CHARACTERIZATION OF WNT SIGNALING IN TAMOXIFEN RESISTANT BREAST CANCER STEM/PROGENITOR CELLS

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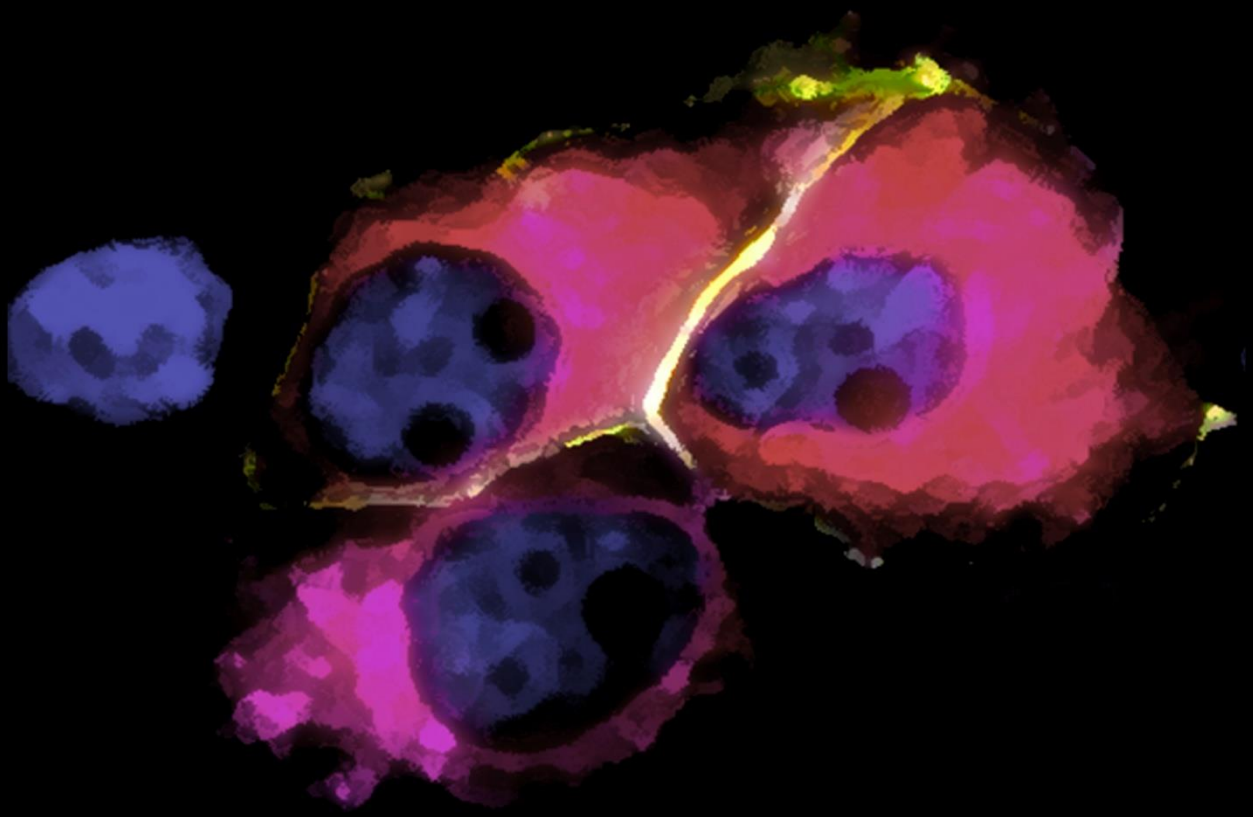

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Characterization of Wnt signaling in tamoxifen resistant breast cancer stem/progenitor cells

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Abbreviations

7AAD	7-aminoactinomycin D	**HRP**	Horseradish Peroxidase
β-cat	β-catenin	**IHC**	Immunohistochemistry
µl	Microliter	**IF**	Immunofluorescence
µM	Micromolar	**JUN**	Jun proto-oncogene, AP-1 transcription factor subunit
°C	Degrees Celsius	**JNK**	c-Jun N-terminal Kinase
Adh	Adherent	**kDa**	Kilodalton
ALDH	Aldehyde Dehydrogenase	**LBD**	Ligand Binding Domain
AP-1	Activator Protein 1	**LEF1**	Lymphoid Enhancer Binding Factor 1
APC	Adenomatous Polyposis Coli	**LGR**	Leucine-Rich Repeat-Containing G-Protein Coupled Receptor
AR	Androgen Receptor	**LRP**	Low Density Lipoprotein Receptor- Related Protein
ATF2	Activating Transcription Factor 2	**M**	Molar
bFGF	Basic Fibroblast Growth Factor	**MFS**	Metastasis free survival
BSA	Bovine Serum Albumin	**min**	Minutes
Ca^{2+}	Calcium ion	**ml**	Milliliter
C CM	Control Conditioned Medium	**mRNA**	Messenger RNA
cDNA	Complementary DNA	**MS**	Mammosphere
CRD	Cysteine Rich Domain	**NLS**	Nuclear Localization Signal
CREB	cAMP Responsive Element Binding Protein	**nm**	nanometer
CSC	Cancer Stem Cell	**NR**	Nuclear Receptor
CSS	Cause Specific Survival	**NTD**	Amino-Terminal-Domain
Ct	Threshold cycle	**OS**	Overall Survival
DBD	DNA Binding Domain	**PBS**	Phosphate-Buffered Saline
DHT	Dihydrotestosterone	**PCR**	Polymerase Chain Reaction
DKK	Dickkopf	**PFS**	Progression free survival
DFS	Disease Free Survival	**PR**	Progesterone Receptor
DMEM	Dulbecco's Modified Eagle's medium	**Q**	Quadrant
DMSO	Dimethyl Sulfoxide	**qRT-PCR**	Quantitative Real Time-PCR
DNA	Deoxynucleotide Acid	**RFS**	Relapse Free Survival
dNTPs	Deoxynucleotide Triphosphates	**RNA**	Ribonucleic acid
DRAQ7	Deep Red Anthraquinone 7	**ROR**	Receptor Tyrosine Kinase-like Orphan Receptor
DVL	Disheveled	**RSPO**	R-spondin
ECM	Extracellular Matrix	**SDS**	Sodium Dodecyl Sulphate
EDTA	Ethylene-Diamine Tetraacetic Acid Disodium Salt	**SERM**	Selective ER Modulator
ER	Estrogen Receptor	**sFRP**	Secreted Frizzled-Related Protein
ERE	Estrogen-Response Elements	**SOX2**	SRY-related HMG-box 2
FACS	Fluorescence Activated Cell Sorting	**TamR**	Tamoxifen Resistant

FZD	Frizzled Class Receptor	**TCF**	Transcription Factor, T-Cell Specific
G418	Geneticin® Disulfate	**TEMED**	Tetramethylethylenediamine
GH	Growth Hormone	**TNBC**	Triple Negative Breast Cancer
GFP	Green Fluorescent Protein	**V**	Volts
h	Hours	**Wnt**	Wingless-type MMTV Integration Site
HER2	Human Epidermal Growth Factor Receptor 2	**Wnt3a-CM**	Wnt-3a-Conditioned Medium
HGF	Hepatocyte Growth Factor		

Abstract

Breast cancer is the most prevalent female cancer and the first cause of death from cancer in women worldwide. Around 75% of breast tumors express estrogen receptor (ER). Tamoxifen, an ER antagonist, is the most common drug used in patients with ER+ breast cancer in both premenopausal and postmenopausal women. However, development of resistance to therapy occurs in approximately 30% of cases, leading to recurrence. Thus, it remains a clinical problem.

Tamoxifen-resistant cells are enriched for stem/progenitor cell populations. The $CD44^{+}CD24^{-/low}$ cell population increase after hormone-therapy is associated with enhanced ability to form mammospheres. In this thesis, I present studies showing that *WNT1* expression is heterogeneous among breast cancer subtypes. In addition, I show that Wnt-1 activates a β-catenin independent signal in tamoxifen-resistant breast cancer cells involving ATF2 and TCF1 and can be potentiated by the expression of USP6, which regulates the degradation and turnover of FZD receptors. In functional assays, Wnt-1 increases the $CD44^{+}CD24^{-/low}$ population and the ability to form mammospheres. Consistent with these observations, tamoxifen treated ER+ breast cancer patients expressing high levels of *WNT1* have poor prognosis. *In silico* analysis identified FZD_5 as a Wnt receptor that is associated with poor prognosis in tamoxifen-treated patients. I demonstrate an interaction between Wnt-1 and FZD_5 using co-immunoprecipitation, immunofluorescence and gene reporter assays.

Together these findings suggest that targeting Wnt-1 signaling by blocking its binding to FZD_5 would be a useful approach for preventing recurrence in a defined group of breast cancer patients.

Resumen

El cáncer de mama es el tipo de cáncer con mayor prevalencia y la primera causa de muerte por cáncer entre las mujeres a nivel mundial. Alrededor del 75% de los tumores mamarios expresan el receptor de estrógeno (ER). El tamoxifen, un antagonista de ER, es el tratamiento más común empleado para tratar a pacientes que expresan ER, tanto en mujeres pre-menopáusicas como en las post-menopáusicas. Sin embargo, en el 30% de los casos se desarrolla resistencia a la terapia incrementando los casos de recurrencia, lo que continúa siendo un problema clínico.

Las células resistentes a tamoxifen están enriquecidas en células madre/progenitoras. El incremento de la población de células $CD44^{+}CD24^{-/low}$ tras la terapia hormonal está asociado con una mayor habilidad de formar mamosferas. En esta tesis se muestra que la expresión de *WNT1* es heterogénea entre los distintos subtipos de cáncer de mama. Además, Wnt-1 activa una señalización independiente de la β-catenina y mediada por ATF2 y TCF1 y es, además, potenciada por la expresión de USP6, que regula la degradación y el reciclaje de los receptores FZD. En los ensayos funcionales se ha observado que Wnt-1 incrementa la población de células $CD44^{+}CD24^{-/low}$ y la habilidad de formar esferas. Estas observaciones son consistentes con el peor pronóstico que presentan los pacientes de cáncer de mama con tumores que expresan ER y han recibido tratamiento con tamoxifen. Por medio de análisis *in silico* se identificó FZD_5 como un receptor de Wnt que se correlaciona con un peor pronóstico de los pacientes tratados con tamoxifen. La interacción de Wnt-1 y FZD_5 se demuestra a través de ensayos de inmunoprecipitación, inmunofluorescencia y ensayos de genes reporteros.

Estas observaciones indican que el bloqueo de la señalización de Wnt-1 evitando su unión a FZD_5 podría ser una buena estrategia para prevenir la recurrencia en un grupo determinado de pacientes de cáncer de mama.

Resumen extendido

El cáncer de mama es el tipo de cáncer con mayor prevalencia entre las mujeres a nivel mundial. El tamoxifen, un antagonista de ER, es el tratamiento más común empleado para tratar a pacientes que expresan ER, tanto en mujeres pre-menopáusicas como en las post-menopáusicas. Sin embargo, el desarrollo de resistencia a la terapia continúa siendo un problema clínico, con el consecuente incremento de los casos de recurrencia.

Las células resistentes a tamoxifen están enriquecidas en células madre/progenitoras. El incremento de la población de células $CD44^{+}CD24^{-/low}$ tras la quimioterapia está asociado con una mayor habilidad de formar mamosferas. Además, la señalización Wnt está estrechamente relacionada con la regulación de la función de las células madre. Por ello, con este proyecto se propuso el objetivo de estudiar los mecanismos por el que la señalización Wnt controla el comportamiento de las CSCs y contribuye a la resistencia a la terapia hormonal. Para ello, se propusieron tres objetivos más específicos:

1. La identificación de las proteínas Wnt involucradas en el mantenimiento de las poblaciones de CSCs en cáncer de mama.

2. Caracterizar la ruta de señalización Wnt en las líneas celulares resistentes a tamoxifen.

3. Desarrollar estrategias para inhibir la vía de señalización Wnt en las CSCs de mama.

Para abordar el primero de los objetivos, se estudió la expresión génica de distintos genes Wnt en las CSCs de dos líneas celulares resistentes a tamoxifen, MCF-7TamR y T47DTamR. Para el enriquecimiento de la población de CSCs se cultivaron las células en condiciones adecuadas para favorecer la formación de mamosferas, donde se ha visto que la proporción de CSCs aumenta. A continuación, se sortearon las células $CD44^{+}CD24^{-/low}$ y se analizó la expresión génica de diferentes genes Wnt. Únicamente el incremento de la expresión de *WNT1* en el conjunto de las esferas, en comparación con la expresión de este gen en células cultivadas en adherencia, fue estadísticamente significativa en las células MCF-7TamR. Esta tendencia se mantuvo en la población $CD44^{+}CD24^{-/low}$ con respecto a su control $CD44^{-}CD24^{+}$. Por otro lado, en las células T47DTamR la expresión tanto de *WNT1* como de *WNT3A* tuvo mucha variabilidad y no resultó

ser estadísticamente significativa. Además, la expresión de *WNT3A* se encontró disminuida en la población de células $CD44^{+}CD24^{-/low}$, al igual que en las MCF-7TamR.

A continuación, se estudió la respuesta de estas células resistentes a tamoxifen a la expresión de los genes Wnt analizados. Para ello, se llevó a cabo el ensayo de reportero empleando una β-catenina/TCF-luciferasa, que refleja la activación de la vía de señalización de Wnt/β-catenina. Las células MCF-7TamR no respondieron a la expresión de ninguno de estos genes ni al medio enriquecido en Wnt-3a, mientras que las células T47DTamR sí que respondieron tanto a la señalización mediada por Wnt-1 como al Wnt-3a presente en el medio. Para determinar los factores que puedan influir en la diferencia de la respuesta observada en las distintas líneas celulares, se consideraron aspectos como la expresión de los receptores ER y AR, así como la expresión de diferentes factores de transcripción implicados en la vía de señalización Wnt. Los resultados mostraron que AR no está implicado en la respuesta a Wnt en las células de cáncer de mama, mientras que la señalización de Wnt/β-catenina parece estar reprimida en las células que expresan ER. Además, estas diferencias se pueden deber a los distintos niveles de expresión de los factores de la familia TCF y LEF que pueden actuar tanto como activadores como represores de la ruta dependiendo del contexto (Sprowl and Waterman, 2013). En general, se observó que los niveles de expresión de los genes de la familia TCF/LEF son muy bajos. En las células MCF-7TamR se comprobó que la expresión de TCF4 no es suficiente para activar la actividad transcripcional, mientras que sí que lo es la expresión de TCF1. La activación mediada por TCF-1 es independiente de la expresión de β-catenina. El uso del mutante dominante-negativo de ATF2 (ΔATF2) redujo la activación mediada por TCF-1, lo que sugiere que ATF2 participa en esta activación. Además, la expresión de *WNT1* incrementa la actividad transcripcional mediada por TCF-1.

Por otro lado, dado que la expresión de *WNT1* no activa la vía de señalización de Wnt/β-catenina y tanto Wnt-1 como ATF2 tienen un papel relevante en la señalización mediada por TCF1, se analizó el efecto de la expresión en la actividad transcripcional mediada por ATF2, utilizando para ello el reportero ATF2-luciferasa. Se observó que la expresión de *WNT1* incrementaba la actividad transcripcional de este reportero. Estos datos fueron confirmados utilizando reporteros que codifican GFP bajo los promotores que responden a TCF o a ATF2. Por tanto, se concluye que entre las células resistentes a tamoxifen, la expresión de *WNT1* está incrementada en algunas poblaciones de células madre con capacidad de formar mamosferas. Además, se ha observado que la señalización mediada por Wnt-1 en estas células puede ser activada independientemente de la β-catenina y mediada por ATF2 y TCF-1.

A continuación, se estudió más en profundidad los niveles de expresión de *WNT1* en el contexto del cáncer de mama utilizando bases de datos disponibles *online*. En esta tesis se muestra que la expresión de *WNT1* es heterogénea entre los distintos subtipos de cáncer de mama y no se correlaciona con el grado ni con la expresión del receptor de estrógenos. En correspondencia con los datos obtenidos de los pacientes, tampoco se observó ninguna diferencia significativa en el nivel de expresión de *WNT1* entre las distintas líneas celulares que se utilizan para los estudios *in vitro*. Sin embargo, sí que se observó una reducción significativa en la supervivencia de pacientes de cáncer de mama que expresan receptores de estrógeno y altos niveles de *WNT1* y que han sido tratados con tamoxifen. Para una caracterización más detallada de los niveles de Wnt-1 en los tumores mamarios, se llevó a cabo un análisis inmunohistoquímico de un array con casi 200 muestras. Los datos obtenidos reflejaron una correlación de los niveles de ER y PR con Wnt-1, pero no de HER2. Los niveles de Wnt-1 también mostraron una correlación inversa con las muestras de metástasis, en las que apenas se apreció la tinción de Wnt-1.

Tras observar el incremento de la expresión de *WNT1* en las células madre con fenotipo $CD44^{+}CD24^{-/low}$ consideramos la posibilidad de estudiar los niveles de expresión en distintos tipos de células madre. Para ello, se utilizaron distintas líneas celulares de ratón de cáncer de mama metastático, 67NR, 4T1 y 4T07. Estas células tienen un alto porcentaje de células madre caracterizadas por la expresión de $ALDH^{+}$, sin embargo, aunque la expresión de *Wnt1* se vio incrementada en el conjunto de las mamosferas formadas por estas células, los niveles de *Wnt1* no cambiaron entre la población $ALDH^{+}$, con respecto al nivel expresado por las células $ALDH^{-}$. Estos datos sugieren que el incremento de la expresión de *WNT1* es exclusivo de determinadas células madre/progenitoras, como por ejemplo, aquellas con fenotipo $CD44^{+}CD24^{-/low}$, que se encuentran enriquecidas en las poblaciones de células resistentes a tamoxifen.

Para una mejor caracterización de los efectos que la expresión de *WNT1* tiene en las células madres, se establecieron líneas celulares estables que expresan *WNT1*. Los niveles de expresión de *WNT1* fueron considerablemente incrementados, así como la producción de proteína Wnt-1, que se detectó por *western blot,* en los extractos celulares y en la matriz extracelular, y por inmunofluorescencia. No se observaron cambios morfológicos ni alteraciones en los niveles de proliferación o ciclo celular de las células que expresan *WNT1*. La actividad transcripcional de las células establecidas fue similar a lo determinado previamente; Wnt-1 no es capaz de activar la actividad transcripcional de β-catenina/TCF pero si la transcripción mediada por ATF2.

En primer lugar, se analizó si la expresión estaba ligada al proceso de adquisición de la resistencia. Sin embargo, la expresión de *WNT1* no incrementó la resistencia a tamoxifen de células sensibles al tratamiento. Las células MCF-7TamR son también menos sensibles que las

células control a otros antagonistas de ER. Por ello, se probó si Wnt-1 afecta al aumento de la resistencia a ICI, un antagonista de ER. Aunque se observó una tendencia a aumentar el tamaño de las colonias formadas por las células resistentes que expresan *WNT1*, no se apreció un aumento en el número de colonias totales. Este resultado indica que Wnt-1 probablemente tenga un efecto en la proliferación de aquellas células con capacidad de formar colonias. Este efecto parece depender de la expresión de ER, aunque no de su actividad transcripcional.

En los ensayos funcionales se observó que la expresión de *WNT1* se traduce en un incremento de la población de células $CD44^{+}CD24^{-/low}$ y en la habilidad de formar esferas en las células resistentes a tamoxifen. Estos datos son consistentes con la peor prognosis que presentan los pacientes de cáncer de mama con tumores que expresan ER y han recibido tratamiento con tamoxifen. Sorprendentemente, cuando se analizaron los niveles de expresión de genes marcadores de CSCs, como *SOX2, NANOG* y *OCT4*, ningún cambio significativo fue detectado. Los niveles de *Wnt1* tampoco aumentan en la población $ALDH^{+}$ analizadas en líneas celulares de cáncer de mama de ratón. Estos resultados sugieren que las células que expresan altos niveles de *WNT1* podrían tener ciertas características correspondientes a grados intermedios entre las células madre y células diferenciadas, por lo que podría tratarse de células progenitoras.

Los cambios en las células con características epiteliales para adquirir propiedades más similares a la de las células mesenquimales están asociadas con una mayor capacidad metastática. El análisis de los niveles de expresión de *Wnt1* en las líneas celulares de ratón muestra que los niveles más altos se encuentran en la línea celular 4T07, que es una línea celular que muestra propiedades tanto epiteliales como mesenquimales. Cuando se analizaron los cambios producidos en los genes característicos de propiedades epiteliales o mesenquimales, se observó que no había cambios en las células resistentes a tamoxifen, mientras que sí que se encontró un aumento significativo de la expresión de *VIMENTIN* y *SNAI2* en las células sensibles al tratamiento tanto en adherencia como en las células que forman esferas. Estos datos indican que la respuesta a la expresión de *WNT1* es diferente en las células en función de su sensibilidad a tamoxifen y que el peor pronóstico de los pacientes resistentes a tamoxifen con altos niveles de expresión de *WNT1* no está asociado a cambios de transición epitelial-mesenquimal (EMT).

Los receptores FZDs son capaces de activar tanto la ruta canónica como la no canónica de la vía de señalización Wnt, por ello, la terapia dirigida al bloqueo de los receptores parece ser prometedora. USP6 es una deubiquitinasa que regula la abundancia de FZDs en la membrana evitando la ubiquitinación mediada por las ligasas RNF43 y ZNRF3 y con ello su reciclaje. En esta tesis, se muestra que la señalización mediada por Wnt-1 puede ser regulada por USP6. La expresión de *USP6* potenció la capacidad de Wnt-1 de activar la transcripción mediada por ATF2.

Además, aumentó la capacidad de las células MCF-7TamR que expresan *WNT1* para formar mamosferas, sugiriendo que la acumulación de receptores en la membrana induce la activación de la señalización mediada por Wnt-1. Sin embargo, dada la baja especificidad de USP6 por regular todos los receptores FZD, pueden surgir efectos secundarios adversos. Todos estos resultados indican la necesidad de conocer el receptor que específicamente reconoce Wnt-1 y activa la señalización. Para determinar cuál de los receptores de Wnt se encuentra implicado en este proceso se llevó a cabo un cribado *in silico*. Se encontró una buena correlación entre los niveles de expresión de *WNT1* y *FZD5*. Es más, un nivel alto de expresión de *FZD5* en pacientes con tumores ER+ tratados con tamoxifen se correlaciona con un peor pronóstico.

La colocalización fue determinada por inmunofluorescencia y la unión entre Wnt-1 y FZD_5 fue confirmada por inmunoprecipitación. Además, la expresión de *FZD5* incrementó la señalización mediada por Wnt-1.

Todas estas observaciones indican que más estudios son necesarios para tratar de bloquear la señalización de Wnt-1 evitando su unión al receptor FZD5 y mejorar con ello el pronóstico y prevenir la recurrencia en un grupo determinado de pacientes de cáncer de mama.

Las conclusiones que se han obtenido de este trabajo son las siguientes:

- La expresión de *WNT1* se encuentra incrementada en las mamosferas y en la población de CSCS con fenotipo $CD44^{+}CD24^{-/low}$ de las células MCF-7TamR, pero no en las CSCs $ALDH^{+}$.
- La actividad de la ruta de señalización Wnt/β-catenina es muy baja en las células MCF-7TamR, lo que puede deberse a bajos niveles de expresión de los genes de la familia TCF/LEF.
- TCF1 recupera la respuesta canónica de la señalización Wnt, y además, permite la activación de la transcripción independiente de β-catenina, que a su vez puede ser bloqueada por ΔATF2.
- Wnt-1 activa la señalización dependiente de ATF2 en las células MCF-7TamR.
- La expresión génica de *WNT1* es heterogénea entre los distintos subtipos de cáncer de mama, pero los niveles de proteína correlacionan con los niveles de ER y PR pero no de HER2. Por otro lado, los pacientes ER+ tratados con tamoxifen y con alta expresión génica de *WNT1* tienen un peor pronóstico. Los niveles de Wnt-1 en metástasis son muy bajos.
- La expresión de *WNT1* no afecta a la resistencia a tamoxifen, sin embargo, puede tener un efecto en la proliferación de poblaciones determinadas de CSCs.

- Wnt-1 tiene un efecto diferente en las células resistentes o sensibles a tamoxifen, favoreciendo la expresión de marcadores de EMT o enriqueciendo la población $CD44^{+}CD24^{-/low}$, respectivamente.
- Wnt-1 se une a FZD_5 y promueve la señalización mediada por ATF2, que puede estar regulada por USP6. Tanto FZD_5 como USP6 son posibles dianas terapéuticas para el bloqueo de la señalización mediada por Wnt-1 en el cáncer de mama.

Introduction

1. HUMAN BREAST

1.1. Human breast structure

Mammary glands are secretory organs composed of different cell types that undergo many stages of development (pubertal growth, pregnancy, lactation and involution) after birth. Changes occurring in the epithelial compartment depend on signals from the mesenchyme during embryogenesis. On the other hand, during puberty and adulthood, these changes depend on circulating hormones released from the pituitary gland and the ovaries (Macias and Hinck, 2013).

Breast tissue is formed by epithelial parenchymal elements and the stroma. Epithelial components occupy 10% to 15% of the breast volume, and the remainder is formed by stromal elements (Bland, 2009). The breast is formed by 15 to 20 lobes, divided into 20 to 40 smaller lobules, which are branched tubule-alveolar glands. Each lobe drains into a lactiferous duct and this into a lactiferous sinus leading to the nipple. The space between lobes is filled by adipose tissue. Mammary glands can vary in size, contour and density between individuals, mostly due to the volume of adipose tissue. The breast is divided into four quadrants, upper inner, upper outer, lower inner and lower outer. Most of the breast volume is in the upper outer quadrants, where most tumors are located (Pandya and Moore, 2011). Tubular structures are bi-layered epithelium mainly composed of basal and luminal cells. Basal epithelium is formed by myoepithelial cells, forming the outer layer of the gland and a small population of stem cells. The luminal epithelium from the inner layer, formed by cells expressing hormone receptors, builds ducts and secretory alveoli (Macias and Hinck, 2013).

1.2. Breast development

Mammary ridges develop at the fifth or sixth week of human embryogenesis. At this stage, two ventral bands of thickened ectoderm, which extend from the axillary to the inguinal regions, are visible. In humans, these mammary ridges of the embryos disappear, except a small portion that remains in the pectoral regions. A primary bud is formed by the ingrowth of the ectoderm allowing the development of each breast and leading to development of 15 to 20 secondary buds that will form the lactiferous ducts and their branches (Moore and Persaud, 2007). The primary lactiferous ducts open a hollow that will transform into a nipple during infancy. In mammals, the rest of the mammary gland does not develop until puberty, under the influence of ovarian estrogen and progesterone production, which will lead to the proliferation of

epithelial and connective tissue elements (Pandya and Moore, 2011), as well as formation of terminal end buds (TEBs) (Paine and Lewis, 2017). TEBs are the structures that direct the growth of ducts through the fat pad, forming the main ductal system of the mammary tree (Williams and Daniel, 1983) (Fig. 1). There are two main structures that form the TEBs; a highly proliferative and low differentiated region located in the bulbous part and a more differentiated and less proliferative region in the ductal part. In the ductal part, two different compartments can be distinguished. There is a single-cell layer of "cap cells" in the outer compartment, which will differentiate into myoepithelial cells as the duct elongates. The inner compartment is formed by a multi-cellular layer of "body cells" (Williams and Daniel, 1983), formed by luminal and alveolar progenitors, which will give rise to more mature luminal cells as the duct elongates (Paine and Lewis, 2017).

During adulthood, side branching and alveolar budding occurs under the influence of ovarian steroids during the menstrual cycle. However, the major changes take place in preparation for lactation during pregnancy and postlactational involution (Howard and Gusterson, 2000). During pregnancy, the mammary gland undergoes maturation and alveologenesis. These changes are controlled mainly by progesterone and prolactin, which lead to an increase in secondary and tertiary ductal branching. Proliferating epithelial cells give rise to alveolar buds and differentiate into alveoli resulting in the formation of milk-secreting lobules. Progesterone induces side-branching and alveologenesis to create a lactation-competent gland in collaboration with prolactin, which also promotes the differentiation of alveoli to synthesize and secrete milk. After lactation, the involution process is initiated to remove milk-producing epithelial cells and restore the ductal architecture back to as it was. There are two involution phases. The first and reversible one takes 48 h, and it is characterized by an apoptotic process, alveolar cell detachment and accumulation of shed cells into the lumen. The transition to the second and the irreversible phase begins when alveoli start to collapse. This involves breakdown of ECM and activation of proteases induce tissue remodeling, which by day six will allow removal of most of the secretory epithelium and its replacement by adipocytes. Although several signaling pathways are involved in the regulation of the switch from lactation to involution, STAT family proteins play a major role, while the second phase of remodeling involves MMPs and plasminogen (Macias and Hinck, 2013) (Fig. 1).

In comparison, age-related lobular involution is a different process that consists of the replacement of glandular epithelium and interlobular connective tissues with fat. In aged breast, only few acini and ducts remains embedded in thin strands of collagen. This reduction of epithelial tissue is important for breast cancer diagnosis, since it increases the effectiveness of

mammography. It also has a protective role, as it has been reported that postmenopausal women, whose breasts have undergone lobular involution have significantly decreased risk of breast cancer. This fact may be related to the depletion of mammary stem cells in the aging breast (Macias and Hinck, 2013).

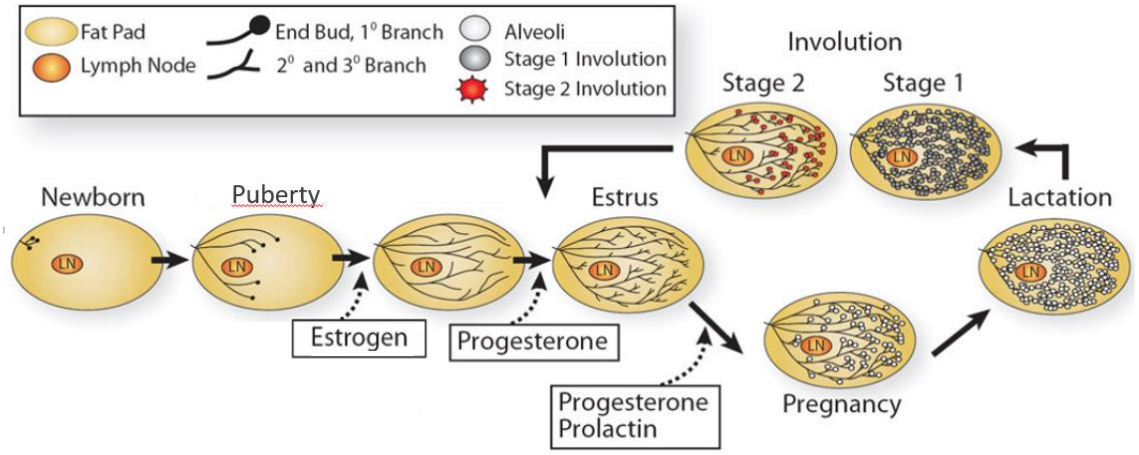

Figure 1. Stages of postnatal mammary gland development. *At birth, there are only few small visible ducts that grow until puberty. During puberty, ductal morphogenesis will fill the fat pad with a mammary epithelial tree under the influence of estrogen. During adulthood, side branches form every menstrual cycle under the influence of ovarian steroids. Alveologenesis occurs during pregnancy, under the effect of prolactin (PRL) and progesterone. PRL stimulation continues until lactogenesis, which ends in milk production until the involution process in which mammary gland is remodeled to its original adult state by the involution process. Adapted from Macias and Hinck, (2013).*

1.3. Estrogen receptors

Nuclear hormone receptors are members of a large family of nuclear receptors that act as transcription factors, and include the androgen receptor, the glucocorticoid receptor, progesterone receptors, mineral-corticoid receptors, and estrogen receptors. The activity of nuclear hormone receptors is modulated by steroid hormones derived from cholesterol, which are able to diffuse through the membrane due to their hydrophobic nature. Estrogens, including estrone, estradiol (E2) and estriol are one class of these steroid hormones. Estradiol is the most potent estrogen hormone and is involved in several physiological functions, such as development and maintenance of reproductive organs, regulation of cardiovascular, skeletal muscle, immune and central nervous system homeostasis, but it has also been involved in the initiation and development of tissue malignancies (Yaşar *et al.*, 2017).

The effect of estradiol is mediated by estrogen receptor-α (ERα) and estrogen receptor-β (ERβ), which are differently distributed and play different roles in each tissue. ERα is the principal

player in the uterus, pituitary gland, skeletal muscle, adipose tissue, bone, and more importantly, in the mammary gland, while ERβ is more important in the ovary, prostate, lung, cardiovascular and central nervous system (Yaşar *et al.*, 2017).

The ERα gene (*ESR1*) is located at q24-q27 of chromosome 6, and comprises 8 exons that encode a 66 kDa protein composed of 595 amino acids. On the other hand, the ERβ gene (*ESR2*) is located at q22-24 of chromosome 14, it also has 8 exons and encodes a smaller protein of 60 kDa with 530 amino-acids. Both ERα and ERβ can be divided into 6 functionally different domains. The A/B domain, encoded by exon 1, is the domain that shares less identity between the ERs, 17% amino-acid identity. The central C region is on the other hand, the one with more identity (97%) and is encoded by exons 2 and 3. This well conserved region contains the DNA binding domain (DBD). Exon 4 encodes part of the C region, D region and part of the E region. The D region contains the nuclear localization signal (NLS) (36% identity) and links the C domain to the multifunctional E region, also called the ligand-binding domain (LBD). The E region (56% identity) is a globular region with a hormone-binding site, a dimerization interface and ligand-independent co-regulator interaction function (activation function, AF-2), encoded by exons 4-8. Exon 8 also encodes the F region (18% identity), located at the carboxyl-terminus (Fig. 2A) (Yaşar *et al.*, 2017). The binding of estradiol to the LBD induce structural reorganization for the formation of dimers or interaction of co-regulators and converts inactive ER to a functionally active form (Mak *et al.*, 1999) (Fig. 2B).

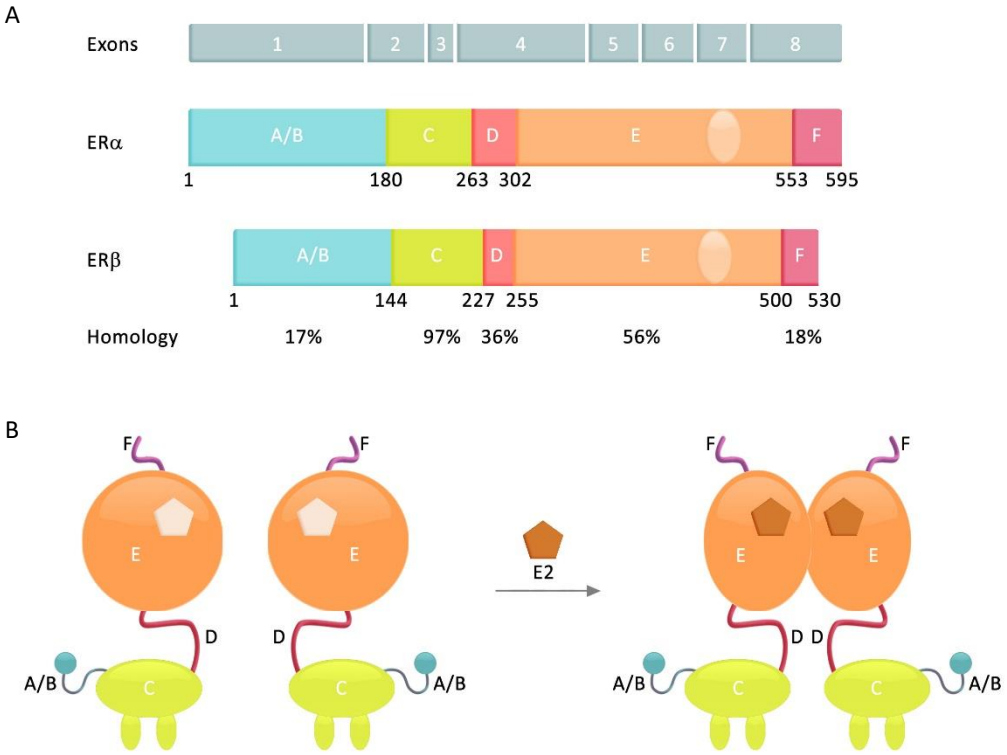

Figure 2. Schematic structure of ERs. A. *ESR genes are composed of 8 exons that encode ER proteins. These proteins are divided into 5 regions, where DBD is in the C region, NLS in the D region and LBD in the E region. The amino acid sequence identities between ERα and ERβ are indicated.* ***B.*** *Representation of estrogen binding and conformational changes that ERs undergo.*

The ER homodimer binds to DNA and other co-regulatory proteins in specific regions called estrogen-response elements (EREs) located in the promoters of estrogen target genes, and enhances their transcription. The binding to these co-regulatory proteins is allowed by the conformational changes induced previously by the binding of the ligand. The most important conformational change takes place in helix 12 (H12) of ER (Shiau *et al.*, 1998). There are many canonical ER target genes described that regulate cell proliferation and survival, such as *pS2* (Stack *et al.*, 1988), *PGR, ESR1, GREB1, MYC* and *GATA3* (Carroll *et al.*, 2006).

1.4. Progesterone receptors

Progesterone is an ovarian hormone with a critical activity during normal breast development, but it has also been linked to increased breast cancer risk (Lydon *et al.*, 1999). Progesterone effects are mediated through the progesterone receptor (PR), which is expressed as two isoforms, PRA and PRB. Both isoforms are transcribed from distinct estrogen-regulated promoters on a single gene (*PGR*) located at the q22-q23 in chromosome 11 (Rousseau-Merck *et al.*, 1987). Both sequences are identical except in the N-terminus, in which PRA lacks 164 amino acids, encoding proteins of 81 kDa (769 amino-acids) for PRA and 115 kDa (933 amino-acids) for PRB (Kastner *et al.*, 1990). Three domains can be distinguished in PRs; an amino-terminal domain (NTD) truncated in the PRA, a central globular DBD and the C-terminal LBD. There are two different AFs; AF-1 is located in the NTD and AF-2 in the LBD (Gronemeyer, Gustafsson and Laudet, 2004; Hill *et al.*, 2012; Kumar and McEwan, 2012). Progesterone enhance conformational changes in the LBD, most notably in helix 12 (H12) to form an AF-2 pocket, which allows co-regulatory protein binding to regulate transcription of target genes (Grimm, Hartig and Edwards, 2016) (Fig. 3A).

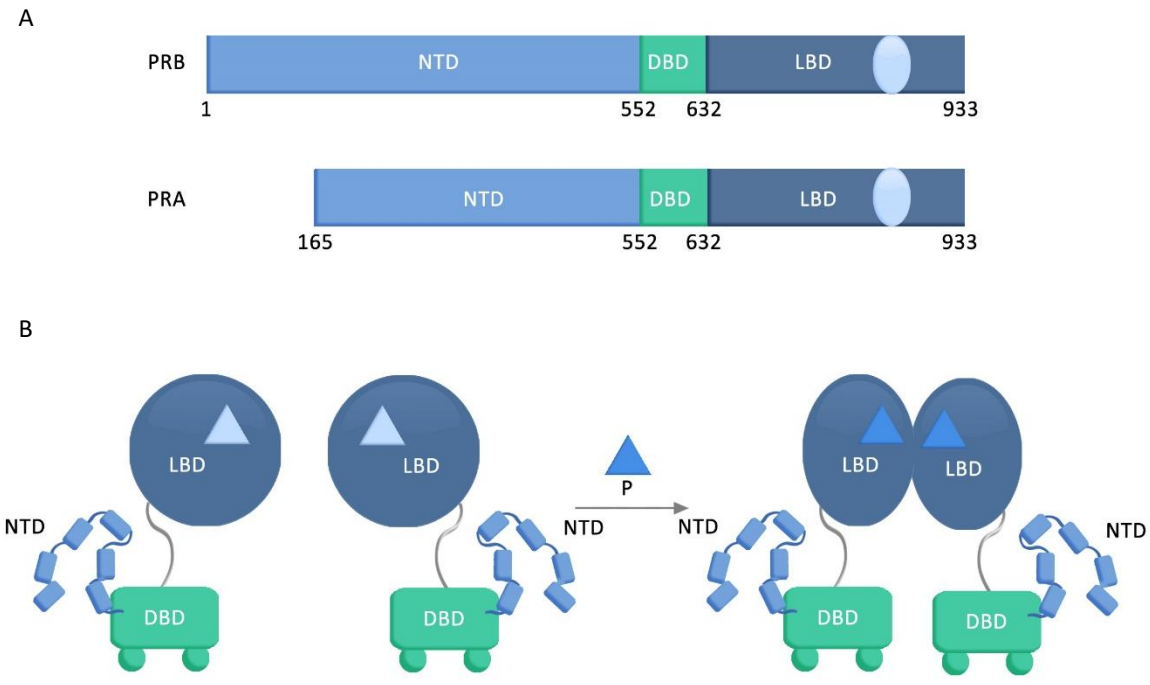

Figure 3. Schematic structure of PRs. A. *PRs are divided into 3 regions, where the DBS is in the central part and the LBD in the C-terminal region.* ***B.*** *Representation of progesterone binding and conformational changes that PRs undergo.*

In the normal breast, PRA and PRB are commonly expressed at the same level. However, imbalance occurs early in breast cancer development and is commonly seen also in premalignant lesions (Mote *et al.*, 2002). The activity of PR is regulated by post-translational modifications, such as phosphorylation, mainly in the N-terminal region of both isoforms by MAPKs and CDK2, for instance (Pierson-mullany and Lange, 2004). But it can also be modified by acetylation or sumoylation which will affect to the localization, stability and activity of PR (Lane, Shen and Horwitz, 2000; Abdel-Hafiz and Horwitz, 2014). PR is expressed in many tissues, including not only mammary glands, uterus and ovaries, but also in brain, the cardiovascular system, bone and central nervous system. However, distribution of PR can vary in these tissues. While it is expressed in every cell in the uterus (epithelial and stromal), it is only expressed in a small subset of epithelial cells in the breast (Hilton, Graham and Clarke, 2015). Binding of progesterone to PR induces conformational changes, receptor dimerization (Fig. 3B), and their binding to progesterone response elements (PREs) in the promoter regions of target genes by recruitment of co-activators and co-repressors (Le Dily *et al.*, 2014). PR target genes mediates cell proliferation, transcription, lipid metabolism and membrane-associated signal transduction through the activation of several paracrine signaling pathways, such as RANKL, Wnt, Notch and GH/cytokines. On the other hand, there is also autocrine regulation of proliferation by progesterone in the mammary gland, induced by the activation of target genes, *CCND1*, for instance (Hilton, Graham and Clarke, 2015).

