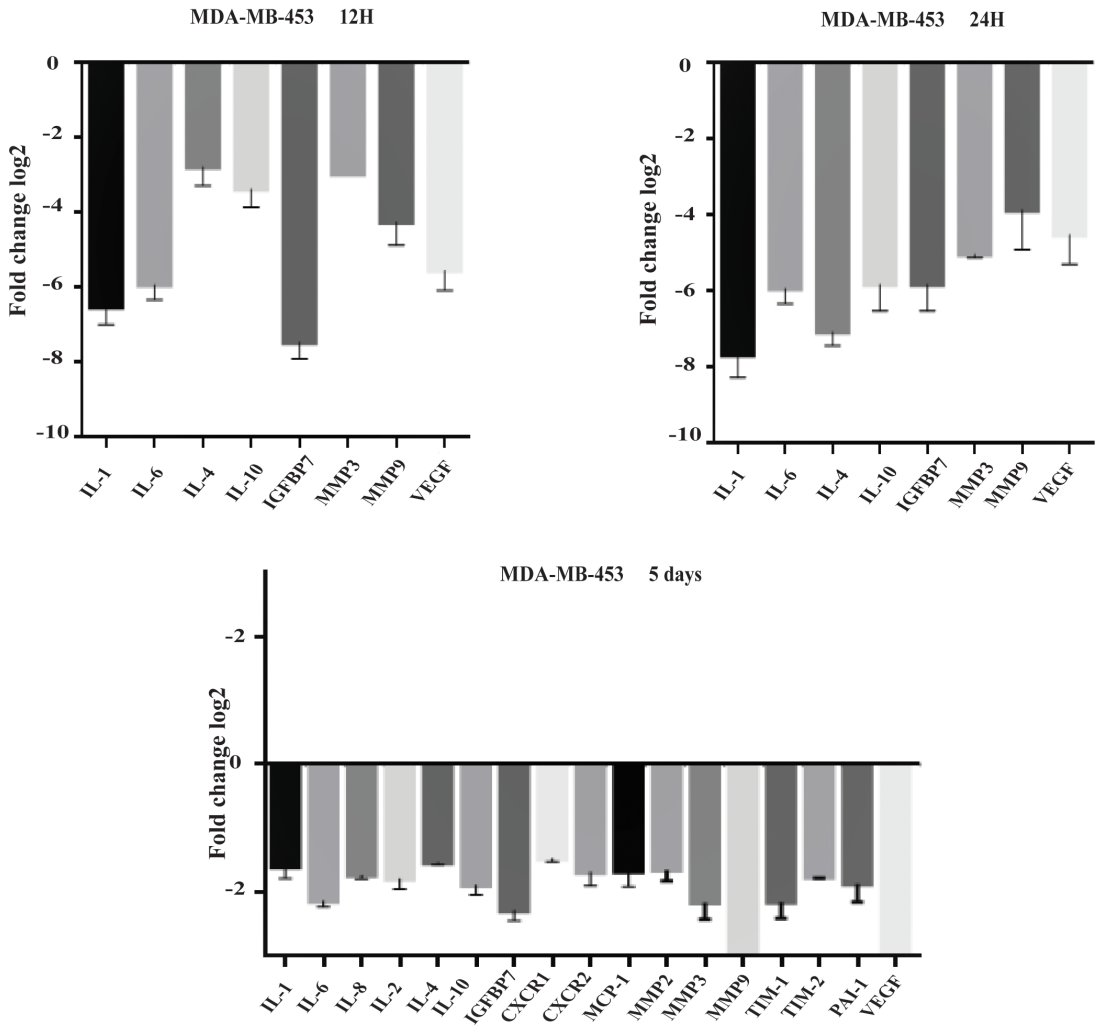

MDA/PRLR 12H
Fold change log2
15
10
5
0
IL-1
IL-6
IL-4
IL-10
IGFBP7
MMP3
MMP9
VEGF
MDA/PRLR 24H
Fold change log2
10
8
6
4
2
0
-2
IL-1
IL-6
IL-4
IL-10
IGFBP7
MMP3
MMP9
VEGF
MDA/PRLR 72H
Fold change log2
1
0
-1
-2
-3
IL-1
IL-6
IL-8
IL-2
IL-4
IL-10
IGFBP7
CXCR1
CXCR2
MCP-1
MMP2
MMP3
MMP9
TIM-1
TIM-2
PAI-1
VEGF

Figure 3.6

B)



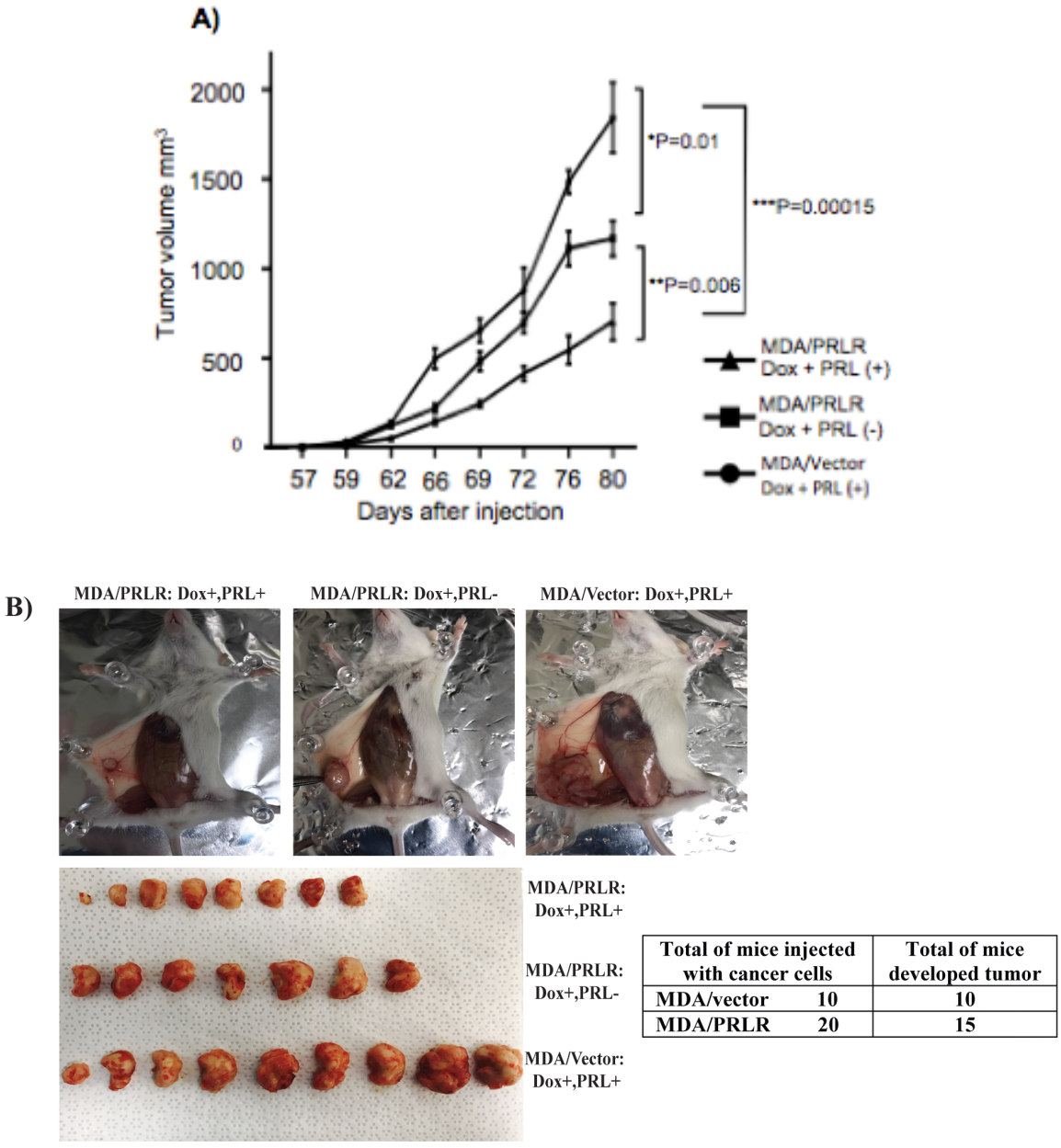
C)

ILs & signaling molecules			IL1A	IL2	IL4	IL6	IL10	CXCL8	CXCR2	CCL2
	Correlation	(r)	-0.05	-0.01	-0.03	-0.28	-0.04	-0.16	-0.14	-0.26
	PRLR	P value	0.3746	0.7823	0.5799	< 0.0001	0.4966	0.0017	0.007	< 0.0001
Microenvironment regulator genes			MMP2	MMP3	MMP9	TIMP1	TIMP2	SERPINE1	VEGFA	
	Correlation	(r)	-0.07	-0.15	-0.2	-0.19	-0.2	-0.11	0.01	
	PRLR	P value	0.1869	0.0042	0.0001	0.0003	0.0001	0.0466	0.8445	

Figure 3.6

Figure 3.6: PRL induces and maintains permanent senescence tumor growth arrest in TNBC

A) MDA-MB-231/PRLR cells were treated or not with dox (100ng/ml) and hPRL (250ng/ml) for 12h, 24h and 72h and the expression of SASP related genes (IL-1, IL-6, IL-8, IL-2, IL-4, IL-10, IGFBP7, CXCR1, CXCR2 and MCP-1; MMPs 2, 3 and 9 and TIM-1 and 2, VEGFA and PAI-1) were examined using qRT-PCR ($P < 0.05$). Results are presented as means ± SEM for triplicates of three independent experiments. B) MDA-MB-453 cells were treated or not with hPRL (250ng/ml) for 12h, 24h and 5 days and the expression of SASP related genes (IL-1, IL-6, IL-8, IL-2, IL-4, IL-10, IGFBP7, CXCR1, CXCR2 and MCP-1; MMPs 2, 3 and 9 and TIM-1 and 2, VEGFA and PAI-1) were examined using qRT-PCR ($P < 0.05$). Results are presented as means ± SEM for triplicates of three independent experiments. C) Table depicting the analysis of PRLR gene expression levels in association with genes involved in SASP in TNBC patients using bc-GenExMiner4.0 databases.