1.5. Other nuclear receptors

Androgen receptors are activated by the androgens testosterone and dihydrotestosterone (DHT). Testosterone is converted to the more potent DHT by 5-α-reductase in peripheral tissues or by aromatase to the 17β-oestradiol in ovaries, bone, brain, adipose tissue and prostate (Li and Rahman, 2008; Ellem and Risbridger, 2010; Smith, Mitchell and McEwan, 2013). In breast, AR is expressed in epithelial glands and stromal/fibroblast cells (Hickey *et al.*, 2012).

The *AR* gene is located at chromosome X at the locus q11-q12. *AR* encodes a protein of 919 amino acids that contains an AF-1 domain in the NTD, a central DBD and a LBD in the C-terminus (Hunter *et al.*, 2017). Normal binding leads to conformational changes, nuclear translocation and binding to DNA androgen response elements (ARE) (Pietri *et al.*, 2016), and regulates transcription of AR-regulated genes, such as *PSA* and *TMPRSS2.* Enzalutamide (MDV3100) is a potent antagonist of AR that blocks the translocation of AR to the nucleus reducing the expression of target genes (Pal k., Stein and Sartor, 2013).

2. BREAST CANCER

2.1. Breast cancer epidemiology

Breast cancer is the most common malignancy in the female population worldwide in terms of incidence, and the first cause of death from cancer in women (Ferlay *et al.*, 2018). Cancer survival has improved since the 1970s for the most common cancers, and this is attributed to improvements in early detection and treatments (Berry *et al.*, 2005). In the case of breast cancer, the death rate has dropped 39% from 1989 to 2015 in the United States (Siegel, Miller and Jemal, 2018).

As for many other cancer types, breast cancer incidence is most common in developed countries, and risk factors are determined by genetic, lifestyle and environmental factors. The first of the risk factors is related to family history and personal characteristics. Women with a family history of breast cancer, mostly in a first-degree relative, show a higher risk for the disease (Collaborative Group on Hormonal Factors in Breast Cancer, 2001). This fact indicates the importance of genetic predisposition. Two of the best known genetic alterations for breast cancer risk are inherited mutations in *BRCA1* and *BRCA2*, which are present in 5-10% of female breast cancers, increasing to 15-20% in familial breast cancers (Turnbull and Rahman, 2008; Tung *et al.*, 2016). Moreover, these mutations increase the risk of developing breast cancer by 80 years of age from 10% in the general population to 70% (Kuchenbaecker *et al.*, 2017). Other personal characteristics, such as height and menstrual cycles (early menstruation and late menopause) increase the risk of breast cancer (American Cancer Society, 2017).

Reproductive factors are also important to consider, since having a first child before age 35 and having more children reduce the probability of luminal breast cancer (Lambertini *et al.*, 2016). On the other hand, although the consumption of fertility drugs has not been related to cancer risk, the use of menopausal hormones showed higher risk of developing breast cancer (Chlebowski *et al.*, 2013; Manson *et al.*, 2013; Brinton, 2017). Regarding lifestyle, obesity in postmenopausal women (La Vecchia *et al.*, 2011), the lack of physical activity (Pizot *et al.*, 2016), diets poor in fruit and/or vegetable intakes (Jung *et al.*, 2013; Emaus *et al.*, 2016; Farvid *et al.*, 2016), alcohol consumption (Liu, Nguyen and Colditz, 2015) and smoking before the first pregnancy (Gaudet *et al.*, 2013; Dossus *et al.*, 2014; Macacu *et al.*, 2015) have been related to increased risk of breast cancer.

2.2. Breast cancer classification

Most breast cancers begin in the lobules, the tissue made up of glands for milk production, or in the ducts that connect lobules to the nipple. When cancer is detected, it is necessary to characterize the type of disease and determine the extent of spread (stage). The prognosis of invasive cancer is strongly influenced by the stage when it is first diagnosed. The TNM classification of tumors provides information about tumor size and whether it has spread to adjacent tissues (T), to nearby lymph nodes (N), or whether there is distant metastasis (M) to other organs (Edge and Compton, 2010). A stage value from 0 to IV is also assigned to determine if it is a carcinoma in situ for stage 0 (cells have not penetrated the ducts or glands), an early-stage invasive cancer (I), or more advanced diseases (II, III and IV) (American Cancer Society, 2017).

Breast cancer can also be classified as *in situ*, including ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS), and invasive cancer. Around 80% of breast cancers are invasive or infiltrating, growing into surrounding breast tissue. There are 21 different histological subtypes, but they can be mainly classified into four molecular subtypes that differ in terms of risk factors, response to treatment and outcomes (Tamimi *et al.*, 2012; The Cancer Genome Atlas Network, 2012; Dieci *et al.*, 2014) (Fig. 4). These subtypes can be classified based on the presence or absence of biological markers, such as the hormone receptors ER and PR and the expression levels of human epidermal growth factor 2 (HER2) (Cheang *et al.*, 2015):

- Luminal A (ER+, PR+, HER2-): This is the most common breast cancer (71%), is usually less aggressive than other subtypes, being associated with the most favorable prognosis, mostly in short term survival due to the hormone therapy (Blows *et al.*, 2010; Haque *et al.*, 2012).

- Luminal B (ER+/-, PR+, HER2+): This cancer subtype is less abundant (12%), it is characterized by the presence of hormone receptors but also by the high expression of Ki67 or HER2, which indicate a high proportion of dividing cells, which is translated into a more aggressive, higher stage and poorer prognosis than luminal A cancers (Haque *et al.*, 2012).

- HER2-enriched (ER-, PR-, HER2+): This is the least abundant group (5%), cancers are more aggressive than the luminal subtypes and are associated with a poorer prognosis.

However, targeted therapies such as Trastuzumab, a monoclonal antibody against HER2, seems to be a promising way to improve outcome among these patients (Romond *et al.*, 2005).

– Basal or triple negative (ER-, PR-, HER2-) (12%): This is the breast cancer type with poorest prognosis as there are no targeted therapies available. These cancers are more common in black women, in premenopausal women and women with a *BRCA1* gene mutation (Perou and Borresen-Dale, 2011).

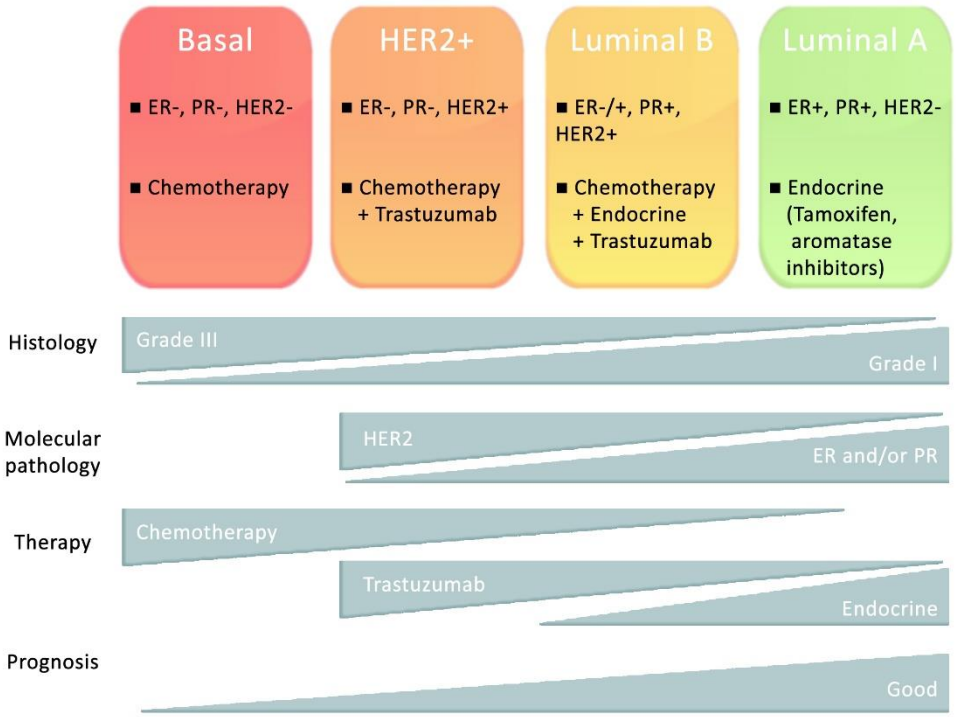

Figure 4. Comparison of the histology, molecular pathology, therapy and prognosis used in different breast cancer tumor subtypes.

2.3. Tamoxifen treatment

Tamoxifen, first known as ICI 46474, is a selective ER modulator (SERM) and the most commonly used therapeutic agent in ER+ breast cancer patients. It belongs to a group of planar compounds with a rigid core region (Fig. 5A). Tamoxifen binds to ER and induces conformational changes as ER does, however, while E2-ER binding allows helix 12 to pack against helix 3 and helix 11 to form a pocket, tamoxifen blocks helix 12 repositioning (Shiau *et al.*, 1998) and thereby blocks coactivator binding to AF-2. Tamoxifen acts as an ER antagonist of genes that depend on AF-2 activation for ER-mediated transcription, but it has also been described to act as an agonist in the uterus, which may be related to the differential expression of co-regulators in tissues, and more importantly it may act as an agonist in the transcription of genes where AF-1 is more important (Chang, 2012).

Diethylstilbestrol (DES) was the first synthetic non-steroidal estrogen with proven clinical relevance and was used in postmenopausal patients with advanced breast cancer in the 1960s. During this time MER-25, an antiestrogen compound was approved to be used as a contraceptive in the UK. This compound led to the discovery of tamoxifen and it showed efficacy in clinical trials for advanced breast cancer by the 1970s, similar to that shown by DES but with fewer side effects. Nevertheless, it did not show the same activity *in vitro* as it did *in vivo*, until it was found that its activity was due to the active metabolites, 4-hydroxytamoxifen and endoxifen (Patel and Bihani, 2018) (Fig. 5).

One of the most relevant problems using tamoxifen and other SERMs is the large degree of resistance that develops after years of treatment. Resistant tumors present activated ER signaling (Dodwell, Wardley and Johnston, 2006; Nardone *et al.*, 2015). This fact led to the search of alternative agents such as selective estrogen receptor degraders (SERDs), which are antiestrogens that destabilize H12 of ER, inducing ER degradation, so that dimers are not formed and ER signaling is completely blocked (McDonnell and Wardell, 2010). One of these compounds is ICI 182,780, also known as fulvestrant, a steroidal compound with a long side chain (Fig. 5B) that binds to ER in the monomeric form, preventing dimerization and ER signaling by inducing ER degradation through the ubiquitin-proteasome pathway (Patel and Bihani, 2018).

Tamoxifen plays a critical role in the treatment of ER+ breast cancer in the adjuvant, advanced and neoadjuvant settings (Chang, 2012). In patients with early stage breast cancer, adjuvant tamoxifen treatment for 5 years delays local and distant relapses and increases overall survival (Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 1998). In women under 40, 5 years of tamoxifen treatment reduced recurrence and mortality rate (Early Breast Cancer Trialists'

Collaborative Group (EBCTCG), 2005). These trials have made tamoxifen the main standard of treatment in premenopausal women with early stage breast cancer.

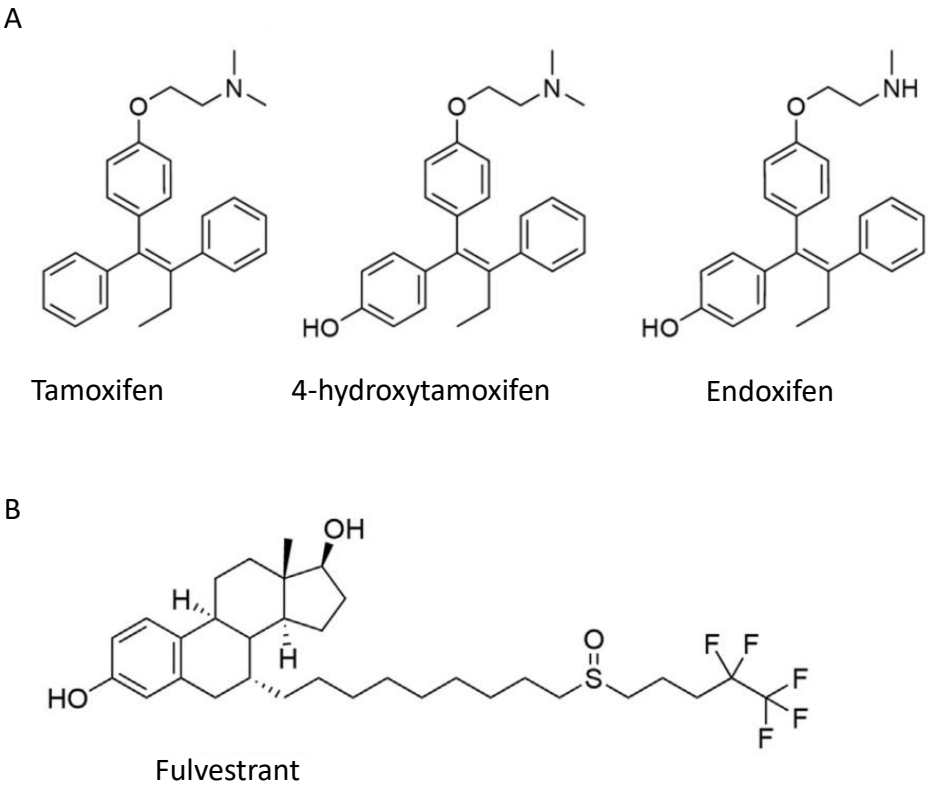

Figure 5. Chemical structures of tamoxifen, its active metabolites, 4-hydroxitamoxifen and endoxifen and fulvestrant. *Adapted from Patel and Bihani, 2018.*

2.4. Tamoxifen resistance

Despite the benefits that tamoxifen shows in the short term, 20-30% of breast cancer patients experience a recurrence, meaning that tumors eventually acquire tamoxifen resistance. Antiestrogen resistance is generally divided into two categories; *de novo* resistance, which are ER+ breast tumors nonresponsive to antiestrogen therapy from the beginning of the treatment, and acquired resistance, developed after a long exposure to antiestrogen therapy in ER+ tumors that initially responded to the treatment (Jordan, 2004).

Several mechanisms that occur in cells lead to antiestrogen resistance. Moreover, taking into account the heterogeneity of breast tumor tissues, mechanisms of resistance within tumors may vary (Chang, 2012). Some of the proposed mechanisms include loss of ER expression and function, alterations in the expression of co-regulatory proteins, activation of growth factor receptor signaling pathways, pharmacological and metabolic changes, regulation of redox status, and enrichment of cancer stem cells (CSCs) and changes in the regulation of cell fate-associated pathways (Chang, 2012).

Recently, it has been proposed a model that combines the last two mechanisms mentioned above. *SOX2* is a member of the Sox family of High mobility Groups (HMG) box transcription factors (Avilion *et al.*, 2003) known to be a stem cell marker. *SOX2* expression is important for keeping ES cells undifferentiated and to maintain self-renewal capacity of pluripotent ES cells (Masui *et al.*, 2007). Piva *et al.*, (2014) proposed that SOX2 plays an important role in the development of tamoxifen resistance by maintaining breast cancer cells in a more stem cell-like state through increased Wnt signaling. Tamoxifen resistant MCF-7 cells showed increased expression of *SOX2*, associated with a $CD44^{+}CD24^{-/low}$ phenotype (See Section 3.2), while *SOX2* silencing significantly reversed tamoxifen resistance in several models. Moreover, clinical relevance was demonstrated with a cohort of ER+ breast cancer patients who received tamoxifen therapy, where high SOX2 levels were correlated with endocrine treatment failure and poor relapse-free survival. In this study, enrichment of CSCs was proposed to be a consequence of activation of Wnt signaling, a well-known pathway involved in cell fate regulation.

3. STEM CELLS

3.1. Adult stem cells

Adult stem cells are responsible for tissue renewal during homeostasis and regeneration. These are cells that represent specific lineages in each organ and give rise to proliferating progenitors responsible for producing more differentiated cells to carry out physiological tissue functions. Stem cells persist long-term, due to their self-renewal capability and ability to generate all differentiated of an organ over the organism´s lifetime. Stem cells can be classified as homeostatic if they are active and responsible for day-to-day maintenance of tissues, or facultative if they are quiescent but perform stem cell functions after injury. These two types of stem cells can coexist in the same tissue, for example $Lgr5^+$ and $Troy^+$ cells are facultative stem cells in the gastric corpus, however $Lgr5^+$ basal cells in the gastric pylorus contribute to homeostasis (Simons and Clevers, 2011; Tan and Barker, 2018).

In the mammary gland, TEBs are made up of cap cells and body cells. Cap cells, functionally referred to as mammary stem cells (MaSCs) (Chen, Liu and Song, 2017), express several markers, including keratin 5 and 14, smooth muscle actin (SMA) and p63. In addition, the stem cell specific isoform of SH2-containing inositol 5'-phosphatase (sSHIP) specifically marks cap cells, although its function in the mammary gland is unknown (Bai and Rohrschneider, 2010). Body cells from TEBs express keratin 6, 8 and 18, and some of them, although they do not respond to ovarian hormones, also express ER and PR (Ismail *et al.*, 2002; Grimm *et al.*, 2006; Paine and Lewis, 2017). Cap cells have been postulated to be examples of multipotent mammary stem cells, as it has been observed by limiting dilution analysis that they can form a complete mammary gland at a higher frequency than other basal lineage cells (Bai and Rohrschneider, 2010). Moreover, cap cells have been reported to show high Wnt signaling, a hallmark of stemness (Roarty *et al.*, 2015).

Since SCs are present in the mammary gland, many strategies have been developed to isolate and purify them (Tiede and Kang, 2011). The first approaches used to identify stem cells were based on morphological and biological properties. Cap cells, hypothesized to represent an undifferentiated mammary stem/progenitor cell population, were initially identified by electron microscopy as pale or light staining cells with low complexity (Smith and Medina, 1988). Another approach, using Hoechst 33342 dye efflux, found a MaSC-enriched fraction with an abundant luminal progenitor population (Welm *et al.*, 2002; Alvi *et al.*, 2003; Asselin-Labat *et al.*, 2008).

3.1.1. Mouse mammary stem cells

A third approach used fluorescence-activated cell sorting (FACS) to detect stem cells that express specific cell-surface markers. Stem cell antigen-1 (Sca1) was used to identify a subpopulation of mammary epithelial cells (MECs). In mouse, MaSCs have been recognized by their expression of CD24 and CD29 (β1-integrin) or CD49f (α6-integrin) (Shackleton *et al.*, 2006; Stingl *et al.*, 2006). This defined a population of $CD24^{+/med}CD29^{hi}CD49f^{hi}$ cells that display hallmarks of stem cells. In addition, protein C receptor (Procr) has been described as a novel Wnt target in mammary epithelia to be present in a subset of multipotent mouse MaSCs (D. Wang *et al.*, 2015). Therefore, the surface marker profile of mouse MaSCs is presently defined as $Lin^{-}Procr^{+}CD24^{+/med}CD29^{hi}CD49f^{hi}Sca1^{low/-}$ (Chen, Liu and Song, 2017).

3.1.2. Human mammary stem cells

Human MaSCs have been more difficult to purify than mouse MaSCs because there are not such reliable markers. Mammosphere culture technology has been used to enrich stem cell populations. However, MaSCs are still less than 1% in cultured mammospheres (Ismail *et al.*, 2002; Grimm *et al.*, 2006; Paine and Lewis, 2017). The PKH26 fluorescent dye labels cells with slow-cycling and quiescent traits during mammosphere formation (Pece *et al.*, 2010). There are also studies claiming that $Lin^{-}CD49f^{+}EpCAM^{low/-}$ or $CD10^{+}$ basal phenotypes define subsets of cells enriched for human mammary repopulating units. Nevertheless, it is still not known whether human mammary stem/progenitor cells only are located within a subset of basal epithelial cells or in both luminal and basal cell populations (Eirew *et al.*, 2008; Lim *et al.*, 2009; Keller *et al.*, 2011; Chen, Liu and Song, 2017).

3.2. Breast Cancer Stem Cells

There is a small subpopulation of undifferentiated cells termed breast cancer stem cells (BCSCs) that are more likely to give rise to tumor progression, and have also been implicated in resistance to conventional therapy (Fillmore and Kuperwasser, 2008; Lin *et al.*, 2012). However, the origin of these cells remains controversial. There is some evidence to suggest these cells arise from MaSC or progenitor cells. Apart from specific properties highly similar to MaSCs, such as self-renewal or tumor initiating ability (Ma *et al.*, 2014), there are some mammary progenitor which express $CD44^{+}CD24^{-}$ cell markers and resemble the $CD44^{+}CD24^{-}Lin^{-}$ population found in BCSCs (S. Liu *et al.*, 2014; Bao *et al.*, 2015). Moreover, since normal stem cells remain for long

periods of time, they are more prone to accumulate genetic alterations, a process that occurs during oncogenic transformation (Sin and Lim, 2017). On the other hand, a second hypothesis postulates the origin of BCSCs in non-stem cells, in differentiated mammary cells, and the exposure to environmental factors as well as to chemotherapy, which leads to genetic alterations giving rise to *de novo* generation of CSCs by a dedifferentiation process (Lagadec *et al.*, 2012; Koren *et al.*, 2015) (Fig. 6).

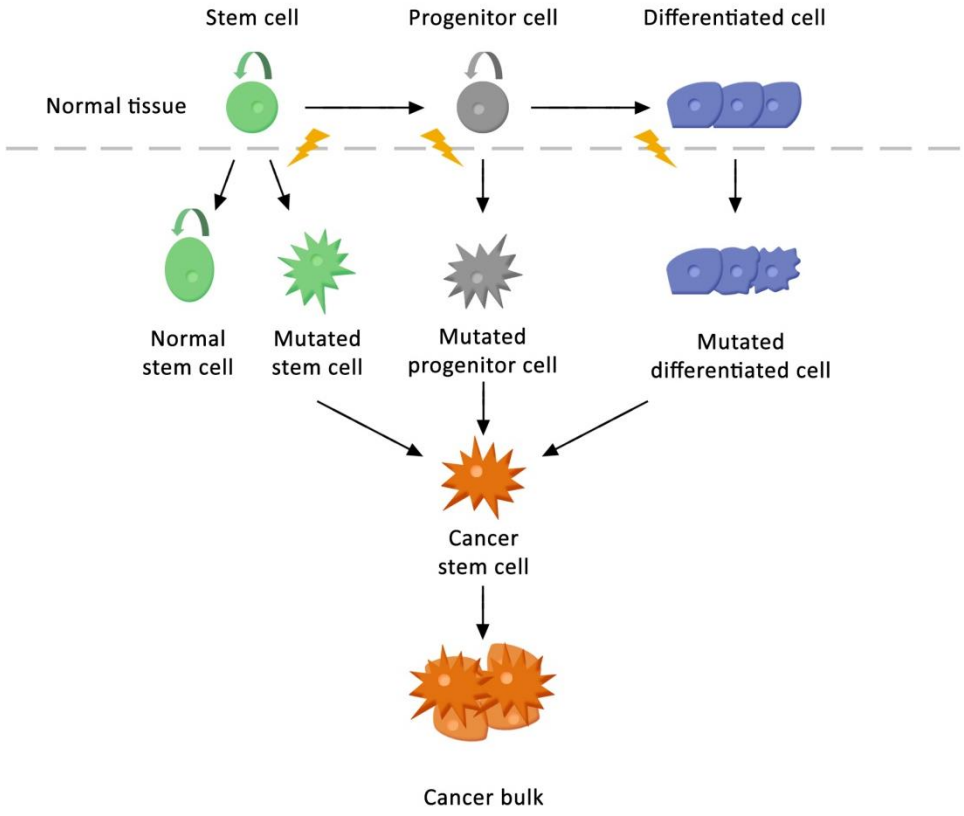

Figure 6. Model of BCSC formation. *BCSC have been proposed to be generated from MaSCs that undergo several mutations during the quiescent state, from mammary progenitor cells that accumulate mutations during cell division, leading to transformation or from mutated and differentiated cells that undergo de-differentiation to re-acquire stem-cell properties.*

The most commonly used markers to identify human BCSCs are CD44, CD24 and ALDH1 (Beça *et al.*, 2013) but others can also be used, such as CD133, CD61 or CD49f, for instance (Sin and Lim, 2017).

- CD44: A transmembrane glycoprotein implicated in several tumor related events, such as adhesion, intracellular signaling, cell proliferation, angiogenesis, migration and invasion properties (Lagadec *et al.*, 2012). Its expression is increased in BCSCs retaining tumorigenicity, multipotency functions and increasing anti-tumor drug resistance (Van Phuc *et al.*, 2011).

- CD24: A surface glycoprotein involved in cell adhesion, upregulation of which reduces stemness in breast cancer cells (Schabath, 2006).
- ALDH1: A detoxifying enzyme that catalyzes oxidation of intracellular aldehydes and mediates conversion of retinol to retinoic acids, which modulates proliferation. ALDH marks both normal and cancerous mammary cells and its expression is related to cell proliferation, differentiation and chemo-resistance (Moreba *et al.*, 2012).

The combined expression of some of these markers has been shown to be much more effective to identify BCSCs. Al-Hajj *et al.*, (2003) showed that $CD44^{+}CD24^{-/low}Lin^{-}$ population from tumors, including breast, exhibit properties of stem cells. However, the size of the $CD44^{+}CD24^{-/low}$ cell population varies among different breast cancer subtypes, basal type is the more enriched in these cells ranging from 0 to 90% while in the luminal subtype it is only between 0 and 15% of the population (Sheridan *et al.*, 2006; Pham *et al.*, 2011). On the other hand, $CD44^{high}$ and ALDH1 activity do not generally coexist in CSCs, suggesting they may be different types of CSCs (S. Liu *et al.*, 2014; Nieto *et al.*, 2016).

Therapy resistance has been correlated with tumors with enrichment of the $CD44^{+}CD24^{-/low}$ cell population (Colak and Medema, 2014; Pattabimaran and Weinberg, 2014; Piva *et al.*, 2014; Cojoc *et al.*, 2015; Gong *et al.*, 2017; Yang *et al.*, 2017) and these cells may be precursors to form secondary tumors (recurrent or metastatic) (Raouf *et al.*, 2012; Medema, 2013; Geng SQ, Alexandrou AT, 2014; Guo, 2014; Nilendu *et al.*, 2018).

3.3. EMT and Cancer Stem Cells

Epithelial to mesenchymal transition (EMT) is a process in which cells acquire new features that allow carcinoma cells to migrate and disseminate to distant sites (Fig. 7). The reverse process, mesenchymal to epithelial transition (MET) is associated with loss of migratory ability, induction of cell proliferation and growth in secondary sites. EMT is a complex process that epithelial cells undergo in response to certain signaling factors, such as TGFβ family growth factors (Nieto *et al.*, 2016).

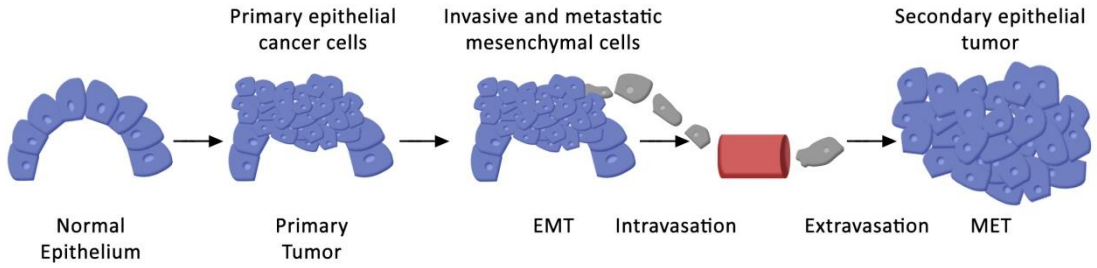

Figure 7. Schematic representation of EMT and MET processes. *Tumor cells at the primary site undergo an EMT process to acquire the ability for intravasation. Cells that survive at metastatic sites undergo MET in order to proliferate and allow secondary tumor growth.*

These changes are induced not only by specific transcription factors (TF), called EMT-TFs, such as Snail, Zeb, and Twist, among others, but also by miRNAs and epigenetic and post-translational regulators (Nieto *et al.*, 2016). However, there is growing evidence that in addition to the mesenchymal and epithelial states, there are intermediate states in which cells undergo a partial EMT. Actually, there have been described many intermediate states with mixed epithelial and mesenchymal phenotypes both in development and in cancer (Blanco *et al.*, 2007; Leroy and Mostov, 2007; Futterman, Garcia and Zamir, 2011; Grigore *et al.*, 2016).

Among the EMT-TFs in breast cancer, *ZEB2* has been involved in regulation of cell fate and stem cell differentiation, and *SNAI1* regulates miR-34. This microRNA also regulates ZEB and SNAI EMT-TF establishing a negative feedback for regulating epithelial and mesenchymal states. In breast, expression of EMT-TFs has been observed in chemo-resistant triple-negative carcinomas, enriched for EMT and stem-cell markers with low level of luminal/epithelial differentiation (Puisieux, Brabletz and Caramel, 2014). ZEBs and SNAI1 EMT-TFs negatively regulate expression of target genes, including *CDH1, CLDN*s and *OCCL* (Lamouille *et al.*, 2013). *CDH1* encodes E-cadherin, a transmembrane protein that forms adherent junctions together with intracellular components such as α-catenin and β-catenin, while claudin and occludin proteins encoded by *CLDN* and *OCCL*, respectively, form tight junctions (Hartsock and Nelson, 2008). PRRX1 and TWIST1 are EMT-TFs thought to be more potent mesenchymal inducers, while SNAI1 and ZEB1 are strong epithelial repressors and weak mesenchymal promoters (Nieto *et al.*, 2016). Reactivation of the EMT program is a physiological event during puberty and pregnancy in the mammary gland (Shamir and Ewald, 2015). Within the tumor, it is important to consider the heterogeneity of phenotypes; there is a small population of invasive cells with mesenchymal properties, while the main bulk of the tumor retains epithelial characteristics. Since the tissue of origin can also affect the heterogeneity shown during EMT program activation, Tan *et al.*, (2014) established a quantitative EMT scoring system based on gene expression profiles, where

VIM and *CDH2,* which encode for Vimentin and N-cadherin, respectively, were used as mesenchymal markers and CK19 and E-cadherin as epithelial markers. They showed that in breast cancer, basal cell lines have an intermediate-high EMT score, whereas luminal cell lines have a lower EMT score, and an intermediate EMT score corresponds to a mixed basal-luminal phenotype.

4. WNT SIGNALING

In 1982, Nusse and Varmus, (1982) found that the mouse mammary tumor virus (MMTV) induced mammary gland tumors in upon insertion upstream of a gene named *Int1*, thereby inducing expression of the Int1 protein. *Wingless* (Wg) was first described in 1976 to be the gene responsible of the phenotype in *Drosophila melanogaster* mutants lacking wings (Sharma and Chopra, 1976), and was then found to be the fly homologue of *Int1*. During the next ten years, major research in Wnt signaling focused on development. Injection of mouse *Wnt1* mRNA was found to cause formation of a secondary body axis in *Xenopus laevis* embryos, providing an assay to characterize new components of the pathway (McMahon and Moon, 1989a). Other components such as TCF and LEF were cloned in 1991 by Clevers and colleagues (Travis *et al.*, 1991; Van De Wetering *et al.*, 1991; Waterman, Fischer and Jones, 1991) and later the Frizzled receptors, LRP5 and 6 co-receptors and also the secreted Wnt inhibitors SFRPs, DKK and WIF1 were identified. Another important milestone in the field was the discovery of the importance of β-catenin stability in Wnt signaling (Peifer *et al.*, 1994; van Leeuwen, Samos and Nusse, 1994; Munemitsu *et al.*, 1995). In the late 1990s the first direct transcriptional target of canonical signaling, the proto-oncogene *MYC*, was described (He *et al.*, 1998). The non-canonical planar cell polarity (PCP) pathway was first described in *Drosophila* and Wnt-5a was found to be involved in calcium signaling in zebrafish and *Xenopus*. Nowadays, it is well known that Wnts are a large family of 19 secreted glycoproteins that have important roles in regulation of cell proliferation, survival, migration, polarity, specification of cell fate and self-renewal in stem cells (Klaus and Birchmeier, 2008; Anastas and Moon, 2013).

4.1. Canonical Wnt signaling

Canonical Wnt signaling is transduced by the binding of canonical Wnt ligands to FZD receptors and LRP5/6 co-receptors, thereby stabilizing β-catenin. In the cytoplasm, β-catenin is recruited into a degradation complex containing Axin and APC proteins. Phosphorylation of β-catenin within this complex by CKI and GSK-3 targets it for ubiquitination and subsequent proteolytic destruction by the proteosomal machinery in unstimulated cells. In the nucleus, TCF/LEF 1

transcription factors are bound to co-repressors and transcription of target genes is blocked. Wnt proteins can be prevented from signaling by binding to secreted Wnt antagonists, such as sFRPs and WIF1 or by binding DKK1. In addition, non-canonical Wnt ligands can compete with canonical Wnt ligands for binding to FZD receptors.

On the other hand, when signaling is activated by the binding of canonical Wnt ligands to FZD receptors and LRP5/6 co-receptors, the latter are phosphorylated and recruit Axin and DVL proteins to the membrane, disrupting the degradation complex and stabilizing β-catenin in the cytoplasm. Cytoplasmic β-catenin can translocate to the nucleus and bind to TCF/LEF1 family proteins, displacing co-repressors. Binding to co-activators such as CBP and Bcl9 enables transcriptional activation of target genes containing TCF/LEF binding sites, for example c-*MYC, VEGF, FGF9, FGF18, CCND1, LBH, SOX2, SOX9* and genes that encode proteins that provide negative feedback, such as *AXIN2, DKK1* and an inhibitory isoform of *LEF1* (He *et al.*, 1998; Tetsu and McCormick, 1999; Yan *et al.*, 2001; Hovanes *et al.*, 2001; Shimokawa *et al.*, 2003; Blache *et al.*, 2004; Niida *et al.*, 2004; Van Raay *et al.*, 2005; Hendrix *et al.*, 2006; Rieger *et al.*, 2010; Kypta and Waxman, 2012) (Fig. 8).

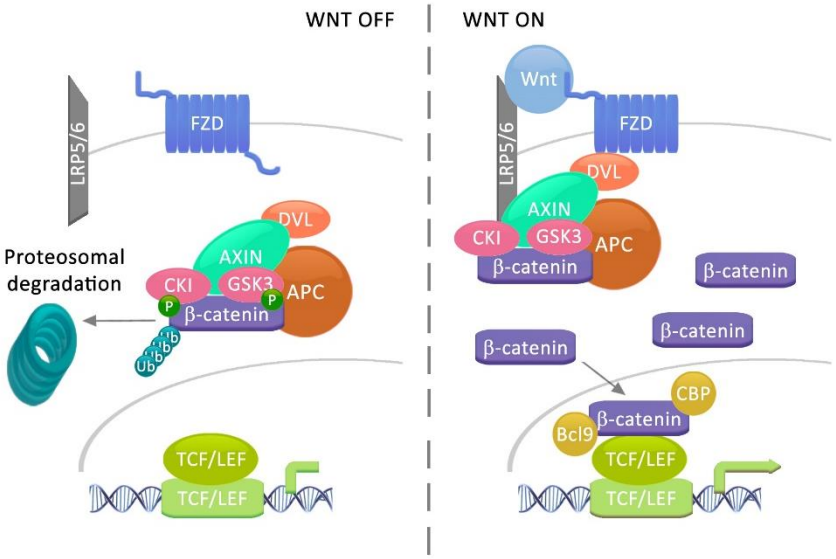

Figure 8. Schematic representation of canonical Wnt signaling. *On the left, when β-catenin signaling is switched off, cytoplasmic β-catenin is recruited into a degradation complex in which CKI and GSK3 kinases phosphorylate β-catenin, which ultimately results in its proteosomal degradation. In the nucleus, TCF/LEF1 family transcription factors are bound to transcriptional co-repressors and transcription is blocked. On the right, when canonical Wnt ligands bind to FZD receptors and LRP5/6 co-receptors, DVL is recruited to the plasma membrane disrupting the degradation complex and enabling stabilization and translocation of β-catenin to the nucleus. In the nucleus, β-catenin binds to TCF/LEF1 transcription factors and other co-activators, such as CBP and Bcl9 to activate transcription of target genes.*

4.2. Non-canonical Wnt signaling

In addition to canonical Wnt signaling, there are a number of other Wnt-regulated signals, including Planar cell Polarity (PCP) pathway, the Wnt Calcium (Ca^{2+}) pathway and other β-catenin independent signals (Fig. 9).

The Wnt/PCP pathway, includes the cytoplasmic signal transduction protein DVL and downstream small GTPases of the Rho family act as mediators of the signal (Strutt, Weber and Mlodzik, 1997), with Rho-associated kinase (ROCK) acting as an effector (Winter *et al.*, 2001). Daam1 is a protein that binds DVL and Rho and is required for both Wnt activation of Rho and cytoskeletal remodeling (Habas, Kato and He, 2001). Another non-canonical signal connected to the PCP pathway involves the Jun N-terminal Kinase (JNK) pathway (Boutros *et al.*, 1998). Various stress stimuli activate Rho family GTPases (Rac, Rho and cdc42) in the cell membrane and other membrane proximal protein components MAP3ks, such as MEKKs, ASK1, TAK1/AB1 or MLK3 that lead to the phosphorylation of JNKs (Kumar *et al.*, 2015). Finally, FZDs can activate intracellular effectors such as p38 through DVL (Bikkavilli, Feigin and Malbon, 2008) to activate JNK, which leads to the activation of Activator Protein-1 (AP-1) family transcription factors (Gomez-Orte *et al.*, 2013). JNKs phosphorylate and activate AP-1, which comprises homo- and heterodimers of Jun and Fos family members that bind and activate target gene promoters (Kumar *et al.*, 2015). JNK also phosphorylates activating transcription factors-2 (ATF2) and cAMP-responsive element-binding protein (CREB). CREB is also activated by other cytoplasmic effectors including adenylate cyclase (AC) and protein kinase A (PKA) (Chen, Ginty and Fan, 2004). The CREB family contains there members ATF1, CREM and CREB, which form dimers within the family and bind the DNA consensus sequence termed the cyclic AMP responsive element (CRE). The ATF2 family also contains three proteins, ATF2, ATF7 and CRE-BPa. ATF2 proteins not only form homodimers but also heterodimers with Jun, Fos and C/EBP family members (Watson, Ronai and Lau, 2018). Binding specificity varies depending on the proteins that form the dimers, as well as their DNA binding specificity (Hai and Curran, 1991).

Other non-canonical Wnt signals involve Ca^{2+} signaling (Veeman, Axelrod and Moon, 2003). Some Wnt ligands can activate the Ca^{2+}-sensitive kinase protein kinase C (PKC) (Sheldahl *et al.*, 1999) and Ca^{2+}/calmodulin-dependent kinase II (CamKII) (Kühl *et al.*, 2000), leading to the activation of the Ca^{2+}-responsive transcription factor nuclear factor of activated T cells (NF-AT) (Murphy and Hughes, 2002; Saneyoshi *et al.*, 2002). It is also known that non-canonical signals can inhibit β-catenin dependent signaling. Inhibition has been proposed to be mediated by the activation of Nemo-like kinase (NLK), which phosphorylates TCF/LEF transcription factors

through CamKII and TAK1 (Ishitani *et al.*, 2003). There are also other mechanisms, involving competition for molecules involved in both canonical and non-canonical signaling, such as DVL or FZD receptors. Activation of the canonical or non-canonical pathway depends on the specificity of the Wnt ligand for coupling FZD to different co-receptors triggering different phenotypic responses. DVL is a component of both canonical and non-canonical Wnt signaling. The N-terminal domain has been reported to be required for canonical Wnt singling, whereas the C-terminal is necessary to display the non-canonical signaling (Grumolato *et al.*, 2010).

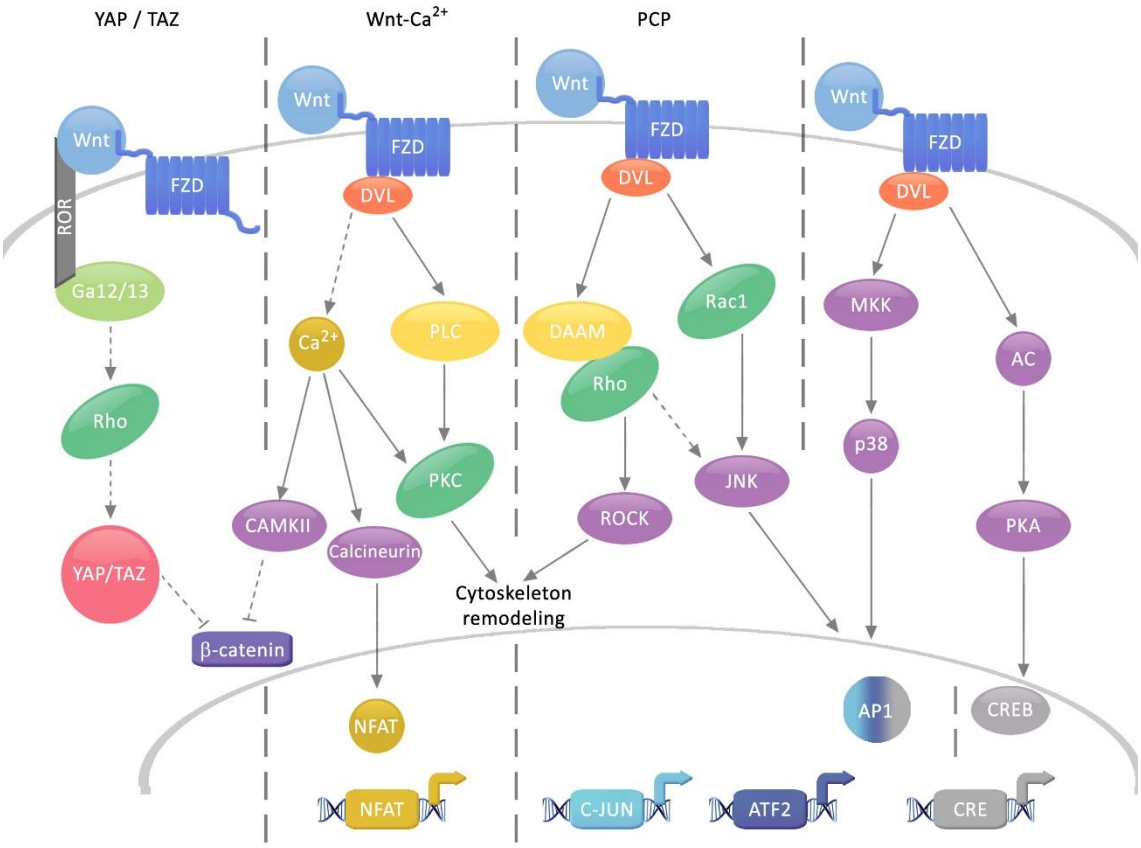

Figure 9. Schematic representation of non-canonical Wnt signaling. *Non-canonical Wnt signaling is able to activate different pathways and intracellular effectors. Wnt and FZD receptors can activate Rho GTPases, which can lead to the activation of YAP/TAZ signaling, or cytoskeletal remodeling through the activation of ROCK. The Wnt-Ca^{2+} pathway can also induce cytoskeletal remodeling and activate nuclear factor of activated T-cells (NFAT)-dependent transcription of target genes, concomitantly inhibiting Wnt/β-catenin signaling. Wnt/PCP signaling not only activates ROCK but also JNK, which leads to the activation of AP-1 family transcription factors c-Jun, ATF2 and CREB. FZD receptors can also activate kinases like PKA and p38. DAAM1, disheveled associated activator of morphogenesis 1; CAMKII, Ca^{2+} and calmodulin kinase II; PLC, phospholipase C; AC, adenylate cyclase; MKK, mitogen-activated protein kinase kinase.*

Wnt/LRP6 signaling has traditionally been described as a linear signal transduction for the stabilization of β-catenin. More recently, it has been reported that downstream events include but are not limited to Wnt/β-catenin signaling. For instance, Acebron *et al.* reported a β-catenin independent signal in proliferating cells. They showed a Wnt-dependent stabilization of many proteins (Wnt/STOP) that peaks during mitosis increasing cellular protein levels and cell size. This signal protects critical proteins such as MYC from GSK3-dependent polyubiquitination and degradation, suggesting its relevance in many cancer cell types (Acebron *et al.*, 2014). Other β-catenin-independent Wnt/LRP6 signaling cascades leads to GSK3 inhibition, which not only targets proteins to degradation but also activates target of rapamycin (TOR) to increase protein translation, modulates the activity of microtubule-associated proteins and inhibits the activity of protein phosphatase 1 (PP1) (Acebron and Niehrs, 2016).

Furthermore, Wnt signals can result in different outputs, depending not only on the specific ligand, receptor or intracellular components that may be acting (Najdi *et al.*, 2012), but also on the crosstalk of all the above-mentioned proteins with other signaling pathways. One example is Wnt-YAP/TAZ signaling, in which Rho GTPases mediate FZD1-$G\alpha_{12/13}$-induced YAP/TAZ activation promoted by Wnt ligands, which results in osteogenesis, cell migration and Wnt/β-catenin signaling blockade as result of induction of secreted Wnt inhibitors (Park *et al.*, 2015) (Fig. 9).

4.3. Wnt ligands

The WNT genes encode 19 Wnt proteins that share between 27 to 83% amino-acid sequence identity. WNT genes are highly conserved among vertebrates. Several human WNT genes are located closely to each other in the genome, for instance *WNT6* and *WNT10A* are located adjacent to each other on chromosome 2, and *WNT1* and *WNT10B* on chromosome 12 and transcribed from the same strand of DNA. The close proximity of these pairs of genes has been conserved in mouse. Since in *Drosophila*, the paralogous genes *wingless (wg)*, *DWnt6* and *DWnt10* are located adjacent on the second chromosome, it is believed that there was an ancient cluster of Wnt genes consisting of *Wnt1*, *Wnt6* and *Wnt10* in a common ancestor of vertebrates and arthropods, but this cluster may have been duplicated losing *Wnt1* from one cluster and *Wnt6* from the other. In vertebrates, orthologues in different species are very similar in sequence. Human Wnt-1 and mouse Wnt-1 are identical in 98% and human Wnt-5a and *Xenopus* Wnt-5a present 85% identity. On the contrary, identity with flies is lower, ranging from 21% between human *Wnt8* and *Drosophila DWnt8* to 42% identity between human Wnt-1 and Drosophila *Wingless (Wg)* (Miller, 2002).

Human Wnt proteins are similar in size, ranging from 39 kDa for Wnt-7a to 46 kDa of Wnt-10a. They all contain 22 to 24 cysteine residues, which are important for correct protein structure and activity. Analysis of the signaling activities point the carboxy-terminal region of Wnt proteins as important for specificity of responses to different Wnts (Du *et al.*, 1995) and the amino-terminal region as mediator for interactions with Wnt receptors (Miller, 2002). Wnts are secreted proteins that can associate with glycosaminoglycans in the ECM and are bound to the cell surface (Bradley and Brown, 1990; Reichsman, Smith and Cumberledge, 1996). Nevertheless, it is also possible to collect active Wnt from the medium of cultured cells (Shibamoto *et al.*, 1998) and several N-linked glycosylated intermediate Wnt protein products in cell lysates (Mikels and Nusse, 2006).

Secretion and recognition of Wnts by receptors is essential for their activity. Wnt proteins were shown by metabolic labeling to be fatty acylated, which is required for secretion of these proteins. Wnt-3a has been described to incorporate fatty acyl groups of different chain lengths at Ser209 but palmitoleate appears to be the elected lipid. Desaturation of palmitoyl-CoA (C16:0) introduces a double bond between the ninth and tenth carbon atoms of the hydrocarbon chain (cis-C16:1n-7) producing a substrate for the Wnt fatty acyl transferase porcupine, which leads to the formation of palmitoleoylated Wnt proteins (Nile and Hannoush, 2016). Independently of their fatty acylation status, Wnt proteins also undergo glycosylation in the ER-Golgi system (23). In the ER, palmitoleoylated Wnts interact with Wntless to traffic to the Golgi and then be transported to the plasma membrane in vesicles (Nile and Hannoush, 2016).

4.3.1 *WNT1*

One of the most studied WNT genes in the context of breast cancer is *WNT1*. *Wnt1* was first discovered in a study of the common integration sites of the mouse mammary tumor virus (MMTV). MMTV infects mouse mammary glands leading to the integration of the viral genome into the host genome; this integration ultimately results in the formation of a mammary tumor because of the transcriptional activation of a cellular oncogene. Two common integration sites of the MMTV were found: *int-1* and *int-2* (van Ooyen and Nusse, 1984). The *int-1* site is located next to the *Wnt1* gene, located in the chromosome 15 in mice. Its coding region consist of 4 exons that encodes a 41 kDa, 370 amino acid protein (McMahon and Moon, 1989b). In humans, the *WNT1* gene is located on chromosome 12. *Wnt-1* is not expressed in normal mammary mouse tissue, but *Wnt1* mRNA can be detected in the neural tube of midgestation embryos suggesting a developmental function.

There are four immature forms of Wnt-1 glycoprotein (molecular weight of 36, 38, 40 and 42 kDa) that are processed to a mature heterogenous glycoprotein with a molecular weight of 44 kDa (Papkoff, 1989). Under normal growth conditions, the 44 kDa protein is hardly detected in the cell culture medium and the majority is found associated with the ECM. *In vitro*, it can be bound to heparin, indicating it might bind glycosaminoglycans in the ECM and suggesting a role in cell-cell communication over short distances (Bradley and Brown, 1990).

Wnt1 expression in female mice induces a potent mitogenic effect on mammary epithelium; ductal hyperplasia is detectable in the end-buds by 18 days of gestation and is very evident 2 weeks after birth. Due to the extensive ductal hyperplasia, female mice are not able to deliver milk to their young. Moreover, breeding females develop tumors earlier than virgin mice, which may be caused either by the hormonal influence or the increased mass of the mammary epithelium during pregnancy and lactation. These primary tumors do not metastasize. However, the majority of female mice expressing *Wnt1* develop lymph node and/or lung metastasis after removal of the primary tumor. The tumors found in these mice are moderately differentiated and are formed of ducts with multiple layer of epithelial cells that show higher nucleus-to-cytoplasm ratio. There is also some necrosis, hemorrhage and extensive fibrosis is present in neoplasms (Li, Hively and Varmus, 2000).

WNT1 over-expression has been associated with tumor proliferation and a poor prognosis in many types of cancer, including breast and lung (Schlange *et al.*, 2007; Nakashima *et al.*, 2008). In breast, *WNT1*-expressing cells were found to have more active β-catenin compared to control cells and treatment with the secreted Wnt antagonist sFRP1 blocked their proliferation. This effect was observed in JIMT-1, SkBr3 and MDA-MB-231 cells but not in BT474 and MCF-7 cells (Schlange *et al.*, 2007; Jamieson *et al.*, 2016). Given the link to MMTV, many studies of Wnt-1 have been carried out using mouse models.

4T1 murine breast adenocarcinoma model is a highly clinically relevant model of spontaneous breast cancer metastasis in multiple sites. 4T1 is a model for triple-negative breast cancer that metastasizes to the lung, liver, bone and brain (H Heppner, R Miller and Malathy Shekhar, 2000; Kau *et al.*, 2012). In mouse 4T1 cell lines, knockdown of *Wnt1* suppressed mammosphere forming ability and ALDH activity, whereas addition of recombinant Wnt-1 enhanced *in vitro* properties of stem cells. Moreover, knockdown of *Wnt1* in 4T1 cells injected into the mammary gland, reduced tumorigenic potential and

tumor initiation capacity *in vivo* (Choi *et al.*, 2012). These effects relate to the ability of *Wnt1* to regulate apoptosis and CSC migration in these metastatic mouse mammary cell lines (Jang *et al.*, 2015).

4.4. FZD receptors

Frizzled genes encode 7 transmembrane (TM) proteins that function in several signal transduction pathways. These genes show essential roles in development, tissue cell polarity, formation of neural synapses and regulation of cell proliferation, among other processes (Huang and Klein, 2004). There are ten human FZD genes described that are classified into four main clusters. FZD_1, FZD_2 and FZD_7 share approximately 75% amino acid identity; FZD_5 and FZD_8 share 70% identity; FZD_4, FZD_9 and FZD_{10} share 65% identity and FZD_2 and FZD_6 share 50% (Fredriksson *et al.*, 2003; Zeng, Chen and Fu, 2018). FZD proteins contain about 500 to 700 amino acids. The N-terminus contains a cysteine-rich domain (CRD) followed by a hydrophilic linker region. The seven TM α-helices contain hydrophobic domains. The intracellular carboxyl-terminal tails are the least conserved among different family members (Wang *et al.*, 1996) (Fig. 10A). It is well known that each Wnt can bind multiple FZDs, and each FZD can respond to multiple Wnts (Hsieh *et al.*, 1999; Holmen *et al.*, 2002; Logan and Nusse, 2004; Wang *et al.*, 2005; Kikuchi, Yamamoto and Kishida, 2007; Smallwood *et al.*, 2007). Crystallization of glycosylated XWnt8/Fz8-CRD allowed Janda *et al.* to determine the structure of the complex. They showed that XWnt8 grasps Fz8-CRD at two opposing sites using extended thumb and index fingers projecting from an extended palmitoleic acid (PAM) group to contact “site 1” and “site 2”, respectively. Wnt lipidation is directly involved in binding site 1, whereas in site 2 protein-protein interactions are dominant and this site has been suggested to be important for discriminating between specific Wnt/Fz pairs (Janda *et al.*, 2012) (Fig. 10B).

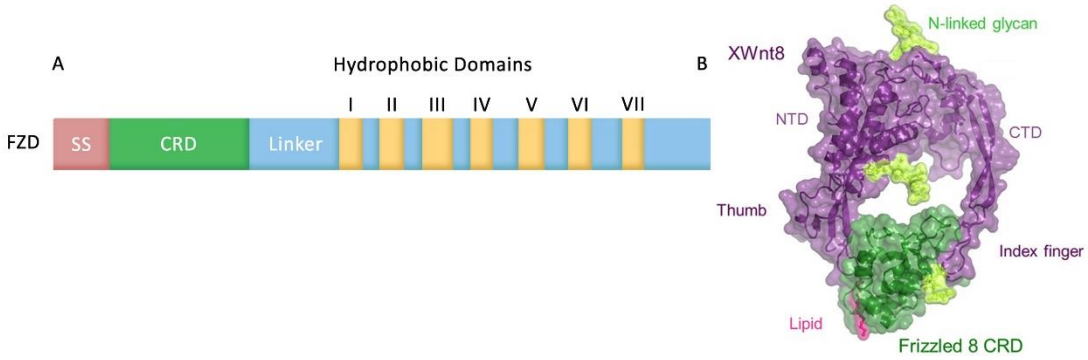

Figure 10. Schematic FZD receptors and crystal structure of XWnt8-Fz8-CRD binding. A. *The N-terminus contains a cysteine-rich domain (CRD) where Wnt ligands bind. This region is followed by a hydrophilic linker region. The seven TM α-helices contain hydrophobic domains.* ***B.*** *Surface representation of XWnt8 structure. The extended palmitoleic acid (PAM) group is shown in red extending from the Wnt thumb (Adapted from Janda et al., 2012).*

4.4.1 FZD5

FZD5 is one of the most highly expressed FZD receptor in the prostate cancer cell lines. Moreover, high *WNT5A* and *FZD5* expression in combination correlates with longer disease-specific survival for prostate cancer patients (Thiele *et al.*, 2018). In breast MCF-7 and MDA-MB-231 cells lines *FZD5* downregulation inhibited Wnt/β-catenin signaling reducing proliferation and migration of breast cancer cells (F. Liu *et al.*, 2014). FZD5 mediates sFRP2-induced angiogenesis *via* calcineurin/NF-AT pathway in endothelial cells (Peterson *et al.*, 2017).

The function of FZD_5 in eye development seems to be species-dependent, it can activate non-canonical signaling in zebrafish and in mice also seems to be independent of Wnt/β-catenin signaling but it activates canonical Wnt signaling in frog embryos (Burns *et al.*, 2008). FZD_5 is also involved in establishment of neural polarity through the activation of non-canonical Wnt signaling involving JNK in rat medullar PC12 tumor cell lines (Slater *et al.,* 2013). On the other hand, using the same cell line, Caricasole *et al.*, (2003) reported that Wnt-7b interacts with FZD_5 and LRP6 to activate β-catenin signaling.

4.5. Wnt signaling in cancer stem cells

There is Wnt signaling cross-talk with FGF, Notch, hedgehog and TGFβ/BMP signaling cascades that regulates stem cell signaling networks (Katoh and Katoh, 2007; Lamb *et al.*, 2015). Both, canonical and non-canonical signals play roles in the development and evolution of CSCs. Canonical Wnt/β-catenin signaling is involved in self-renewal of stem cells and proliferation or differentiation of progenitor cells (Lui, Hansen and Kriegstein, 2011; Barker, 2014; Van Camp *et al.*, 2014; Yang *et al.*, 2016). On the other hand, non-canonical Wnt signals promote invasion, survival and metastasis of CSCs, cell polarity and inhibition of canonical signaling (Qin *et al.*, 2015; Webster, Kugel and Weeraratna, 2015; Kumawat and Gosens, 2016; Katoh, 2017).

Wnt/β-catenin signaling is activated by canonical ligands, such as Wnt-3a (Zhang *et al.*, 2017) or Wnt-3, among others (Katoh *et al.*, 2001), but there are also genetic alterations in many components of the Wnt pathway that can induce signaling. For instance, EIF3E-RSPO2 or PTPRK-RSPO3 fusions, gain of function mutations in *CTNNB1* (β-catenin) and loss of function mutations in *APC, AXIN, RNF43* and *ZNRF3* genes. The activation of this pathway directly promotes CSC proliferation through the regulation of *CCND1*, *MYC* and YAP/TAZ, but also by inducing expression of secreted growth factors in CSCs that modify the microenvironment (Katoh, 2017). *MET* (which encodes the HGF receptor) is upregulated in human basal-like breast cancers with *TP53* mutations (Chiche *et al.*, 2017), and combined activation of Wnt/β-catenin and HGF/MET signals induces sonic hedgehog (SHH) upregulation in mouse mammary CSCs, which has been reported to activate cancer-associated fibroblasts for the synergistic proliferation of CSCs and cancer-associated fibroblasts (Valenti *et al.*, 2017). Therefore, Wnt/β-catenin signaling does not only induce CSC maintenance directly, but also through indirect effects (Katoh, 2017).

Non-canonical Wnt signaling in CSCs are activated by Wnt-5a, Wnt-11 and other non-canonical ligands secreted by cancer cells (Sheldahl *et al.*, 1999) or by stromal/immune cells (Blumenthal *et al.*, 2009), and also as a result of genetic alterations, such as *MET* amplification (Gentile *et al.*, 2014). Moreover, FZD and ROR family Wnt receptors can activate non-canonical signaling. For example, both FZD_7 and ROR1 have been reported to activate the phosphatidylinositol-3-kinase (PI3k)-AKT signaling cascade (Zhang *et al.*, 2012). The activation of these signals leads to promotion of invasion, survival and metastasis of melanoma CSCs or prostate circulating tumor cells (CTCs) (Miyamoto *et al.*, 2015; W. Wang *et al.*, 2015; Webster, Kugel and Weeraratna, 2015; Katoh, 2017).

4.6. Wnt signaling in breast

4.6.1 Wnt signaling in breast development

Wnt signals are essential to initiate specification of cells that will form the mammary lines during embryogenesis and later these signals become restricted to the cells forming placodes (Yu, Verheyen and Zeng, 2016). Chu (2004) reported that over-expression of *Dkk1* in the ectoderm blocks the formation of mammary placodes and antagonizes the expression of *Wnt10b*. It is thought that *Wnt3, Wnt6* and *Wnt10b* are expressed in a band of the ectoderm flanking the mammary line to initiate specification, while non-canonical Wnts, such as *Wnt5a* and *Wnt11* are expressed in the underlying flank mesenchyme, suggesting that non-canonical signaling regulates canonical signaling, limiting the response to the ectodermal ridge that becomes the mammary lines (Chu, 2004; Veltmaat et al., 2004; Macias and Hinck, 2013).

Wnt pathway components are temporally and spatially differentially expressed in the mammary gland (Buhler *et al.*, 1993; Lane and Leder, 1997). *Lrp6* co-receptors are expressed in both layers of the epithelium and stroma during embryogenesis; however, in young and adult mice expression is limited to the basal layer (Lindvall *et al.*, 2009). In TEBs, although Wnt-4 and Wnt-5a ligands are localized in the luminal compartment, canonical signaling is mostly active in the cap cell layer (MaSCs), where Wnt-6 is localized (Paine and Lewis, 2017). *Rspo1* is regulated by estrogen and progesterone in luminal epithelial cells and collaborates with Wnt-4 to expand these MaSCs (Pierce *et al.*, 1993). LRG5 and 6 (receptors for R-spondins) are also localized in the basal layer and are required for TEB formation and side branching (Badders *et al.*, 2009; Lindvall *et al.*, 2009). Canonical Wnt signaling is required for stem cell maintenance, branching and alveologenesis.

Non-canonical Wnt signaling is also crucial in mammary gland development. Wnt-2, Wnt-5a and Wnt-7b ligands and ROR2 receptor are expressed in TEBs during puberty to regulate branching and proliferation by inhibiting canonical signaling (Roarty and Serra, 2007; Roarty *et al.*, 2015).

4.6.2 Wnt signaling in breast cancer

Several components of the Wnt pathway have been defined as oncogenes or tumor suppressors in human cancer. The most evident example is loss-of-function mutations in APC, a scaffold protein that is essential for the destabilization complex that promotes β-catenin degradation. APC mutations are found in 85% of colon cancer tumors. Mutations in β-catenin itself, which affect phosphorylation sites and result in its stabilization, have been reported in several human cancers, including colorectal, medulloblastomas, hepatoblastomas, hepatocellular carcinomas, endometrial, and Wilm´s tumors (Polakis, 2012). However, genetic mutations in the Wnt pathway are not typically associated with breast cancer. Only 6% of breast tumors contain mutations in the APC gene and there are no further reports indicating mutations in β-catenin in breast cancer (Sorlie, Bukholm and Borresen-Dale, 1998; Schlosshauer *et al.*, 2000; Geyer *et al.*, 2010; Yu, Verheyen and Zeng, 2016). However, epigenetic inactivation of extracellular Wnt antagonists seems to be a frequent event. Methylation of *DKK1* and *DKK3* promoters have been reported, as well as epigenetic silencing of genes encoding *sFRP*s (Suzuki *et al.*, 2008; Veeck *et al.*, 2009), which is associated with worse prognosis. In addition, expression of the secreted Wnt antagonist *WIF1* is reduced in 60% of breast cancers (Wissmann *et al.*, 2003). Furthermore, modulation of FZD receptors, such as FZD_7 (Sorlie, Bukholm and Borresen-Dale, 1998) in TNBC and increases in Wnt-2, Wnt-4, Wnt-5a, Wnt-7b, Wnt-10b and Wnt-13 (now called Wnt-2b) proteins have been reported in breast tumors (Huguet *et al.*, 1994; Lejeune *et al.*, 1995; Bui *et al.*, 1997; Bergstein and Brown, 1999). Many and Brown (2014) showed that both canonical and non-canonical Wnt pathways are important for promotion of stem cell growth in mammospheres. Moreover, autocrine activation of Wnt signaling has been reported to regulate the proliferation and survival of human basal and luminal breast cancer cell lines (Schlange *et al.*, 2007). Regarding treatment resistance, Wnt signaling mediates radiation resistance of mouse mammary progenitor cells (Woodward *et al.*, 2007) and inhibition of Wnt signaling restores tamoxifen sensitivity in breast cancer cell lines (Piva et al., 2014).

Studies in mice have been relevant to demonstrate that Wnt signaling over-activation increases breast cancer risk. Overexpression of *Lrp6* (Zhang *et al.*, 2010) or of a stabilized form of β-catenin (Hatsell *et al.*, 2003) causes mouse mammary hyperplasia. Overexpression of *Wnt1* and *Rspo* in mouse mammary epithelial cells resulted in mammary tumor formation, and cells exhibit a strong EMT phenotype with high

metastatic capacity to form secondary tumors in lung or spleen (Klauzinska *et al.*, 2012). These effects may be related to the ability of *Wnt1* expression to enrich the cancer stem cell population (Choi *et al.*, 2012). Moreover, both CSC enrichment and metastasis can be suppressed by silencing *Wnt1* (Jang *et al.*, 2015). It is noticeable that the murine MMTV-Wnt1 model of mammary cancer exhibits hallmarks and shares transcriptional patterns with human TNBC. Some of these genes are known to be direct targets of β-catenin/TCF binding (Herschkowitz *et al.*, 2007; Yu, Verheyen and Zeng, 2016).

4.7. Wnt inhibition approaches

Due to the aberrant expression of Wnt signaling in many cancers, targeting this pathway has been a goal in biomedical research for the last 30 years. However, no drugs have been approved for targeting this pathway yet. One of the key issues is that inhibition of Wnt signaling may affect normal stem cell populations and regeneration of tissues and organs. Deeper knowledge of cross-talk between signaling pathways is necessary for designing efficient therapeutic approaches (Krishnamurthy and Kurzrock, 2018).

A widespread approach used to block Wnt signaling, is to target porcupine (PORCN), a membrane-bound O-acyltransferase that is required for Wnt ligand secretion as it palmitoylates Wnts, which is essential for their secretion and biological activity (Wang *et al.*, 2013). LGK974 is a Porcupine-selective inhibitor that blocks Wnt signaling and tumor growth *in vivo* (Liu *et al.*, 2013). There are ongoing clinical trials for this drug in metastatic colorectal and head and neck cancers. ETC-159 is another PORCN inhibitor in clinical trials for colorectal cancer (Teneggi *et al.*, 2016).

Another approach is based on the use of antibodies against Wnt ligands. Antibodies have been developed against Wnt-1 and Wnt-2 and have been reported to show tumor suppression activity in melanoma, sarcoma, colorectal cancer, non-small cell lung carcinoma and mesothelioma (He *et al.*, 2004; Mikami *et al.*, 2005). There are also monoclonal antibodies that target FZD receptors, such as OMP-18R5 (Vantictumab), which targets five out of ten FZD receptors ($FZD_{1/2/5/7/8}$). The safety and efficacy of this antibody in non-small cell lung cancer, pancreatic, breast (Gurney *et al.*, 2012) and ovarian cancer (Fischer *et al.*, 2017) are being evaluated alone and in combination with chemotherapy.

Wnt inhibitors have been proposed to be good candidates to eradicate drug resistant cancer stem cells as a second line therapy (Fischer *et al.*, 2017). Other approaches that have been proposed for cancer therapy include Tankyrase inhibitors, which stabilize Axin, thereby

increasing β-catenin degradation, Dishevelled inhibitors, TCF/β-catenin transcription complex inhibitors, Wnt co-activator antagonists that inhibit binding between β-catenin and its transcriptional co-activator CBP, and Notch and Hedgehog inhibitors that have been reported to cross-talk with the Wnt pathway (Krishnamurthy and Kurzrock, 2018).

4.7.1 DUBs as targets for cancer therapeutics

The therapeutic potential of targeting deubiquitinases (DUBs) is an emerging approach as an option for treatment for cancer. Several DUBs have been reported to play a role in the regulation of cellular processes, such as cell cycle control, cell signaling and apoptosis. The ubiquitin-proteasome system (UPS) is one of the main systems for controlling protein function and stability. Actually, more than 80% of cellular proteins are degraded by the UPS. Ubiquitination is a process of covalent modification of a protein with the small molecule ubiquitin. This process is dependent on the activity of three enzymes, Ub-activating (E1), Ub-conjugating (E2) and Ub-ligating (E3). Substrate specificity is conferred by E3 ligases, which bind and coordinate the covalent attachment of ubiquitin to target substrates. Ubiquitination can be reversed by specialized enzymes known as deubiquitinases (DUBs), which oppose the action of E3 ligases by cleaving the bond between target proteins and ubiquitin (D'Arcy, Wang and Linder, 2015).

Several DUBs directly regulate Wnt pathway components. For instance, USP4 modulates Wnt signaling through interactions with TCF4 but also can directly regulate β-catenin deubiquitylation and USP6 increases FZD abundance in the membrane by opposing the activity of the ubiquitin ligase RNF43 pointing to USP6 as a potential target to modulate Wnt signaling (Madan *et al.*, 2016).

Hypothesis and aims

Since CSCs have been linked to resistance to therapy in cancer, and more specifically to tamoxifen resistance in breast cancer, and given the importance of Wnt signaling in the regulation of stem cell function, the overall aim of this project is to study how Wnt signals affect proliferation and tamoxifen resistance in breast cancer stem cell populations. A better understanding of the mechanisms involved could lead to novel strategies to overcome resistance and provide a basis for new breast cancer treatment options. I therefore had the following aims:

1. Identification of Wnt proteins involved in the maintenance of CSC populations in breast cancer.

2. Characterization of Wnt signaling pathway activity in tamoxifen resistant cell lines.

3. Development of strategies to regulate Wnt signaling in breast CSCs.

Material and Methods

1. CELL CULTURE AND DRUG TREATMENTS

1.1. Human and mouse cell lines culture

MCF-7 and T47D cell lines were obtained from American Tissue Culture Collection (ATCC) and their resistant derivatives were developed by the culture of cells in the presence of 5 x 10^{-7} M 4-OH-tamoxifen or ethanol for six months, as described in Piva *et al.*, (2014). Cells were continuously cultured in the presence of 5 x 10^{-7} M tamoxifen (Table 1). LNCaP C4-2B cells were obtained from Charlotte Bevan (Imperial College London) and HEK293 cells from ATCC. Mouse 67NR, 4T07 and 4T1 breast cancer cell lines were a gift from Beatrice Howard (Institute of Cancer Research, London). All cells were grown in media supplemented with 8% fetal bovine serum (FBS) (Life Techonologies) and 1% penicillin/streptomycin (Life Technologies) in incubators at 37°C and 5% CO_2.

Cells over-expressing *WNT1* were obtained by transfecting 3 x 10^{-5} MCF-7C or MCF-7TamR cells in 6 cm plates with 3 μg of a plasmid encoding pcDNA-*WNT1* (GeneCopoeia, Frederick, MD) or with an empty pcDNA3 vector, as control, using Lipofectamine LTX and Plus Reagent (Life Technologies) following the instructions provided by the manufacturer. These vectors also contain a gene that confers resistance for Geneticin (G418), the drug that was used for selection. Transfected cells were exposed to a high concentration (400 μg/ml) of G418 (Life Techonologies) for two weeks and later maintained in culture with 200 μg/ml G418.

Cell line	Specie	Disease	Molecular classification	Receptors expression	Drug in culture
MCF-7C	Human	Adenocarcinoma	Luminal A	ER+ PR+ HER2-	EtOH
MCF-7TamR	Human			ER+ PR- HER2-	tamoxifen
MCF-7C-V	Human			ER+ PR+ HER2-	EtOH+G418
MCF-7C-W1	Human			ER+ PR+ HER2-	EtOH+G418
MCF-7TamR-V	Human			ER+ PR- HER2-	tamoxifen+G418
MCF-7TamR-W1	Human			ER+ PR- HER2-	tamoxifen+G418
T47D-C	Human	Ductal Carcinoma	Luminal A	ER+ PR- HER2-	EtOH
T47DTamR	Human			ER-PR- HER2-	tamoxifen
67NR	Mouse	Breast cancer	Basal like	ER-PR- HER2-	
4T07	Mouse			ER-PR- HER2-	
4T1	Mouse			ER-PR- HER2-	

Table 1: Description of breast cell lines characteristics and drugs kept in culture. *Concentration of tamoxifen was 5 x 10^{-7} M and 200 μg/ml for G418. EtOH, ethanol; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor 2.*

1.2. Drug treatments

Drugs for normal maintenance of cells in culture are described in Table 1.

MCF-7TamR and T47DTamR cells were treated with Wnt-3a CM, added at 1:2.5 dilution in normal medium. Conditioned media (CM) were prepared using L-*Wnt3a* cells obtained from ATCC. Cells were grown for 4 days and then media were harvested and filtered to obtain the first batch. Fresh media was added to the cells for another 3 days and a second batch of medium, after filtering, was mixed 1:1 with the first batch. This Wnt-3a CM was stored at 4ºC. Cells were also treated with IWP-2 (Calbiochem) at 5 μM, diluted and filtered before use; the same volume of DMSO (Sigma) was used as a control. Fulvestran (ICI 182,780) (Tocris) was used at 5 x 10^{-7} M.

1.3. Mammosphere formation

MCF-7TamR and T47DTamR cells were detached with Tryple 1X (Gibco) and seeded in Poly(2-hydroxyethyl methacrylate) (pHEMA)-coated (Sigma) ultralow attachment 6-well plates (Corning) at 10^3 cells/well or at 10^4 cells/ml in flasks (Corning) for primary mammosphere formation or 5 x 10^3 cells/ml for secondary mammospheres. Cells were cultured in serum-free DMEM:F12 supplemented with B27 (Invitrogen), 20 ng/mL EGF (Life Technologies) and 20 ng/mL bFGF (BD Biosciences).

Mammospheres were dissociated with Tryple 1X after 3 days for passaging or 5 days for analysis. The numbers of mammospheres were calculated as the average of 3 wells for each cell line in 3 independent experiments. All the samples were analyzed using a FACSAria™ flow cytometer (BD Biosciences).

1.4. Colony formation assay

Colony formation is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony that consists of at least 50 cells. The assay tests the ability of cells to retain the capacity for giving rise to progeny after a treatment (Franken *et al.*, 2006). To perform the assay, a bottom layer made of 0.7% agarose/DMEM without cells was prepared in 6-well plates and kept at 4°C for 1 h. In the upper part 10^3 cells/well treated with tamoxifen 5 x 10^{-7} M, ICI 10^{-9} M or ethanol were embedded into a 0.35% agarose/DMEM layer. Colonies were grown for 10 days, during that time 250 μl of fresh media with treatments were added every 3 days. Colonies were counted taking into account the average of 9 fields for each well and counting the colonies using a 10X magnification lens on an Olympus CKX31 inverted microscope in triplicates and in 3

independent experiments. The sizes of the colonies were also considered using an Olympus WHB 10x/20 eyepiece with micrometer, defining colonies with a diameter greater than 50 μm as large colonies and those smaller than this as small.

1.5. Cell proliferation assays

MCF-7C and MCF-7TamR cells, selected with G418 after transfection of pcDNA or pcDNA-WNT1, were seeded in normal media at 10^3 cells/well in 96-well plates. Tamoxifen dilutions starting at 10^{-6} M were added after 24 hours and medium was changed every 3 days. Cell proliferation was determined after 7 days by staining with crystal violet (Sigma). For staining, cells were washed twice with PBS and stained with 100 μl of crystal violet for 20 min on a rocker to ensure all the surface was covered and after washing another two times with PBS, plates allowed to dry overnight. Once plates were dry, the crystal violet-stained cells were dissolved in 10% acetic acid solution for 5 minutes and then absorbance was measured at 595 nm.

1.6. Cell cycle analysis

2 x 10^5 cells were seeded in 6 cm plates and treated with 5 x 10^{-7} M tamoxifen. After 48 h, cells were detached with 0.25% trypsin and centrifuged at 0.4 x 10^3 g for 5 min. After washing with PBS, cells were re-suspended in 1 ml of EtOH 70%, vortexed and frozen for at least 24 h at -20°C. Propidium iodide (PI) (Life Technologies) staining was used to determine DNA content in cell cycle analysis. To determine cell viability and analyze only live cells, DRAQ7 (Biostatus) was used, a far-red fluorescent DNA dye that only stains the nuclei in dead and permeabilized cells. All the samples were analyzed using a BD FACSCanto™ II system flow cytometer.

2. CELL TRANSFECTION

Assay	Plate (wells)	Volume of transfection (µl)	DNA (ng)	Lipo LTX (µl)	Plus Reagent (µl)	GeneJuice (µl)
GFP Reporters	6	200	1000	3	1.5	3
Gene Reporter Assays	12	100	500	1.5	0.5	1.5
Immunofluorescence	24	50	250	0.5	0.5	1

Table 2: Amounts of reagents used for transfection in different types of assay.

2.1. Plasmids

The plasmids used were pcDNA hWNT1, hWNT3A, hWNT7B, hWNT10B (GeneCopoeia, Frederick, MD), pRL-tk (Promega), Super8XTOPFlash (Veeman, Axelrod and Moon, 2003), ATF2-luciferase (Van Der Sanden *et al*., 2004), kindly provided by Christof Niehrs (Mainz, Germany), ARR2-Pb-luciferase (Clegg *et al*., 2012), MMTV-luciferase (Truica, Byers and Gelmann, 2000) from C. Bevan (Imperial College London), TOPdGFP (Boitard *et al.*, 2015), ATF2dGFP (Ohkawara and Niehrs, 2011) and its empty vector UBI-GFP (Giry-Laterrière, Verhoeyen and Salmon, 2011), pRK5 mFzd1-10-1D4 (Yu *et al.*, 2012) were from Chris Garcia and Jeremy Nathans, pcDNA3.2-FZD5-V5 (Voloshanenko *et al*., 2017) was from MM Maurice, CMV500 ΔATF2, ΔCREB, ΔFos and its empty vector CMV500 (Ahn *et al.*, 1998) were from Charles Vinson, TCF1 (Van de Wetering *et al.*, 1996) and HA-tagged TCF4 were provided by Marc van de Wetering and Hans Clevers, β-catenin (Giannini, Vivanco and Kypta, 2000), pcDNA5D FRT/TO GFP USP6 (MRC PPU Reagents and Services, University of Dundee) and pEGFP-C1 (Clontech).

2.2. Transfections for gene reporter assays

8 x 10^4 cells were plated in normal growth medium without tamoxifen or G418. After 24 h, cells were washed twice with PBS to remove antibiotics and serum, and 400 µl of OptiMEM (Gibco) was added to each well. Cells were transfected using the amount of DNA indicated in Table 2. The reporters used were Super8xTOPFlash (TOP-luc), ATF2-luciferase, the AR-dependent reporters ARR2-Pb-luciferase and MMTV-luciferase, a constitutive Renilla luciferase reporter (pRL-tk) and plasmids encoding genes related to Wnt signaling. The ratios of plasmids used are indicated in Table 3. Cells were transfected using Lipofectamine LTX and Plus Reagent (Life Technologies) following the instructions by the supplier (Table. 2). *WNT1*-expressing cell lines

were transfected with GeneJuice (Millipore), following manufacturers' instructions. Transfection mix was removed after 4 h and fresh medium was added to the cells. After 24 h, cells were washed with PBS and lysed in 110 µl of Passive Lysis Buffer (Promega). Luminescence was determined using Glow-Juice (PJK GmbH) following manufacturer´s guidelines and measured using a luminometer (Turner Biosystem). Results are shown as relative light units of luciferase activity, normalized to Renilla.

2.3. Transfections using GFP reporter plasmids

2.5×10^5 cells were plated in normal medium for 24 h. Cells were then washed twice with PBS and 800 µl of OptiMEM added to each well. Transfection conditions using Lipofectamine LTX are indicated in Table 2 and the quantity of each plasmid in Table 3. *WNT1*-expressing cell lines were transfected using GeneJuice. The reporters used were TOPdGFP, ATF2dGFP (Section 2.1); which are plasmids that encode GFP driven by TCF-responsive or ATF2-responsive promoters, respectively. UBI-GFP was used as a control. The transfection mix was removed after 4 h and fresh media added. After 72 h, cells were washed and detached using Tryple 1X and centrifuged for 5 min at 0.4×10^3 g. Cells were re-suspended in FACSFlow medium (PBS 1X, HEPES 25 mM pH 7, EDTA 5 mM, BSA 1%) and DRAQ7 was added as a marker of cell viability. All the samples were analyzed using a FACSAria™ flow cytometer.

Reagent	Gene Reporter Assays DNA (ng)/well	GFP Reporters DNA (ng)/well	Immunofluorescence DNA (ng)/well
Reporter	350	500	-
Renilla	50	-	-
Test Gene	100	500	250

Table 3: Amounts of plasmids used for each type of assay.

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3. FACS ANALYSIS

3.1. Sorting of $CD44^{+}CD24^{-/low}$ cells

Mammospheres were dissociated into single cells using Tryple 1X and placed into polystyrene V-bottom-96-well plates (Sarstedt). Cells were blocked for 15 min in PBS with 40% FBS at room temperature, washed twice with 1% BSA in PBS buffer and then incubated for 30 min at 4°C with PE-CD24 (BD, ML5) and APC-CD44 (BD, G44-26) antibodies diluted in PBS with 1% BSA. Control samples were stained with isotype-matched control antibodies. More information about the antibodies is detailed in Table 7. After staining, cells were washed twice with 1% BSA in PBS and re-suspended in 300 µl of homemade FACSFlow media (PBS with 1% BSA) with 1.67 µl of the viability dye 7AAD (BD), a ready-to-use nucleic acid dye for dead cell exclusion. This dye can be used in conjunction with PE- and FITC-labelled monoclonal antibodies, with minimal spectral overlap between 7AAD, PE and FITC fluorescence emissions.

Control cell populations (cells collected from adherent cultures) were analyzed to gate the population by selecting populations based on their granularity (side scatter, SSC), size (forward scatter, FSC) and to exclude cells positive for 7AAD (See populations P1, P2 and not P3 in Fig. 11). Compensation process is performed to correct spillover, the physical overlap among the emission spectra of fluorochromes. Cells forming mammospheres were then analyzed using the same parameters. Cells in quadrants Q4 and with the phenotype $CD44^{+}CD24^{-/low}$ were selected for sorting, the counterparts in the quadrant Q1 were also sorted as control cells.

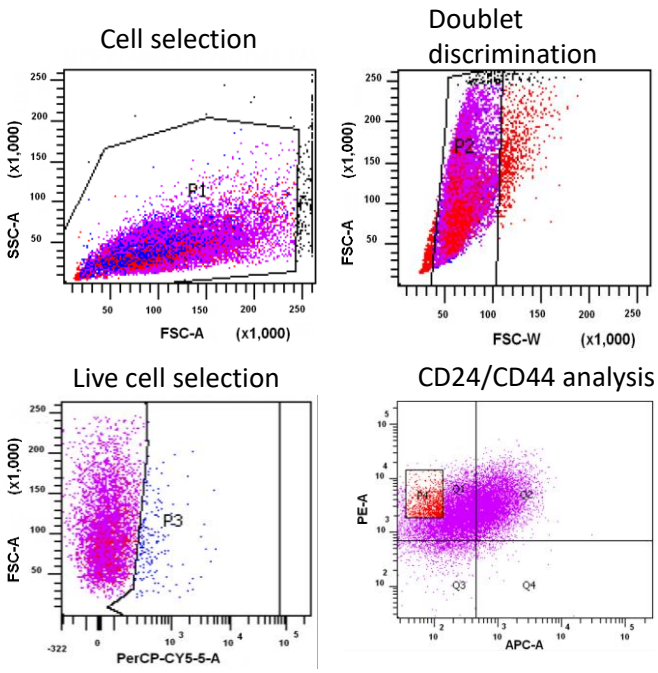


Figure 11. Gating strategy used to select the population of interest for FACS analysis. *First population is selected based on size and granularity (FSC-A and SSC-A) (P1). Then single cells (P2) and finally live cells (not P3) are selected. After compensation process of fluorochromes, gates were established for PE-CD24 and APC-CD44 using MCF-7TamR cells from adherent cultures.*

3.2. ALDEFLUOR analysis

ALDH enzymatic activity was measured using the ALDEFLUOR assay (Stemcell Technologies) according to manufacturer's guidelines. High expression of ALDH identifies stem and progenitor breast cells providing a complementary method to the one that uses antibodies to stain for cell surface antigens. During FACS analysis, control cells treated with 4-(N, N-diethylamino) benzaldehyde (DEAB), which blocks the reaction by formation of stable, covalent acyl-enzyme intermediate species (Luo *et al.*, 2015), were used to adjust FCS and SSC voltages and define the region indicating cells that are negative for ALDH activity. $ALDH^+$ cells have different SSC properties, so maintaining the same gate as used in the control test, probe cells were analyzed for FITC-A staining (Fig. 12). The viability of all samples was determined using DRAQ7.