Total of mice injected with cancer cells		Total of mice developed tumor
MDA/vector	10	10
MDA/PRLR	20	15

Figure 3.7

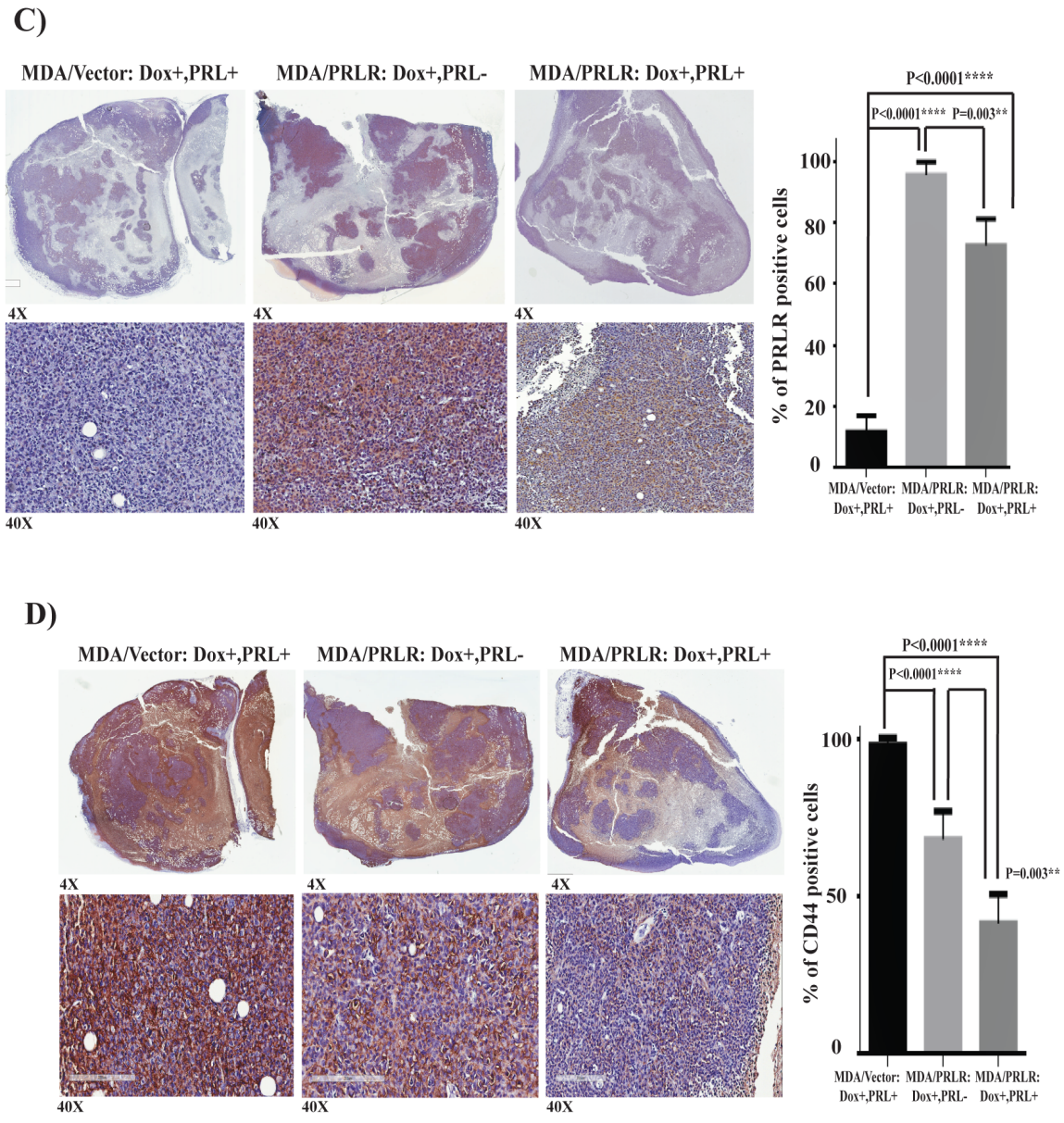

C)
MDA/Vector: Dox+,PRL+
MDA/PRLR: Dox+,PRL-
MDA/PRLR: Dox+,PRL+
4X
40X
P<0.0001****
P<0.0001**** P=0.003**
% of PRLR positive cells
100
80
60
40
20
0
MDA/Vector: Dox+,PRL+
MDA/PRLR: Dox+,PRL-
MDA/PRLR: Dox+,PRL+
D)
MDA/Vector: Dox+,PRL+
MDA/PRLR: Dox+,PRL-
MDA/PRLR: Dox+,PRL+
4X
40X
P<0.0001****
P<0.0001****
P=0.003**
% of CD44 positive cells
100
50
0
MDA/Vector: Dox+,PRL+
MDA/PRLR: Dox+,PRL-
MDA/PRLR: Dox+,PRL+

Figure 3.7

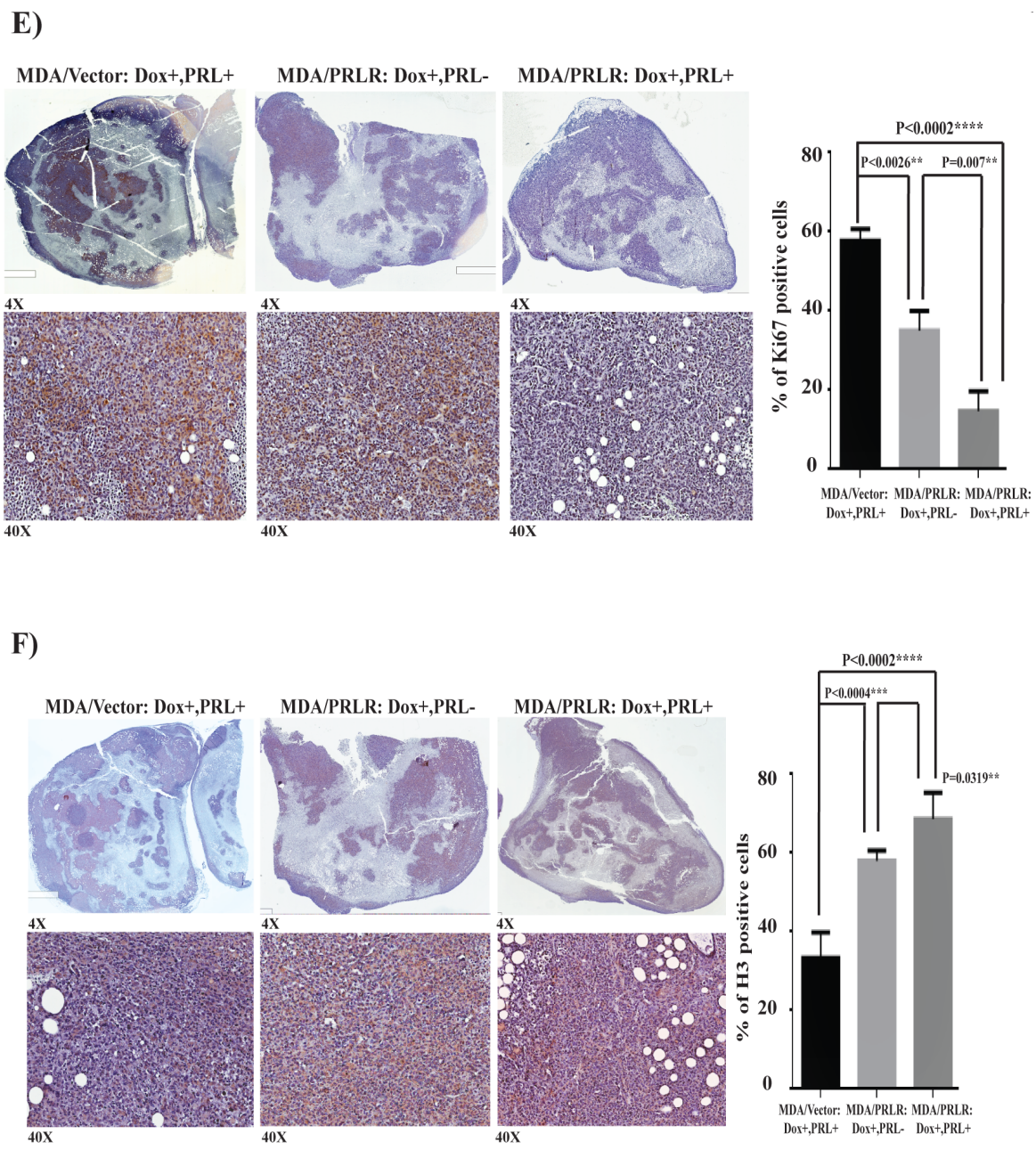

E)
MDA/Vector: Dox+,PRL+
MDA/PRLR: Dox+,PRL-
MDA/PRLR: Dox+,PRL+
4X
4X
4X
40X
40X
40X
P<0.0002****
P<0.0026**
P=0.007**
% of Ki67 positive cells
80
60
40
20
0
MDA/Vector: Dox+,PRL+
MDA/PRLR: Dox+,PRL-
MDA/PRLR: Dox+,PRL+
F)
MDA/Vector: Dox+,PRL+
MDA/PRLR: Dox+,PRL-
MDA/PRLR: Dox+,PRL+
4X
4X
4X
40X
40X
40X
P<0.0002****
P<0.0004***
P=0.0319**
% of H3 positive cells
80
60
40
20
0
MDA/Vector: Dox+,PRL+
MDA/PRLR: Dox+,PRL-
MDA/PRLR: Dox+,PRL+