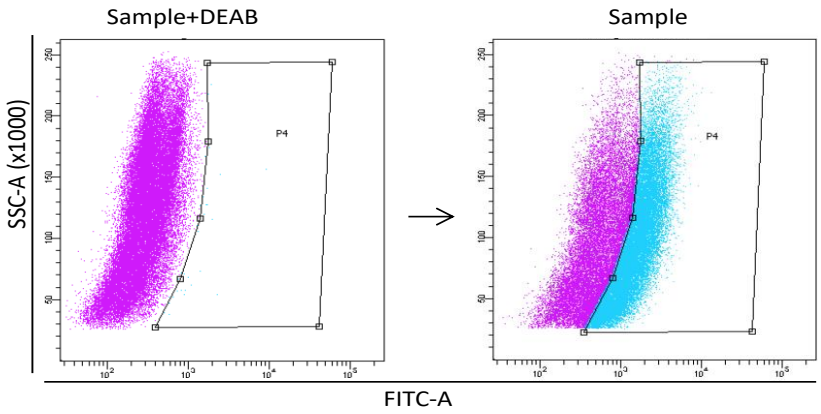


Figure 12. Gating strategy used to select the population of interest for ALDH assay. *First, cells treated with DEAB are used to define cells negative for ALDH activity. Then probe cells are analyzed maintaining the same gate to identify ALDH⁺ cells.*

3.3. Quantification of GFP reporters

Results are shown as relative percent of GFP positive cells transfected with WNT1 or TCF1 plasmids with respect to cells transfected with pcDNA3, for each of TOPdGFP and ATF2dGFP reporters, as compared to cells transfected with the same plasmid and the control UBI-GFP reporter. Calculation formulas are indicated below. Since the GFP signal of cells transfected with each UBI-GFP, TOPdGFP or ATF2dGFP differed considerably, it was necessary to gate independently for every reporter co-transfected with pcDNA.

$$\frac{\text{WNT1-TOPdGFP/pcDNA-TOPdGFP}}{\text{WNT1-UBI-GFP/pcDNA-UBI-GFP}} = \frac{\Delta\text{WNT1-TOPdGFP}}{\Delta\text{WNT1-UBI-GFP}} = \Delta\Delta\text{WNT1-TOPdGFP}$$

$$\frac{\text{TCF1-TOPdGFP/pcDNA-TOPdGFP}}{\text{TCF1-UBI-GFP/pcDNA-UBI-GFP}} = \frac{\Delta\text{TCF1-TOPdGFP}}{\Delta\text{TCF1-UBI-GFP}} = \Delta\Delta\text{TCF1-TOPdGFP}$$

$$\frac{\text{WNT1-ATF2dGFP/pcDNA-ATF2dGFP}}{\text{WNT1-UBI-GFP/pcDNA-UBI-GFP}} = \frac{\Delta\text{WNT1-ATF2dGFP}}{\Delta\text{WNT1-UBI-GFP}} = \Delta\Delta\text{WNT1-ATF2dGFP}$$

4. BIOINFORMATIC DATABASE ANALYSIS

WNT1 mRNA expression levels in breast cancer were examined using GOBO (Gene Expression-Based Outcome for Breast Cancer Online; http://co.bmc.lu.se/gobo) (Ringnér *et al.*, 2011), which stratifies breast cancer according to molecular subtypes and also shows the expression levels of *WNT1* mRNA in breast cancer cell lines. Breast-Mark (http://glados.ucd.ie/BreastMark/mRNA_custom.html) was used to correlate WNT gene expression and survival in breast cancer patients and bc-GenEXMiner v4.1 (http://bcgenex.centregauducheau.fr) was used to determine the correlations between *WNT1*, FZD receptors and *USP6* expression.

5. RNA EXTRACTION AND ANALYSIS

5.1. RNA extraction and cDNA synthesis

RNA from adherent cells and mammospheres was extracted using PureLink RNA Mini Kit (Life Technologies), according to manufacturer´s instructions. The concentration and quality of RNA was measured using a ND-1000 spectrophotometer (NanoDrop Technologies). 2 μg of RNA diluted in 22 μl of H_2O was used for cDNA synthesis using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) (Life Technologies) in a final volume of 40 μl. Thermocycler conditions were set up for 1 h at 37 °C followed by 1 minute at 95 °C to inactivate the enzyme. The cDNA samples were stored at -20 °C.

RNA from FACS sorted cells was extracted using PureLink RNA Micro Kit (Life Technologies), according to manufacturer´s instructions. RNA was eluted from columns in 14 μl H_2O and the whole sample used for cDNA synthesis using SuperScript™ VILO™ cDNA Synthesis Kit (Life Technologies) in a final volume of 18 μl. Thermocycler conditions were set up for 10 min at 25 °C, followed by 1 h at 42 °C and enzyme inactivation was carried out for 5 min at 85 °C. The cDNA samples were stored at -20 °C.

5.2. Primer design and set up

Primers were designed using Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Selection parameters included primers targeting a unique sequence for each gene of 80-150 bp, if possible, primers were separated by an intron to avoid genomic DNA amplification, and an optimal melting temperature of 62 °C. When possible, self-complementary, GC% above

70 and differences in melting temperature between forward and reverse primer were avoided. Primer amplification efficiency at different concentrations was determined by serial dilutions of cDNA and standard curve analysis. Amplicons resulted from the PCR were run on agarose gels to confirm the size was the same as the product length predicted by Primer-BLAST.

5.3. qRT-PCR

Real-Time PCR was performed either in a ViiA7 System or QuantStudio 6 Flex Real-Time PCR System (Life Technologies) using Perfecta SYBR Green SuperMix Low Rox (Quanta Bio). All reactions were run in a final volume of 6 μl, of which 1 μl was cDNA and the annealing temperature was set at 62°C. The ΔΔCt quantitation method was used to determine the changes in gene expression. In all cases, 36B4 was used as a housekeeping gene. Details of the primers are in Table 4.

Gene	sequence 5>3	Specie	Primer Concentration (nM)
SOX2 F	GCACATGAACGGCTGGAGCAACG	Human	900
SOX2 R	TGCTGCGAGTAGGACATGCTGTAGG	Human	900
NANOG F	CAGCTGTGTGTACTCAATGATAGATTT	Human	300
NANOG R	ACACCATTGCTATTCTTCGGCCAGTTG	Human	900
OCT 4 F	GACAACAATGAAAATCTTCAGGAG	Human	900
OCT 4 R	CTGGCGCCGGTTACAGAACCA	Human	900
WNT1 nonCDS F	ATGGTGTCATTCTGCCTGCT	Human	300
WNT1 nonCDS R	GACTTAGGAGGACCCGGAGA	Human	300
WNT1 CDS F	CTTCGGCAAGATCGTCAACC	Human	900
WNT1 CDS R	TAGTCACACGTGCAGGATTCG	Human	900
WNT3A F	GTGGAACTGCACCACCGT	Human	900
WNT3A R	ATGAGCGTGTCACTGCAAAG	Human	900
WNT7B F	TGGCGTCCTGTACGTGAAGCTC	Human	900
WNT7B R	CGGGGCTAGGCCAGGAATCTT	Human	900
WNT10B F	TGCGAATCCACAACAACAGG	Human	900
WNT10B R	CATGACACTTGCATTTCCGCT	Human	900
WNT11 F	AGACCGGCGTGTGCTATG	Human	900
WNT11 R	CACCTGTGCAGACACCAGAC	Human	900
TCF1 (TCF7) F	CCGTCTACTCCGCCTTCAAT	Human	100
TCF1 (TCF7) R	CGTAGAGAGAGAGTTGGGGGA	Human	100
TCF3 (TCF7L1) F	GTCACCATCTCCAGCACACTT	Human	300
TCF3 (TCF7L1) R	CCGGGGGAGAAGTGGTCATT	Human	300
TCF4 (TCF7L2) F	TGCCTTTCACTTCCTCCGAT	Human	900
TCF4 (TCF7L2) R	CAAGGGCCGCACCAGTTATT	Human	900
LEF1 F	AGCACGGAAAGAAAGACAGC	Human	300

LEF1 R	TCGTTTTCCACCTGATGCAGA	Human	300
ERα F	CCACCAACCAGTGCACCATT	Human	300
ERα R	GGTCTTTTCGTATCCCACCTTTC	Human	900
PR F	CGCGCTCTACCCTGCACTC	Human	900
PR R	TGAATCCGGCCTCAGGTAGTT	Human	900
PS2 F	TCGGGGTCGCCTTTGGAGCAG	Human	300
PS2 R	GAGGGCGTGACACCAGGAAAACCA	Human	300
AR F	GGTGTCACTATGGAGCTCTCACAT	Human	900
AR R	GCAATCATTTCTGCTGGCG	Human	900
TMEPAI F	CGAGATGGTGGGTGGCAGGTC	Human	900
TMEPAI R	CGCACAGTGTCAGGCAACGG	Human	900
TMPRSS2 F	CACGGACTGGATCTATCGACAA	Human	900
TMPRSS2 R	CGTCAAGGACGAAGACCATGT	Human	900
VIM F	GCTTCAGAGAGAGGAAGCCG	Human	600
VIM R	AAGGTCAAGACGTGCCAGAG	Human	600
SNAI2 F	GCCAAACTACAGCGAACTGG	Human	300
SNAI2 R	AGTGATGGGGCTGTATGCTC	Human	300
ZEB1 F	AAGAATTCACAGTGGAGAGAAGCCA	Human	300
ZEB1 R	CGTTTCTTGCAGTTTGGGCATT	Human	300
CDH1 F	AGCAGAACTAACACACGGGG	Human	600
CDH1 R	ACCCACCTCTAAGGCCATCT	Human	600
OCCL F	TCTCCCTCCCTGCTTCCT	Human	600
OCCL R	GAGCAATGCCCTTTAGCTTCC	Human	600
36B4 F	GTGTTCGACAATGGCAGCAT	Human	300
36B4 R	AGACACTGGCAACATTGCGGA	Human	300
Wnt1 F	CTGTGCGAGAGTGCAAATGG	Mouse	600
Wnt1 R	GATGAACGCTGTTTCTCGGC	Mouse	600
36B4 F	TCCAGGCTTTGGGCATCA	Mouse	900
36B4 R	CTTTATCAGCTGCACATCACTCAG	Mouse	900

Table 4. Table summarizing the primers used in this study.

6. PROTEIN ANALYSIS

6.1. Sample preparation

Cell extracts were obtained by lysing cells in radioimmunoprecipitation assay (RIPA) lysis buffer (Millipore) with phosStop (Roche), Protease Inhibitors (Roche) and 0.1% SDS (Life Technologies) and centrifuged for 10 min at 15 x 10^3 g. The supernatants were mixed with 2X Laemmli Buffer (Sigma) and heated at 95 ºC for 5 min. The amounts of lysis buffer used for each plate when 70-80% confluent are shown in Table 5.

Plate size	12-well	6-well	6 cm	10 cm
RIPA 1X (µl)	100	200	300	800

Table 5. Volume of RIPA lysis buffer used for different plate size.

Extracts from extracellular matrix (ECM) were obtained as described by Howe *et al.*, (1999). Briefly, cells were removed by incubating with PBS containing 2 mM EDTA for 10 min and proteins remaining on the plate were directly solubilized by boiling in 2X Laemmli Buffer (Sigma). A parallel gel was run with the same amount of sample stained using Coomassie.

6.2. Immunoprecipitation

HEK293 cells (3.5 x 10^5 cells) were plated in 6 cm plates and transfected with 1250 ng of DNA. Cells were harvested 24 h following transfection and extracted using lysis buffer (50 mM TrisHCl (pH 8), 1% Triton X-100, 150 mM NaCl, 1mM EDTA) supplemented with Complete, EDTA-free protease Inhibitor cocktail tablets (Roche) and phosStop (Roche). Cell extracts were clarified by centrifugation for 12 min at 15 x 10^3 g at 4 ºC and incubated with anti-1D4 antibody on a rotating wheel at 4 ºC for 90 min before another incubation with protein A/G-Plus agarose beads for 1 h. After three washes in lysis buffer, the beads were resuspended in SDS sample buffer and heated at 37 ºC for western blotting.

6.3. Western blotting

Extracts were separated on SDS polyacrylamide gels, with the acrylamide/bis-acrylamide (Sigma) concentration between 6 and 12% depending on the size of the target protein. The amounts of the other components used for the gels are shown in Table 6.

Reagent	Volume (ml) for a X % resolving gel	Volume (ml) for stacking gel
H_2O	3.767 – $V_{acrylamide}$	0.68
30% Acrylamide/Bis-acrylamide	X * 0.03 * 5	0.17
1,5 M Tris (pH 8,8)	1.3	0.13
10 % SDS	0.05	0.01
10 % Ammonium persulfate	0.05	0.01
TEMED	0.003	0.002

Table 6: Reagents used for preparing SDS polyacrylamide gels.

Gels were run at 15 V/gel for 2 h and transferred to 0.45-micron filter nitrocellulose membranes (Millipore) at 10 V for 30 min using a Semi-Dry Transfer Cell (BioRad). Membranes were washed in TBST (Tris-buffered saline, 0.05% Tween (Sigma)) and incubated in blocking buffer 3% BSA in TBST for 1 h. Primary antibodies diluted in blocking buffer were incubated overnight and after washing with TBST three times, HRP-conjugated secondary antibodies diluted at 1:20,000 in blocking buffer were incubated for 1 h. After washing in TBST, membranes were developed using chemiluminescence (Clarity Western ECL Substrate, BioRad). Information about the antibodies used is detailed in Table 7.

Antibody	Company	Specie	Application	Concentration
WNT1	**Santa Cruz**	**Rabbit**	**WB, ECM extracts**	**1:200**
WNT1	**Enzo Life Sciences**	**Rabbit**	**WB, IF, IHC**	**1:500; 1:25; 1:200**
ER	**Novocastra**	**Mouse**	**WB**	**1:2000**
PR	**Novocastra**	**Mouse**	**WB**	**1:1000**
1D4	**Santa Cruz**	**Mouse**	**WB, IF**	**1:1000; 1:200**
V5	**Invitrogen**	**Mouse**	**WB, IF**	**1:1000; 1:200**
GFP	**Roche**	**Mouse**	**WB**	**1:1500**
GAPDH	**Santa Cruz**	**Mouse**	**WB**	**1:1000**
HSP60	**Santa Cruz**	**Rabbit**	**WB**	**1:5000**
APC-CD44	**BD**	**Mouse**	**FACS**	**1.5 µg/ml**
PE-CD24	**BD**	**Mouse**	**FACS**	**25 µg/ml**

Table 7: Table summarizing the antibodies used in this study.

7. IMMUNOFLUORESCENCE

5 x 10^4 cells/well were seeded in 24-well plates containing cover slips and cultured for 24 h. Transfection was performed after 24 h (See section 2). After 24 h, cells were washed twice with PBS and fixed with 4% paraformaldehyde (Santa Cruz) for 20 min and permeabilized with 0.1% TritonX-100 (Sigma) in PBS for 10 min. Blocking buffer was prepared with 2% BSA, 0.01% NaN_3 and 50 mM Glycine (Sigma). After blocking samples for 1 h, antibodies diluted in blocking buffer were incubated overnight and washed three times with PBS. AlexaFluor conjugated antibodies diluted at 1:500 were incubated for 1 h. Details of the primary antibodies used are in Table 7.

Vectashield mounting medium with DAPI (Vector laboratories Laboratories Inc.) was used to mount coverslips and samples were stored at 4 °C in the dark. Pictures were taken using a Zeiss fluorescence microscope Axioimager D1 and a Leica Confocal microscope.

8. IMMUNOHISTOCHEMISTRY

To determine the conditions for detection of Wnt-1 by IHC, two breast invasive ductal carcinoma tissue arrays (T089a), including 3 cases of breast invasive ductal carcinoma, medullary carcinoma and invasive lobular carcinoma, were used to test different conditions for antibody staining. Figure 13 shows an example of images from the array stained with Wnt-1 antibody, which was found to be optimal at 1:200 dilution in pH 6.0 citrate buffer, and the respective negative control of the condition sample.

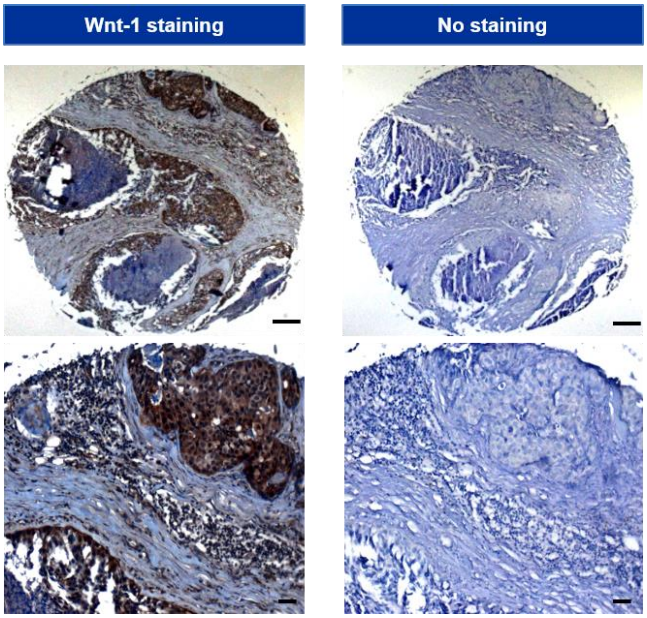


Figure 13. Immunohistochemistry of T089a tissue array used for tritation of Wnt-1 antibody. *On the left a sample stained with antibody at 1:200 and on the right the same sample only stained with secondary antibody. DAB agent was used for 10 sec in both cases. Low scale bars = 100 μm and high scale bars = 20 μm.*

Tissue microarrays were obtained from US Biomax Inc. BR2082b TMA contains 192 specimens, including 32 cases of metastatic carcinoma, 69 invasive ductal carcinomas, 21 lobular carcinomas, 4 squamous cell carcinomas, 17 intraductal carcinomas, 1 lobular carcinoma *in situ*, 9 fibroadenomas, 8 hyperplasias, 12 inflammatory tissues, 17 adjacent normal breast tissues and 2 normal breast tissues, that had been classified according to patient pathology, grade (I-IV), TNM grading and ER, PR and HER2 expression levels. Detailed information of the tumor samples can be found at https://www.biomax.us/tissue-arrays/Breast/BR2082b.

Paraffin-embedded tissue sections were deparaffinized with Histo-Clear II (National Diagnosis) and then rehydrated through citrosol and transferred through four changes of 100, 96, 70% ethanol and water. Antigen retrieval was performed using sodium citrate buffer at pH 6.0 in a pressure cooker for 20 min and endogenous peroxidase was blocked with 3% H_2O_2 for 10 min and washing three times in PBS for 5 min. Avidin/Biotin blocking kit (Thermo Fisher Scientific) was used, following the manufacturer´s instructions. Sections were incubated for 30 min in blocking solution containing 5% horse serum in PBS. Staining was performed overnight with rabbit-anti human Wnt-1 antibody (See Table 7) in Antibody Diluent (DAKO North America) at 4 ºC. Afterwards, sections were incubated with biotinylated secondary antibody by Vectastin® Elite ABC Kit (1:200, Vector Laboratories Inc.) in blocking buffer for 30 min and diaminobenzidine (DAB) (DAKO) was used as a chromogenic agent for detection. Sections were counterstained with Mayer´s hematoxylin (Sigma) for 10 sec and mounted with DPX (Sigma). Stained samples were visualized using a Zeiss light microscope Axioimager A1.

9. STATISTICAL ANALYSIS

Data shown are the averages of 3 or 4 independent experiments. In the case of luciferase assays, RT-PCR, proliferation assays, mammosphere and colony formation assays, each experiment was the result of the average of triplicates. Two-sided student´s t test for single comparison or one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test (post-hoc analysis) was applied to determine significance and $P < 0.05$ was considered to be statistically significant, unless otherwise specified. For TMA analysis, patients were divided into low (0, 1) and high (2, 3) expression and analyzed by one-way v2 test, Pearson Chi-square test with correction or Fisher’s exact test, two-sided, available on the VassarStats website (http://vassarstats.net/). If the contrary is not indicated error bars show SD. Microsoft Excel and Graph-pad Prism 6.0 were used for statistical analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Results

I. WNT SIGNALING IN TAMOXIFEN RESISTANT MODELS

1. WNT gene expression in CSC enrichment conditions in two tamoxifen resistant cell lines

Several tamoxifen resistant breast cancer cell lines have been developed and characterized in our laboratory. Both MCF-7 and T47D parental cell lines are ER+. However, when T47D cells became resistant to tamoxifen ER expression is lost. The mammosphere formation assay is a widely used technique that has been described to increase the enrichment of CSCs (Dontu, El-Ashry and Wicha, 2004). Tamoxifen resistant MCF-7 breast cancer cells show increased efficiency for formation of primary (MS I) and secondary (MS II) mammospheres. Moreover, MCF-7TamR and T47DTamR cell lines showed a significant increase in the proportion of $CD44^{+}CD24^{-/low}$ cells and an increase in *SOX2* mRNA and protein levels (Piva *et al.*, 2014).

The first aim of this project was to identify Wnt ligands that might be involved in the maintenance of CSCs in tamoxifen resistant cells. For this point, we used two cell lines MCF-7TamR and T47DTamR, which were cultured in low attachment conditions to promote mammosphere formation and increase the proportion of CSCs. As expected, under these conditions, we found a clear increase in the $CD44^{+}CD24^{-/low}$ CSC population in both cell lines (Fig. 14A). On average, the increase was 5-fold for MCF-7TamR cells and 2-fold for T47DTamR cells (Fig. 14B).

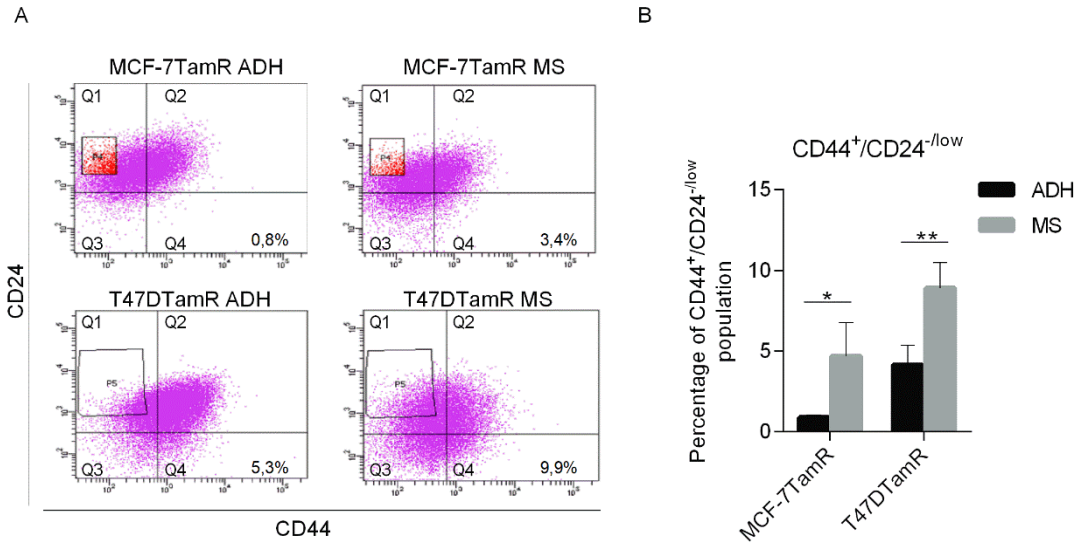


Figure 14. FACS analysis of tamoxifen resistant breast cancer cell lines. A. *Representative analysis of MCF-7TamR and T47DTamR cells stained for CD24 and CD44. $CD44^{+}CD24^{-/low}$ population was increased in all experiments when cells were grown in low attachment conditions (MS). NonCSC population were selected for sorting cells from Q1 corresponding to the phenotype $CD44^{-}CD24^{+}$ and CSC population from Q4 for the phenotype $CD44^{+}CD24^{-/low}$.* ***B.*** *Percentage of $CD44^{+}CD24^{-/low}$ cells in adherence (ADH) and in mammospheres (MS) for MCF-7TamR and T47DTamR cells; n=4,* P < 0.05; ** P < 0.01, Student´s t test. ADH, adherent; MS, mammospheres.*

In order to determine the expression levels of Wnt family genes in CSCs, cells expressing high levels of CD44 and low levels of CD24 were FACS-sorted and WNT gene expression levels were analyzed by qRT-PCR. WNT gene expression was also analyzed in adherent cells and in the bulk cell population that form mammospheres, which is a mixture of CSCs and cells that may provide a CSC niche. At least 8 WNT genes have been reported to be expressed in human breast tissue, WNTs 2, 3, 4, 5A, 7B, 10B, 13 (2B) and 14 (9B) (Kirikoshi, Sekihara and Katoh, 2001). Huguet *et al.*, (1994) studied WNT gene expression in human breast cancer, non-tumorous breast tissue and in human breast and breast cancer cell lines. They observed expression of *WNT3, WNT4* and *WNT7B* in human breast cell lines, and Wnt-2, Wnt-3, Wnt-4 and Wnt-7b proteins in human breast tissues. *Wnt2, Wnt4* and *Wnt7b* over-expression was associated with abnormal proliferation while *Wnt1* (Lee *et al.*, 1995) and *Wnt3* (Callahan, 1996) were found to be involved with tumorigenesis in mice. These different functions of Wnt family members in the mammary gland were later corroborated by Naylor *et al.*, (2000), who observed that *Wnt4, Wnt5b* and *Wnt7b* were expressed during normal mammary gland development, but not *Wnt1*, expression of which induces ductal branching and hyperplasia.

It was also observed that *Wnt7b* does not affect mammary gland development although in cell culture it behaves in a similar way to *Wnt1*. *Wnt1* was the first oncogene identified in naturally occurring mammary tumors in mice (Nusse R*et al*., 1984) and is now used in genetically manipulated mouse models of breast cancer using mouse mammary tumor virus (MMTV), although other Wnts, such as *Wnt3a* (Roelink *et al.*, 1990) and *Wnt10b* (Roelink *et al.*, 1990), are also used for insertional activation in some tumors (Li, Hively and Varmus, 2000). There are publications that claim that the activation of canonical Wnt signaling, mostly by Wnt-1 or Wnt-3a, in breast cancer and breast stem cells increases the population of stem-like cells (Lamb *et al.*, 2013); Wnt-7b (Wang *et al.*, 2005) and Wnt-10b (Wend *et al.*, 2013) have also been described to activate canonical Wnt signaling. For these reasons, *WNT1, WNT3A, WNT7B* and *WNT10B* were selected to be analyzed in the context of CSCs in tamoxifen resistant cell lines.

In MCF-7TamR cells, *WNT1* levels were significantly higher in mammospheres than in adherent cells (Fig. 15A) and there was a trend for an increase in the $CD44^{+}CD24^{-/low}$ cells, compared to the $CD44^{-}CD24^{+}$ cells (Fig. 15B). There was also a trend for increased *WNT3A* expression in mammospheres, compared to adherent cells. However, *WNT3A* expression was similar in $CD44^{+}CD24^{-/low}$ cells and $CD44^{-}CD24^{+}$ cells (Fig. 15A, B). *WNT7B* expression levels in mammospheres were unchanged and were lower in the $CD44^{+}CD24^{-/low}$ cells and *WNT10B* expression was not altered in mammospheres or in CSCs (Fig. 15A, B). In T47DTamR cells, *WNT1* and *WNT3A* expression levels were highly variable and did not increase significantly in mammospheres (Fig. 15C). Moreover, *WNT3A* levels were significantly lower in the $CD44^{+}CD24^{-/low}$ population and as observed in MCF-7TamR cells, *WNT7B* levels also showed a tendency to be lower, while the expression of *WNT1* and *WNT10B* was unchanged (Fig. 15D).

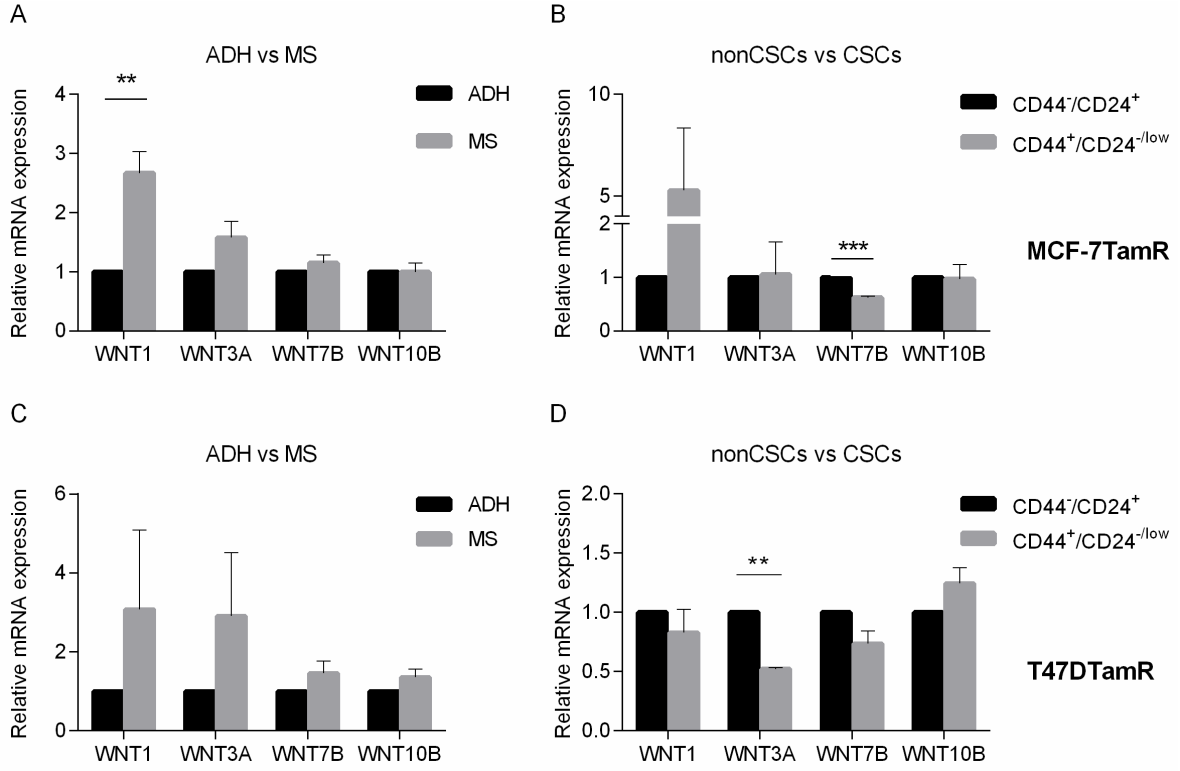


Figure 15. Analysis of mRNA level of WNT genes. A. *Relative mRNA expression of the indicated WNT genes, comparing adherent (ADH) MCF-7TamR cells and mammospheres (MS); n=4.* ***B.*** *Relative mRNA expression of WNT genes comparing sorted $CD44^{-}CD24^{+}$ population (nonCSCs) and $CD44^{+}CD24^{-/low}$ population (CSCs) in MCF-7TamR cells; n=4.* ***C.*** *Relative mRNA expression of the indicated WNT genes, comparing adherent (ADH) T47DTamR cells and mammospheres (MS); n=4.* ***D.*** *Relative mRNA levels of WNT genes comparing sorted $CD44^{-}CD24^{+}$ population (nonCSCs) and $CD44^{+}CD24^{-/low}$ population (CSCs) in T47DTamR cells; n=4, ** P < 0.01, *** P < 0.001, Student´s t test, (error bars represent SEM). ADH, adherent; MS, mammospheres.*

2. Differences in canonical Wnt signaling in MCF-7 and T47D cells

To study the effect of Wnt ligands in both cell lines and the impact they may have in the activation of the canonical Wnt pathway, I carried out gene reporter assays using the Super8xTopFlash plasmid (β-catenin/TCF luciferase), which encodes eight LEF/TCF binding sites linked to firefly luciferase and provides a measure of Wnt/β-catenin (canonical) signaling activity. Renilla luciferase reporter (pRL-tk) was used as a control. Wnt signaling was activated using Wnt-3a-conditioned-medium (Wnt-3a CM) or by transfecting cells with plasmids expressing *WNT1, WNT3A, WNT7B* or *WNT10B* genes. Cells treated with Wnt-3a CM were also treated with IWP-2, which inhibits Wnt lipidation, preventing their secretion (Chen *et al.*, 2009), as a control for possible effects of endogenous Wnt ligands. Surprisingly, the β-catenin/TCF luciferase reporter was not activated by transfection of any of the WNT gene expression plasmids in MCF-7TamR cells (Fig. 16A) or by Wnt-3a CM (Fig. 16B). On the other hand, in T47DTamR cells the canonical pathway was significantly activated by transfection of *WNT1* and *WNT3A* plasmids. Transfection of *WNT10B* plasmid showed a trend for activation, while *WNT7B* had no effect (Fig. 16C). In addition, Wnt-3a CM strongly activated β-catenin/TCF luciferase in T47DTamR cells (Fig. 16D). These results indicated that MCF-7TamR and T47DTamR cells respond differently to Wnt ligands, with respect to activation of β-catenin/TCF luciferase.

MCF-7 and T47D cells have been reported to respond differently to sFRP1 treatment and DVL knockdown (Schlange *et al.*, 2007). Actually, sFRP1 treatment reduced the level of active β-catenin measured by western blotting in T47D cells but not in MCF-7 cells. These data are consistent with our observation that MCF-7TamR do not show a canonical response to Wnts. Therefore, differences in the response of MCF-7TamR and T47DTamR to Wnt ligands are unlikely to be due to different mechanisms of resistance acquisition. However, others have shown that Wnt-3a stimulates *AXIN2* expression in MCF-7 cells (Wang *et al.*, 2014). In order to determine the reason for the different responses, we considered the roles of ER, AR and TCF family members. T47DTamR cells have lost expression of ER, which could affect Wnt signaling (El-Tanani *et al.*, 2001), and AR has a role in tamoxifen resistance (De Amicis, F.*et al*., 2010) and canonical *WNT7B* is an AR target gene (Zheng, D. *et al.,* 2013). TCF family members are key mediators of canonical Wnt signaling and during the course of my studies were reported to be differentially expressed in MCF-7 and T47D cells (Jamieson *et al.*, 2016).

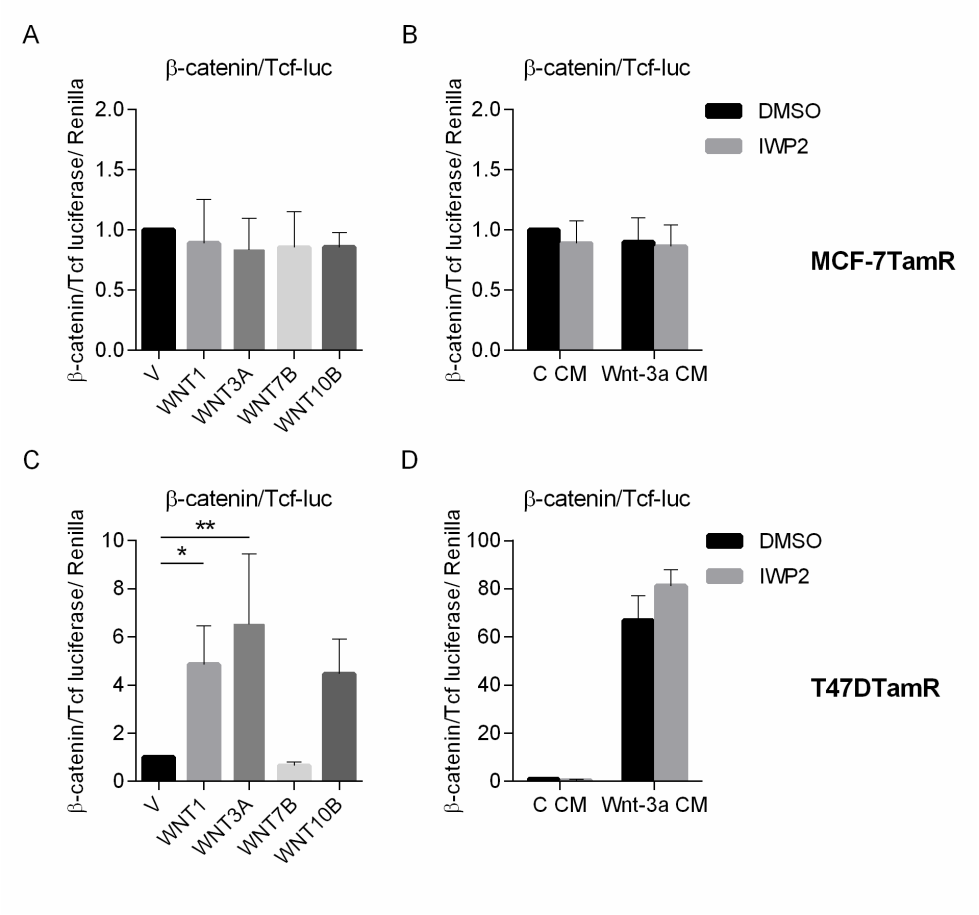


Figure 16. β-catenin/TCF luciferase activity in tamoxifen resistant breast cancer cell lines. A. *Relative β-catenin/TCF luciferase activity mediated by transfection of plasmids encoding different WNTs in MCF-7TamR cells; n=3.* ***B****. Relative β-catenin/TCF luciferase activity in MCF-7TamR cells treated with C CM or Wnt-3a CM; n=3.* ***C.*** *Relative β-catenin/TCF luciferase activity mediated by transfection of plasmids encoding different WNTs in T47DTamR cells; n=3.* ***D.*** *Relative β-catenin/TCF luciferase activity in T47DTamR cells treated with control CM or Wnt-3a CM; n=2, * P < 0.05; ** P < 0.01, Student´s t test (error bars represent SD). C CM, control conditioned-medium; Wnt-3a CM, Wnt-3a-conditioned-medium.*

2.1 Effects of ER on the Wnt response

Since ER expression was lost in one of the cell lines during the acquisition of resistance, we investigated whether ER expression was involved in the canonical response to Wnt-1 and Wnt-3a. In mesenchymal progenitor cells, E2 does not affect β-catenin/TCF reporter activity, but *WNT3A* up-regulates ERα expression to induce osteogenic differentiation (Gao *et al.* 2013). Moreover, a genetic interaction between human ERα and β-catenin signaling has been reported (Kouzmenko *et al.*, 2004).

First, I determined if the different Wnt responses of T47DTamR and MCF-7TamR cells resulted from differential expression of ER. ER levels in T47DTamR cells were lower than in parental T47D-C cells (Fig. 17A). This was not the case in MCF-7TamR cells, although ER transcriptional activity was reduced, as indicated by reduced expression of its target gene PR (Fig. 17B). In order to test the hypothesis that high ER levels repress the Wnt response, β-catenin/TCF gene reporter assays were carried out in MCF-7C and T47D-C cells. The reporter was not activated by transfection of any of the WNT gene expression plasmids in MCF-7C cells. In fact, *WNT1* and *WNT7B* reduced β-catenin/TCF reporter activity. Reporter activity in T47D-C cells was inhibited by *WNT7B*, as in MCF-7C cells. However, it was activated by both *WNT1* and *WNT3A*. The strongest response of T47DTamR cells to Wnt-1, as compared to T47D-C cells, suggests that ER levels may affect the Wnt-1 response in these cells. However, MCF-7C and T47D-C cells express similar levels of ER and show different responses to Wnt-1, so the different responses of MCF-7 and T47D cells are unlikely to be related to ER.

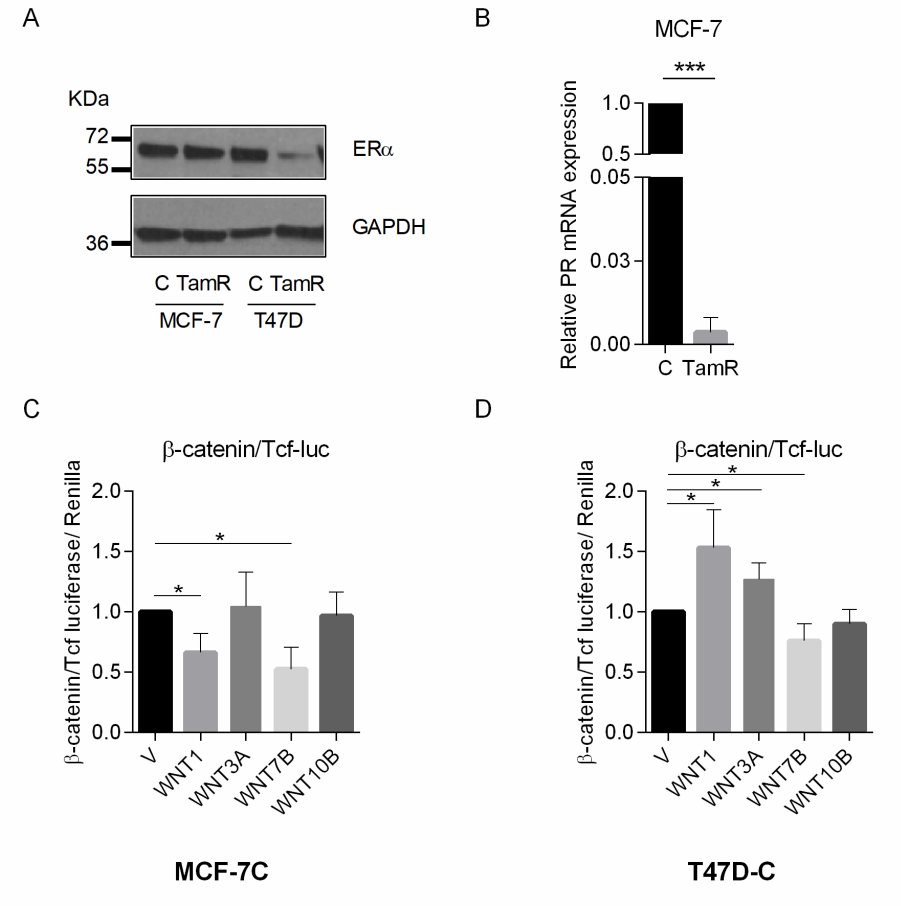


Figure 17. Effect of the ER on the Wnt response. A. *Protein expression of ER in human breast cancer cell lines.* ***B.*** *Relative mRNA expression level of PR in MCF-7 cell lines; n=3.* ***C.*** *Relative β-catenin/TCF luciferase activity mediated by transfection of plasmids encoding different WNTs in MCF-7C cells; n=4.* ***D.*** *Relative β-catenin/TCF luciferase activity mediated by transfection of plasmids encoding different WNTs in T47D-C cells; n=4, * P < 0.05; *** P < 0.001, Student´s t test (error bars represent SD).*

2.2 Effect of AR on the Wnt response

AR is a nuclear receptor whose link to Wnt signaling has been studied in more depth than that of ER. As previously described, β-catenin modulates AR (Verras, M. Brown, J. Li, X. Nusse, R. Sun, 2004), but there is also evidence of AR modulating Wnt. For example, AR provides a vehicle for trafficking β-catenin to the nucleus (Mulholland *et al.*, 2002). In addition, AR signals regulate the expression of several WNT genes in prostate cancer, including *WNT7B* (Zheng *et al.*, 2013), and can contribute to ER transcriptional activity in aromatase inhibitor-resistant breast cancer cells (Rechoum *et al.*, 2014). Importantly, AR competes with TCF/LEF for binding to β-catenin (Kypta and Waxman, 2012). Therefore, high AR activity could be responsible for the low β-catenin/TCF activity in MCF-7TamR cells.

In order to study whether endogenous AR affected canonical Wnt signaling in MCF-7TamR cells, cells were treated with the AR antagonist MDV3100, and examined for changes in expression of WNT genes (Fig. 18A) and AR and ER target genes (Fig. 18C). Because androgens have been reported to negatively regulate *WNT11* expression in hormone-dependent LNCaP cells (Zhu *et al.*, 2004) and this can be inhibited by MDV3100, I also checked *WNT11* gene expression levels. MDV3100 treatment did not significantly affect the expression of *WNT1, WNT3A, WNT7B, WNT10B* or *WNT11* (Fig. 18A). However, *WNT11* levels were significantly lower in TamR cells, compared to control MCF-7C cells (Fig. 18B). MDV3100 did not affect expression of *AR* or its target genes in prostate cancer (*TMEPAI* and *TMPPRSS2)* or of the ER target gene *PS2*. In the case of PR, which is also an ER target gene, there was a tendency for reduction in MCF-7TamR cells (Fig. 18C). On the other hand, lower expression of PR, as shown previously in Fig. 17B, and *PS2* was observed in MCF-7TamR cells, compared to MCF-7C cells (Fig. 18D). In this comparison, *TMPPRSS2* levels were also higher but were not affected by MDV3100. These results suggest endogenous AR is not involved in the regulation of Wnt gene expression in TamR cells.

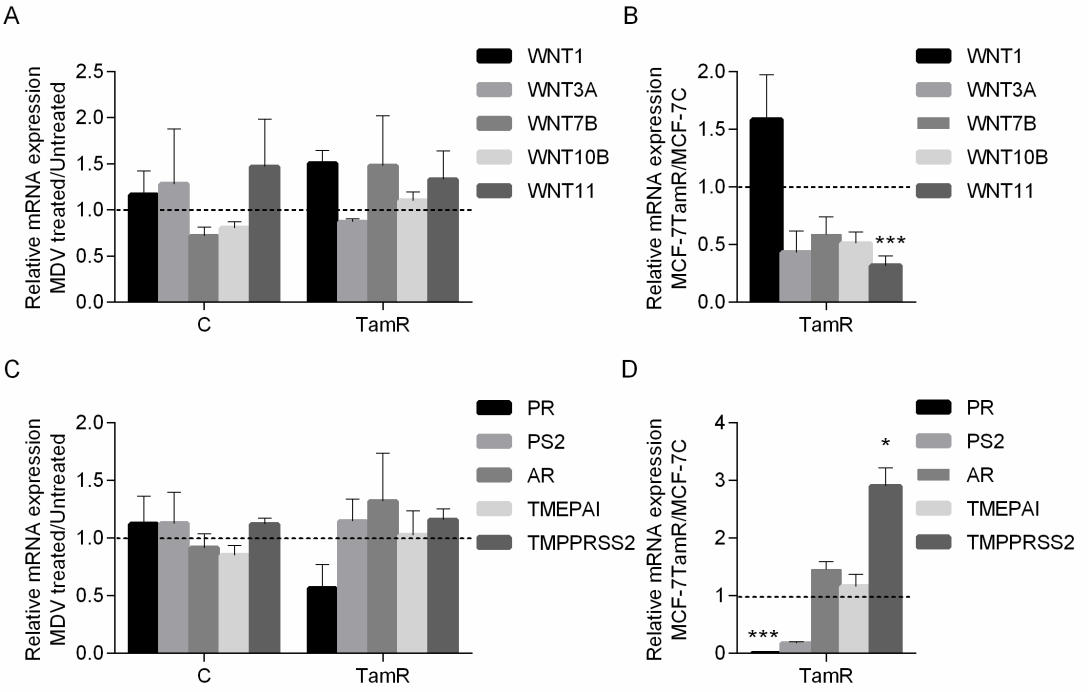


Figure 18. Effect of the AR antagonist MDV3100 on gene expression. A. *Relative mRNA expression of WNT genes in MCF-7 cells treated with MDV3100, normalized to untreated cells.* ***B.*** *Relative mRNA expression of WNT genes in MCF-7TamR cells compared to control cells.* ***C.*** *Relative mRNA expression of ER and AR target genes in MCF-7 cells after MDV3100 treatment, normalized to untreated cells.* ***D.*** *Relative mRNA expression of ER and AR target genes in MCF-7TamR cells compared to control cells; n=3, * P < 0.05; *** P < 0.001, Student´s t test (error bars represent SEM).*

In order to determine AR activity in MCF-7TamR cells, gene reporter assays were carried out using MMTV-luc and ARR2-Pb-luc reporters, which are reporters used to measure transcriptional activity of AR. As a control for MDV3100 activity, gene reporter assays were carried out in the prostate cancer cell line C4-2B. In the presence of MDV3100, the transcriptional activity of both reporters was significantly reduced, as expected (Fig. 19A). AR reporter activation was stimulated in MCF-7TamR cells by the AR ligand DHT (Fig. 19B). However, MDV3100 did not affect basal AR reporter activity in cells (Fig. 19C). These results indicate that although AR is expressed and can be activated, it is not normally active in MCF-7TamR cells and so is unlikely to be responsible for the low β-catenin/TCF activity in these cells.

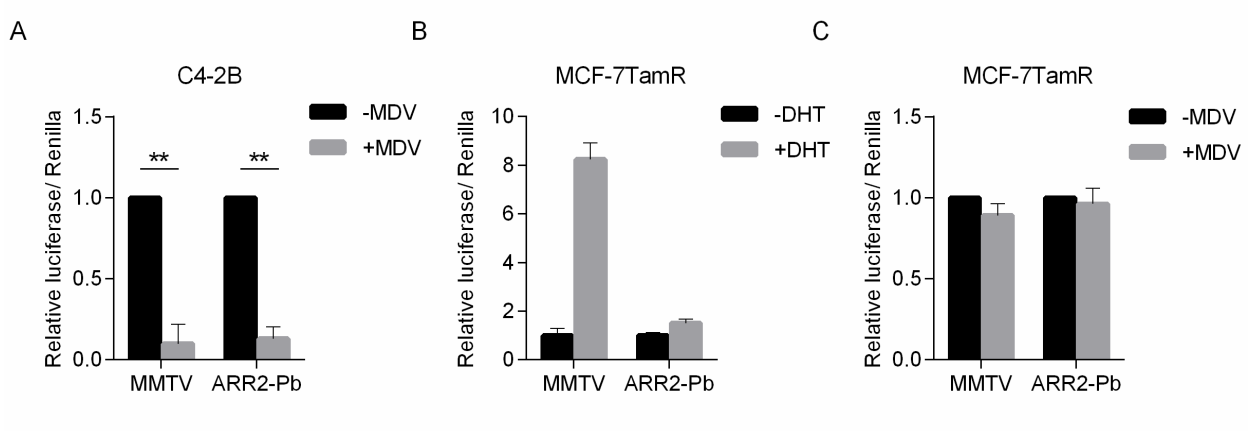


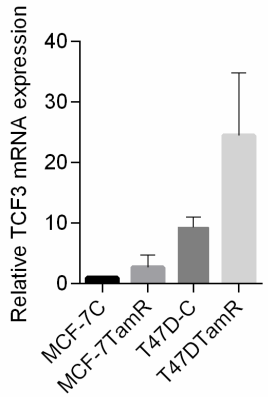
Figure 19. Effect of the AR antagonist MDV3100 on transcriptional activity of AR. A. *Relative luciferase activity using MMTV-luc and ARR2-Pb-luc reporters in C4-2B cells treated with MDV3100; n=3.* ***B.*** *Relative luciferase activity using MMTV-luc and ARR2-Pb-luc reporters in MCF-7TamR cells stimulated with the AR ligand DHT; n=2.* ***C.*** *Relative luciferase activity using MMTV-luc and ARR2-Pb-luc reporters in MCF-7TamR cells treated with MDV3100; n=3, ** P < 0.01, Student´s t test (error bars represent SD).*

2.3 Effect of TCF/LEF family factors on the Wnt response

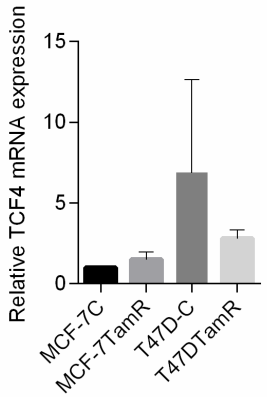
In order to explore other factors that could explain differences in the response to Wnt signaling in MCF-7TamR and T47DTamR cells, I examined differences in the mRNA expression levels of TCF/LEF family transcription factors, which can act as activators or repressors of Wnt signaling, depending on context (Sprowl and Waterman, 2013). In humans, high levels of *LEF1* mRNA have been reported in testis, adrenal gland, blood cells, spleen and ileum, *TCF1* is highly expressed in blood, spleen and small intestine, while *TCF3* is abundant in cervix, breast, colon, adipose tissue and colorectal cells and *TCF4* is found in a more ubiquitous pattern, although it is most highly expressed in breast (Hrckulak *et al.*, 2016).

LEF1 mRNA was undetectable in MCF-7 cells and *TCF1* (*TCF7*) was barely detected in any of the cell lines (Ct values were about 38); note ΔCT values in Fig. 20C. On the other hand, *TCF3* (*TCF7L1*) mRNA was abundant in all cell lines (Fig. 20C), and was more highly expressed in T47D cells than in MCF-7 cells (Fig. 20A) and more in TamR cell lines compared to the control cells. These data was consistent with the RNAseq data, which was performed after the generation of cell lines resistant to tamoxifen. Jamieson *et al.*, (2016) previously reported that TCF-3 protein is present in T47D cells but not in MCF-7 cells. Our results indicated that *TCF4* mRNA (*TCF7L2*) was less abundant than *TCF3* in T47D and in MCF-7 cells (Fig. 20 B and C). Nevertheless, there are different *TCF4* mRNA variants (Mao and Byers, 2011) that might account for the *TCF4* levels detected.

A



B



C

ΔCT	TCF1	TCF3	TCF4
MCF-7C	22,80	8,72	10,10
MCF-7TamR	23,59	8,08	9,48
T47D-C	22,02	4,43	7,82
T47DTamR	16,61	3,56	8,06

Figure 20. Relative RNA expression of TCF factors. A. *Relative mRNA levels of TCF3 in breast cancer cell lines.* ***B.*** *Relative mRNA levels of TCF4 in breast cancer cell lines.* ***C.*** *ΔCT values obtained for each TCF gene normalized with respect to 36B4; TCF1 (TCF7); TCF3 (TCF7L1); TCF4 (TCF7L2).*

Since MCF-7TamR cells do not respond to Wnt ligands it was considered that this might be due to the low expression of TCF/LEF family proteins. In order to determine if expression of TCF/LEF family members could restore the response to exogenous Wnt and activate β-catenin/TCF transcriptional activity, MCF-7TamR cells were transfected

with plasmids encoding β-catenin and TCF family genes. *TCF1* was chosen because of the availability of a *TCF1* expression plasmid and the low basal expression of *TCF1* in MCF-7 cells. In addition, I used *TCF4* as its levels were higher in T47D than MCF-7 cells and a *TCF4* plasmid was also available. β-catenin/TCF luciferase assays were carried out using cells transfected with plasmids encoding β-catenin, *TCF1* and *TCF4* and the β-catenin/TCF-responsive SUPER8XTOPFlash reporter. Co-expression of β-catenin with either *TCF1* or *TCF4* activated this reporter in MCF-7TamR cells (Fig. 21A). Surprisingly, *TCF1*, but not *TCF4* also activated the gene reporter independently of β-catenin (Fig. 21A). This result is reminiscent of a study in hematopoietic cells, in which TCF1 but not TCF4 associates with ATF2 to activate this reporter independently of β-catenin (Grumolato *et al.*, 2013). To test if a similar mechanism was taking place in TCF1-transfected MCF-7TamR cells, we used a dominant-negative form of ATF2, ΔATF2 (Steinmüller and Thiel, 2003). ΔATF2 mutant retains the basic leucine zipper domain needed for dimerization and DNA-binding, but lacks the N-terminal transcriptional activation domain. β-catenin independent activation of the reporter by TCF-1 was reduced when ΔATF2 was expressed (Fig. 21B) suggesting a role for ATF2.

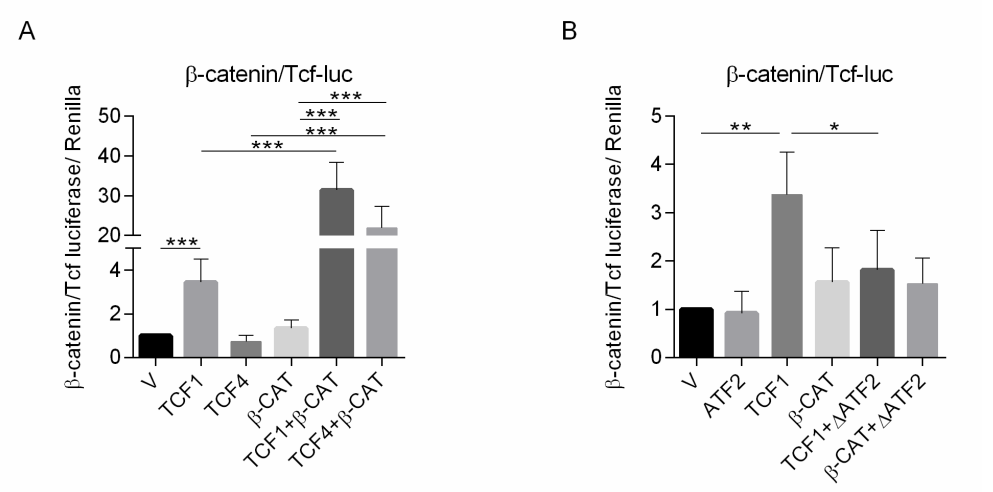


Figure 21. Effect of TCF/LEF family factors on the Wnt response. A. *Gene reporter assays measuring β-catenin/TCF-dependent transcriptional activity in MCF-7TamR cells expressing β-catenin and/or TCF1 and TCF4; n=4.* ***B.*** *Gene reporter assays measuring β-catenin/TCF-dependent transcriptional activity in MCF-7TamR cells expressing TCF1, β-catenin, ATF2 and ΔATF2; n=4, * P < 0.05; ** P < 0.01; *** P < 0.001, Student´s t test (error bars represent SD). β-CAT, β-catenin; ΔATF2, Dominant- Negative ATF2.*

3. WNT1 signaling in tamoxifen resistant cell lines

Wnt1 expression has been linked to enrichment of CSCs in a mouse model of breast cancer (Choi *et al.*, 2012) and has also been described to be over-expressed in human breast tumors (Ayyanan *et al.*, 2006). In C57MG mouse mammary epithelial cells, ectopic expression of *Wnt1* expression increases accumulation of cytosolic β-catenin (Shimizu *et al.*, 1997). Our results demonstrated that *WNT1* is the most upregulated WNT gene in the CSCs compared to the non-CSCs in MCF-7TamR cells, while none of the WNTs examined stood out in T47DTamR cells. However, ectopic expression of *WNT1* did not increase β-catenin/TCF luciferase activity in MCF-7TamR cells. Therefore, Wnt-1 signaling was studied in more detail.

In MCF-7TamR cells, canonical Wnt signaling was activated by TCF1 (Fig. 21 and 22A). In order to determine whether Wnt-1 is involved in TCF1-mediated signaling, both *WNT1* and *TCF1* were co-expressed in MCF-7TamR cells. As shown in Fig. 22A, expression of *WNT1* potentiated TCF1-activation of the TOPFlash reporter. In order to determine the effects of *WNT1* and *TCF1* using an alternative approach, I used TOPdGFP, which encodes GFP driven by a TCF-responsive promoter, and measured the number of GFP-positive cells by flow cytometry. As a control, a GFP driven by a control promoter (CMV) was used. The GFP signal obtained for each reporter was normalized to the signal captured for the same reporter co-transfected with an empty vector. The results revealed that expression of *TCF1* but not *WNT1* increased the number of GFP-positive cells when using the TOPdGFP reporter (Fig. 22 B).

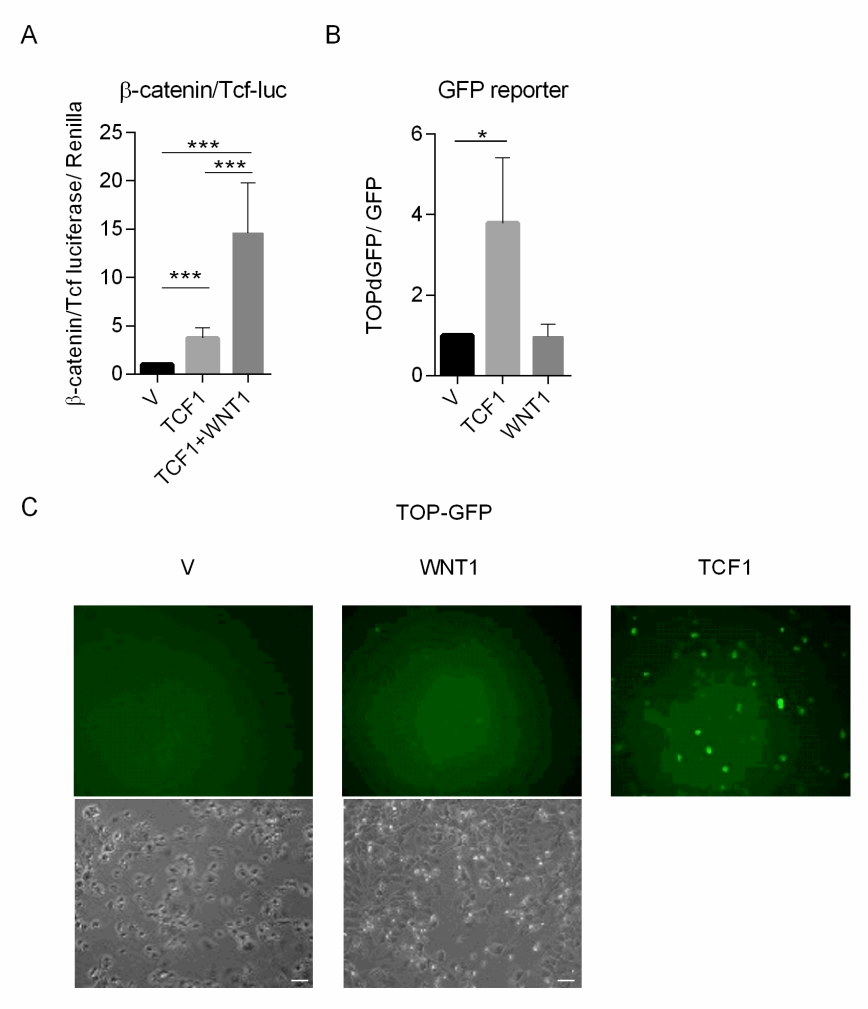


Figure 22. Effect of WNT1 expression in TCF1 mediated signaling. A. *Gene reporter assay measuring β-catenin/TCF-dependent transcriptional activity in MCF-7TamR cells co-expressing WNT1 and TCF1; n=3.* ***B.*** *GFP based reporter assay measuring TOPdGFP reporter by FACS. GFP signal was normalized using an empty GFP vector; n=5, * P < 0.05; *** P < 0.001, Student´s t test (error bars represent SD).* ***C.*** *Pictures of GFP positive cells transfected with TCF1 or WNT1 using TOPdGFP reporters. Bright field images are shown in insets to reveal cells.*

Although ATF2 has been found to be a partner of TCF1 for activation of canonical Wnt signaling, it also mediates non-canonical signaling. ATF2 forms dimers with other AP-1 family components and different heterodimers have different DNA binding specificities (Hai and Curran, 1991) and roles in cancer (van Dam and Castellazzi, 2001). To better understand the role of ATF2, the effect of *WNT1* expression on ATF2-mediated transcription was determined. The ATF2-dependent luciferase reporter is based on the C/EBP-activating transcription factor-responsive element that provides a readout for non-canonical WNT/JNK signaling (Ohkawara and Niehrs, 2011). Expression of *WNT1* in MCF-7TamR cells activated the ATF2-dependent luciferase reporter and also an ATF2-based GFP reporter (Fig. 23A and B). AP-1 family members can form homo- or heterodimers at ATF/CRE binding sites. Although Jun-Jun and Jun-Fos dimers can bind ATF/CRE

sites (van Dam and Castellazzi, 2001), ATF/CREB heterodimers have a higher affinity for these sites (Hai and Curran, 1991). Here, I have used dominant-negative constructs to block AP-1 family transcription factors. Cells were co-transfected with the ATF2 reporter and WNT1 together with dominant-negative forms of ATF2 (ΔATF2), CREB (ΔCREB) and Fos (ΔFOS). ΔATF2 binds and inhibits endogenous ATF2 and Jun (Hai and Curran, 1991), whereas ΔFOS binds Jun (Abate, Luk and Curran, 1991), and ΔCREB inhibits gene expression *via* inhibition of the DNA binding activity of CREB and is considered to be a specific inhibitor of CREB-mediated gene transcription (Ahn *et al.*, 1998). ΔATF2 showed the strongest inhibition of gene reporter activity (Fig. 23C), suggesting that ATF2 and its partners are important for Wnt-1 activation of the ATF2 reporter. Although dominant-negative ATF2, c-Jun and c-Fos all reduced the response to Wnt-1, ATF2 or its binding proteins had the strongest effect on Wnt-1 activation of ATF2-luciferase (Fig. 23C).

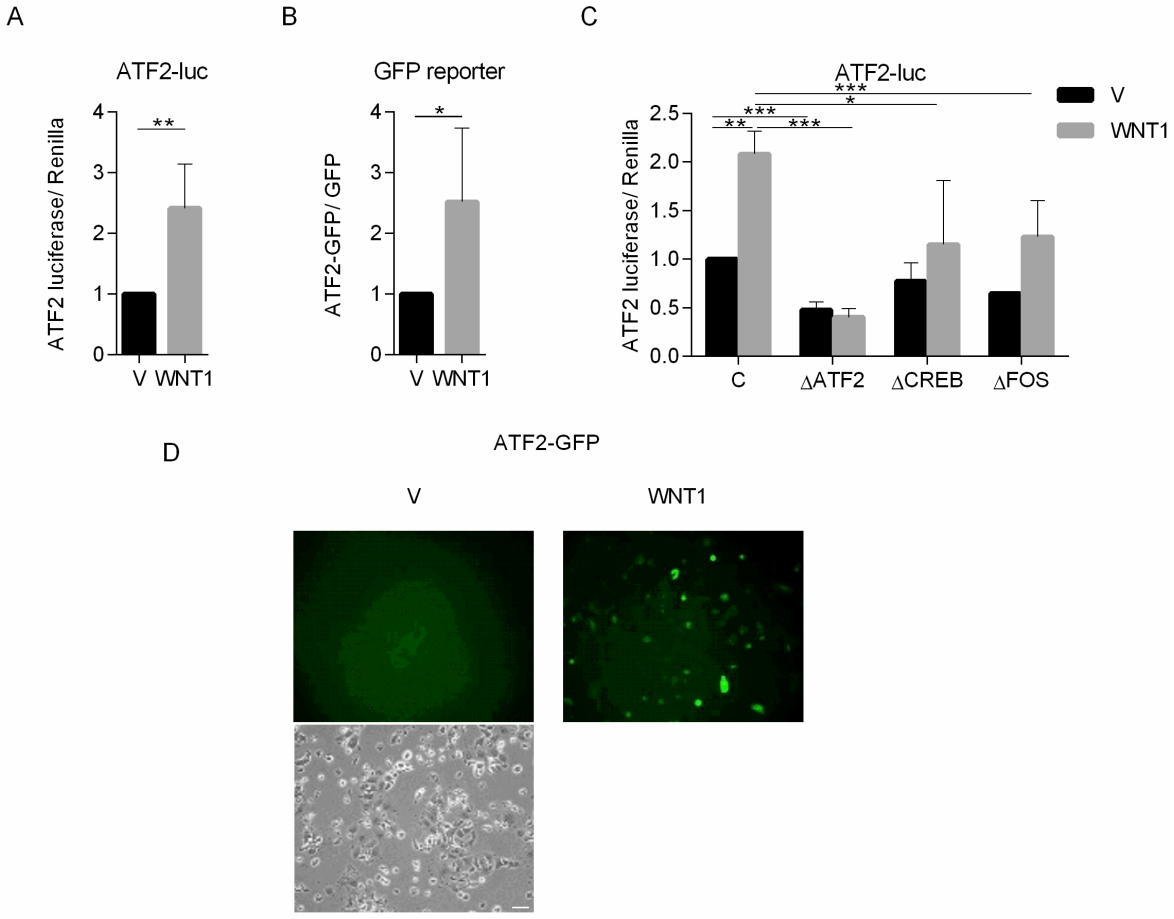


Figure 23. Effect of WNT1 on ATF2-dependent transcriptional activation in MCF-7TamR cells. ***A.*** *Gene reporter assay measuring ATF2-dependent transcriptional activity in MCF-7TamR cells expressing WNT1; n=4.* ***B.*** *GFP based reporter assays measuring ATF2dGFP reporter by FACS. GFP signal was normalized using a vector expressing GFP driven by the CMV promoter; n=5.* ***C.*** *Gene reporter assay measuring ATF2-dependent transcriptional activity in MCF-7TamR cells expressing dominant negative forms of ATF2, c-Jun and c-Fos in the presence or the absence of WNT1 expression; n=3, * P < 0.05, ** P < 0.01; *** P < 0.001, Student´s t test (error bars represent SD).* ***D.*** *Pictures of GFP-positive cells transfected with WNT1 using ATF2dGFP reporters. Bright field images are shown in insets to reveal cells. ΔATF2, dominant-negative ATF2; ΔCREB, dominant-negative CREB; ΔFOS, dominant-negative FOS.*

II. CHARACTERIZATION OF WNT1 EXPRESSION IN BREAST CANCER AND BREAST CANCER STEM CELLS

1. WNT1 expression in ER+ breast cancer patients

Based on our observations of *WNT1* gene expression levels in CSCs in MCF-7TamR cells and the interest of Wnt-1-mediated signaling in this context, we characterized the expression of *WNT1* in breast cancer. We examined publically available data from patients to analyze *WNT1* expression changes in patients, according to clinical characteristics that might identify some differences using the GOBO Gene set online tool (Ringnér et al., 2011). Results showed that there are no significant differences in *WNT1* expression with respect to molecular subtypes, ER status or tumor grade (Fig. 24).

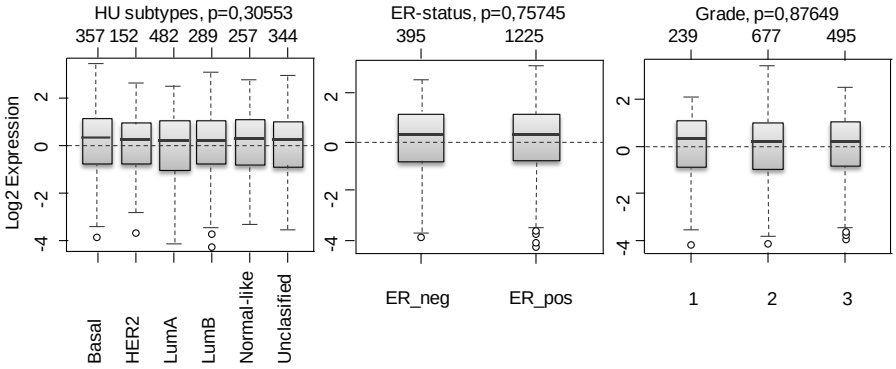


Figure 24. WNT1 expression in human breast tumor. *Box plot of WNT1 gene expression for tumor samples stratified according to HU subtypes* (Hu *et al.*, 2006)*, according to ER status or according to histological grade. Obtained from GOBO Gene set online tool. Dots represent outliers and bars minimum and maximum values excluding outliers.*

Afterwards, we analyzed possible links between *WNT1* expression and patient survival. Fig. 25A represents the disease free survival (DFS) of all breast cancer patients and patients classified according to breast cancer tumor subtypes. There were no differences in survival related to *WNT1* expression in any of these subgroups. However, more detailed analysis revealed a worse prognosis and a significant decrease in the overall survival (OS) and distant disease free survival (DDFS) among those patients expressing high levels of *WNT1* and presenting ER+ tumors who had undergone treatment with tamoxifen (Fig. 25B). In contrast, the whole patient population did not show any variation in the DFS rates related to the expression of *WNT1*. In order to reconsider other WNTs as possibly being linked to survival, we checked the other WNT genes (Table 8). Kaplan-Meier analysis using OS as endpoint is shown in Fig. 25C. Apart from *WNT1*, the expression levels of *WNT6, WNT7A* and *LEF1* were also correlated with poor prognosis. On

the contrary, low expression of *WNT7B*, *WNT5A, WNT11, TCF1 and TCF3* were associated with good prognosis (Table 8). These results indicate that *WNT1* expression might be important in the context of tamoxifen-treated patients.

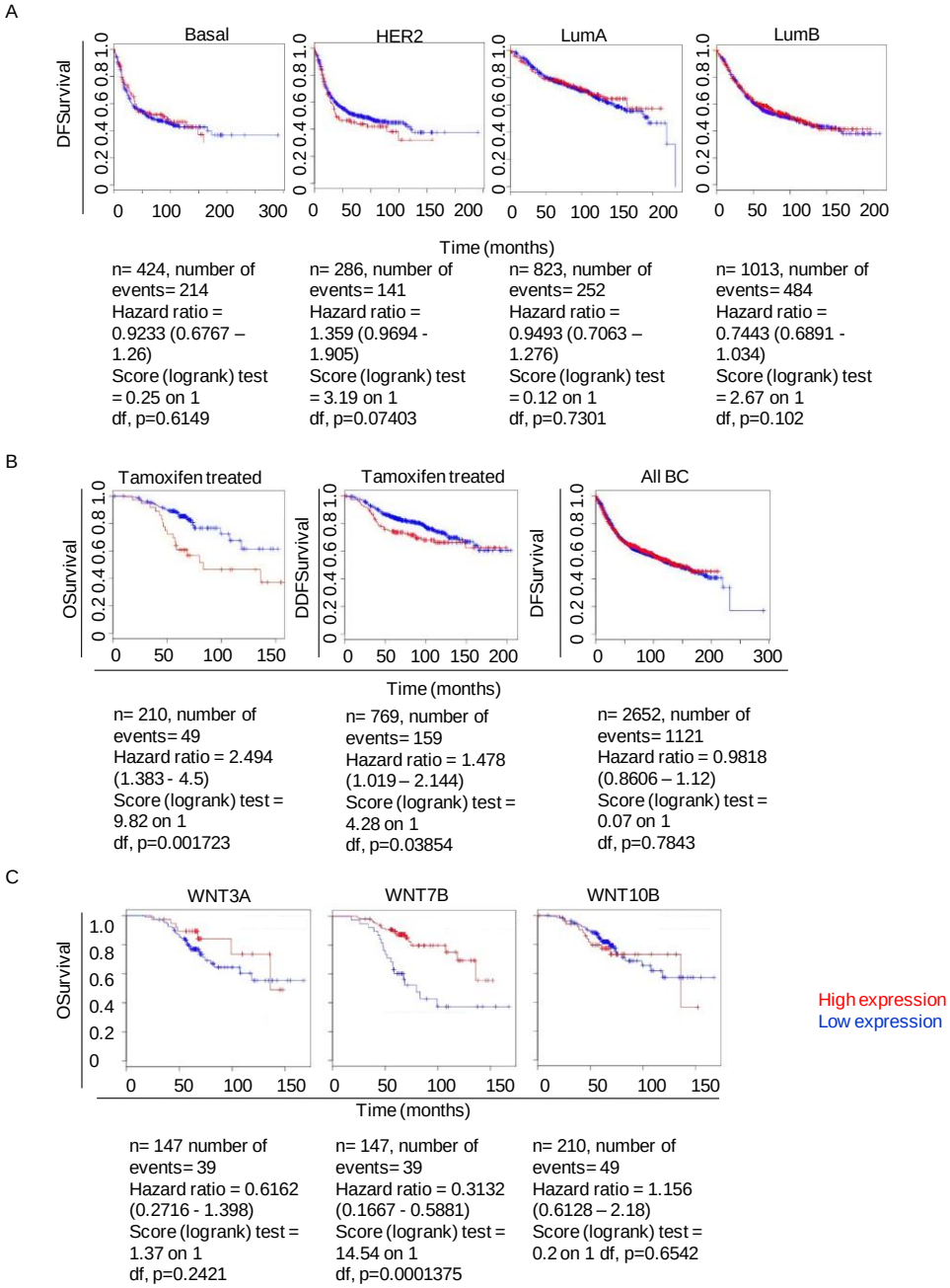


Figure 25. WNT1 expression in human breast tumors and survival effect. A. *Kaplan-Meier analysis, using DFS as endpoint for basal, HER2+, luminal A and luminal B tumors stratified into high and low WNT1 gene expression level.* ***B.*** *Kaplan-Meier analysis, using OS and DDFS as endpoint for ER+ tumors in patients treated with tamoxifen, stratified into high and low WNT1 gene expression level and using DFS for all BC patients stratified into high and low WNT1 gene expression level.* ***C.*** *Kaplan-Meier analysis, using OS as endpoint for ER+ tumors in patients treated with tamoxifen, stratified into high (red) and low (blue) WNT3A, WNT7B and WNT10B gene expression. DFS, disease free survival; OS, overall survival; LumA, luminal A; LumB, luminal B.*

Gene	OS	DDFS	DFS	Gene	OS	DDFS	DFS
WNT1	0.00172	0.03854		WNT8A			
WNT2				WNT8B			
WNT2B				WNT9A			
WNT3				WNT9B			
WNT3A				WNT10A			
WNT4				WNT10B			
WNT5A		0.02907		WNT11		0.0142	
WNT5B				WNT16			
WNT6	0.01495			TCF1	0.04609		
WNT7A	0.00027	0.01062		TCF3	0.00196	0.00163	0.00113
WNT7B	0.00013			TCF4			
				LEF1	0.00166	0.04741	

Table 8. Survival effect of WNT genes expression. *Kaplan-Meier analysis, using OS, DDFS and DFS as endpoint for ER+ tumors in patients treated with tamoxifen, stratified into high and low WNT gene expression level. Statistically significant p-values are indicated in red when high expression level and in blue when low expression level is associated with poor prognosis. OS, overall survival; DDFS, distant disease free survival; DFS, disease free survival.*

To better characterize the expression of *WNT1* in breast cancer, Wnt-1 protein levels were examined by immunohistochemistry (IHC) in a tissue array with a range of breast cancer pathologies (BR2082b-TMA). This array contains 192 different samples, including metastatic carcinomas, ductal and lobular carcinomas, fibroadenomas, hyperplasias and inflammatory tissues and also adjacent normal and normal breast tissues. Information provided for TNM, clinical stage, pathology grade, and ER, PR and HER2 IHC results were used for correlation analysis. We selected a Wnt-1 antibody that was previously validated for use in IHC in several publications (Königshoff *et al.*, 2008; Xie *et al.*, 2012). Prior to staining BR2082b-TMA, the antibody was tested and optimized using a smaller TMA (T089a) (See Fig. 13 in Material and Methods, Section 8).

Scoring of BR2082b-TMA IHC was performed using two approaches. First, in collaboration with a group of mathematicians, an algorithm was designed to automatically quantify and classify Wnt-1 staining. This method consists of four steps that are repeated for every sample. First, each tissue core is divided into 64 blocks, next DAB positive areas are identified and a color deconvolution process is performed. Then, an algorithm is used to cluster each block into four different intensities based on the Allred score, which calculates and combines the percentage of positive cells with the intensity of staining (Henriksen *et al.*, 2007). Finally, classification of

each sample is obtained from the reconstitution of all segments, providing a new image with quantification.

These results using this method were then compared with a second approach, in which staining intensities were scored independently by three people (MV, RK and EO) using the quickscoring method (Henriksen *et al.*, 2007). Divergences in scores between the two methods were then re-evaluated until consent was found. Scoring for Wnt-1 was further analyzed by a histopathologist (Dr. Ignacio Zabalza, Department of Pathology, Galdakao-Usansolo Hospital). Each score was based on the staining intensity as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (strong staining). A representative example for each staining intensity is shown in Fig. 26.