Figure 3.7

Figure 3.7: PRL suppresses tumor growth and markers of proliferation and stemness while induces genomic heterochromatin hyper-methylation in vivo

A) Graph depicting tumor volume of MDA-MB-231/Vector or MDA-MB-231/PRLR treated and untreated mice xenografts for 3 weeks after PRL treatment. B) Representative pictures of NOD/SCID mice bearing tumors of MDA-MB-231/Vector and MDA-MB-231/PRLR in treated and un-treated mice (upper panel). Pictures of dissected tumors from the different experimental groups and table indicating the number of mice injected with cancer cells and the number of mice that showed tumor development. C) Immunohistochemical staining of PRLR in tumors obtained from MDA-MB-231/Vector or MDA-MB-231/PRLR (4X and 40X). The right panel represents the quantification of PRLR positive cells in each group (Materials and Methods). D) Immunohistochemical staining of Ki67 in tumors obtained from MDA-MB-231/Vector or MDA-MB-231/PRLR treated or untreated with PRL for three weeks (4X and 40X). The right panel represents the quantification of Ki67 positive cells in each group (Materials and Methods). E) Immunohistochemical staining of CD44 in tumors obtained from MDA-MB-231/Vector or MDA-MB-231/PRLR treated or untreated with PRL for three weeks (4X and 40X). The right panel represents the quantification of CD44 positive cells in each group (Materials and Methods). F) Immunohistochemical staining of H3K9me3 in tumors obtained from MDA-MB-231/Vector or MDA-MB-231/PRLR treated or untreated with PRL for three weeks (4X and 40X). The right panel

represent the quantification of H3K9me3 positive cells in each group (Materials and Methods).

	MDA-MB-231/WT	MDA-MB-231/vector	MDA-MB-231/PRLR	MDA-MB-453
CD44+/CD24-	75%	67%	77%	8.5%
CD44+/CD24+	27%	36%	24%	1%
CD44-/CD24-	1%	1%	1%	89.2%
CD44-/CD24+	0	0	0	1.3%
ALDH+	1.65%	2.05%	0.92%	2.6%

Figure S1: Basal levels of cancer stem cells in TNBC cells

Basal expression levels of CSC populations present in MDA-MB-231 WT, MDA-MB-231/vector, MDA-MB-231/PRLR and MDA-MB-453 cells assessed by CD44-CD24-ALDH triple staining followed by flow cytometry analysis. Percentages of $ALDH^+$ population basal levels in MDA-MB-231/PRLR compared with MDA-MB-231/vector cells.

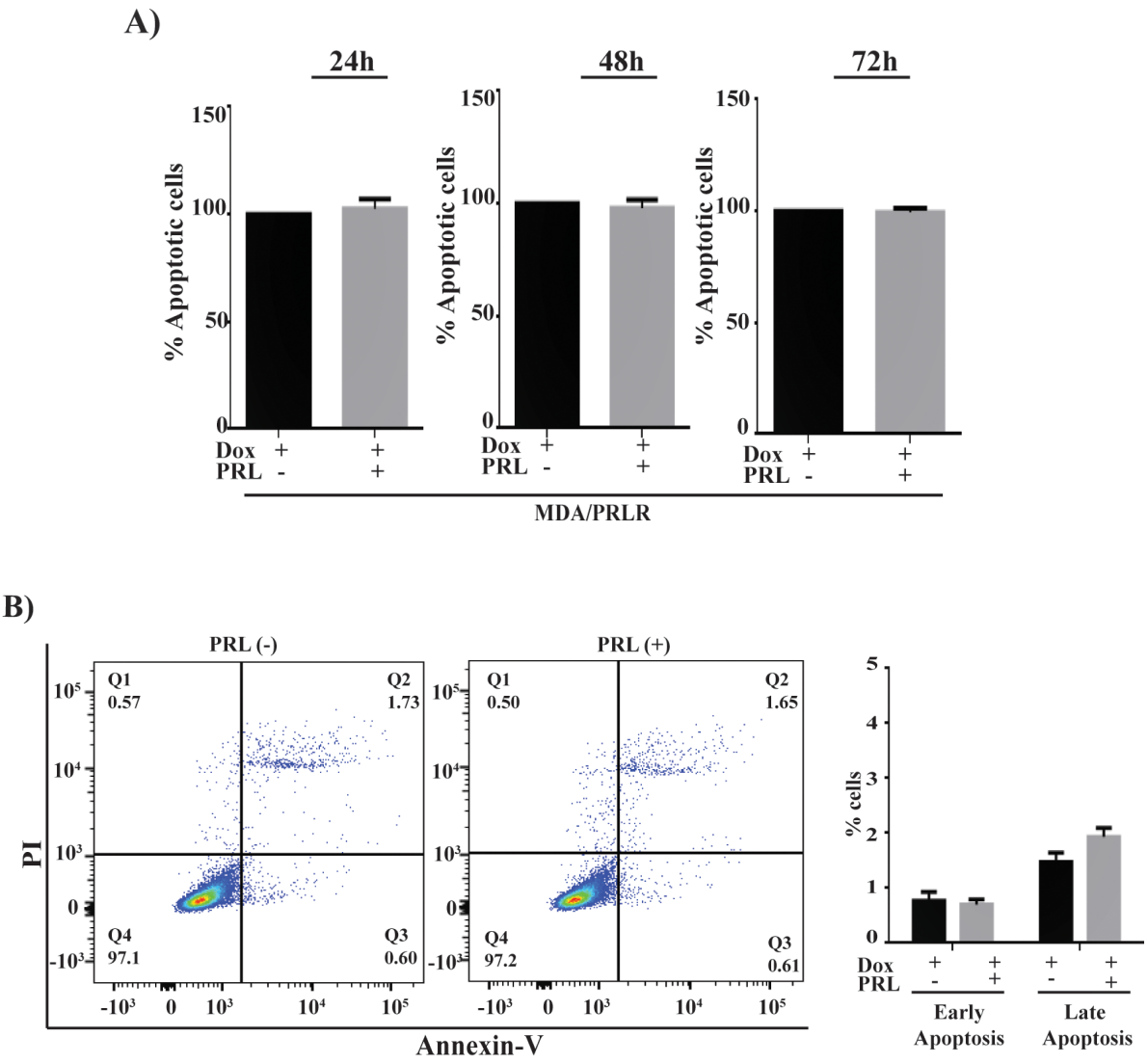

A)
24h
48h
72h
% Apoptotic cells
150
100
50
0
Dox + +
PRL - +
MDA/PRLR
B)
PRL (-)
PRL (+)
Q1
0.57
Q2
1.73
Q4
97.1
Q3
0.60
Q1
0.50
Q2
1.65
Q4
97.2
Q3
0.61
PI
Annexin-V
% cells
Early Apoptosis
Late Apoptosis

Figure S2

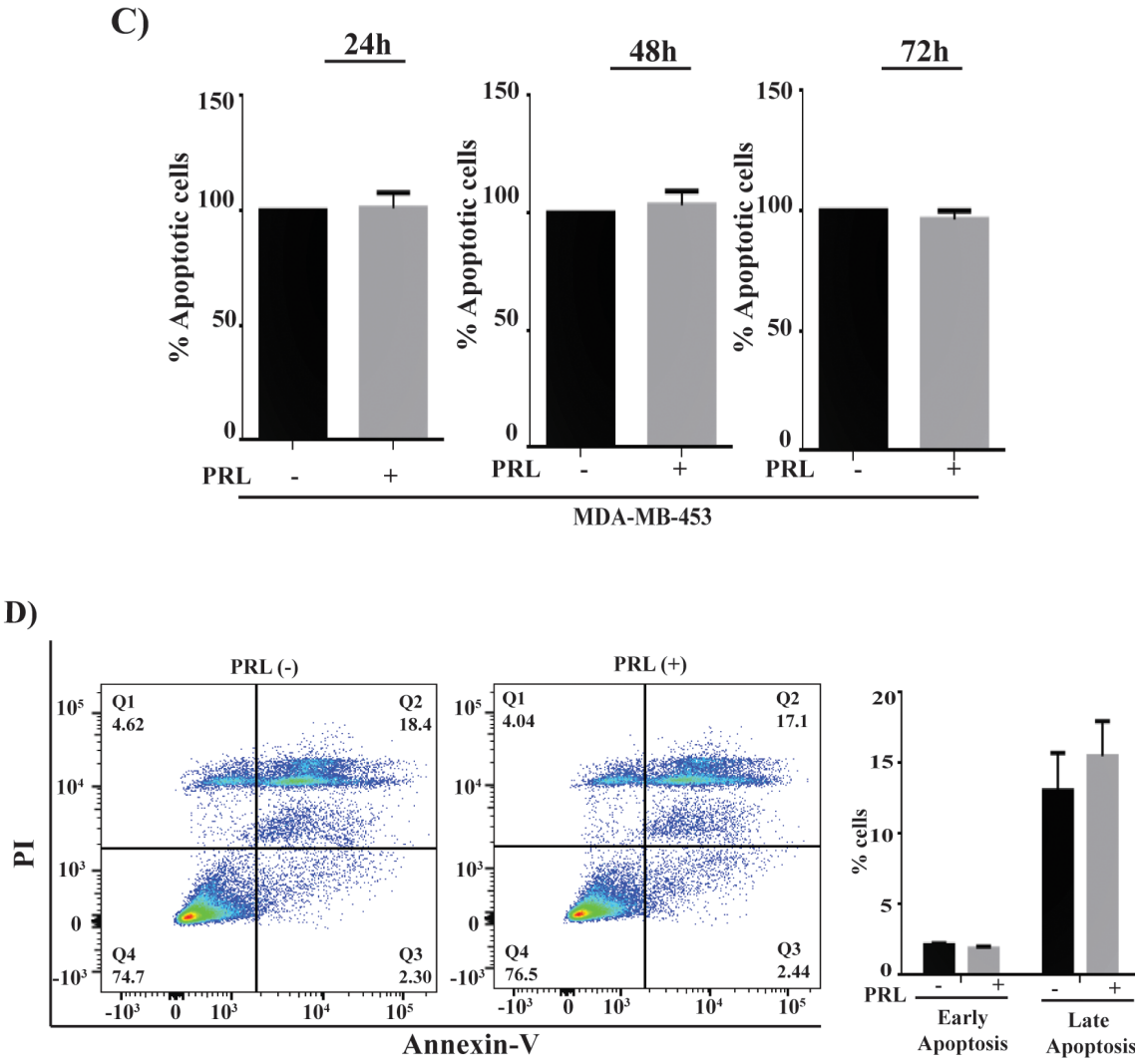
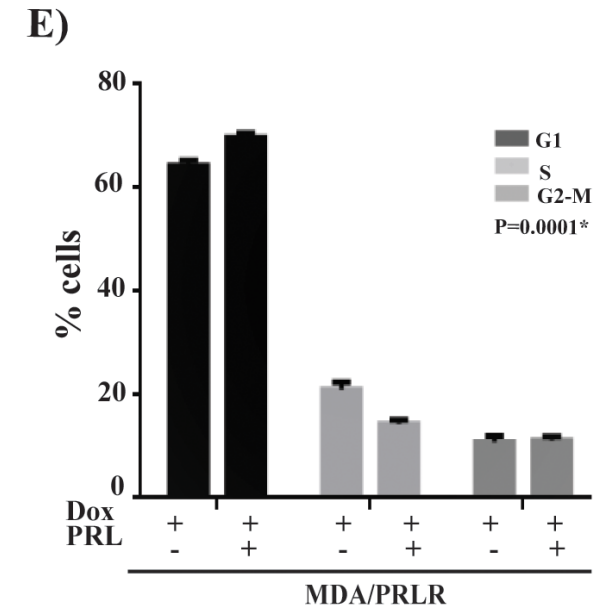

C)
24h
48h
72h
% Apoptotic cells
150
100
50
0
PRL - +
MDA-MB-453
D)
PRL (-)
PRL (+)
Q1 4.62
Q2 18.4
Q4 74.7
Q3 2.30
Q1 4.04
Q2 17.1
Q4 76.5
Q3 2.44
PI
Annexin-V
% cells
20
15
10
5
0
Early Apoptosis
Late Apoptosis

Figure S2


E)
80
60
40
20
0
% cells
G1
S
G2-M
P=0.0001*
Dox
PRL
+ + + + + +
- + - + - +
MDA/PRLR

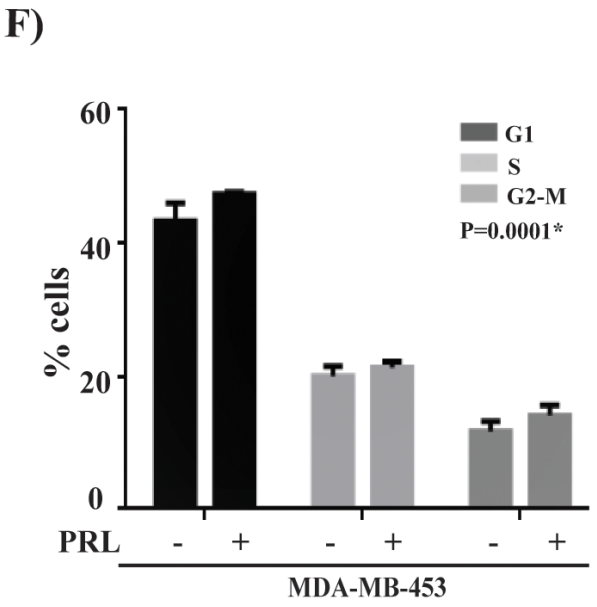

F)
60
40
20
0
% cells
G1
S
G2-M
P=0.0001*
PRL
- + - + - +
MDA-MB-453

Figure S2

Figure S2: Effect of PRL treatment in apoptosis and cell cycle

A) MDA-MB-231/PRLR cells were plated and treated with dox (100ng/ml) or not treated for over night period. Cells were then treated or not with hPRL (250 ng/ml) for 24h, 48h and 72h and subjected to Caspase 3/7-activity assay. B) MDA-MB-231/PRLR cells were with dox (100ng/ml) for over night period. Cells were treated or not with hPRL (250 ng/ml) for 72h, then apoptotic rates were assessed using Annexin V and PI double staining followed by flow cytometry analysis. C) MDA-MB-453 cells were plated and treated or not treated with hPRL (250 ng/ml) for 24h, 48h and 72h and subjected to Caspase 3/7-activity assay. D) MDA-MB-453 cells were treated or not with hPRL (250 ng/ml) for 5 days and apoptotic rates were assessed by Annexin V and PI double staining followed by flow cytometry analysis. E) MDA-MB-231/PRLR cells were plated and treated with dox (100ng/ml) or not for over night period. Cells were then treated or not with hPRL (250 ng/ml) previous 24h synchronization in starvation media. Cell cycle analysis of MDA-MB-231/PRLR treated and un-treated cells was performed after 72h of PRL treatment. F) MDA-MB-453 cells were plated and treated or not treated with hPRL (250 ng/ml) previous 24h synchronization in starvation media. Cell cycle analysis of MDA-MB-453 treated and un-treated cells was performed after 5 days of PRL treatment. Data represent mean ± SEM of three independent experiments.

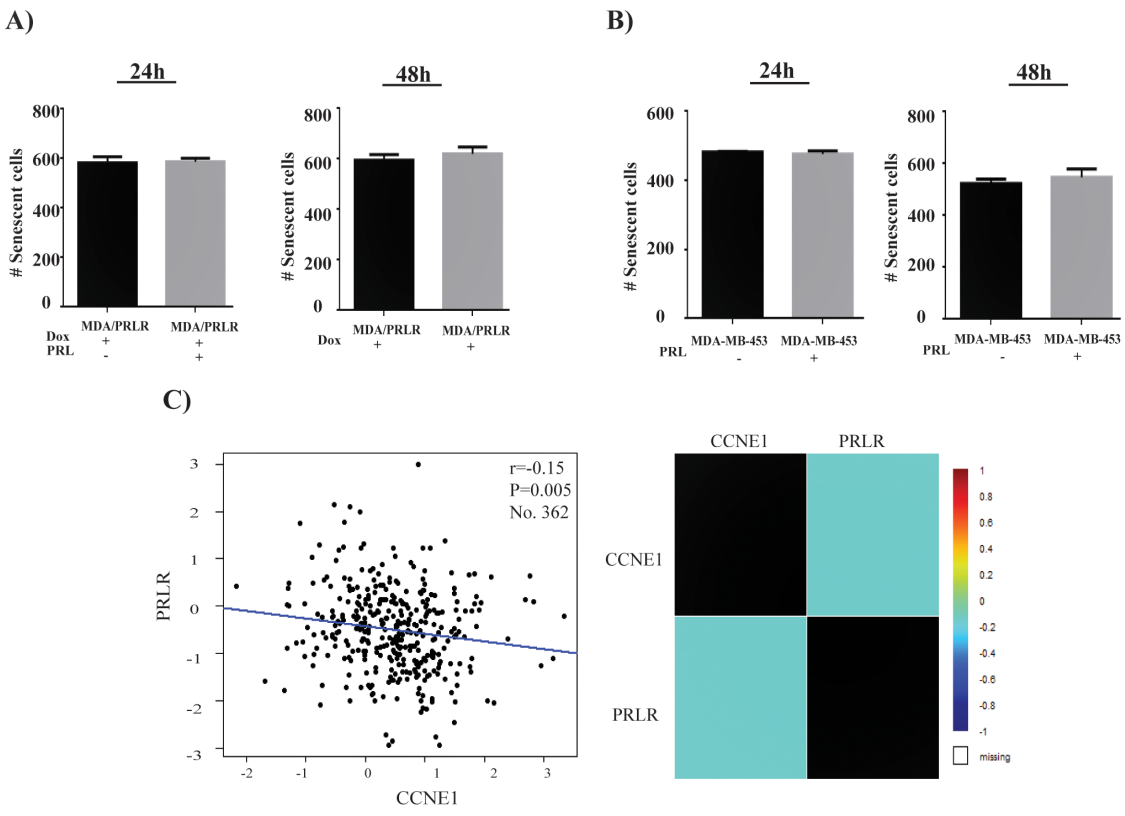


Figure S3: PRL treatment effects in cellular senescence regulators

A) MDA-MB-231/PRLR cells were treated or not with dox (100ng/ml) and hPRL (250ng/ml) for 24h and 48h following assessment of positive SAβ-gal staining. B) MDA-MB-453 cells were treated or not with hPRL (250ng/ml) for 24h and 48h following assessment of positive SAβ-gal staining. C) Correlation between PRLR gene expression levels and cyclin E in TNBC patient samples using Pearson's pairwise correlation plot and heat map in bc-GenExMiner4.0 database.