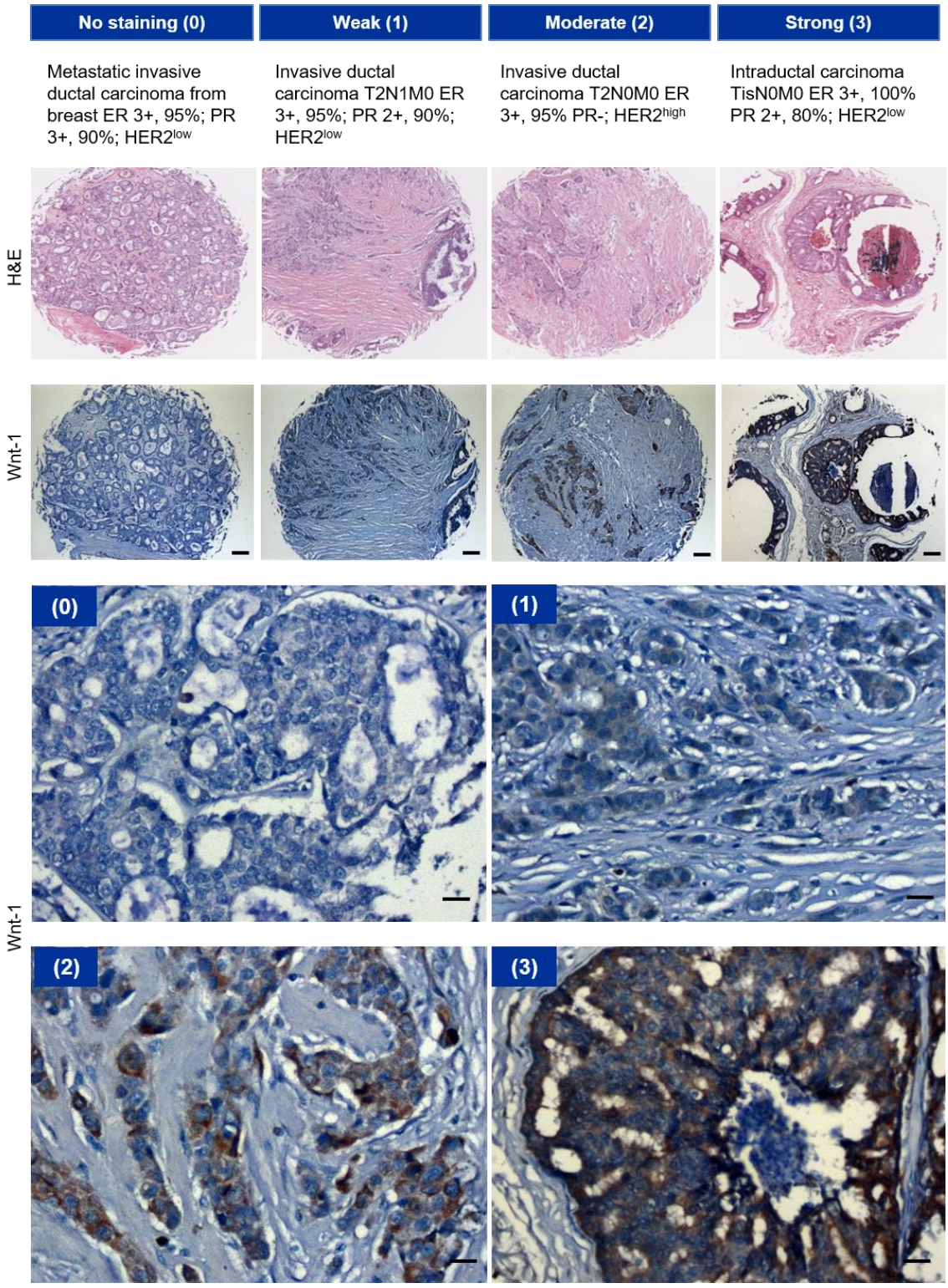
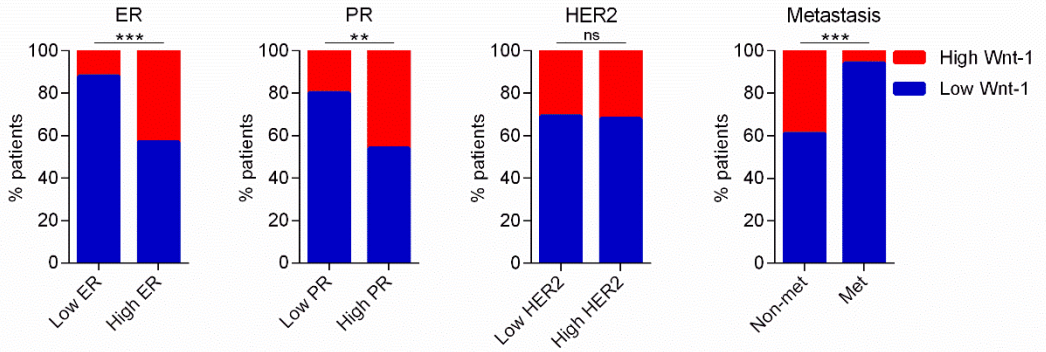


Figure 26. Scoring system for Wnt-1 staining in human breast tissue array. *Examples of Wnt-1 immunohistochemistry in breast cancer cells using the quick score method as 0 (negative), 1 (weak), 2 (moderate), 3 (strong). Staining of samples using H&E are shown in the upper row. These H&E images are available in [https://www.biomax.us/tissue-arrays/Breast/BR2082b.](https://www.biomax.us/tissue-arrays/Breast/BR2082b) The second row shows staining for Wnt-1 in low magnification. Scale bars = 100 μm. In the bottom part higher magnification images are shown. Scale bars = 20 μm. H&E, hematoxylin and eosin stain.*

In order to determine whether there was an association between Wnt-1 and expression of ER, PR or HER2, the expression levels of Wnt-1 by IHC were compared with the data provided in the specification sheet for the patient tumors. Wnt-1 was significantly higher in ER+ tumors than in ER- tumors. There was also a positive correlation for Wnt-1 and PR whereas there was no correlation between Wnt-1 and HER2. Low Wnt-1 levels were noticeable in metastatic tumors, suggesting an inverse correlation with metastasis. Statistical analysis confirmed positive correlations between Wnt-1 and both ER and PR and an inverse correlation between Wnt-1 and metastasis (Fig. 27A and B). In Fig. 27C some examples that are representative of these observations are shown. Regarding ER, Wnt-1 staining is higher in ER+ patients, in which strong Wnt-1 can be found in 43% of patients, compared to in 12% of ER- patients. From the selected sections, the first two columns represent ER+ tumors and the third and fourth columns ER- tumors, and examples of strong and a weak Wnt-1 staining are shown in each cases. With respect to PR, high Wnt-1 was found in 20% of PR- tumors and 46% in PR+ tumors. In a and b, two invasive ductal carcinomas are shown, both with the same TNM, ER and HER2 status but differing in PR status. Strong staining is detected in the PR+ sample (a) and weak staining in the PR- one (b), but there were also examples where high Wnt-1 staining was found in PR- tumors (c). HER2 expression did not correlate with Wnt-1, Wnt-1 staining being high in around 30% of patients. Examples a, b and d are invasive ductal carcinomas with low HER2, one with high Wnt-1 (a) and two with low Wnt-1 (b and d). Strong staining in a $HER2^{high}$ sample is shown in panel c. Finally, only 2 of the 31 metastatic expressed higher levels of Wnt-1, with the vast majority being negative, as is shown in Fig. 26 (example of no staining).

A



B

	ER+	ER -	PR+	PR -	$HER2^{high}$	$HER2^{low}$	Metastasis	No-metastasis
Wnt-1 high	36 (42.85%)	6 (11.76%)	27 (45.76%)	15 (19.73%)	27 (31.39%)	15 (30.61%)	2 (6.45%)	40 (38.46%)
Wnt-1 low	48 (57.14%)	45 (88.23%)	32 (54.23%)	61 (80.26%)	59 (68.60%)	34 (69.38%)	29 (93.54%)	64 (61.53%)
	ER+ v. ER-		PR+ v. PR-		HER^{high} v. HER^{low}		Met v. Non-Met	
p Chi Sq. Pearson	0.0002		0.0012		0.92		0.0007	
p Fisher 2t	0.00021		0.0015		1		0.00067	

C

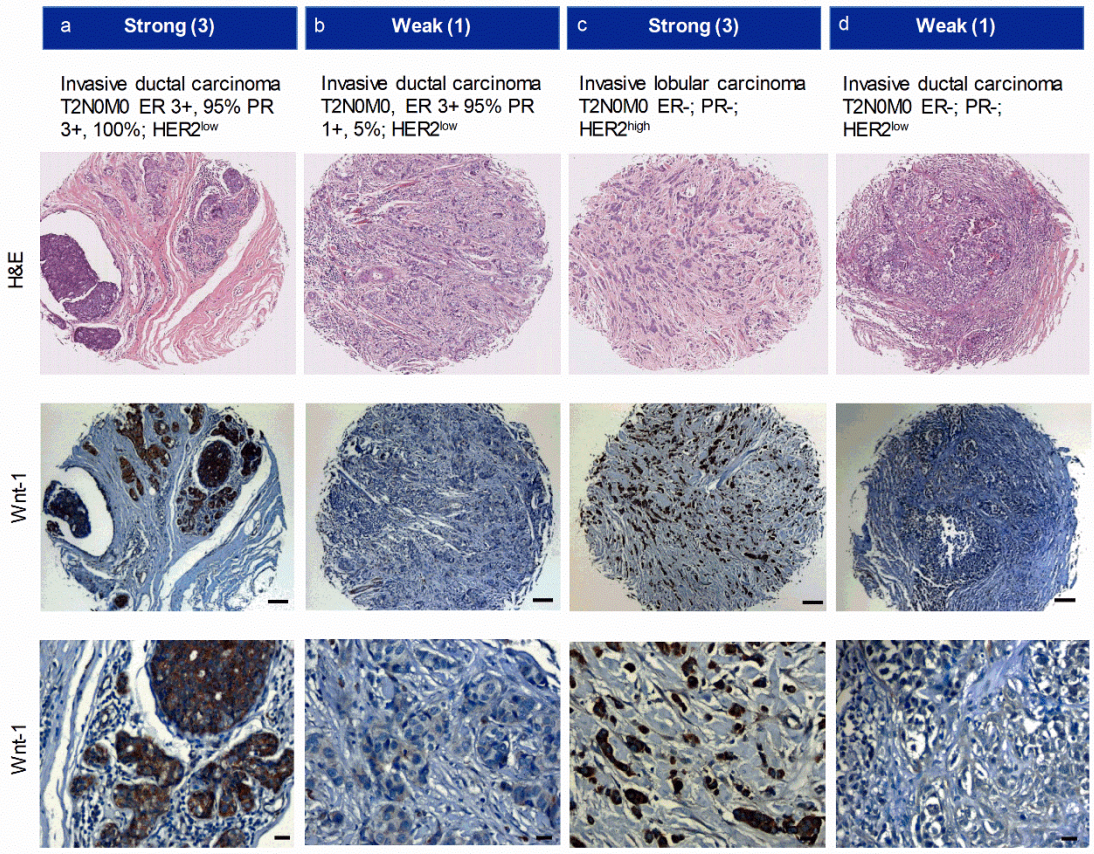


Figure 27. Immunohistochemical analysis of Wnt-1 in a human breast tissue array. A. *Stratification of Wnt-1 according to ER, PR, HER2 levels and in metastatic and non-metastatic tumors, ** P < 0.01; *** P < 0.001.* ***B.*** *Statistical analysis of Wnt-1 staining in breast cancer.* ***C.*** *Tumor sections from patients stained for H&E (top) and Wnt-1. Low (scale bars = 100 μm) and high (scale bars = 20 μm) magnification images are shown. Chi Sq. Pearson, Pearson Chi-square test with correction; Fisher 2t, Fisher's exact test, two-sided. ns, not significant.*

2. WNT1 expression in breast cancer cell lines

Expression of *WNT1* in a range of breast cancer cell lines was also important to consider given the differences observed in the signaling mediated by Wnt-1 in MCF-7TamR and T47DTamR cells. The GOBO Gene set online tool (Ringnér et al., 2011) was used to examine *WNT1* expression in human breast cancer cell lines commonly used as models of different breast cancer molecular types. *WNT1* was not differentially expressed in breast cancer cells of different molecular subtypes mimicking basal and luminal tumors (Fig. 28). In our MCF-7TamR and T47DTamR cell lines, expression of *WNT1* was very low (ΔCt values on average were 16.87 for MCF-7TamR and 13.67 for T47DTamR), and not detectable at the protein level. RNAseq data, available in the lab, showed that it was not detected at all in MCF-7TamR cells and barely detected in T47DTamR cells (read numbers 0 and 0.7, respectively).

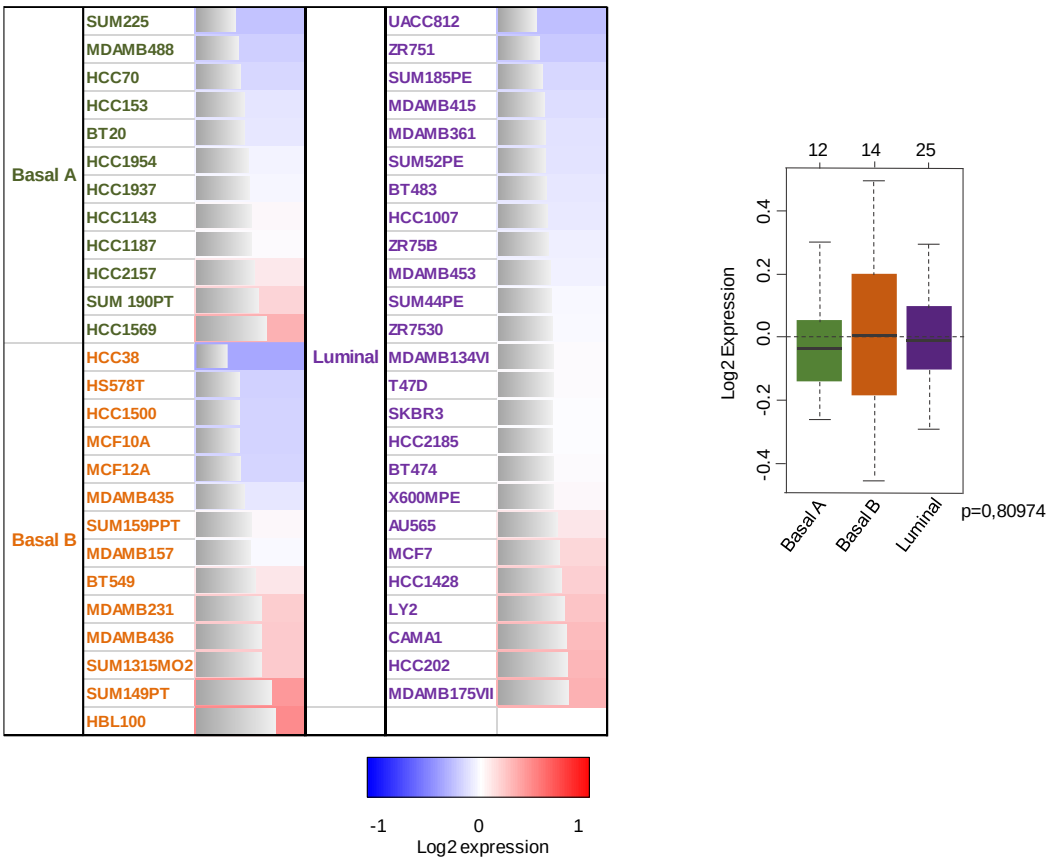


Figure 28. WNT1 expression in human breast cancer cell lines. *Expression of WNT1 across 51 individual cell lines and box plots of WNT1 gene expression across cell lines grouped in the basal A (green), basal B (orange) and luminal (purple) subgroups. Obtained from GOBO Gene set online tool. (error bars represent SD)*

Since many Wnt-1 studies in breast cancer have been carried out using mouse models, *Wnt1* mRNA levels and protein expression were evaluated in a panel of mouse metastatic breast cancer cell lines. These cell lines are isolated from a single, spontaneously arising mammary tumor (H Heppner, R Miller and Malathy Shekhar, 2000) and are classified on the basis of their ability to metastasize spontaneously from the orthotopic site. 4T1 cells metastasize *via* the hematogenous route forming visible metastases in several organs. 4T07 are highly tumorigenic, but fail to metastasize; cells may be recovered from the blood and lungs but visible metastases do not develop. Non-metastatic 67NR cells fail to leave the primary site. In adherent conditions, 4T07 cells expressed the highest levels of *Wnt1* mRNA (Fig. 29A). Total cell extracts expressed higher levels of Wnt-1, after normalization to the loading control HSP60 (Fig. 29B). Consistent with this, extracts from the ECM of 4T07 cells also contained more Wnt-1 than ECM from 67NR or 4T1 cells (Fig. 29). These results are consistent with the reduced expression of *WNT1* in human metastatic tumors.

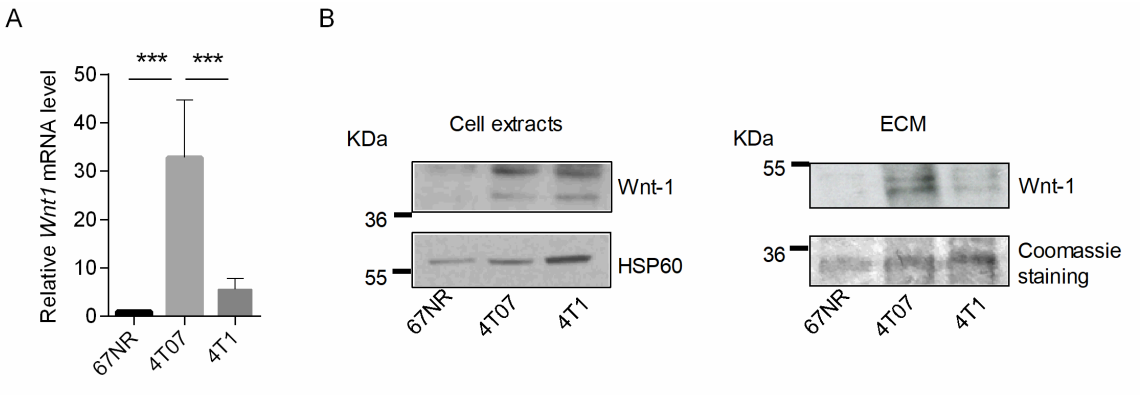


Figure 29. Wnt1 expression in mouse mammary cancer cell lines. A. *Expression of Wnt1 mRNA in the indicated mouse mammary cancer cell lines, normalized to expression in 67NR cells; n=3, *** P < 0.001, Student´s t test (error bars represent SD).* ***B.*** *Western blots for Wnt1 protein levels detected in cell extracts and in the ECM from indicated mouse mammary cancer cell lines.*

3. WNT1 expression in breast cancer stem cells

Considering the importance of *WNT1* expression in the context of CSCs, it was considered to be relevant to study *WNT1* expression in other CSC populations in addition to $CD44^{+}CD24^{-/low}$ cells (previously shown in Fig. 15). To this end, I examined *WNT1* expression by PCR in the $ALDH^{+}$ cells using MCF-7TamR cells FACS sorted for $ALDH^{+}$ and $ALDH^{-}$ populations using the ALDEFLUOR assay. However, *WNT1* expression could not be detected in this CSC population, possibly owing to low expression and the low proportion of $ALDH^{+}$ cells. To try to resolve this issue, I used mouse

metastatic breast cancer cells, where the proportion of ALDH$^+$ population and basal *Wnt1* mRNA levels are higher (Fig. 30A and C). The results shown in Fig. 30A indicate that there was no enrichment of *Wnt1* in the ALDH$^+$ populations derived from these cells.

On the other hand, *Wnt1* mRNA levels were increased in mammospheres formed by 4T07 cells, but not in non-metastatic 67NR cells; there was a trend for an increase in 4T1 cells (Fig. 30B). The results in 4T07 cells are consistent with those in MCF-7TamR cell mammospheres, where *WNT1* expression was also elevated (Fig. 15A).

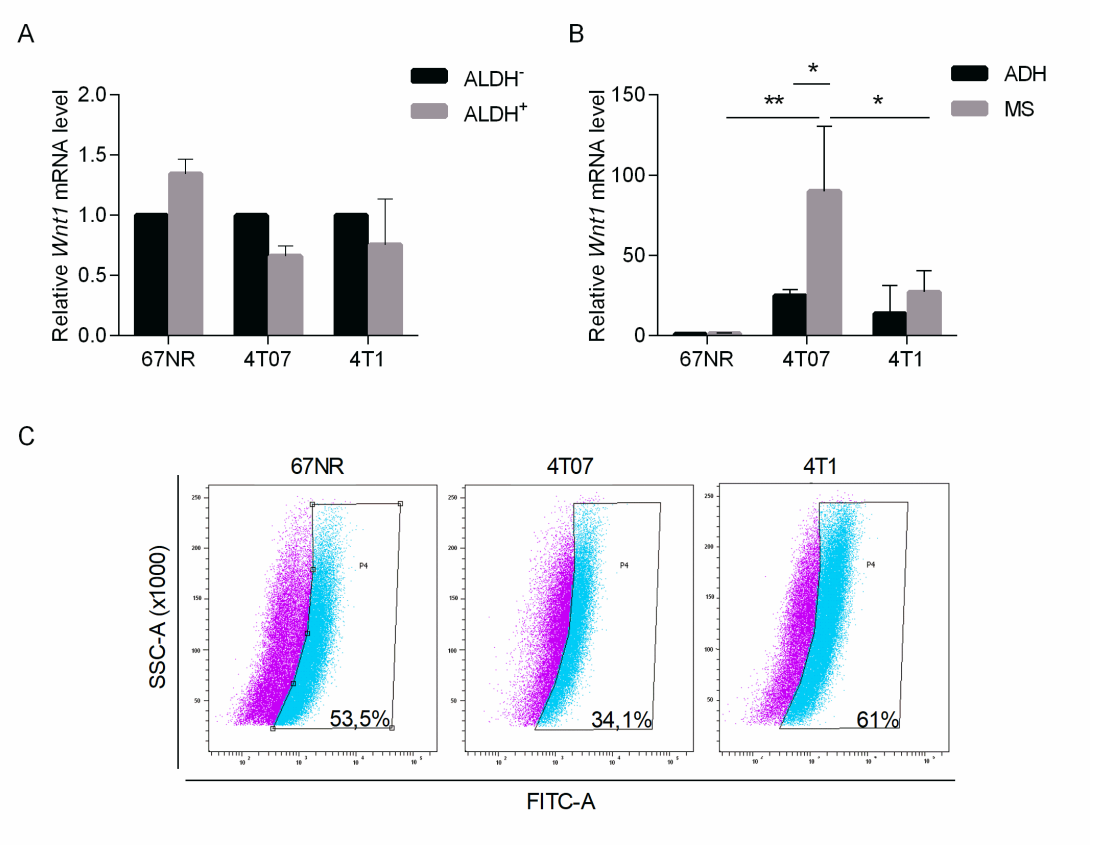


Figure 30. Wnt1 expression in mouse mammary cancer stem cells. A. *Relative mRNA expression of Wnt1 in ALDH+ cells sorted from mouse mammary cancer cell lines, compared to ALDH- cells; n=2.* ***B.*** *Relative mRNA expression of Wnt1 in comparing adherent (ADH) and mammospheres (MS) from mouse mammary cancer cell lines; n=3, * P < 0.05; ** P < 0.01, Student´s t test (error bars represent SD).* ***C.*** *Representative analysis of ALDH+ populations. The ALDH+ population selected for sorting is shown in blue. ADH, adherent; MS, mammospheres.*

III. EFFECTS OF ECTOPIC EXPRESSION OF WNT1 IN MCF-7 AND MCF-7TAMR CELLS

1. Generation of cells over-expressing WNT1

Considering the potential importance of Wnt-1 in the context of stem cells and tamoxifen resistance, we required a system in which cells could grow long-term in the presence of Wnt-1. There are several approaches to activate Wnt signaling *in vitro*. One option is to add recombinant Wnt proteins, supplemented with RSPO1 (Huggins, Brafman and Willert, 2016). Bengoa-Vergniory et al., (2014), used recombinant Wnt-7a, Wnt-5a, and Wnt-3a to restore signaling to cells treated with IWP-2, and Dkk-1 to block activation of canonical signaling in human embryonic stem-derived neural progenitor cells. Jang *et al.*, (2015) used Wnt-3a-CM for reporter assays. Recombinant Wnt-1 was used for breast tumor mammosphere formation assays (Choi *et al.*, 2012). Similarly, for long term expansion of MaSCs, Zeng and Nusse (2010) used purified Wnt-3a protein, and Cimetta *et al.*, (2013) used microfluidic bioreactors to expose human pluripotent stem cells in 3D cultures to a concentration gradient of Wnt-3a. Alternatively, WNT genes can be stably expressed. In breast, Karow *et al.*, (2009), expressed *Wnt3a* under a constitutively active promoter in mouse mesenchymal stem cells. This approach was also used by Miranda-Carboni *et al.*, (2008) to express *WNT10B* in MCF-7 cells and by Schlange *et al.*, (2007), who transfected T47D and SkBr3 cells with a *WNT1*-encoding plasmid and harvested the conditioned media from these cells for co-culture assays. This is a cost-effective alternative to using recombinant proteins. Since over-expression of WNT genes has been reported in breast cancer cell lines, and our MCF-7TamR cells responded to transiently transfected *WNT1* plasmid (Fig. 23), this approach was chosen.

MCF-7 cells, both C and TamR, were transfected with a plasmid encoding *WNT1* and selected with G418 to generate pooled cell lines stably expressing *WNT1* (MCF-7C-W1 and MCF-7TamR-W1) or empty vector (MCF-7C-V and MCF-7TamR-V). Expression of *WNT1* mRNA was confirmed by qRT-PCR using a primer for the cDNA sequence (CDS), which is the sequence present in the plasmid. As a negative control a primer for the *WNT1* non-coding region was used, which confirmed that endogenous *WNT1* levels were not affected (Fig. 31A). Wnt-1 protein was detected by western blotting in cell extracts (Fig. 31B). Although Wnt-1 was not detectable in cell-conditioned medium, it was detected in the ECM (Fig. 31B). Wnt-1 protein was also detected by immunofluorescence (Fig. 31C).

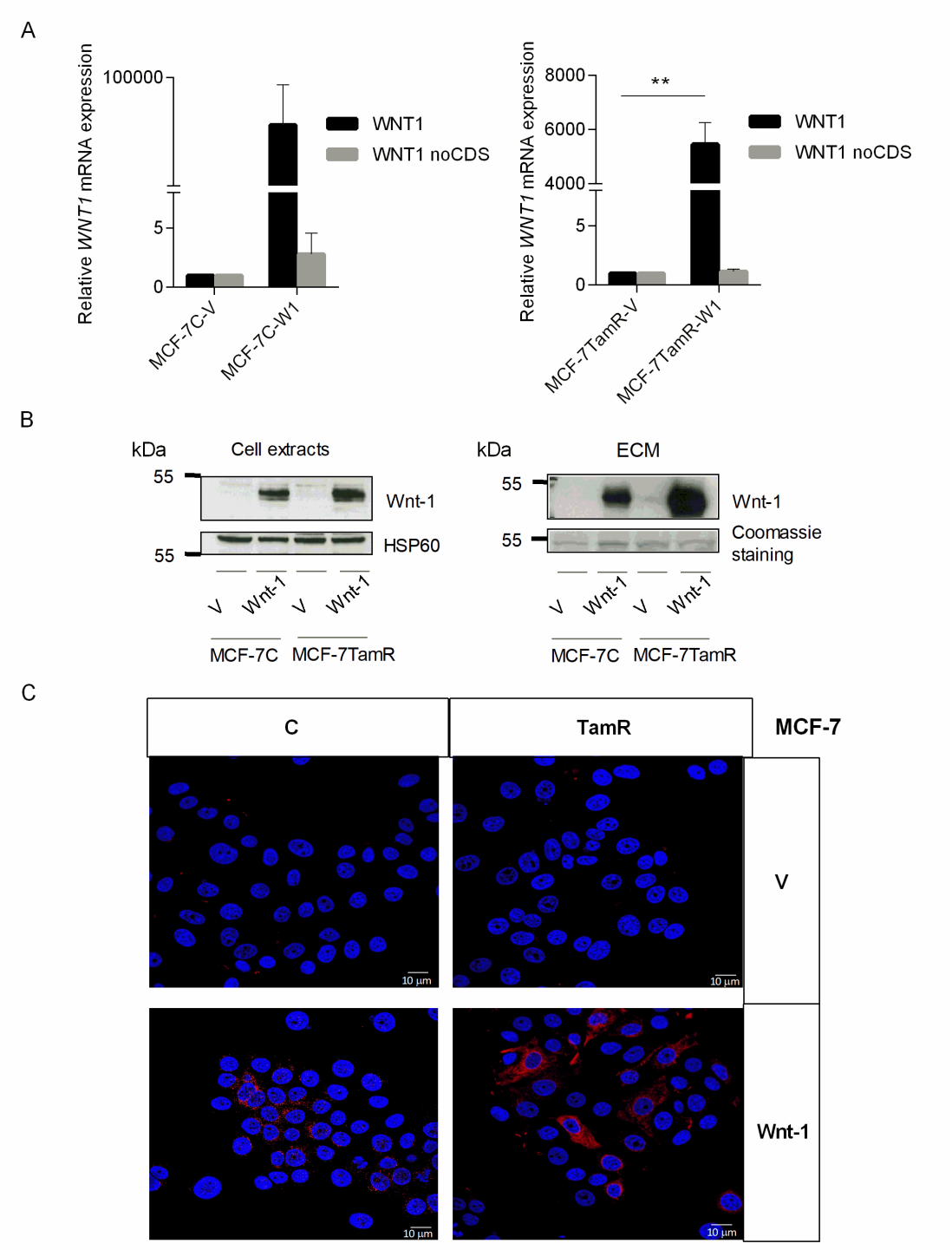


Figure 31. Characterization of WNT1-expressing cell lines. A*. Expression of WNT1 mRNA in MCF-7C-V vs MCF-7C-W1 and in MCF-7TamR-V vs MCF-7TamR-W1 using WNT1 primers to amplify WNT1 coding region and non-coding region (noCDS); n=3, ** P < 0.01, Student´s t test (error bars represent SEM).* ***B.*** *Western blots for Wnt-1 protein in cell extracts and in the ECM in the indicated cell lines.* ***C****. Wnt-1 detection by immunofluorescence in the indicated MCF-7 cell lines. Scale bars are indicated. CDS, cDNA sequence; noCDS, no cDNA sequence.*

Next, in order to determine the effects of stable overexpression of *WNT1* in canonical and non-canonical Wnt signaling in MCF-7TamR cells, gene reporter assays and GFP reporters were carried out. In luciferase assays, consistent with the results of transient transfection assays (Fig. 22 and 23), stable expression of *WNT1* did not activate β-catenin/TCF-dependent transcription but did increase ATF2-dependent transcription, as compared to control MCF-7TamR-V cells (Fig. 32A). These results indicated that stable expression of *WNT1* had similar effects as transient expression. However, we noted that the effect of stably expressed *WNT1* were not as strong as in cells transiently expressing *WNT1*. Moreover, in experiments using canonical and non-canonical GFP reporters, stable expression of *WNT1* reduced β-catenin/TCF-dependent activity and did not affect ATF2-dependent activity (Fig. 32B). The reasons for this are unclear but may reflect differences in the ways the results are normalized using the two approaches. In the luciferase assay, renilla is used and provides a more accurate value, as it reflects signal in cells expressing the specific reporter. The GFP reporter experiments rely on GFP levels expressed by a CMV promoter in other cells.

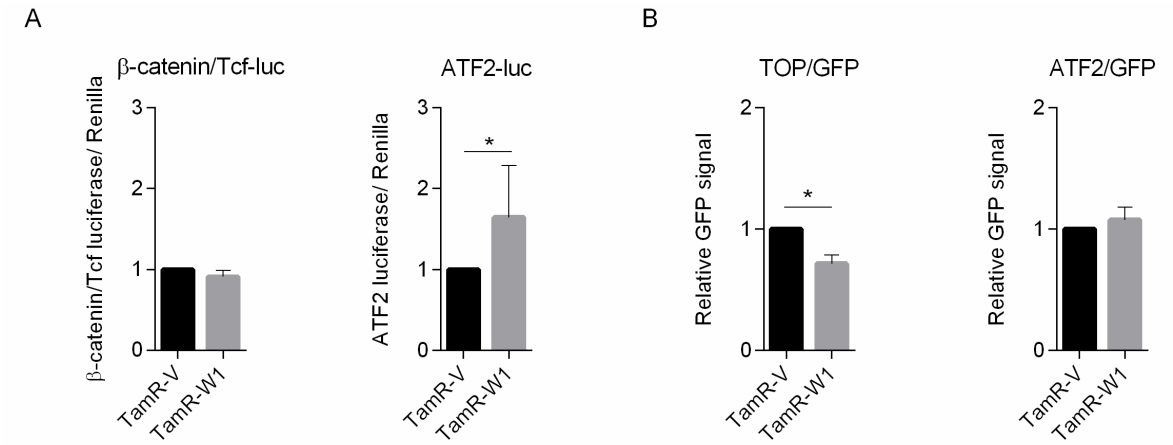


Figure 32. Transcriptional activity of MCF-7TamR-W1 cells. A. *Relative β-catenin/TCF luciferase and ATF2-luciferase activity mediated by WNT1 in MCF-7TamR-W1 cells; n=3.* ***B.*** *WNT1 mediated transcriptional activity measured by the quantification of GFP⁺ cells by FACS using a TOPdGFP and ATF2dGFP reporters normalized to GFP; n=3, * P < 0.05, Student´s t test (error bars represent SD).*

2. Effects of Wnt-1 on the acquisition of tamoxifen resistance

Comparison of the control and *WNT1*-overexpressing cells did not reveal any differences in their morphologies (Fig. 33A). Given the link between *WNT1* expression and survival of tamoxifen treated patients (Fig. 25), we next compared the effects of tamoxifen on the control and *WNT1*-expressing cell lines. As expected, MCF-7TamR cells, which are routinely cultured in the presence of tamoxifen, remained resistant to tamoxifen at all tested dilutions and *WNT1* expression did not alter this resistance. On the other hand, as previously reported for MCF-7 cells (Piva et al., 2014), tamoxifen reduced control cell proliferation by 30% at higher doses. This inhibition was also seen in MCF-7C-W1 cells (Fig. 33B), suggesting that ectopic expression of *WNT1* is not sufficient to confer resistance to MCF-7 cells. Tamoxifen is known to inhibit proliferation of MCF-7 cells by increasing accumulation of cells in G_1 phase, with a concomitant reduction of S and G_2/M phase cells (Osborne *et al.*, 1983). To further analyze whether Wnt-1 was having an effect on tamoxifen sensitivity that was not measurable using proliferation assays, cell cycle assays were carried out in the presence and the absence of tamoxifen. In the presence of tamoxifen (5×10^{-7} M), the proportion of control cells arrested in G_0/G_1 increased from 60% to 80%. Cells in S phase were reduced to 10% and there was little variation in G_2/M cells. Similar results were observed in MCF-7C-W1 cells, suggesting that *WNT1* expression does not affect tamoxifen-induced cell cycle effects (Fig. 33C).

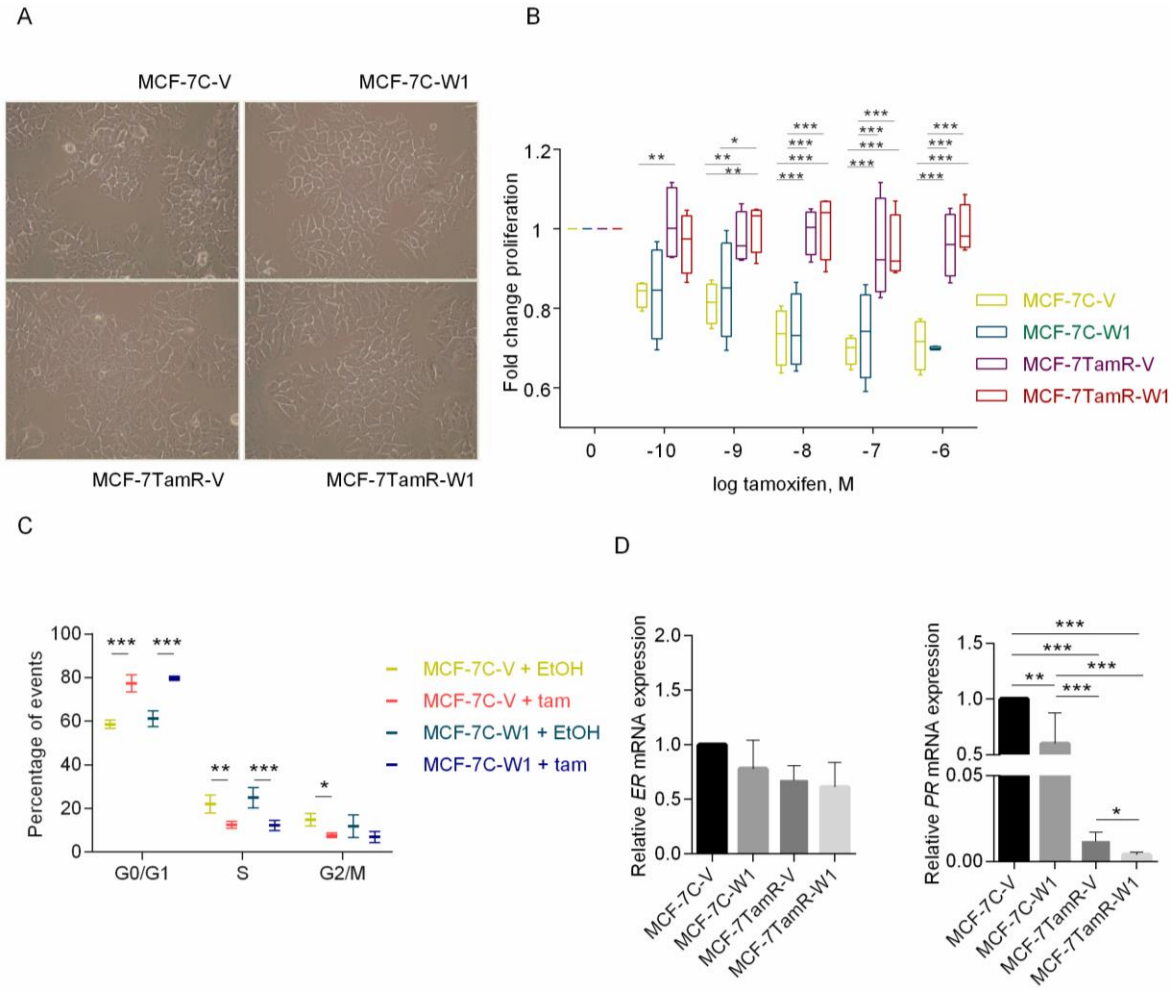


Figure 33. Tamoxifen sensitivity in WNT1 over-expressing cells. A. *Pictures of MCF-7 derived cells in normal culture conditions (error bars represent SD).* ***B.*** *Proliferation assay with WNT1 expressing MCF-7C and MCF-7TamR cells respect to their control cell lines using serial dilutions of tamoxifen; n=5, * P < 0.05; ** P < 0.01; *** P < 0.001, Two-way ANOVA test (The median in shown as a line in the center of the box, whiskers represent the maximum and minimum values).* ***C.*** *Cell cycle analysis of MCF-7C cells after 5 x 10^{-7} M tamoxifen treatment; n=3, * P < 0.05; ** P < 0.01; *** P < 0.001, Two-way ANOVA test (error bars represent SD).* ***D.*** *ER and PR expression levels in generated new cell lines; n=3, * P < 0.05; ** P < 0.01; *** P < 0.001, Student´s t test (error bars represent SD).*

Owing to the importance of ER expression in the context of tamoxifen resistance, the effect of stable expression of *WNT1* on ER and PR was analyzed by qRT-PCR. There were no significant differences in ER mRNA levels among the cell lines. As expected, PR was lower in MCF-7TamR cells than in control MCF-7 cells. In addition, PR expression was lower in cells expressing *WNT1*, even in TamR cells (Fig. 33D). These data suggest that Wnt-1 inhibits ER mediated signaling.

TamR cells are also less sensitive to other ER antagonists, such as fulvestrant (ICI 182,780) (Domenici et al., 2014). As a control for ICI activity, adherent cells were treated at 5 x 10^{-7} M ICI for 48 h and expression levels of SOX9, ER and PR were determined. In the presence of ICI, *SOX9* expression levels were increased in all cell lines. Regarding ER, although ER mRNA expression was not affected, ER protein levels were reduced. This reduction in ER protein results in the diminution of PR, both at the mRNA and protein level (Fig. 34A and B). However, *WNT1* expression did not affect the levels of ER or SOX9 in MCF-7C or MCF-7TamR cells. To examine whether expression of *WNT1* affects resistance to ICI, colony formation assays were performed using a concentration of ICI (10^{-9} M) at which MCF-7C cells are sensitive and MCF-7TamR cells are resistant. The colony formation assay is an *in vitro* survival assay based on the ability of a single cell to undergo unlimited divisions and grow into a colony. After a treatment, only a fraction of seeded cells retains the capacity to produce colonies (Franken *et al.*, 2006). However, in the colony formation assays there were no significant differences in the response to ICI between control cells and cells expressing *WNT1* with respect to the numbers of colonies (Fig. 34C). On the other hand, there was a tendency for *WNT1*-expressing cells to form colonies larger than 50 μm in the presence of ICI. Moreover, *WNT1* expression increased the numbers of large colonies formed by TamR cells. Together, these results indicate Wnt-1 does not affect the number of single cells able to undergo unlimited divisions but it may increase proliferation of those cells that form colonies.

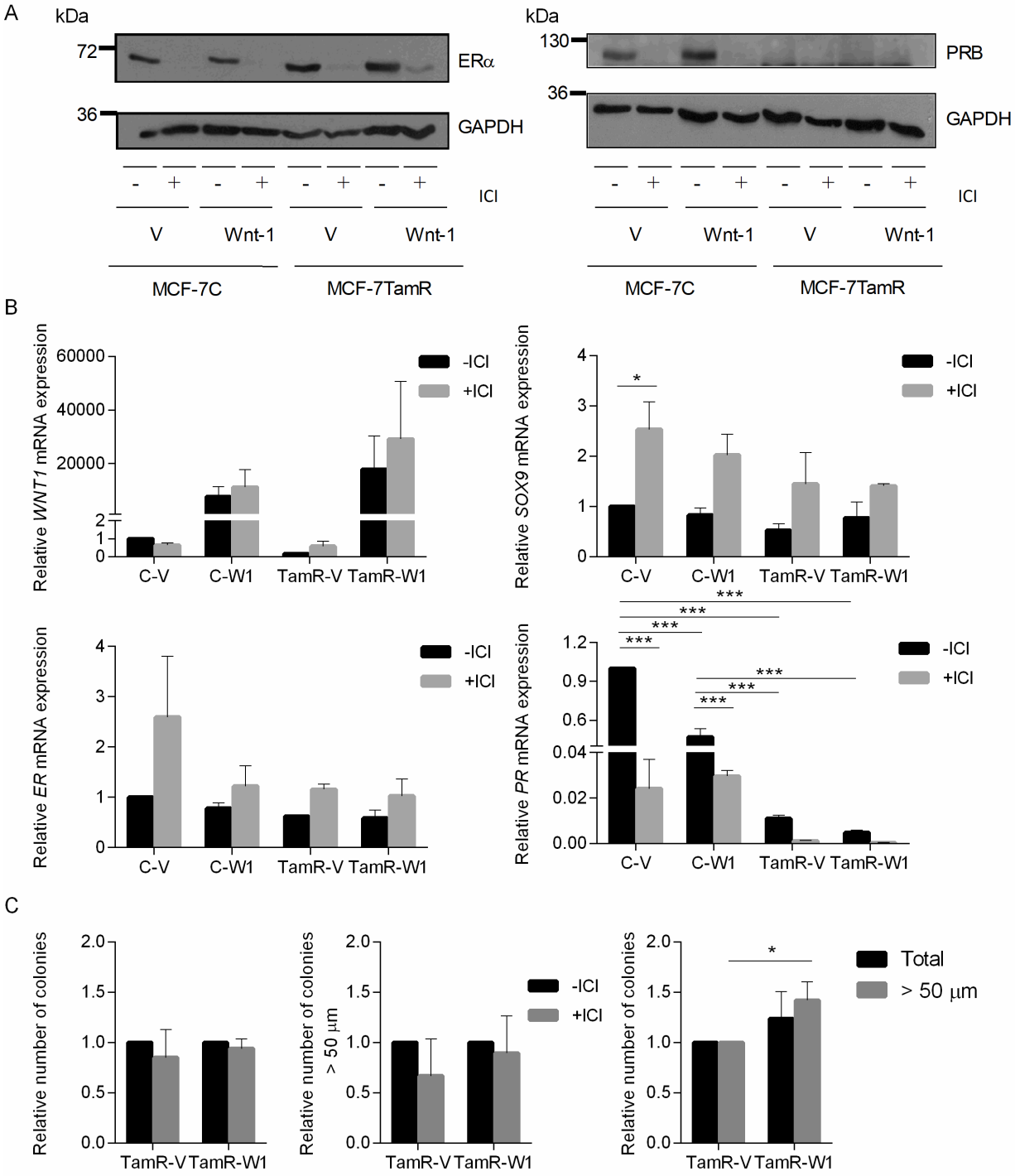


Figure 34. ER blockade in WNT1 expressing cells. A. *Western blot for ER and PR proteins after ICI treatment in the indicated cell lines. GAPDH in cell extracts was used as a loading control. Representative experiment of n=3.* ***B.*** *Relative mRNA levels of WNT1, SOX9, ER and PR in response to ICI treatment; n=3.* ***C.*** *Colony formation assays in MCF-7TamR cells in the presence of ICI and comparisons between the numbers of colonies > 50 µm in TamR-V and TamR-W1 cells in the absence of treatment; n=3, * P < 0.05; ** P < 0.01; *** P < 0.001, Two-way ANOVA test (error bars represent SD).*

3. Effects of Wnt-1 in breast CSCs

Considering the potential importance of *WNT1* expression in the context of CSCs and taking into account the previous results, the effect of stable expression of *WNT1* on the CSC population was monitored. For this, MCF-7C and MCF-7TamR cells expressing empty vector or *WNT1* were stained for CD44 and CD24 and FACS-analyzed. As shown in Fig. 35A, MCF-7-W1 cells were more enriched for $CD44^{+}CD24^{-/low}$ cells compared to their respective control cells, both in adherent conditions and in mammospheres. This increase was significant in the case of MCF-7TamR cells (See an example of analysis in Fig. 35E), in which the $CD44^{+}CD24^{-/low}$ population is increased compared to MCF-7C cells, and more clearly in the mammospheres formed by these cells (Fig. 35B).