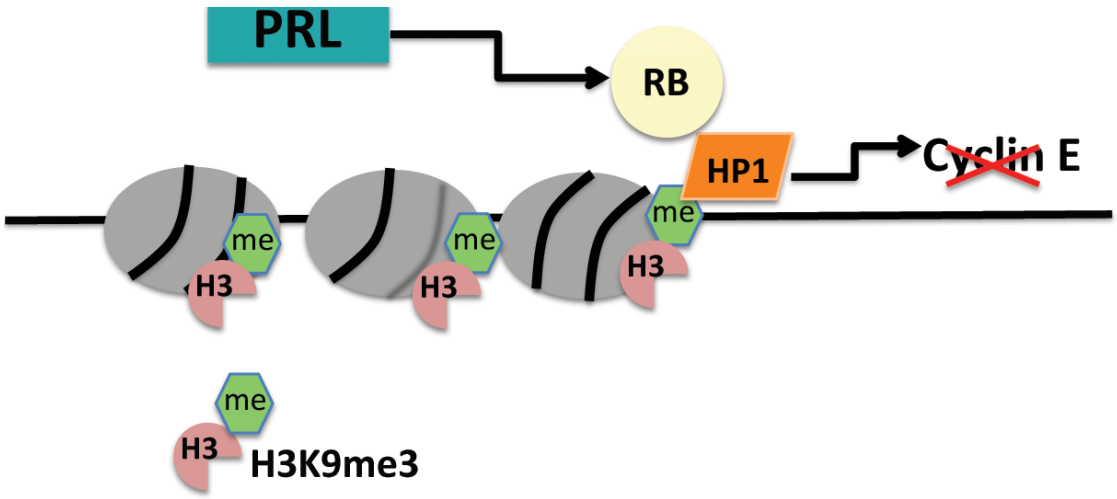


Figure S4: Prolactin treatment induces Heterochromatin formation

Figure depicting heterochromatin formation mechanism induced by PRL treatment in TNBC cells.

Chapter 4: General discussion and conclusions

Chapter 4:

Discussion and Conclusions

4.1 TNBC features and challenges

TNBC tumors account for approximately 15% of all breast cancers and their management represents one of the major clinical challenges. This is mainly attributed to heterogeneity, aggressive clinical features and lack of molecular targets (Malorni, Shetty et al. 2012).

TNBC tumors are heterogeneous and are classified into 6 different subtypes: Basal-like 1, basal-like 2, immunomodulatory, mesenchymal, mesenchymal stem-like and luminal androgen receptor. All lack ER, PRG and HER2 (Dent, Trudeau et al. 2007). However, all display diversity at the biological, molecular and clinical behavior (Lehmann, Bauer et al. 2011, Metzger-Filho, Tutt et al. 2012, Shah, Roth et al. 2012). Recently, Claudin-low subgroup was also categorized. This TNBC subgroup is characterized by high levels of EMT, immune response as well as stem cells associated genes (Prat, Parker et al. 2010).

The aggressive clinical behavior of TNBC is attributed to its high-grade and poorly differentiated tumors (Rakha, El-Sayed et al. 2007, Thike, Cheok et al. 2010). Moreover, this subtype contains the worst prognosis and patients are at high risk of relapse due to the high levels of stem-like cells (Honeth, Bendahl et al. 2008). These cells are believed to play an important role in TNBC

aggressiveness, progression (Yu, Zhu et al. 2013), resistance to therapy and metastasis (Sheridan, Kishimoto et al. 2006, Dalerba, Cho et al. 2007, Li, Lewis et al. 2008, Charafe-Jauffret, Ginestier et al. 2009). For that reason, identification of novel biomarkers in this breast cancer subtype is critically needed to help understand the biology of TNBC and the development of new tools for prognosis and therapy (Wang, Hu et al. 2016).

4.2 The Role of PRL signaling pathway in breast cancer

Previous studies showed a possible link between PRL and breast tumorigenicity and metastasis (Wennbo, Gebre-Medhin et al. 1997, Chen, Ramamoorthy et al. 1999, Vonderhaar 1999, Chen, Holle et al. 2002, Clevenger, Furth et al. 2003, Rose-Hellekant, Arendt et al. 2003, Oakes, Robertson et al. 2007, Sutherland, Forsyth et al. 2016). In contrast, PRL signalling was recently proposed to prevent breast cancer cell dissemination and to predict a favorable clinical outcome (Sultan, Xie et al. 2005, Nouhi, Chughtai et al. 2006) (Hachim, Hachim et al. 2016, Hachim, Shams et al. 2016).

The reasons of variability in these findings might be attributed to different factors including the using of different probes, antibodies, techniques and animal models.

Animal studies that involved genetically modified mice was shown to induce mammary tumors in agreement with the pro-tumorigenic role of PRL signalling pathway. Transgenic model over-expressing PRL systemically was shown to develop mammary tumors with a high latency of tumor development

(15 months) indicating that PRL is not a strong oncogene (Wennbo, Kindblom, Isaksson, & Tornell, 1997). Other example is the delayed of tumor initiation in mammary gland after the deletion of PRL (Vomachka, Pratt, Lockefeer, & Horseman, 2000) or PRLR gene in mice (Oakes et al., 2007). The reason for these findings could be the fact that mice genetically engineered to induce mammary tumorigenesis, might minimize their resemblance to the clinical scenarios, firstly because it is well know that the knockout of PRLR inhibits the formation of lobular-alveolar unit in which breast cancer arise (Lee & Ormandy, 2012; Ormandy, Camus, et al., 1997) and secondly because PRLR can be down-regulated due to high levels of its ligand (PRL) (Genty, Paly et al. 1994).

Another finding that supports the tumor promoter role of PRL in breast cancer is the finding of Clevenger and his colleagues (Clevenger, Chang et al. 1995, Reynolds, Montone et al. 1997) that showed PRL expression in human breast cancer tumors using an in situ hybridization (ISH) protocol. However, Nitze and his group mentioned that the probes used in this report were not validated (Nitze, Galsgaard et al. 2013). Moreover, when they use validated probes and validated ISH protocol, they were unable to detect any significant levels of PRL expression in human breast cancer samples as well as in breast cancer cell lines (Nitze, Galsgaard et al. 2013).

In addition, the use of different antibodies to detect PRLR expression in breast cancer samples and cell lines was another reason for this variation in results. For example, many of the reports that showed high PRLR expression in breast cancer samples and breast cancer cell lines used antibodies that later

were found to be non-specific for PRLR and did not even cross-react with human PRLR. Moreover, the use of validated PRLR antibodies revealed undetectable or low PRLR expression levels in breast cancer samples (Galsgaard, Rasmussen et al. 2009).

Finally, most of the reports studied before investigated the effect of PRL in breast cancer without highlighting the effect of breast cancer heterogeneity. Indeed, investigating the role of PRL in various and heterogeneous breast cancer subtypes might help in the better evaluation of its role in tumorigenicity and might explain the diversity in results observed before. For example, recent report by Ali and her group found PRLR to be expressed in ~20% in non-TNBC breast cancer subtypes and to be very low/undetectable in TNBC cases. This highlights the need to investigate the distinct role of PRL in each breast cancer subtype, which might help in better understanding the role of PRL hormone in breast cancer biology.

4.3 The prognostic value of PRL pathway in TNBC tumors

Loss of cellular differentiation is a common feature of TNBC tumors. In addition, TNBC tumor cells are thought to originate from a progenitor mammary population (Luo X, et al., 2010). Therefore, elucidating the role of mammary differentiation pathways in TNBC biology might provide novel approach in advancing classification, prognosis and treatment. PRL pathway is critical for mammary gland development and terminal differentiation of mammary epithelial cells (Hennighausen and Robinson 2005). While previous reports showed a

possible link between PRL and breast cancer initiation, promotion and metastasis (Wennbo, Gebre-Medhin et al. 1997, Chen, Ramamoorthy et al. 1999, Vonderhaar 1999, Chen, Holle et al. 2002, Clevenger, Furth et al. 2003, Rose-Hellekant, Arendt et al. 2003, Oakes, Robertson et al. 2007, Sutherland, Forsyth et al. 2016), more recent evidence suggests a tumor suppressor role in breast cancer (Sultan, Xie et al. 2005, Nouhi, Chughtai et al. 2006). This role is mainly though suppression of the EMT process, essential for cancer cell migration and metastasis (Sultan, Xie et al. 2005, Nouhi, Chughtai et al. 2006).

This was supported by other reports, which showed a strong association between PRL pathway members and favourable clinicopathological parameters including differentiated tumors, LN negativity as well as prolonged patient survival (Hachim, Hachim et al. 2016, Hachim, Shams et al. 2016) (Faupel-Badger, Duggan et al. 2014). The role of PRL pathway in TNBC is not well illustrated, however, epidemiological study on more than 500 breast cancer patients showed that women with prolonged breastfeeding, seemed to reduce the probabilities of having TNBC (Redondo, Gago-Dominguez et al. 2012). Moreover, another report from our lab investigating the prognostic value of PRLR in different molecular subtypes revelaed PRLR receptor expression to be significantly lower/undetectable in TNBC tumors compared to non-TNBC subtypes (Hachim, Hachim et al. 2016).

In the present study, we investigated the prognostic role of PRL and its signaling pathway in TNBC using both TMA as well as large publically available databases. Interesintgly, while our results confirm that TNBC tumors express

lower levels of PRLR compared to other breast cancer subtypes, PRLR mRNA levels were shown to be preserved at high or intermediate levels in approximately one third of TNB patients. Those patients with higher PRLR expression showed a significant association with two distinct metagenes, one with luminal-like differentiation (FOXA1 and AR related genes) and the other associated with epithelial phenotype (claudin-CD24 related genes). This suggests that PRLR can identify a subgroup of TNBC tumors with epithelial and luminal-like differentiation. The clinical behaviour of this subgroup was also found to be distinct, with siginficant favorable prognosis compared to patients with lower PRLR expression. This is supported by recent reports that showed patients with TNBC tumors and higher levels of luminal-like genes (AR and GATA3) and better prognosis compared with other TNBC patients.

Alltogether, highlights the possible use of PRLR as a sub-classifier that identify patients with good overall survival that can benefit from less aggreessive treatment appoaches.

4.4 Biological effect of PRL in TNBC tumors

There are many previous reports that investigated the role of PRL in breast cancer. Some of them deal with PRL as a promoter of breast tumorigenesis (Clevenger, Chang et al. 1995, Ginsburg and Vonderhaar 1995, Touraine, Martini et al. 1998) and recent reports propose PRL as a tumor suppresor (Sultan, Xie et al. 2005, Nouhi, Chughtai et al. 2006). However, the role of PRL in TNBC cell behaviour was not well illustrated.

Here we evaluated the role of PRL in TNBC cell biology using two different models. PRLR was restored in MDA-MB-231, claudin low-TNBC cell line and activated in MDA-MB-453, PRLR-TNBC cell line. Interestningly, *in vitro* and *in vivo* models reavelaed that PRLR restoration/activation was not only sufficient to reduce TNBC cell viability, but also reverse their mesenchymal phenotype and induce an epithelial phenotype. Moreover, it causes reduction in their *in vivo* tumor formation capacity.

Our work supports that autocrine PRL as well as PRLR gain of function causes either no change or reduces breast cancer cell proliferation (Nitze, Galsgaard et al. 2013, Zhang, Cherifi et al. 2015). In addition, the abililty of PRL signalling pathway to induce epithelial phenotype and suppress the mesenchymal phenotype was observed in previous reports (Nouhi, Chughtai et al. 2006).

Moreover, PRL pathway through STAT5 activation lead to relocaliztion of E-cadherin reducing invasion and migration of breast cance cell lines (Sultan, Xie et al. 2005). These results provide a strong evidence to support the tumor suppressor role of PRL pathway in TNBC and highlight the possible theraputic application.

4.5 Mechanisms of PRL action in TNBC cell lines

In the previous chapters we provided undeniable evidence regarding the role of PRL pathway in inducing cell differentiation and as a suppressor of breast carcinogenesis. In addition, there are no studies that evaluated the anti-tumorigenic effects and the final cell fate after PRL treatment. For that reason in

chapter 3, we evaluated several mechanisms though which PRL might induce its effects.

Interestingly, while PRL showed no effect on cellular apoptosis, PRL was able to induce two mechanisms believed to be essential in determining the cell fate: differentiation and cellular senescence.

Terminal differentiation is one of the major mechanism through which PRL exerts its biological effects (Horseman, Zhao et al. 1997, Lee and Ormandy 2012). Here we confirmed that this pro-differentiation role of PRL is still preserved in cancer cells and can cause a significant depletion in stem cells populations $CD44^{+}/CD24^{-}$ and $ALDH^{+}$. These CSC are believed to play an essential role in TNBC aggressive behaviour through promotion of invasion, metastasis as well as chemo and radio-chemotherapy resistance (Sheridan, Kishimoto et al. 2006, Dalerba, Cho et al. 2007, Li, Lewis et al. 2008, Charafe-Jauffret, Ginestier et al. 2009).

We also found, PRL treatment to mediate cellular senescence, a mechanism characterized by irreversible cell proliferation arrest (Buttitta and Edgar 2007) (Sledge and Pegram 2015) (Prieur and Peeper 2008, Campisi 2013). PRL induced cellular senescence was achieved through down regulation of several senescence associated genes and markers, including β-galactosidase and cell cycle regulatory genes like RB, p21, p15, 53BP1, INK4C and cyclin E. In addition, PRL treatment is able to induce morphological changes as well as chromatin condensation, features associated with the senescence phenotype.

Significantly, PRL treatment also was able to inhibit the production of inflammatory cytokines as well as growth factors, known to be associated with the senescence-associated secretory phenotype (SASP) (Liu, Uppal et al. 2015).

These finding highlight the role of PRL hormone in inducing cellular senescence as a protective mechanism in TNBC cells, leading to a prolonged anti-proliferative stage without the induction SASP, believed to have pro-tumorigenic and tumor progression role as well as chemotherapy resistance (Perez-Mancera, Young et al. 2014) (Jackson, Pant et al. 2012, Sun, Campisi et al. 2012).

Interestingly, our data implicates both mechanisms differentiation and senescence through which PRL produce its anti-tumorigenic effects in TNBC are potentially linked to RB function (Panteleeva, Boutillier et al. 2007). RB, which is an important gene for chromatin cohesion and structure, was found to be required for cell cycle-regulated H3K9me3 at the cyclin E promoter through activation of HP1. In our study, we found PRL treatment to increase H3K9me3 and to correlate positively with RB and HP1.

H3K9me3, a histone modification associated with heterochromatin, contributes to gene regulation by forming large repressive domains to induce silencing of genes (Justin S. Becker et al., 2015). This is supported by reports that showed that H3K9me3 heterochromatin is important for controlling terminal differentiation, determination of cell fate through epigenetic regulation and cell type specific silencing of genes (Allan, R. S. et al. 2012, Liu, J. et al. 2015).

These indicate that the induction of both processes by PRL might be parallel or overlapping.

Altogether, we speculate that PRL initiates a series of events starting by inducing cell differentiation that converts cancer cells into less aggressive and leads to an activation of tumor suppressor pathways. Furthermore, these cells will undergo cellular senescence leading to permanent arrest and more resistance to oncogenic signals.

The above-mentioned data highlights the ability of PRL to induce reprograming of the TNBC cells and convert them into more differentiated and non-tumorigenic. This highlights the great potential to use PRL hormone as a novel and alternative therapeutic approach for TNBC patients that will not only help to eradicate these tumors, but also will help in reducing their likelihood to recur and be resistant to the conventional treatments.

More importantly, our work is proposing a new management approach for TNBC patients based on PRL pathway. This approach is based on screening for PRLR expression in patients that may benefit from the use of PRL hormone as a novel pro-differentiation therapy as a single agent or in combination with the standar theraphy. This will ensure the improvement of patient overall survival and decrease tumor recurrance.

Appendix: Extended Material and Methods

Appendix:

Extended Material and Methods

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

Antibodies and reagents

Anti-PRLR antibody (polyclonal antibody) (H-300) against the intracellular domain of the PRLR long form (Santa Cruz #sc-20992), CD44 (Abcam #Ab6123) and Ki67 (Abcam #Ab16667) was used for IHC. H3K9me3 (Abcam #Ab8898cam) was used for IHC, IF and WB. Phospho-Stat5a (Invitrogen #71-6900) and β-tubulin (Santa Cruz #sc-53140) (monoclonal antibodies) were used for WB. APC/Cy7 Anti-CD44 (Biolegend # 103028), APC Anti-CD24 (Biolegend #311118) were used for flow cytometry analysis. Goat anti-mouse horseradish peroxidase (HRP) and goat anti-rabbit HRP purchased from Santa Cruz Biotechnology. Recombinant Human Prolactin (rhPRL) was purchased from Feldan Therapeutics (1F-02-008). According to manufacturer report the purity of the preparation is greater than 95% as determined by SDS-PAGE and endotoxin level is <0.01 EU per 1 μg of the protein by the LAL method (indicating the certainty that there is no possibility of the product to cause any bacterial contamination that can produce tissue injury or death). Other reagents and materials include: doxycycline hyclate (Sigma #D9891-56), B27 Supplement (Life Technologies #17504-044), nitrocellulose membranes (Whatman) and enhanced chemiluminescence hyperfilm, ultra-low attachment 12 well plates for

tumorsphere assay (Corning #CLS3471); 24-well plates HTS multiwell insert system format (BD Falcon), 8.0μm pore size were used for invasion assays; 96-well plates (Corning #3753 and Fisher #7201216) were used for MTT assays.

Cell culture and generation of stable cell lines

MDA-MB-231 parental cells were used to generate stable cell lines overexpressing the human long form PRLR cDNA using doxycycline (dox)–dependent lentiviral system designated as MDA-MB-231/vector and MDA-MB-231/PRLR according to manufacturer's instructions (Clontech) (Nouhi Z, etal., 2006). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (Multicell, #095150). MDA-MB-453 cells obtained from Dr. Morag Park (McGill University) were cultured in DMEM supplemented with 10% fetal bovine serum (ATCC 30-2008).

Cell lysis, immunoprecipitations and western blotting

For whole cell lysates and immunoprecipitations, cells were lysed in lysis/SDS buffer as described previously (Brouckaert, O., et al., 2012). Immunoprecipitations were done for 3 hrs at 4°C using a pAb to PRLR and protein A-Sepharose beads. Proteins were run on SDS-PAGE and transferred to nitrocellulose membrane for western blotting analysis using the appropriate antibodies.

Tissue microarray

Tissue microarray of TNBC cases (BR487) was commercially purchased from US Biomax (Rockville, MD). The TMA includes 42 cases of invasive ductal carcinoma, 2 cases of intraductal carcinoma and medullary carcinoma, 1 case of infiltrating lobular carcinoma and lobular ductal mixed carcinoma. Clinicopathological characteristics including tumor stage, grade, estrogen receptor (ER), progesterone receptor (PR), HER-2 status was also available.

Immunohistochemistry

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 #sc-20992), or a rabbit polyclonal antibody to AR (Santa Cruz #CO215), CD44 (Abcam #Ab6123) Ki67 (Abcam #Ab16667) and H3K9me3 (Abcam #Ab8898). UltraVision LP Detection System HRP Polymer & DAP Plus Chromogen (Thermo Fisher Scientific, Fremont CA) was used for detection. The TMA slides were scanned using Aperio XT slide scanner (Leica Biosystems).