The increase in CSCs can be translated into the ability of breast cancer cells to form mammospheres. To further analyze whether the increase in CSC was correlated with an increased ability to form mammospheres, mammosphere formation assays were carried out. The increase in mammosphere formation ability of MCF-7 cell lines expressing *WNT1* is represented in Fig. 35C (Fig. 35F shows an example of the mammospheres). This ability was maintained in the second passage of cells forming those mammospheres.

To validate the WNT-1-mediated increase in the CSC population, mRNA expression levels of the CSC markers *SOX2*, *OCT4* and *NANOG* were analyzed. Surprisingly, no significant differences were observed in adherent cells. Indeed, there was a tendency for these genes to be reduced in the mammospheres formed by MCF-7C-W1 cells (Fig. 36A). Regarding MCF-7TamR cells, no significant differences were apparent in adherent cells or mammospheres, but there was a significant reduction in *SOX2* levels after mammosphere passage (Fig. 36A, B and C). Notably, although *WNT1* was highly expressed in primary and secondary mammospheres, it was lower in secondary mammospheres (Fig. 35D).

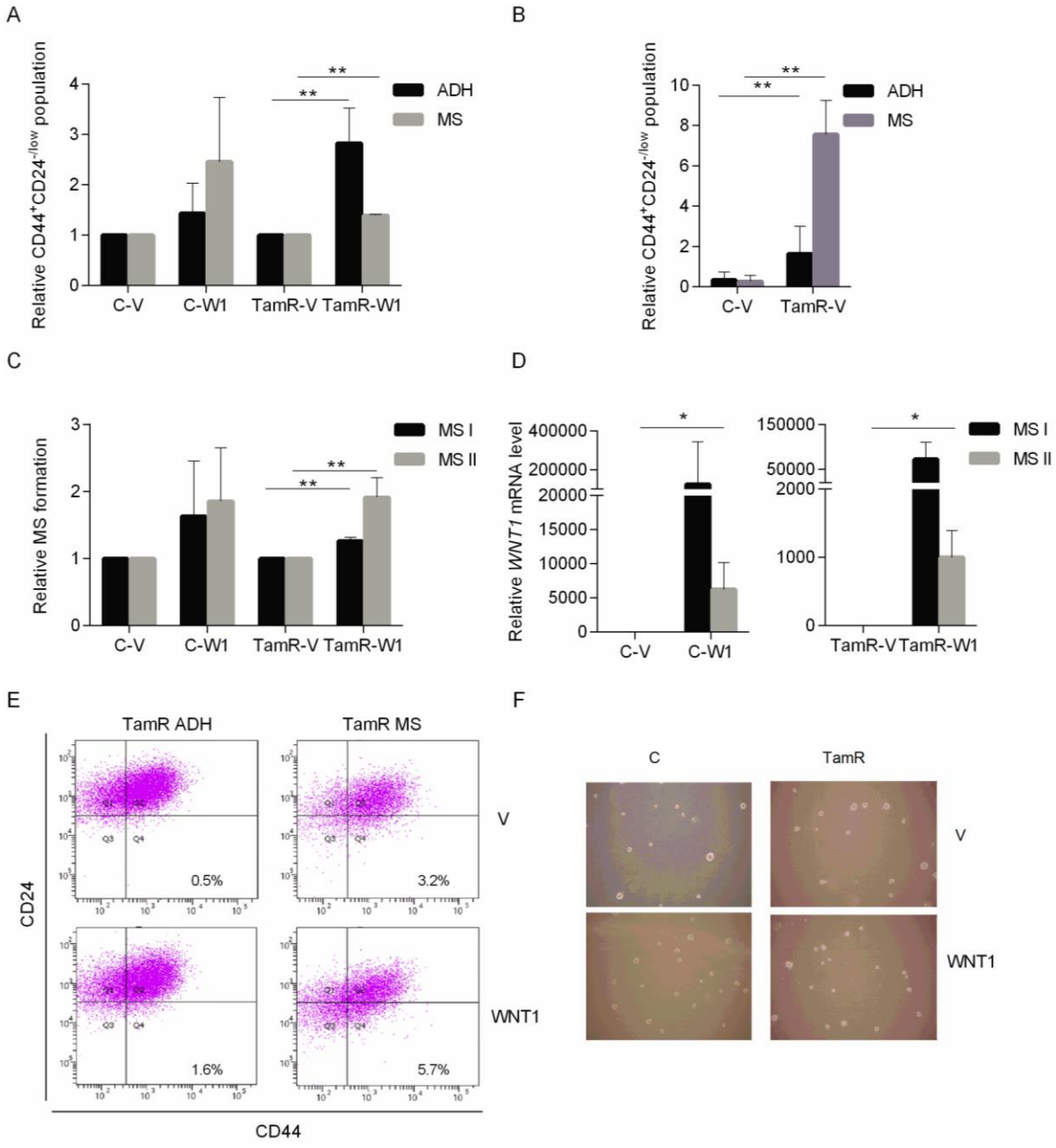


Figure 35. Effects of WNT1 expression in CSCs. A. *Relative $CD44^{+}CD24^{-/low}$ population in adherent and mammospheres in WNT1-expressing cells; n=3.* ***B.*** *Relative $CD44^{+}CD24^{-/low}$ population in adherent and mammospheres in TamR cells relative to C cells; n=3.* ***C.*** *Relative number of mammospheres formed by cells expressing WNT1 and mammospheres formed after passage of mammospheres; n=3.* ***D.*** *Relative WNT1 expression in the mammospheres formed by WNT1-expressing cells; n=3, * P < 0.05; ** P < 0.01, Student´s t test (error bars represent SD).* ***E.*** *Representative analysis of MCF-7TamR-V and MCF-7TamR-W1 cells stained for CD24 and CD44. The percentage of $CD44^{+}CD24^{-/low}$ population for each cell line is indicated.* ***F.*** *Representative pictures of mammospheres formed by the indicated cell lines. ADH, adherent; MSI, primary mammospheres; MS II, secondary mammospheres.*

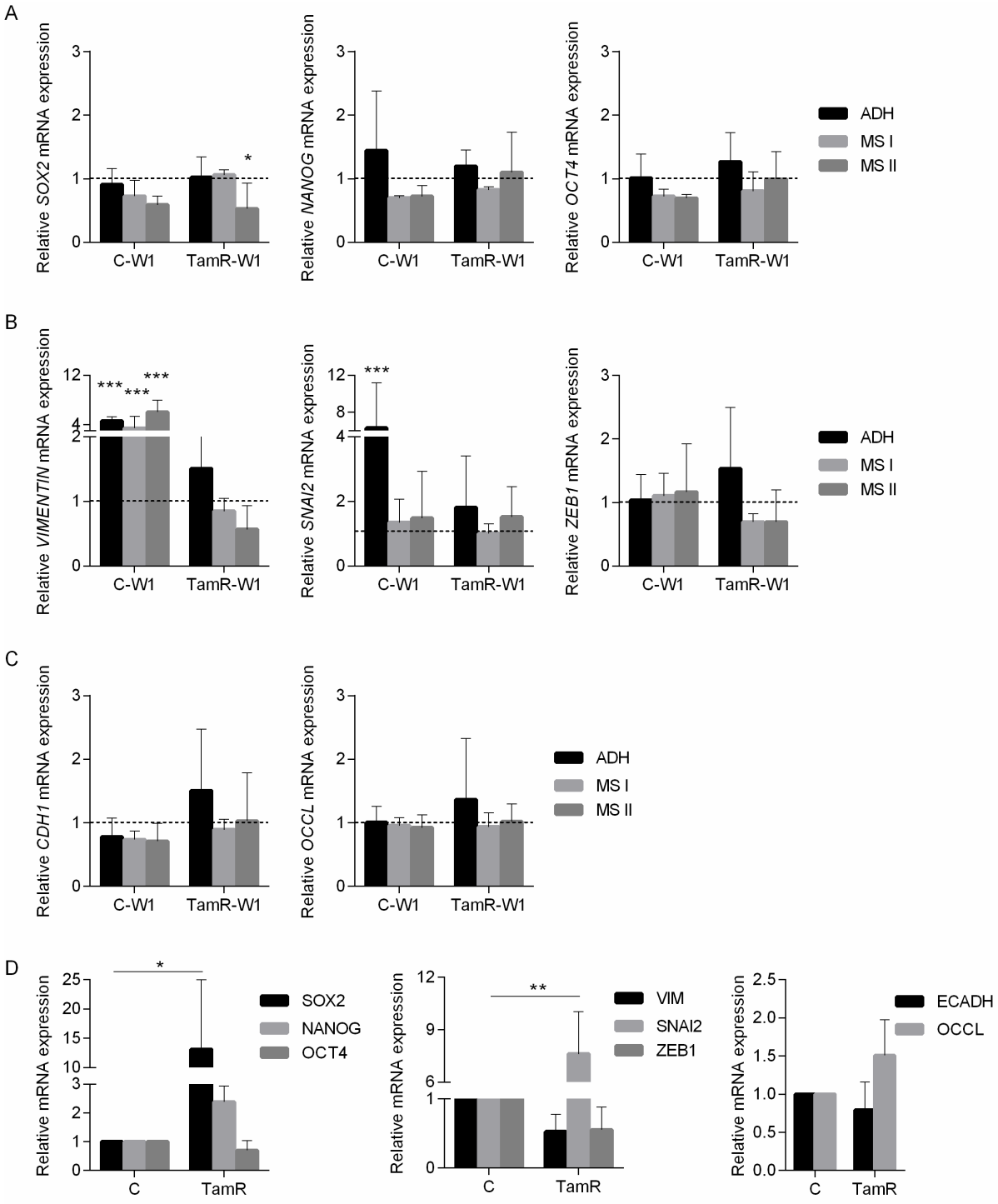


Figure 36. Effects of WNT1 expression on the expression of CSC and EMT markers. A. *Relative mRNA expression of CSC markers in MCF-7C-W1 and MCF-7TamR-W1 cells compared to MCF-7C-V and MCF-7TamR-V cells, respectively.* ***B.*** *Relative mRNA expression of EMT markers in MCF-7C-W1 and MCF-7TamR-W1 cells compared to MCF-7C-V and MCF-7TamR-V cells, respectively.* ***C.*** *Relative mRNA expression of epithelial markers in MCF-7C-W1 and MCF-7TamR-W1 cells compared to MCF-7C-V and MCF-7TamR-V cells, respectively.* ***D.*** *Relative mRNA expression of CSCS, mesenchymal and epithelial markers in adherent MCF-7TamR cells compared to MCF-7C cells. (A, B, C, D) n=3, * P < 0.05; ** P < 0.01; *** P < 0.001, Student´s t test (error bars represent SD).*

4. Effects of Wnt-1 on EMT markers

In connection with this, EMT is another hallmark of CSCs in which Wnt signaling also plays a role (DiMeo et al., 2009; Ksiazkiewicz, Markiewicz and Zaczek, 2012). Moreover, *WNT1* is a direct target of miR-148a, a microRNA involved in regulation of invasion and migration in MCF-7 and MDA-MB-231 breast cancer cells (Jiang *et al.*, 2016). To examine whether expression of *WNT1* affects EMT, mRNA levels of genes related to EMT were analyzed, in adherent and mammosphere conditions. Our results showed that there were no significant differences in the expression of mesenchymal (*VIMENTIN, SNAI2, ZEB1*) or epithelial (*CDH1 and OCCLUDIN*) markers in MCF-7TamR cell lines (Fig. 36B and C, respectively). However, in MCF-7C cells there was a significant increase in the expression of *VIMENTIN*, in adherent as well as in mammospheres in *WNT1*-expressing cells. *SNAI2* expression was also significantly increased in adherent cells. However, there were no significant differences in the expression of the epithelial markers.

As a control, expression level of these genes in MCF-7TamR cells were compared to MCF-7C cells (Fig. 36D). *SOX2* and *SNAI2* gene expression levels were significantly higher in MCF-7TamR cells than in MCF-7C cells. There was also a trend for *NANOG* and *OCCL,* expression to be higher and for *VIM* and *ZEB1* expression to be lower in MCF-7TamR cells. These results suggest that MCF-7TamR cells do not show more mesenchymal features compared to MCF-7C cells. Expression of *VIMENTIN* and *SNAI2 was* significantly increased in MCF-7C-W1 cells, but not in MCF-7TamR-W1 cells compared to MCF-7C-V and MCF-7TamR-V cells, respectively. Together, these results indicate that the response to *WNT1* in MCF-7 cells may differ in tamoxifen treated cells.

IV. IDENTIFICATION OF WNT1 RECEPTORS AS SIGNALING MEDIATORS AND POSSIBLE THERAPEUTIC TARGETS

1. Targeting FZD receptors

Each Wnt can bind to different FZD receptors, and each FZD can respond to multiple Wnt ligands. To date, the selectivity of each Wnt to specifically bind a FZD receptor remains poorly understood. FZD receptors can activate canonical and non-canonical Wnt signals, so targeting FZDs might be a promising approach for cancer therapy (Zeng, Chen and Fu, 2018). USP6 is a deubiquitylase that increases the membrane abundance of FZDs when the gene is over-expressed. FZD protein accumulation in the membrane increases cellular sensitivity to Wnt ligands by opposing the activity of the ubiquitin ligases RNF43 and ZNRF3 (Madan *et al.*, 2016). USP6 prevents the turnover of FZD receptors by blocking FZD ubiquitylation, which ultimately prevents endocytosis and lysosomal degradation. FZD abundance in the membrane has been determined measuring endogenous FZD levels in HEK293 cells by flow cytometry using the monoclonal antibody OMP-18R5, which recognizes FZD_1, FZD_2, FZD_5, FZD_7, and FZD_8 (Gurney *et al.*, 2012).

In order to analyze whether USP6 enhances Wnt-1 mediated signaling in MCF-7TamR cells, an ATF2-luciferase reporter assay was performed. MCF-7TamR cells were transfected with *WNT1* and *USP6* expression plasmids. USP6 did not significantly alter ATF2-mediated signaling. However, it enhanced the effects of Wnt-1 (Fig. 37A). β-catenin/TCF-dependent activity was not altered, consistent with these cells do not display β-catenin/TCF transcriptional activity (Fig. 37B).

Wnt-1 increases the capacity for mammosphere formation in MCF-7 TamR cells (Fig. 35). Mammosphere formation assays were carried out to determine if USP6 changes the ability of *WNT1*-expressing cells to form spheres. MCF-7C-W1, MCF-7TamR-W1 cells and their respective control cell lines were cultured in low attachment conditions after transfection with the USP6 expression plasmid. As shown in Fig. 37C, USP6 showed a tendency to increase the ability of MCF-7C-W1 cells to form mammospheres, and significantly increased the number of mammospheres formed by MCF-7TamR-W1 cells, but not by MCF-7C-V or MCF-7TamR-V cells, suggesting that USP6 enhances activation of Wnt-1-mediated signaling.

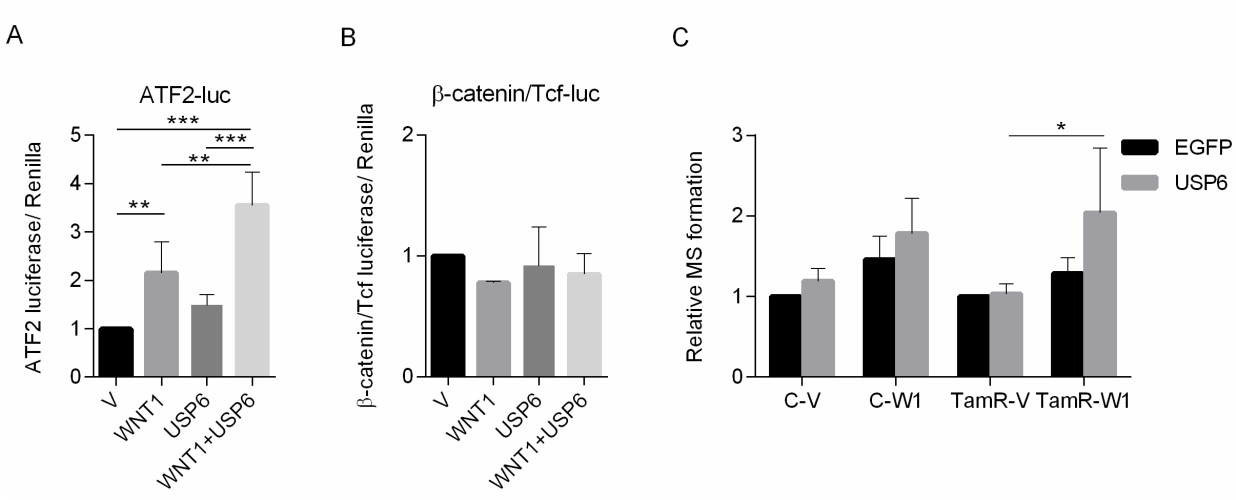


Figure 37. USP6 effect on WNT1 mediated signaling. A. *Gene reporter assay measuring ATF2-dependent transcriptional activity in MCF-7TamR cells expressing WNT1 and USP6; n=4.* ***B.*** *Gene reporter assay measuring β-catenin/TCF-dependent transcriptional activity in MCF-7TamR cells co-expressing WNT1 and USP6; n=4.* ***C****. Relative number of mammospheres formed by MCF-7C and MCF-7TamR cells expressing WNT1 and/or USP6 compared to MCF-7C-V and MCF-7TamR-V cells; n=3, * P <0.05; ** P <0.01; *** P <0.001, Student´s t test (error bars represent SD).*

However, as happens with the OMP-18R5a monoclonal antibody, a strategy targeting many FZDs by USP6 might present many side effects due to its universality and non-specificity. Therefore, to better understand how Wnt-1 signaling is transduced and to address a more specific therapeutic approach, I focused on the identification of the FZD receptor that transduces Wnt-1 signal in MCF-7 cells, since this would also address Wnt specificity in the context of the response of breast CSCs.

2. Identification of Specific Wnt-1 receptors

Benhaj, Akcali and Ozturk, (2006) analyzed the expression profile of FZD receptors in breast cancer cell lines. In MCF-7 cells, *FZD1, FZD2, FZD3, FZD4* and *FZD6* were the most abundant receptors. Lamb *et al.*, (2013) also described that *FZD4* was over-expressed compared to normal breast cell lines. *FZD1* and *FZD8* expression has been reported to be involved in drug resistance, *FZD5, FZD6* and *FZD10* have been related to EMT and *FZD7* is involved in cell proliferation (Zeng, Chen and Fu, 2018). On the other hand, *FZD3* is less well studied and there is no antibody to target FZD_3 for cancer therapy. However, FZD_3 has been claimed to reduce migration in breast cancer through interaction with Wnt-5a (Hansen *et al.*, 2009).

Regarding Wnt-1, no direct binding of Wnt-1 to FZD receptors in breast cancer has been published to date. A systematic map of Wnt-FZD interactions in human HEK293 cells studied different combinations and measured the activation of β-catenin/TCF-luciferase. The strongest

activation by Wnt-1 was *via* FZD_5 and FZD_8, although there was also a strong induction with FZD_4, a medium induction with FZD_2 and weak effects with FZD_1, FZD_7 and FZD_{10} (Voloshanenko *et al.*, 2017).

For an initial approach, an *in silico* analysis with bc-GenEXMiner v4.1 was used to examine targeted gene correlations and the linear dependence between each pair formed by *WNT1* and FZD family genes. Fig. 38 shows all correlation plots obtained for the 10 different FZDs. Five of the receptors (*FZD4, FZD5, FZD8, FZD9* and *FZD10*) showed a significant p-value. However, Pearson´s correlation coefficient was in all cases weak, showing the highest values for positive correlation between *WNT1* with *FZD5* and *FZD9*.

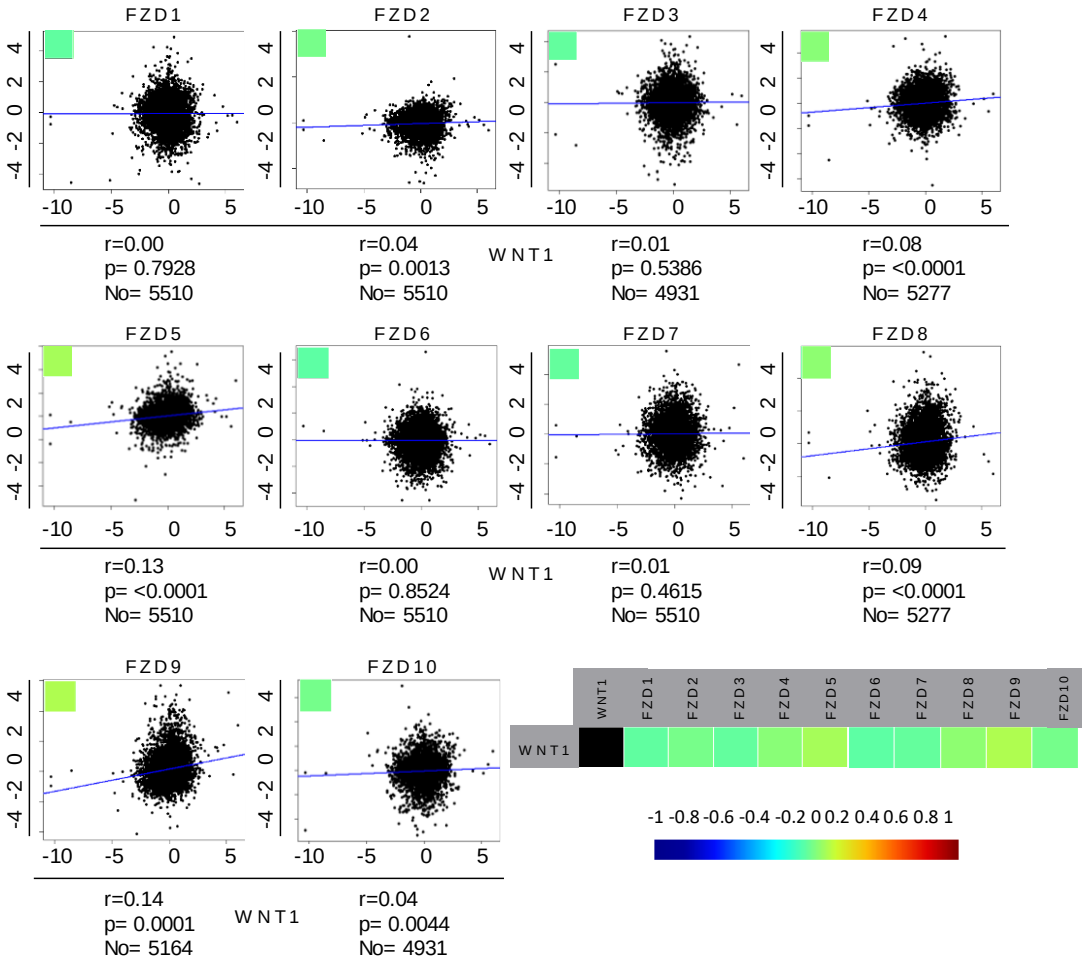


Figure 38. Targeted gene correlation analysis. *For each pair of genes, a correlation plot is displayed, along with correlation coefficient value (r), corresponding p-value (p) and number of patients involved (No). A summary of the Correlation maps in color code obtained from bc-GenEXMiner v4.1 is shown at the bottom of the figure. The analysis was performed by selecting the data from ER+ patients in bc-GenEXMiner v4.1.*

Next, we tested which of the receptors can co-localize with Wnt-1 and so could be involved in the transduction of the signal. For this, co-localization analysis was performed using immunofluorescence. MCF-7TamR-W1 cells were transfected with 1D4-tagged FZDs. Initial screening showed possible co-localization between Wnt-1 and FZD_3, FZD_4, FZD_5 and FZD_{10}. (Fig. 39).

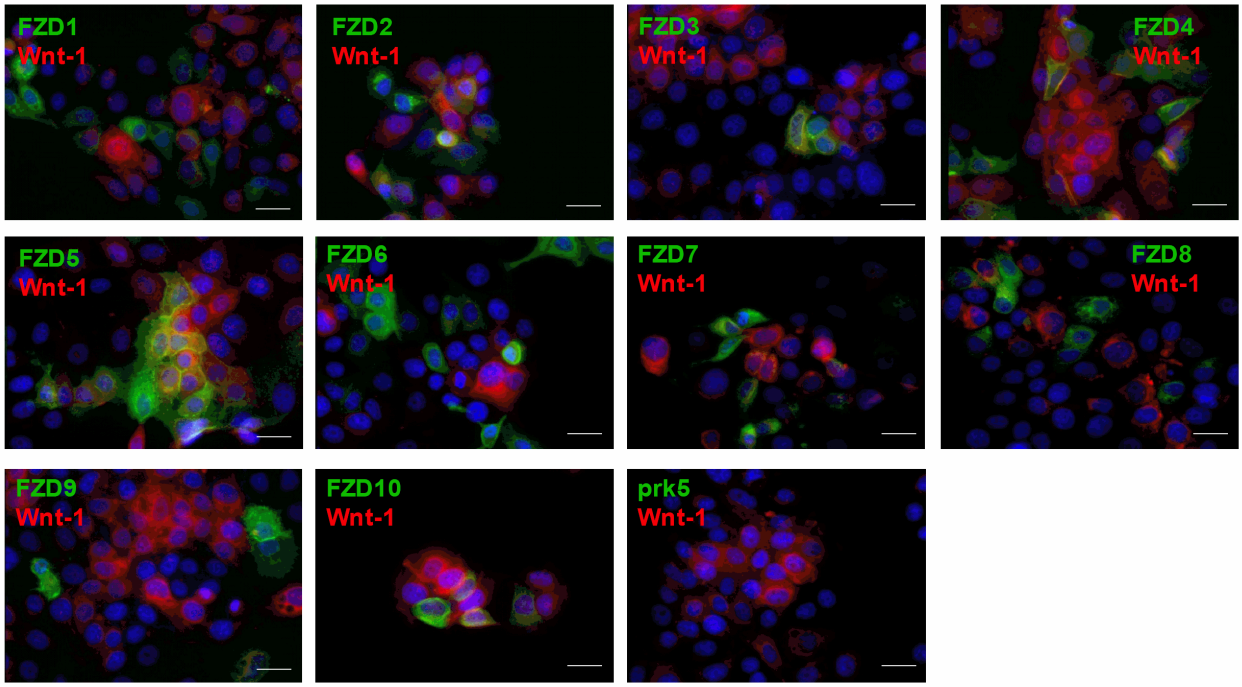


Figure 39. Co-localization assay of Wnt-1 and FZD receptors by immunofluorescence. *Immunofluorescence staining for Wnt-1 in red and different 1D4-tagged FZD receptors in green. DAPI staining of nuclei is in blue, scale bars= 40 μm.*

However, more detailed images using a confocal microscope showed that the strongest co-localization of Wnt-1 in the membrane was with FZD_3, FZD_4 and FZD_5 (Fig. 40).

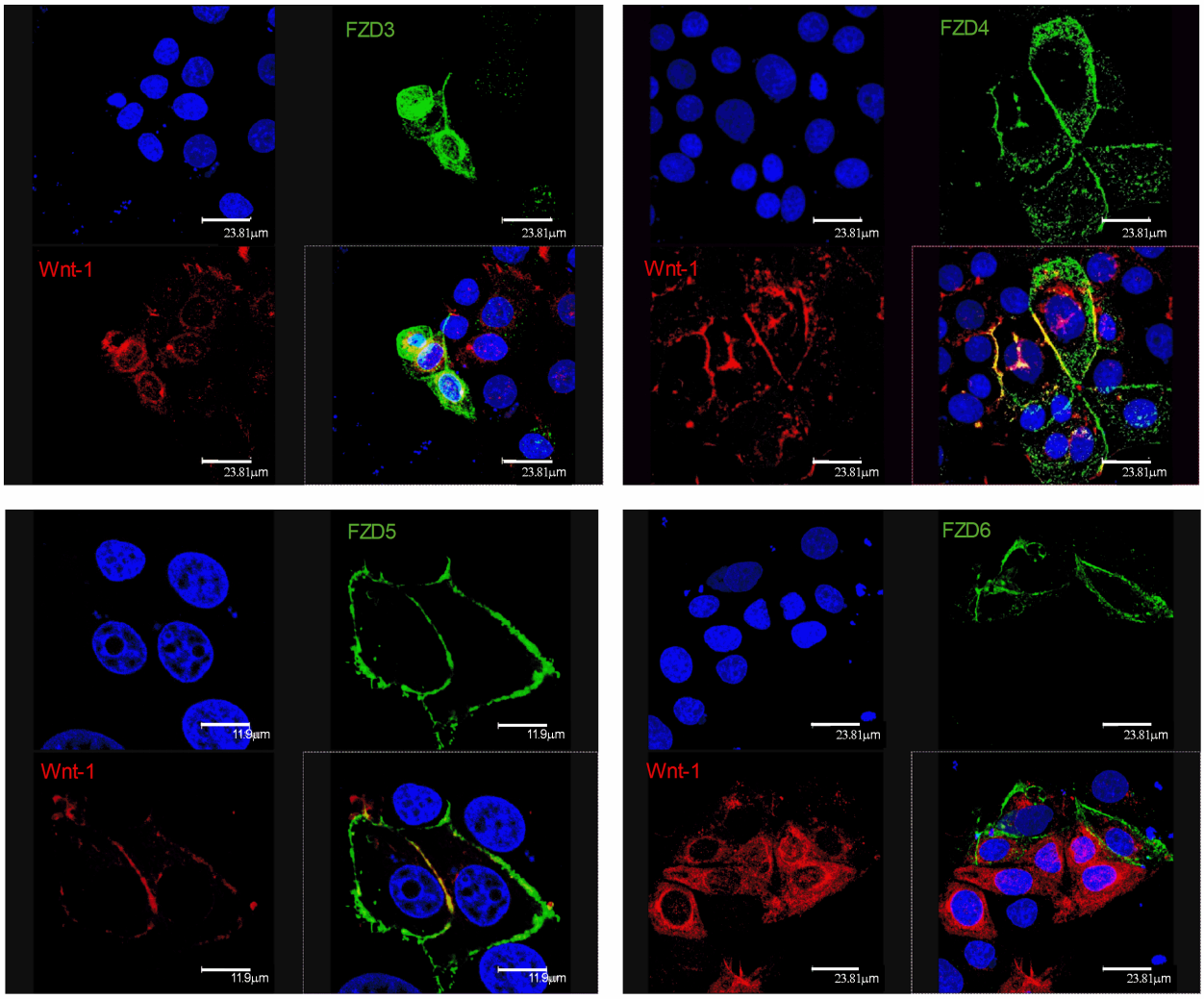


Figure 40. Confocal images of co-localization assay of Wnt-1 and FZD receptors. *Wnt-1 in red and FZD_3, FZD_4, FZD_5 and FZD_6 in green. Note the co-localization at cell borders in yellow for FZD_3, FZD_4 and FZD_5. FZD_6 is shown as sample of non-colocalization. DAPI staining of nuclei is in blue, scale bars are indicated.*

Given the potential link between Wnt-1 and tamoxifen resistance (Fig. 25), the possible links between expression of FZD receptors and the survival of tamoxifen-treated breast cancer patients were analyzed. As shown in Fig. 41, among all FZD receptors, only high *FZD5* expression correlated with reduced patient overall survival, demonstrating the potential importance of *FZD5* in tamoxifen resistance. On the contrary, high expression of *FZD1* and *FZD10* were associated with good prognosis using DDFS as an endpoint (Table 9).

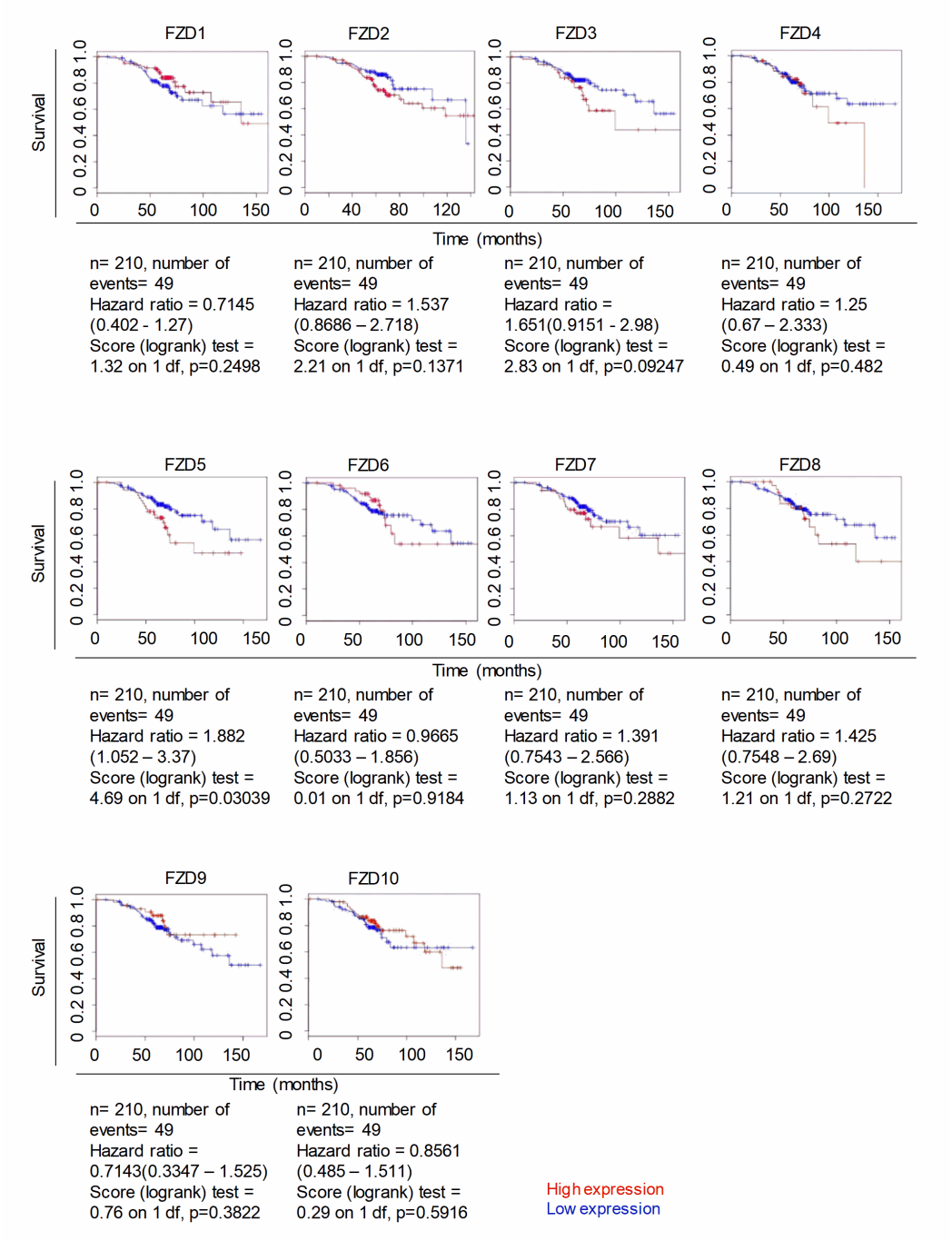


Figure 41. Expression of FZD receptors and survival effect in human breast tumors. *Kaplan-Meier analysis, using OS as endpoint for ER+ tumors in patients treated with tamoxifen, stratified into high (red) and low (blue) gene expression level.*

Gene	OS	DDFS	DFS
FZD1		0.0009	0.00239
FZD2			
FZD3			
FZD4			
FZD5	0.03039		
FZD6			
FZD7			
FZD8			
FZD9			
FZD10		0.0434	
USP6	0.02925	0.02687	

Table 9. Survival effect of Wnt receptor genes expression. *Kaplan-Meier analysis, using OS, DDFS and DFS as endpoint for ER+ tumors in patients treated with tamoxifen, stratified into high and low Wnt receptor gene expression level. Statistically significant p-values are indicated in red when high expression level and in blue when low expression level is associated with poor prognosis.*

Following the strategy used for USP6, to analyze whether FZD_5 enhances Wnt-1 mediated signaling in MCF-7TamR cells, an ATF2-luciferase reporter assay was performed. The cells were very responsive to FZD transfection with only 5 ng of *FZD5* plasmid being sufficient for an additive effect on Wnt-1 mediated ATF2-dependent gene reporter activity (Fig. 42A). To corroborate these results, an immunoprecipitation assay was performed in HEK293 cells to confirm the binding of Wnt-1 to FZD_5. HEK293 cells were co-transfected with V5-WNT1 and 1D4-FZD5, and cell lysates were incubated with 1D4 antibody and immunoprecipitated using protein A/G agarose beads. Fig. 42B shows that when 1D4-FZD_5 is immunoprecipitated a V5-tagged Wnt-1 is detected.

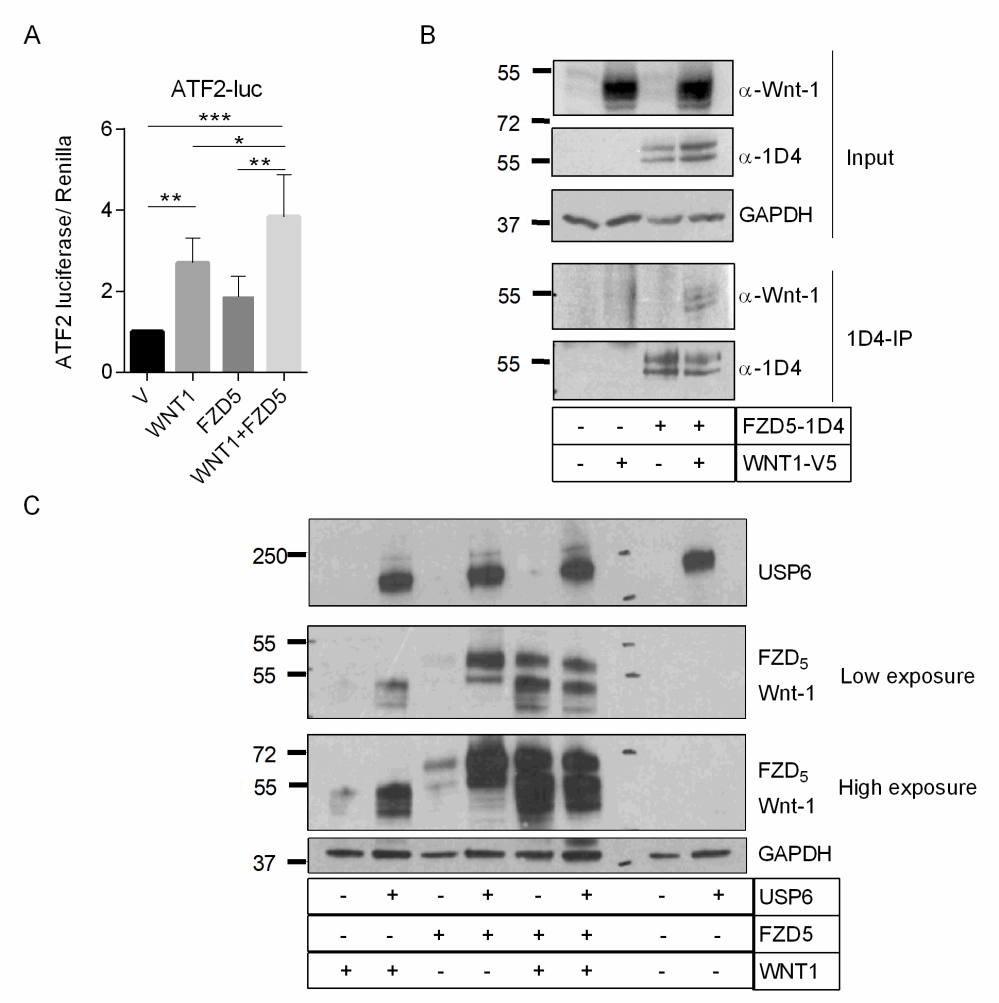


Figure 42. FZD_5 as possible Wnt-1 receptor in MCF-7TamR cells. A. *Gene reporter assay measuring ATF2-dependent transcriptional activity in MCF-7TamR cells expressing WNT1; n=4, * P < 0.05; ** P < 0.01; *** P < 0.001, Student´s t test (error bars represent SD).* ***B.*** *293 cells were co-transfected with WNT1 and 1D4-FZD5, then cell lysates were analyzed by immunoprecipitation with protein A/G agarose and western blotting with Wnt-1 directly (bottom). To confirm immunoprecipitation of Frizzled-IgG, the immunoprecipitated sample was analyzed by western blotting with anti-1D4 antibody. To confirm expression of WNT1 and FZD5 in cells, total lysate was analyzed by western blotting with anti-Wnt-1 and anti-1D4 antibody (top). GAPDH was used as a loading control. Representative blot of n=3.* ***C.*** *Western blot for the indicated antigens in MCF-7TamR cells; FZD5 and Wnt-1 top short exposure, bottom long exposure; GAPDH was used as loading control. Representative blot of n=3.*

Together, these results are consistent with a model in which USP6 increases membrane levels of FZD5 to enhance the response to Wnt-1. To better determine whether FZD5 is a USP6 target, FZD5 levels were measured by western blot in the presence or absence of USP6 (Fig. 42C). USP6 increased the levels of FZD5, but also increased Wnt-1 levels, suggesting a more general effects of USP6. However, immunofluorescence analysis did not find membrane co-localization of USP6 with Wnt-1 although this was observed between USP6 and FZD5 (Fig. 43).