Immunohistochemistry Scoring

For prolactin receptor (PRLR) and H3K9me3, the staining was considered positive, when the malignant cells showed granular cytoplasmic staining, while CD44 staining was considered positive only if the malignant cells showed positive membranous staining. In comparison, Ki-67 positive cells where

considered only if the malignant cells showed nuclear stain. AR immunostaining was assessed using Allred score (Sutherland A. et al., 2016). Quantification of positive cells was done through measuring of the positive cells in at least 4 different fields. The mean of positive malignant cells for each stain was measure and statistical analysis between the groups was measured using t-test in the GraphPad Prism6 software. Two investigators including one anatomical pathologist, blindly from the clinical data evaluated the slides independently. If there is any discordance, simultaneous examination was performed to solve the differences.

Human breast cancer gene profiling databases and in silica analyses

The publicly available Breast Cancer Gene-Expression Miner Version 3.0 was used to evaluate the mRNA levels of PRLR in different molecular subtypes using robust single sample predictor 3 classification (RSSPC) (Burstein, M. D. et al., 2015). This program divides PRLR mRNA expression levels into three equal quantiles. The upper quantile group represents high PRLR mRNA levels, middle quantile represent intermediate PRLR mRNA levels and lower quantile represent low PRLR expression levels. Furthermore, the prognosis gene expression analysis tool of Breast Cancer Gene-Expression Miner Version 3.0 was used to assess the association between PRL, PRLR, Jak2 and Stat5a mRNA levels and patient outcome. The gene expression correlation analysis tool of Breast Cancer Gene-Expression Miner Version 3.0 was used to study the correlation between PRLR mRNA level and members of the different metagenes within TNBC.

Correlation coefficient value between PRLR mRNA expression and each member of the metagenes as well as p-value and number of patients involved was calculated (indicated in Figure 2.3). Analysis of the association between PRL, PRLR, Jak2 and Stat5a was done using Breast Cancer Kaplan-Meier plotter. 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.

The Breast Cancer Gene-Expression Miner Version 4.0 (bc-GenExMiner 4.0) correlation module was used to evaluate the association between PRLR and different genes including cyclin E1, self-regulatory factor, microenvironment regulator genes and different interleukins and signalling molecules.

The prognostic module of the Breast Cancer Gene-Expression Miner v4.0 was also used to evaluate the association between different genes and patient outcome represent as any event free survival (AEFS) and metastasis free survival (MFS) in TNBC patients. In addition, the expression module of the same program was also used to investigate the expression levels of CD44 in TNBC breast cancer patients compared to non-TNBC patients. The Breast Cancer Gene-Expression Miner v4.0 is publicly available user-friendly web-based application that allow investigators to analyse the expression levels of different gene in around 5000 breast cancer patients sample belong to different breast cancer subtypes.

The KM plotter database was also used to investigate the association between the expression levels of some genes with patient outcome represented as relapse free survival (RFS). This is another publically available database that also allow investigating the association between gene expression levels and patient outcome in more than 5000 patients.

RNA extraction and qRT-PCR

MDA-MB-231/PRLR and MDA-MB-231/vector cells grown to confluence then were pre-treated with doxycycline (100ng/ml) before stimulation. Before ligand stimulation, cells were starved in DMEM (2% FBS) and stimulated with rhPRL 250ng/ml for a period of 72h. MDA-MB-453 cells were starved in DMEM (2% FBS) and stimulated with rhPRL 250ng/ml for a period of 5 days. Cells were lysed in 500 μl of trizol. Total RNA was isolated as described by the manufacturer (Invitrogen Life Technologies, Burlington, Ontario, Canada). Samples were quantified by absorbance at 260 nm. Aliquots of 300 to 400 ng of total RNA were used for reverse transcription and PCR amplification in one step using Brilliant II SYBR green quantitative real- time PCR (qRT-PCR) Master Mix kit, 1-Step (Stratagene Amsterdam, Zui-doost, The Netherlands) according to the manufacturer's recommendation. RT-qPCR of EMT markers (slug, snail, twist, FN1, vimentin, e-cadherin, zeb1), CSC transcription factors (oct4, sox2 and nanog), senescent-associated genes (RB, p21, p16, p15, INKC4, Cyclin E, H2AX, 53BP1, HP1 protein and Lamin B1) and senescence-associated secretory phenotype genes (IL-1, IL-6, IL-8, IL-2, IL-4, IL-10, IGFBP7, CXCR1, CXCR2 and

MCP-1; matrix metalloproteinases (MMPs) 2, 3 and 9 and its inhibitors (TIM-1, 2), VEGFA and PAI-1) was performed. The specificity of the primers was then tested by a dissociation program at 95°C for 1 min, with a ramp-down to 65°C and then a ramp-up to 95°C (at the instrument default rate of 0.2°C/s). Dissociation curve analysis was performed after the completed q-PCR. Data were obtained by slowly ramping up the temperatures of reaction solutions from 65 to 95°C.The quantitative gene expression analysis was performed using the following primers:

Primer	**Forward Sequence**	**Reverse Sequence**
Slug	CTGGTCAAGAAGCATTTC	GGGGAAATAATCACTGTAT
Snail	GAAAAGGGACTGTGAGTA	GAATAGTTCTGGGAGACA
Twist	GGAGACCTAGATGTCATTGTT	ACGCCCTGTTTCTTTGAA
FN1	TGTGGTTAGTGTCTATGC	GCGATCAATGTTGGTTAC
Vimentin	AACCTGAGGGAAACTAAT	TTGATAACCTGTCCATC
E-cadherin	ACATACACTCTCTTCTCTC	GTCATTCTGATCGGTTAC
Zeb1	GAAAGTGTTACAGATGCAG	TTCCTTTCCTGTGTCATC
Oct4	ATCCTGCCTTTTCACACCAC	CTCCAGGTTGCCTCTCACTC
Sox2	CATCACCCACAGCAAATGA	TGCAAAGCTCCTACCGTACC
Nanog	TTCCTTCCTCCATGGATCTG	TCTGCTGGAGGCTGAGGTAT
CD24	AAAGGAGCTGGTGCTGATGT	GAGTTTTCAGCCCTCACTGC
CD44	AGCCACCTTGGGGTTCTAGT	ACCGACAGAGAAGGCAAGAA
GAPDH	AGGGCCCTGACAACTCTTTT	AGGGGTCTACATGGCAACTG

RB	CCCCAAGCTCAAAGTAGCAG	CCAGACCAGCAGCATCAGTA
P21	CTCTCCAATTCCCTCCTTCC	AAGCACCTGGAGCACCTAGA
P16	AGCAGTCCGACTCTCCAAAA	GGGTGTTTGGTGTCATAGGG
P15	GACCGGGAATAACCTTCCAT	CACCAGGTCCAGTCAAGGAT
INCK4C	CAATGGCTCAGTTTTGCTGA	TTTTTCCCCTTTCCTTTGCT
Cyclin E	GTTGAACCCTGGAAGTGGAA	GGGCTTTGTCCTGTTGGTAA
H2AX	AACTCCCCAATGCCTAAGG	TCCCTTCCAGCAAACTCAAC
TP53BP1	TTTAAGGCAGCTCTGGCAAT	CACGCCTCTCTCTGGGTAAG
HP1	CTTTGCTCCTCCCACCATTA	AAGGCCTCAAGACTGCAAAA
Lamin B1	TCCAGGCCAGCAGAGTAGTT	GCCCAGTCAAACCACAGAAT
IL-1alfa	CAGTTCTGCTGACTGGGTGA	AGGTGCTGACCTAGGCTTGA
IL-6	GCAGAAAAAGGTGGGTGTGT	GCAGAAGAGAGCCAACCAAC
IL-8	TAGCAAAATTGAGGCCAAGG	AAACCAAGGCACAGTGGAAC
IL-2	TTAAGCCTAGGGAGGGTGGT	TCCCAGCAGGAAATAGATGG
IL-4	GCCTTCAGCACATCTTCACA	ATCATCGCTTCTCTGCACCT
IL-10	AGGGAATTGGGTTTGTTTCC	GGTAACCCTAAGGGCAGGAG
IGFBP7	CTGCTTGGTAGCTCCTGGTC	CTGCTTGGTAGCTCCTGGT
CXCR1	GGAGCCGTTGGTCAGAAATA	CCTACTGGGCCTCAAATGAA
CXCR2	ACATGGGCAACAATACAGCA	TGAGGACGACAGCAAAGATG
MCP-1	CAGCTCTGGGAACACACTCA	GAGTCACCGTCTCTGGAAGC
MMP2	TCAAGGAGCTGGTAGGCAGT	TCTCCCCTGAGCTTGTGAGT
MMP3	CTGGGAAAATCAGCCATTGT	AGGTTCTGGAGGGACAGGTT
MMP9	GTCTTGTGGAGGCTTTGAGC	CAGGGATCTCCCCTCCTTAG

TIM-1	GAAAAAGCTGGGTCTTGCTG	AGTGTCCTGGAGGCTGAGAA
TIM-2	TCCCCAATCCCCTTAAAATC	GCTATCAGCCACAGCAACAA
VEGFA	CTGCCTCCTGACACTTCCTC	TTTCTTGCGCTTTCGTTTTT
PAI-1	AACCCTACCCTGCTTCCTGT	AGAAGGAGTGGGACAGCTC

MTT assay

2.5x10^3 cells of MDA-MB-231/vector and MDA-MB-231/PRLR were seeded into 96-well plate overnight and grown in 2% FBS in DMEM media. Cells were treated with rhPRL 250ng/ml for a period of 24-72h. Then, cells were incubated with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) at 37°C for 2h as previously described (Cocolakis, E, et al., 2001). Results are presented as means ± standard errors of the means (SEM) for triplicates of three separate experiments. Statistical significance was assessed using student's t test analysis. 5 x 10^3 cells of MDA-MB-453 were seeded into 96-well plate overnight and grown in 2%FBS in DMEM media. Cells were then starved in 2% FBS starvation media and either treated or not with hPRL 250ng/ml for a period of 24-72h following the same procedure as mentioned above. Results are presented as means ± standard errors of the means (SEM) performed as triplicates of five independent experiments. Statistical significance was assessed using student's t test analysis.

MDA-MB-231 xenograft animal models

Subcutaneous Tumor implantation: Female NOD/SCID mice (18 in total) were purchased from Charles River Laboratories (Saint-Constant, QC, Canada), housed and maintained under specific pathogen-free conditions (RI-MUHC animal facility). The mice were randomly assigned into three groups (n=6 mice/group): MDA-MB-231/vector (dox+rhPRL), MDA-MB-231/PRLR (dox+rhPRL) and MDA-MB-231/PRLR (dox). Cells (3×10^6/ml) were re-suspended in Matrigel and implanted subcutaneously into the right flank of each mouse. The mice were injected intra-peritoneal with doxycycline (20 mg/kg) daily. MDA-MB-231/PRLR treated mice were injected intra-peritoneal every second day with rhPRL (0.1mg/g). Tumor growth was monitored up to 8 weeks after implantation. Tumor volume was measured in two dimensions with a vernier caliper (Mitutoyo, Kawasaki, Japan) and calculated using the formula [length x width^2] /2. Mice were euthanized by cervical dislocation following 8 weeks of treatments. For measuring serum levels of injected rhPRL, mice were anesthetised and blood was collected by cardiac puncture. Levels of serum rhPRL were determined using radioimmunoassay at 2h and 4h post injection (Table S1).

MDA-MB-453 xenograft animal models

Subcutaneous Tumor implantation: Female NOD/SCID mice (12 in total) were purchased from Charles River Laboratories (Saint-Constant, QC, Canada) housed and maintained under specific pathogen-free conditions (RI-MUHC animal facility). The mice were randomly assigned into two groups (n=6

mice/group): MDA-MB-453 untreated and MDA-MB-453 rhPRL treated. Cells (5×10^6/ml) were re-suspended in matrigel and implanted subcutaneously into the right flank of each mouse. The mice were treated intra-peritoneal with either vehicle or hPRL (0.1mg/g) each second day. Tumor growth was monitored up to 8 weeks after implantation. PET/SPECT/CT scan was performed (please see below) on three mice from each group. At the end of the experiment mice were sacrificed by CO2 asphyxiation and subjected to necropsy.

MDA-MB-231 xenograft animal models

Mammary fad pat tumor implantation: 30 Female NOD/SCID mice were purchased from Charles River Laboratories (Saint-Constant, QC, Canada) and randomly assigned into three groups (n=10 mice/group) according to PRL treatment: MDA-MB-231/vector, MDA-MB-231/PRLR untreated and MDA-MB-231/PRLR treated. The mice were injected in the mammary fad pat. After the tumors reach a minimum volume of 0.3-0.5cc (after 8 weeks of cell implantation), animals were treated with doxycycline (20 mg/kg) daily. MDA-MB-231/vector, MDA-MB-231/PRLR treated group were injected intra-peritoneal every second day with rhPRL (0.1mg/g). Tumor growth was monitored for 3 weeks during the treatment. At the end of the experiment mice were sacrificed by CO2 asphyxiation and subjected to necropsy. Tumors were collected from mice at the time of the necropsy and fixed with 4% paraformaldehyde.

Whole-body imagines of NOD/SCID/xenograft mice with PET/SPECT/CT scan analysis

The animals were kept fasting for approximately 12hrs before undergoing scanning. They were anesthetized using 1 L/min of 2% isoflurane in 100% oxygen. After receiving the anesthesia, they were injected in the tail vein with the radiotracer (fludeoxyglucose [^{18}F]) (FDG). Following an appropriate uptake period of 45 minutes, animals were re-anesthetized (isoflurane, 5% induction, 1.5-2% maintenance throughout the scan), moved to the Mediso nanoScan and placed in the prone position on an animal bed covered with absorbent paper. Following completion of PET and/or SPECT scans; a CT scan was performed for anatomical localization and attenuation correction. Respiration rate and body temperature was continuously monitored and the temperature maintained at ~37°C throughout the study using a feedback-regulated warming system. Following completion of scanning, the animals were euthanized. Data acquired in list-mode format for 60min, full 3D sonograms with corrected efficiency, scattering, attenuation, count losses and decay were reconstructed using an iterative 3D dynamic raw-action maximum likehood algorithm (Drama). After PET scanning a CT scan was performed. Analysis of the PET/SPECT and the CT datasets were imported using the nonproprietary AMIDE software, version 0.8.2 (http://amide.sourceforge.net)(Loening and Gambhir 2003). The imaging system used was the nanoScan pre-clinical SPECT/CT/PET (Mediso medical imaging systems, Hungary). The imaging was performed by RI-MUHC Small Animal

Imaging Labs (SAIL) Platform, Centre for Translational Biology, Research Institute of the McGill University Health Centre, Montreal, Canada.

Tumorsphere formation assay

MDA-MB-231/PRLR and MDA-MB-453 cells were treated for 72h and 5 days with or without PRL respectively. Cells were trypsinized and 1000 cells per well were plated in a 12-well low-attachment plate (Corning). Cells were grown for 7 days in DMEM-free serum supplemented with B27 (Invitrogen) and PRL (250ng/ml).

Flow cytometry analysis

Adherent cells were dissociated into single cells by trypsin-EDTA and filtered through a 40µm nylon mesh (BD Biosciences, San Diego, CA). 1×10^6 cells were washed with PBS containing 0.5% FBS, incubated with anti-CD44 conjugated to APC-cy7, anti-CD24 conjugated to APC (BD Biosciences). Cells were then washed with 0.5%PBS-FBS for 3 times. After washing, cells were analyzed with Accuri C6 flow cytometer (BD Biosciences) and Flowjo software (Tree Star Inc). Single $CD44^+/CD24^-$, $CD44^+/CD24^+$, $CD44^-/CD24^-$ and $CD44^-/CD24^+$ cell was sorted using FACSAria into either 12-well low-attachment plate to perform tumorsphere analysis or to 96-well low attachment plate to perform MTT assay.

ALDEFLUOR assay

The assay was performed as manufacturer's protocol. 1 × 10^6 MDA-MB-453 cells were centrifuged and resuspended in 1 ml ALDH assay buffer. 5µl substrate was added into the cell suspension. DEAB was used as negative control. Cells were then incubated for 40 min at 37 °C. Percentage of $ALDH^+$ cells were analyzed with Accuri C6 flow cytometer and Flowjo software.

Caspase Glo3/7 Assay

Cells were plated in 96-well plates at 5000 cells/100µL in medium supplemented with 2% FBS. Caspase 3/7 activity was measured using the Caspase Glo 3/7 Assay (Promega) according to manufacturer's instructions. Cells were incubated with equal volumes of medium and Caspase Glo reagent for 30 minutes at room temperature, and luminescence was measured using the EG & G Berthold luminometer (Bad Wildbad, Germany).

Annexin V Apoptosis Detection assay

The assay was performed as manufacturer's protocol. 1X10^6 cells were washed twice and resuspended with PBS-2%FBS (Assay buffer). Cells were then double-stained with Annexin V conjugated to FITC and PI for 15 minutes in the dark at room temperature, and then analyzed by flow cytometry.

Cell cycle analysis

Cells were harvested and washed with cold PBS, and then fixed with 70% ethanol for 30 min at RT. The fixed cells were washed with cold PBS twice, added 500μL DNA staining solution (including 200 μg/mL RNase A and 20 μg/mL propidium iodide staining solution) and incubated for 30 minutes. Finally, cells were analyzed by flow cytometry in the presence of the dye.

Senescence-associated β-galactosidase assay

The assay was performed as manufacturer's protocol. Senescence β-gal staining was performed using Senescence β-Galactosidase staining kit (Cell signalling). MDA-MB-231/PRLR, MDA-MB-231/vector (treated and untreated with PRL for 72h) and MDA-MB-453 (treated and untreated with PRL for 5 days) cells were fixed by Fixative Solution for 15 min, followed by β-Galactosidase solution incubation overnight at 37 °C. The staining was checked under microscope for the development of green color.

Immunofluorescence microscopy

MDA-MB-453 was treated for 5 days with or without PRL. Cells were trypsinized and grown on coverslips for 1 day, next, were fixed with 3.7% formaldehyde for 15 minutes and permeabilized in 0.1% Triton X-100 for 5 minutes. Cell were then washed with PBS and blocked for 1h in 2% normal donkey serum. Cells were incubated with an anti-H3K9me3 specific antibody for 1h, washed with PBS and incubated with Alexa Fluor®456 goat anti-rabbit

antibody and DAPI for 1h. Stained coverslips were mounted with SlowFade® Gold antifade reagent. Confocal analysis was performed using a Zeiss LSM 510 Meta Axiovert confocal microscope using 63× objectives.

Electron microscopy

Cells of each group were harvested and fixed at 1×10^6 with 2.5% glutaraldehyde for 6h at 4°C. Then the cells were processed for ultra structure analysis in the Facility for Electron Microscopy Research (FEMR), McGill University.

Statistical analysis:

Pearson correlation coefficient was used to evaluate the correlation between PRLR and different members of the metagenes used to distinguish molecular heterogeneity of TNBC. Correlation coefficient was denoted as (r). P value is also provided to evaluate the linear dependence between the two genes. Any event free survivals (AEFS), relapse free survival (RFS) curves in different databases were plotted using the Kaplan-Meier method. In vitro assays were all performed in triplicates of at least three independent experiments. Results were shown as means ± SEM. Student's t-test was used to evaluate the statistical significance. The difference between groups was analyzed using Student's *t*-test, and $*P < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism software.

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