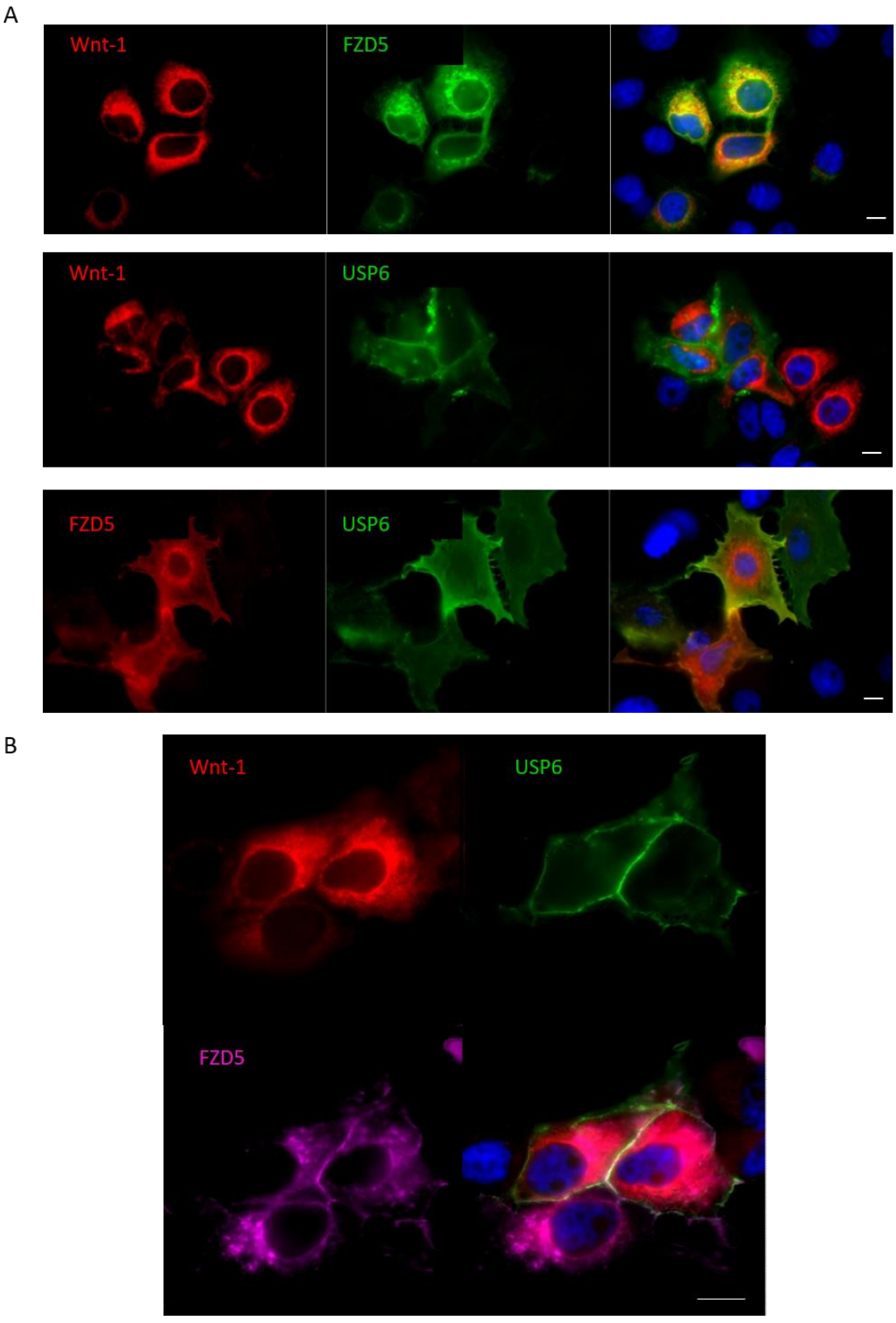


Figure 43. Four color immunofluorescence staining for Wnt-1, FZD_5, USP6 and DAPI in MCF-7TamR cells. A. *Staining for two by two combinations of Wnt-1, FZD_5 and USP6. FZD5 was detected using a far red filter and is represented in green or in red for a better visualization.* ***B.*** *Immunofluorescence staining for Wnt-1 in red, FZD_5 in purple and USP6 in green. DAPI staining of nuclei is in blue, scale bars= 10 μm.*

Discussion

I. WNT SIGNALING IN TAMOXIFEN RESISTANT MODELS

Tamoxifen is the most widely used therapy for ER+ breast cancer patients, which represent almost the 70% of the total. However, many patients will develop resistance to hormonal therapies (Chang, 2012). Wnt signaling has been shown to be important in the context of CSCs (Zeng and Nusse, 2010) and therapy resistance in many tissues (Yang *et al.*, 2008; Flahaut *et al.*, 2009; Noda *et al.*, 2009; Chau *et al.*, 2012), including breast cancer (Zhang *et al.*, 2016). Tumors are composed of very heterogeneous populations of cells, and only a small fraction of the cells have the capacity for renewal and differentiation. The most aggressive and less differentiated tumors have been confirmed to contain a higher proportion of CSCs, whereas more differentiated tumors have a better prognosis (Vivanco, 2010). Previous studies have demonstrated the relevance of hormones and CSCs in the development of therapy resistance (Simões *et al.*, 2011; Piva *et al.*, 2014). MCF-7TamR cells have an increased population of CSCs (cells with $CD44^{+}CD24^{-/low}$ and $ALDH^{+}$ phenotype), increased invasion capacity and a more aggressive phenotype than MCF-7C cells *in vitro* and *in vivo*. Functional assays demonstrated that Sox2 can be used not only as a biomarker, but is also involved in the process of tamoxifen resistance acquisition (Piva *et al.*, 2014). Since Wnt signaling is important for the renewal and differentiation of CSCs, it may be a good target for new therapies. Our group has previously shown that Wnt signaling is inhibited in human embryonal carcinoma cell lines by retinoic acid (Elizalde *et al.*, 2011) and it has also been reported that Wnt signaling is involved in the normal physiology of breast stem cells and that its inhibition reduces tumor growth *in vivo* (Proffitt *et al.*, 2013). It was therefore considered relevant to study the mechanism of Wnt signaling in breast CSCs in more depth to try to block this pathway and inhibit or delay possible tumor recurrence in patients.

Many studies have reported up-regulation of Wnt signaling in *in vitro* models of acquired tamoxifen resistance in breast cancer (Sim *et al.*, 2014, Loh *et al.*, 2013). These models show an increase in the CSC population, and it has also been observed that the Wnt pathway is activated in breast CSCs but suppressed in normal breast stem-like cells (Lamb *et al.*, 2013). Moreover, WNT gene expression levels have been documented to differ in various breast cancer cell lines (Benhaj, Akcali and Ozturk, 2006). Consistent with this, I observed differences in the expression levels of WNT genes in two different tamoxifen resistant breast cancer cell line models enriched for CSCs. While MCF-7TamR cells showed increased levels of *WNT1* expression, both in mammospheres and in $CD44^{+}CD24^{-/low}$ cells, T47DTamR cells did not show any significant increases among any of the WNT genes examined.

Activation of canonical Wnt signaling, by expression of *WNT1* or using recombinant Wnt-3a in breast cancer and breast stem cells increases the population of stem-like cells (Lamb *et al.*, 2013). Moreover, inhibition of Wnt signaling by generation of *Wnt1* knockdown cell lines or using small-molecule Wnt/β-catenin signaling inhibitor, not only reduces the CSC population but also tumor cell invasion and migration (Jang *et al.*, 2015), suggesting that WNT genes are good targets for therapy. Some of the effects of Wnt have been related to other pathways, such as EGFR/ERK1/2 activation (Schlange *et al.*, 2007), but other than this and β-catenin/TCF signaling, downstream Wnt signal transduction in breast cancer has not been studied. I found that different breast cancer cell lines respond differently to the same Wnt ligands. On the one hand, T47DTamR cells respond to *WNT3A* expression by activating β-catenin/TCF signaling. On the other hand, MCF-7TamR cells do not respond to *WNT3A* expression in this way, although they do activate a TCF1-mediated β-catenin-independent activation of β-catenin/TCF luciferase involving ATF2, as previously described in hematopoietic cells (Grumolato *et al.*, 2013). One possibility to explain this could be cell-type specific differences in the metabolic response to stress, which has been observed in MCF-7 and T47D cells and is regulated by tamoxifen (Radde *et al.*, 2015). Radde *et al.*, (2015) reported that T47D cells have lower mitochondrial activity relative to MCF-7 cells, suggesting that T47D cells adapt better to stress caused by tamoxifen, which likely may reflect cancer cell avoidance of apoptosis and different activation of related signaling pathways. Inhibition of mitochondrial function in epithelial cancer cells is a strategy for conferring drug resistance (Martinez-Outschoorn *et al.*, 2011).

Type I nuclear receptors (NRs), such as ER, AR or PR, characterized by the ability to form homodimers, mediate their effects by binding ligands to activate gene expression. The target-gene specificity for a given hormone depends on nucleotide repeats in the hormone response element (HRE) of the promoter of the target gene and the DBD of the receptor that recognizes it. NRs are also affected by posttranslational modifications as a consequence of interactions with other pathways, including Wnt, for instance, by phosphorylation of the the N-terminal A/B region of the receptors by JNKs (Tata, 2002; Rochette-Egly, 2003). Several components of the Wnt pathway modulate NR functions in tissues regulated by ER, PR and AR. For example, transfection of *WNT3A* enhances whereas transfection of *WNT11* inhibits AR-mediated transcription in prostate cancer (Verras, M. Brown, J. Li, X. Nusse, R. Sun, 2004; Zhu *et al.*, 2004). Moreover, activation of Wnt/β-catenin signaling in AR^+ prostate cancer cells is not TCF-dependent but it is mainly through AR-dependent mechanisms (Verras, M. Brown, J. Li, X. Nusse, R. Sun, 2004; Cronauer *et al.*, 2005). Expression of *Wnt1* has also been linked to the induction of retinoic acid-responsive genes (Szeto *et al.*, 2001). Other Wnt pathway components such as

β–catenin are co-activators of AR in LNCaP and PC3 prostate cancer cells (Truica, Byers and Gelmann, 2000; Mulholland *et al.*, 2002; Yang *et al.*, 2002). Finally, TCF factors have also been studied in this context. For example, TCF1 stimulates the effects of ERα, whereas TCF4 acts as a co-repressor in rat mammary epithelial cells (El-Tanani *et al.*, 2001). Having this and the importance of hormone regulation in breast in mind, it was considered that expression of different TCF family members might have distinct effects in breast cancer cells.

In this study, we have observed that the loss of ER may enhance canonical Wnt signaling. T47DTamR cells might respond to Wnt signals as basal cancer lines do, in which it is described that β-catenin accumulation is an early marker of the basal-like phenotype (Khramtsov *et al.*, 2010). Moreover, Wnt-3a may crosstalk with ER signaling by up-regulating ERα and down-regulating ERβ expression in mesenchymal progenitor cells (Gao *et al.*, 2013). Crosstalk between Wnt-3a and ER may explain the reduction of *WNT3A* gene expression in CSCs of T47DTamR cells, which have lost ER expression. Control luminal cells generally have low basal β-catenin/TCF activity. T47D-C cells respond to *WNT1* and *WNT3A*, but to a lesser extent than T47DTamR cells. In the case of MCF-7 control cells, there is no activation of β-catenin/TCF activity; what is more, there is a decrease in the responses to transfection of *WNT1* and *WNT7B*.

AR signaling is important in the regulation of Wnt signaling in some contexts, such as prostate cancer (Pakula, Xiang and Li, 2017). However, AR signaling was not active in our breast cancer cell models, as shown by the lack of response to MDV3100 (Fig. 19).

Another perspective directed us to further study differences in the expression of Wnt signaling components. We observed clear differences in the expression of LEF and TCF transcription factors between MCF-7TamR and T47DTamR cell lines. TCF/LEF members are a small family of DNA binding factors that switch from transcriptional repression to activation in response to Wnt signals. The specificity of this transcriptional switch depends on cell context and expression of TCF/LEF factors and their variants. These variants are generated by alternative promoters, alternative exon cassettes and alternative splicing sites, allowing combinatorial insertion/exclusion of functional and regulatory domains (Arce, Yokoyama and Waterman, 2006), and differences in affinity for DNA. LEF1 protein levels have been reported to be increased in breast cancer cell lines, compared to normal breast cell lines (Lamb *et al.*, 2013). On the contrary, Jamieson *et al.*, (2016) reported that LEF1 protein is undetectable in MCF-7 cells. I did not detect LEF1 expression by qRT-PCR. However, the RNAseq data indicated that the increases of LEF1 in tamoxifen resistant cells was not significant in MCF-7 but it was in T47D cells, suggesting that could be a factor involved in their different responses to Wnt/β-catenin.

TCF1 was the least expressed family member in both cell lines, although the expression in T47DTamR was higher. On the contrary, results from qRT-PCR analysis showed that TCF3 was the most highly expressed family member in both cell lines. These data suggest that TCF family expression patterns could be important to determine how cells respond to Wnt-1. However, it is important to note that the different variants have not been studied in depth. There are no many variants of TCF3, but LEF1 (not detectable in our cell lines) can be expressed with alternative N- and C- termini and there are more than 10 versions of TCF4 that could have different biological roles have been found (Mao and Byers, 2011). Context-dependent activation may provide for other differences. A TCF3/TCF1 switch has an important role in *WNT3A* induced stem-cell renewal, regulating stem cell marker Nanog, depending on the availability of TCF1 and how it competes with TCF4 variants (Yi *et al.*, 2012) or how it is exported from the nucleus (Najdi *et al*., 2009). RNAseq data also showed increased levels of TCF1 and TCF3 in T47DTamR cells, compared to control T47D cells. These data suggest that changes in the balance of TCFs and/or expression of different TCF variants could affect how each cell line responds to Wnt ligands.

Schlange *et al.*, (2007) have described differences in the levels of active β-catenin in response of MCF-7 and T47DTamR cell to sFRPs. I also observed differences in the response to activation of Wnt signaling. After analyzing the roles of ER and AR, which have been implicated in the acquisition of tamoxifen resistance, we considered whether differences could be related to the different expression patterns of TCF family factors that could have been altered during acquisition of resistance. However, Wnt signaling did not appear to depend on either ER or AR signaling. Clearer differences were observed in the expression of different TCF factors. Altogether, it seems that the differences in Wnt signaling are cell type dependent and that TCF factors could play a role.

ATF2 is involved in non-canonical Wnt signaling (Ohkawara and Niehrs, 2011). In light of previous studies showing a switch from canonical to non-canonical ATF2-dependent Wnt signaling (Bengoa-Vergniory *et al.*, 2014) and that canonical and non-canonical Wnt signals can be important at different steps of differentiation (Many and Brown, 2014), I analyzed ATF2 activation using gene reporter assays in cells expressing *WNT1*. D'Amico *et al.,* (2000) showed that expression of *CCND1*, a WNT target gene, is induced through a CREB/ATF2 binding site in MCF-7 cells. In mesothelioma cells *WNT1* expression has also been implicated in non-canonical Wnt/JNK signaling (You *et al.*, 2004). However, the same group published that canonical Wnt signals predominate in MCF-7 cells (He *et al.*, 2004). Moreover, considering the importance of TCF family proteins, a synergistic cooperation has been observed between AP-1 and LEF1 transcription factors to activate *MMP7* gene expression (Najdi. *et al*., 2009). I found activation

of ATF2 in response to Wnt-1 using luciferase and GFP reporters. AP-1 family members can form homo- or heterodimers at ATF/CRE binding sites. Jun-Jun and Jun-Fos dimers can bind to ATF/CRE sites (van Dam and Castellazzi, 2001), but ATF/CREB heterodimers have higher affinity than Fos/Jun dimers for these sites (Hai and Curran, 1991). Jun forms stable heterodimers with ATF2 and although its affinity for CRE sites is lower than that of ATF2, ATF2/Jun heterodimers can bind to ATF/CRE sites (Hai and Curran, 1991). Dominant negative forms of AP-1 family members that are mutated either in the DNA binding region or that cannot bind other regulatory proteins, inhibit the activation of the wild-protein (Steinmüller and Thiel, 2003). Experiments using a set of dominant-negative constructs that block DNA binding by formation of inactive dimers suggested that heterodimers and homodimers containing ATF2 are likely important in the response to Wnt-1, since the ΔATF2, which binds ATF2 and Jun had the strongest inhibitory effects.

II. CHARACTERIZATION OF WNT1 EXPRESSION IN BREAST CANCER AND BREAST CANCER STEM CELLS

Tumor recurrence and relapse have been reported to occur as a result of the presence of CSCs (Bao et al., 2013), and in the case of breast cancer the presence of CSCs is also related to worse prognosis (Carrasco et al., 2014). Having observed the increased gene expression of *WNT1* in the context of CSCs present in tamoxifen resistant cells, the importance of *WNT1* in terms of prognosis was analyzed. Clinical significance of Wnt-1 has been studied in different tissues by IHC analysis. Wang *et al*., (2009) showed by IHC that Wnt-1 levels are increased in 61% of nasopharyngeal carcinoma patients, however, this high expression had no effect on RFS, MFS or PFS of patients. In renal cell carcinoma, Kruck et al., (2013) found a correlation between Wnt-1 staining with increased tumor diameter, stage and vascular invasion, but only high cytoplasmic β-catenin levels were related to decreased OS and CSS, while Wnt-1 expression itself could not be correlated. In the case of ovarian cancer, another hormone-dependent type of tumor, Bodnar et al., (2014) observed increased levels of E-cadherin together with β-catenin and Wnt-1, but again, only high β-catenin expression levels correlated with decreased OS. Only in non-small cell lung (NSCL) carcinoma, in which Wnt-1 is highly expressed in less than 50% of tumors, was there a worse prognosis, namely for OS of patients with high levels of Wnt-1 in tumors (Nakashima et al., 2008).

In the present study, the expression of *WNT1* among different subsets of breast cancer patients and the possible correlation with prognosis was analyzed using online tools. Firstly, heterogeneous expression of *WNT1* was observed among all breast cancer patients that did not correlate with molecular subtype, tumor grade, ER status or DFS. Interestingly, there was a negative correlation between *WNT1* expression and OS and DDFS of patients treated with tamoxifen. Since CSCs have been linked to poor prognosis, and we have observed that *WNT1* expression is increased in the subset of $CD44^{+}CD24^{-/low}$ cells in tamoxifen-resistant cells, Wnt signaling seems to be relevant for regulating the properties of CSCs, which ultimately may be translated into worse OS and DDFS. On the other hand, mouse cell lines that have not been treated with tamoxifen also have high levels of *Wnt1* in mammospheres, hence, *WNT1* expression may be increased in environments appropriate for CSC proliferation. Thus, tamoxifen treatment is not the only condition that increases *WNT1* expression, but it may be a relevant factor in ER+ breast cancer patients.

To better understand expression of *WNT1* in different types of breast cancer, immunohistochemistry was performed on a breast cancer tissue array. Analysis of the staining

indicated that Wnt-1 expression is quite heterogeneous in breast tumors. In normal breast samples, Wnt-1 staining was detected in some basal epithelial cells. Wnt target genes have also been found to be localized in the basal layer and to mark mouse multipotent stem cells with epithelial-to-mesenchymal transition characteristics and high regenerative capacity that can differentiate into all linages of the mammary epithelium (D. Wang *et al.*, 2015). Since *WNT1* expression is increased in CSCs, it could also be expressed in some MaSCs. Wnt-1 staining was not correlated with HER2 levels, strong staining was found in around 30% of samples independently of the HER2 expression. On the other hand, a positive correlation between Wnt-1 and ER and PR levels was found. In the case of ER, high Wnt-1 levels increased from 12 to 43%, and from 20 to 46% for PR levels. With respect to pathology, Wnt-1 levels were very low in almost all metastatic cases. These results suggest that Wnt-1 is important in the context of ER+ and PR+ patients, which correlates with the molecular characterization of luminal breast tumors that are those more prone to receive hormonal therapy. However, *WNT1* expression in those patients after tamoxifen treatment is also correlated with worse prognosis. Moreover, *WNT1* expression shows an inverse correlation with metastasis, suggesting that *WNT1* expression might be important at early stages of the disease and not in metastatic tumors.

In silico analysis of gene expression in commonly used breast cancer cell lines showed that *WNT1* is not highly expressed in any of the cell lines representing different types of human breast cancer, and was rather heterogeneous among human breast cancer cell lines. Moreover, in mouse breast cancer cell lines, the highest *Wnt1* expression was found in 4T07 cells, which represent tumors at an intermediate state, with EMT and MET features, suggesting that *Wnt1* expression may be increased at intermediate stages of the disease and confirming IHC data of human tumor samples.

The MMTV-Wnt1 mouse breast cancer model forms tumors that contain CSCs and so is useful to study the role of Wnt-1 in breast cancer. In this model, *Wnt1* expression is related to an increased population of $Thy1^{+}CD24^{+}$ cancer cells (Cho et al., 2008), $ALDH^{+}$ cells (Choi et al., 2012), $CD29^{low}CD24^{+}CD61^{+}$ luminal progenitor cells (Vaillant et al., 2008) and epithelial cells expressing the progenitor cell markers keratin 6 and Sca-1 (Li *et al.*, 2003). To the best of our knowledge, to date, no such characterization has been done using human cells. It was not possible to determine *WNT1* expression in MCF-7TamR $ALDH^{+}$ cells, although an increase of *WNT1* expression in the $CD44^{+}CD24^{-/low}$ population was observed. Many studies have reported a high percentage of $CD44^{+}CD24^{-/low}$ and $ALDH^{+}$ cells in triple negative tumors but only $CD44^{+}CD24^{-/low}$ cells in luminal subtype tumors (Ricardo et al., 2011; Beça et al., 2013). 67NR, 4T07 and 4T1 mouse metastatic cell lines, derived from a single spontaneously arising mammary tumor in a

BALB/c mouse have a higher frequency of $ALDH^+$ cells than human luminal-like cell lines (Kim et al., 2013). These cells show increased expression of *Wnt1* in mammospheres (Fig. 30B), but this was not related to the $ALDH^+$ population. These data suggest that *WNT1* may be expressed in a subset of CSCs with the $CD44^+CD24^{-/low}$ phenotype that are not $ALDH^+$. Indeed, the $ALDH^+$ population has been shown to be different from the $CD44^+CD24^{-/low}$ population (S. Liu *et al.*, 2014). Proteomics analysis showed that the $ALDH^+$ population could be distinguished from the $CD44^+CD24^{-/low}$ population by differential expression of proteins in three major categories; cell-cell junctions, glucose metabolism and signal transduction (Nie *et al.*, 2015). The cell-cell junctions category modulates critical events, including cell motility and surface remodeling involved in cell migration and invasion (Azios et al., 2007) and with CSC features (Nie et al., 2015), in which Wnt-1 could also be involved.

III. EFFECTS OF ECTOPIC EXPRESSION OF WNT1 IN MCF-7 AND MCF-7TAMR CELLS

To better understand the role of *WNT1* expression in CSCs and whether it is important in the acquisition of tamoxifen resistance, cells over-expressing *WNT1* were selected. Stably transfected *WNT1* was detected at high levels by qRT-PCR and protein level by immunofluorescence and western blotting. Western blot assays showed that Wnt-1 protein was found in cell lysates and in the ECM but not in the CM. This confirmed the findings made by Bradley and Brown (1990), who found Wnt-1 was barely detected in the CM and associated with the ECM, where it is thought to act in cell-cell communication over short distances, as may happen in mammosphere formation assays. On the other hand, transcriptional activity of stably transfected *WNT1* was not as strong as that of transiently transfected *WNT1*. The canonical Wnt pathway was not altered in luciferase gene reporter assays, although a modest but significant reduction in activity was observed using GFP reporters. Regarding ATF2 mediated transcriptional activation, a small but still significant effect was observed using the ATF2-luciferase reporter, but no changes were observed in the ATF2-based GFP reporter. This suggests that stably transfected Wnt-1 may accumulate in the ECM in an inactive form. Galli *et al.*, (2018) showed that biologically active Wnt-1 present in the conditioned media from cells expressing *WNT1* does not induce paracrine signaling, unlike in cell co-cultures, indicating that bioavailable Wnt-1 is cell-associated. Therefore, whether transfection of reporters is the best method to study the transcriptional activation in these cells needs to be reconsidered. On the other hand, working in three-dimensional cultures, as is done in the stem cell field, in which CSC growth is optimized and where the cell-cell contact is more important may be reliable. In addition, according to studies of colonic crypts, Wnts are distributed as gradients during successive cell divisions (Farin *et al.*, 2016). Hence, we considered that this 3D cell culture system would be more relevant to study the effects of Wnt-1 in CSCs.

Antiestrogen resistance can be separated into two categories: *de novo* resistance and acquired resistance. *De novo* resistance occurs in ER+ tumors, which are nonresponsive to antiestrogen treatment from the beginning (Chang, 2012). This type of resistance and the role of Wnt-1 can be studied in MCF-7 control (MCF-7C) cells, whereas acquired resistance is developed after long term therapy in ER+ tumors, which corresponds to the MCF-7TamR cell model (Domenici *et al.*, 2014). First, to determine if Wnt-1 is related to acquisition of tamoxifen resistance, I tested whether cell proliferation was altered by Wnt-1 at different concentrations of tamoxifen. A 30% reduction was observed in MCF-7C cells for tamoxifen concentrations higher than 10^{-8} M,

independently of *WNT1* expression. On the contrary, as expected, MCF-7TamR cells do not show any significant change in proliferation in response to Wnt-1, and cells maintained their proliferation rates around 100%, even at the higher concentrations of tamoxifen. I also confirmed that tamoxifen induces cell cycle arrest at G_0/G_1 and that Wnt-1 does not affect this, suggesting that *WNT1* expression is not sufficient for acquisition of *de novo* resistance to tamoxifen.

Other mechanisms for acquisition of tamoxifen resistance are related to the loss of ER expression and function. I confirmed that ER mRNA expression and protein levels are not significantly affected by Wnt-1, however, ER transcriptional activity may be altered since both MCF-7C-W1 and MCF-7TamR-W1 had reduced PR expression levels (Fig. 33D). To further analyze whether the ER response is different in TamR-W1 cells and whether this affects tamoxifen sensitivity, I analyzed the ability of cells to grow in anchorage independent conditions. ICI, which antagonizes ER, MCF-7TamR-V and MCF-7TamR-W1 cells, did not affect the numbers of colonies formed. There was, however, a difference in the colony size for these two cell lines in the absence of treatment, indicating that Wnt-1 may not affect the number of cells that retain the capacity to form colonies but it may affect their proliferation. In summary, it seems that *WNT1* expression is not sufficient for acquisition of tamoxifen resistance but it may have a role in long-term proliferation of tamoxifen resistant cells, most importantly in those cells that retain ER expression, even though ER is not completely active.

In fact, *WNT1* expression was found in CSCs isolated from MCF-7TamR cells (Fig. 15). I therefore analyzed the populations of CSCs in the *WNT1* expressing cell lines. Interestingly, Wnt-1 increased the $CD44^+CD24^{-/low}$ population in both MCF-7C and MCF-7TamR cells (Fig. 35A). Consequently, the number of mammospheres formed by these cells was also increased by Wnt-1. This was statistically significant in MCF-7TamR cells, in which the proportion of CSCs and the number of mammospheres formed is already elevated compared to in MCF-7C cells. Surprisingly, although *SOX2* and *NANOG* levels were increased in MCF-7TamR cells in comparison to MCF-7C cells, *SOX2, NANOG* or *OCT4* expression levels were not increased in *WNT1*-expressing cell lines (Fig. 36A). Moreover, expression of *SOX2* was significantly reduced in MCF-7TamR-W1 cells in the mammospheres formed after passage, where the proportion of CSCs is enriched. In mice, *Wnt1* silencing has been reported to suppress mammosphere formation and reduce the $ALDH^+$ population *in vitro* and reduce tumorigenic potential and tumor initiation capacity of cells *in vivo* (Choi *et al.*, 2012). Moreover, *Wnt1* knockdown suppresses breast cancer capacity for invasion and migration, which translates into reduced tumor metastasis in a murine xenograft model (Jang *et al.*, 2015). However, characterization of the Wnt

expression profile of these CSCs has not been published. In colon, the highest expression of Wnt targets is in the bottom of the crypts, where $Lgr5^+$ stem cells are located between Paneth cells, which are the cells that produce high levels of Wnt-3. In this system, Wnt-3 is transferred in a cell-bound manner through cell division, and not through diffusion (Farin *et al.*, 2016). Despite epithelial production of Wnt-3a, mesenchymal cells are also a redundant source of Wnt-3 in the niche (Puschhof and Clevers, 2018). Wnt-1 appears to accumulate in the extracellular matrix, where it may act on surrounding cells. Moreover, *WNT1* expression increases the $CD44^+CD24^{-/low}$ population, but does not affect the number of single cells able to undergo unlimited divisions or the expression of *SOX2, NANOG* and *OCT4* (Fig. 34 and 36), suggesting that the enriched population may be an intermediate stage between the complete stem and differentiated cells. *WNT1* has been previously described not to affect the number progenitor cells under adherent and mammosphere conditions, but to significantly increase acinar colony formation in collagen, suggesting Wnt signaling is necessary for luminal acinar progenitor activity (Arendt *et al.*, 2014). Thus, identification of cells and their differentiation into intermediate populations of mammary stem cells and/or progenitor to mammary luminal epithelial and myoepithelial cells would be worth studying in more detail. For that, identification of the population using several markers that have been used to identify these populations including $Ep\text{-}CAM^{-/low}CD49f^+$, $CD10^+$, or $Ep\text{-}CAM^+/MUC\text{-}1^{neg}$ (Ghebeh *et al.*, 2013) would be recommendable to do. Primary human mammary epithelial cells (MECs) can form luminal acinar and basal ductal colonies on collagen gels. Wnt-1 has been reported to regulate both types of progenitor while progesterone favors ductal progenitor cells and estrogen affects acinar ones. However, both hormones stimulates paracrine Wnt signaling (Arendt *et al.*, 2014). Since we have observed that Wnt-1 correlates with ER and PR levels and it is important in the context of tamoxifen treatment, it would also be interesting to further study the link between hormonal regulation and Wnt-1 signaling.

As several types of breast cancer stem cells have been described, the possibility was considered that Wnt-1 enriches for other CSC phenotypes. Among the different types of breast CSCs, $CD44^+CD24^{-/low}$ cells display a mesenchymal phenotype while $ALDH^+$ cells are more epithelial-like (S. Liu *et al.*, 2014). In mouse breast cancer cell lines, 67NR cells are more mesenchymal than 4T1 cells, which undergo MET and have more epithelial characteristics, while 4T07 cells have an intermediate phenotype with low levels of *CDH2, VIM, CDH1* and *ZEB2* (Dykxhoorn *et al.*, 2009). I found that Wnt-1 is most highly expressed in 4T07 (Fig. 29), whereas 4T1 cells have a higher proportion of ALDH positive cells (Fig. 30). Analysis of the expression profiles of EMT-related genes found that *VIMENTIN* and *ZEB1* levels tend to be lower in MCF-7TamR cells than in MCF-7C cells, while *SNAI2* is higher. On the other hand, there were no significant changes in epithelial

markers, suggesting that tamoxifen resistance does not induce EMT. When analysis was focused on changes induced by Wnt-1, the only significant change in MCF-7C-W1 cells were *VIMENTIN* in adherent cells and mammospheres and *SNAI2* expression in adherent cells. On the other hand, no significant changes were found in MCF-7TamR cells, but Wnt-1 further reduced *VIMENTIN* and *ZEB1* levels in mammospheres. Wnt/β-catenin signaling has been reported to be active in TNBC, where Wnt target gene expression is much higher than in luminal cell lines (Dey *et al.*, 2013). Wnt/β-catenin signaling pathways in TNBC have been related to metastasis progression (Dey and Barwick, 2013). Moreover, Jiang *et al.,* (2016) reported that overexpression of miR-148a reduced expression of *WNT1* and other Wnt/β-catenin components, such as β-catenin and TCF4, blocking migration and invasion in breast cancer cell lines. This effect of Wnt-1 on invasion has also been reported *in vivo* and linked to the induction of Snai1 protein and repression of *CDH1* transcription through an Axin2-dependent pathway (Yook *et al.*, 2006). This was not observed in our system, in which MCF-7C cells showed a tendency to induce EMT markers in response to Wnt-1, but not tamoxifen resistant ER+ tumor cells.

If we conclude that *WNT1* expression is more important in early stages of breast cancer, and it is related to the enrichment of TamR CSCs, Wnt-1 may facilitate the adaptation of $CD44^{+}CD24^{-/low}$ cells to the niche in the presence of tamoxifen. On the other hand, the modest changes observed in transcriptional activation and CSCs and EMT-related gene expression can be due to the accumulation of Wnt-1 in the cytoplasm and ECM. Galli *et al.*, (2018) reported that overexpressed Wnt-1 protein accumulates in the endoplasmic reticulum and Golgi, and that elevated expression of the upstream regulator of trafficking WLS is necessary to enhance Wnt-1 signaling. Therefore, it would be interesting to upregulate this regulator, in order to determine if this increases the effects of Wnt-1 on breast cancer cell function.

These results point out two aspects to consider. First, that the changes and responses to Wnt-1 in stably *WNT1* transfected cells are quite modest and second, that there may be differences in the response to Wnt-1 in cells that are resistant to tamoxifen, compared to those that are not. The effects observed are more clear and consistent in MCF-7TamR cells, were Wnt-1 seems to have a more important role, while in MCF-7C cells, Wnt-1 may affect EMT. However, expression of *WNT1* in the latter cells is not sufficient to increase the CSC population or to confer resistance to tamoxifen.

IV. IDENTIFICATION OF WNT1 RECEPTORS AS SIGNALING MEDIATORS AND POSSIBLE THERAPEUTIC TARGETS

I observed that Wnt-1 appears to increase the proportion of CSCs. The analyzed population does not express more *SOX2, OCT4* or *NANOG.* Although we were not able to determine a specific phenotype, Wnt-1 does increase the number of cells that form mammospheres. These findings suggest that targeting Wnt-1 mediated signaling could be a promising alternative strategy to prevent recurrence.

Over the last decade, targeting CSCs has been a goal to overcome poor prognosis in breast cancer, leading to better patient survival. Currently used approaches for targeting CSCs include phenotypic marker based targeting, including CD44. However, most markers do not distinguish normal stem cells from CSCs. Furthermore, CSCs are believed to be the cause of tumor heterogeneity, which is a key factor for therapy resistance and complicates targeting of cell surface markers (Annett and Robson, 2018). Selective targeting of CSC signaling networks to impair self-renewal, proliferation and differentiation is a challenge. Targeting Wnt signaling seems promising, and there are several approaches used in clinical trials, such as anti-FZD10 antibodies for synovial sarcoma and OMP-54F28, which is a recombinant fusion protein of the cysteine-rich domain of FZD_8 and the immunoglobulin Fc domain that competes with the native FZD receptors for Wnt ligands, as well as the previously mentioned Vantictumab (OMP-18R5) (Phi *et al.*, 2018).

Wnt-1 silencing has been reported to have apoptotic effects on MCF-7 cells (Wieczorek *et al.*, 2008). Another general approach for targeting Wnt signaling pathway is acting through regulators. Inhibition of ZNRF3 and RNF43 E3 ligases enhances Wnt/β-catenin signaling, as well as Wnt/PCP signaling, in agreement with the role of FZD receptors in both canonical and non-canonical Wnt signaling (Hao *et al.*, 2012). Several USPs have been found to regulate Wnt signaling, specifically, FZD receptors are targets of USP6 (Madan *et al.*, 2016). I found that USP6 enhanced Wnt-1 mediated signaling and mammosphere formation in MCF-7TamR-W1 cells, consistent with a role in the regulation of the Wnt-1 response. Therefore, USP6 could be a good candidate to be blocked in tumors of patients with high expression of *WNT1*. The USP6-related USP32 is over-expressed in breast cancer cell lines and primary breast tumors (Akhavantabasi *et al.*, 2010). Moreover, USP6 is classified as cancer causative gene by Cancer Gene Census (Gao *et al.*, 2012). Nevertheless, regulating the abundance of receptors at the plasma membrane through the regulation of ZNRF43 may also affect other targets and have side effects. For this

reason, it may be therapeutically more interesting to determine the specific receptor that binds Wnt-1 and try to block that binding, for example using peptides or monoclonal antibodies. For example, LRP6 antibodies have been described, YW210.09 inhibits Wnt-1 induced signaling in HEK293 and breast Hs578T cell lines, whereas another potentiates Wnt signaling (Gong *et al.*, 2010). LRP6 is a specific Wnt co-receptor that activates Wnt/β-catenin signaling when phosphorylated, whereas Ror1/2 Wnt co-receptors trigger non-canonical Wnt signaling (Grumolato *et al.*, 2010). Since Wnt-1 increases ATF-2-dependent transcription in MCF-7TamR cells but not in T47DTamR cells, identification of FZD receptors that transduce the Wnt-1 signal would provide targets to inhibit both canonical and non-canonical Wnt signaling in tamoxifen-resistant cells.

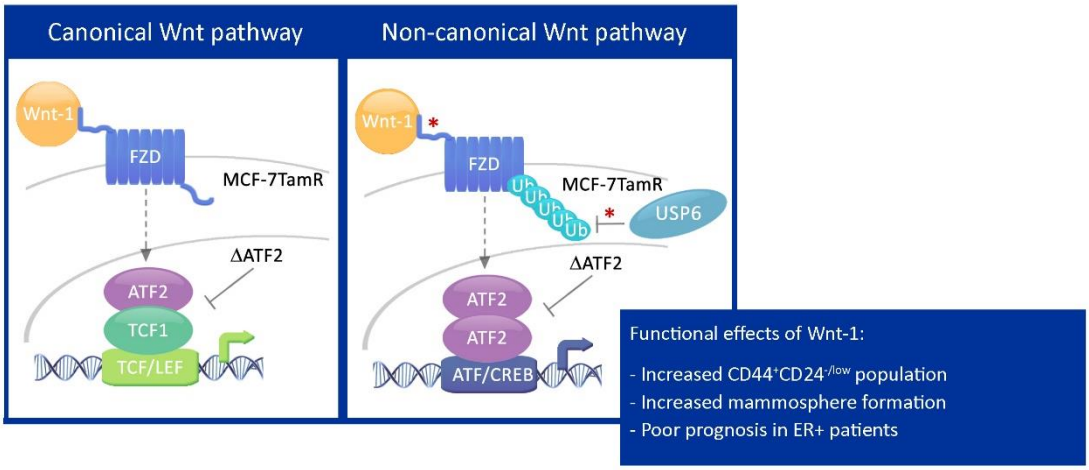
In silico analysis was used to identify the Wnt-1 receptor candidate. Gene correlation analysis found weak correlation of *WNT1* expression and all FZD receptors, with the highest positive correlations for *FZD9* and *FZD5*. On the other hand, breast cancer patient data showed that *FZD5* correlated with poor prognosis in patients treated with tamoxifen. Furthermore, a general screen to detect co-localization of FZD receptors and Wnt-1 showed a good co-localization at the membrane for Wnt-1 with FZD_3, FZD_4 and FZD_5. Taken together, these observations suggested that studies of *FZD5* in the context of Wnt-1 signaling may be relevant. Indeed, Voloshanenko *et al.*, (2017) showed that Wnt-1 activates TCF4-dependent luciferase reporter activity *via* FZD_5 in HEK 293 cells. I found that FZD_5 enhanced TCF/β-catenin independent signaling. Moreover, expression of both *WNT1* and *FZD5* are related with poor patient survival, and both proteins co-localize at the membrane and increase the activation of ATF2-dependent signaling. Finally, since USP6 regulates FZD receptor abundance at the membrane. I tried to confirm the accumulation of FZD_5 at the membrane by co-expressing *USP6* and *FZD5*. Although, higher levels of FZD_5 were observed by western blot, it was not possible to demonstrate that its accumulation was exclusively at the membrane. Nevertheless, there is also evidence to support the hypothesis that USP6 regulating FZD_5, since antibodies targeting FZD_5 have been observed to inhibit the growth of *RNF43*-mutant cells *in vitro* and in *in vivo* xenografts in pancreatic ductal adenocarcinomas (Steinhart *et al.*, 2016), supporting the idea of the importance of posttranslational regulation of FZD_5 by ubiquitination. Moreover, the antibodies used by Steinhart *et al.* (IgG-2919 and IgG-2921) showed high affinity binding to the human FZD_5 CRD and some cross reactivity to the FZD_8 CRD. This, compared to the cross reactivity of OMP-18R5 for FZD_1, FZD_2, FZD_5, FZD_7 and FZD_8, may present a better option for tamoxifen resistant patients. However, these antibodies have not yet been tested in clinical trials.

In summary, this work suggests that blockade of Wnt-1-mediated signaling in tamoxifen resistant patients has therapeutic interest for inhibition of the proliferation of $CD44^{+}CD24^{-/low}$ population and of breast cancer cells with the capacity to form mammospheres, and therefore improve patient prognosis. Moreover, blocking Wnt-1 signaling may also be relevant before acquisition of resistance, since it seems that Wnt-1 mediated signaling may be related to early events of EMT, leading to a higher possibility of metastasis. The pathway may be blocked by inhibiting the binding of Wnt-1 to FZD_5, which can be regulated by USP6. In conclusion, in future, it would be interesting to determine whether targeting USP6 or FZD_5 can improve patient prognosis.

Conclusions

As a summary of these results, we can draw the following conclusions:

1. *WNT1* mRNA levels are elevated in mammospheres and $CD44^{+}CD24^{-/low}$ MCF-7TamR breast CSCs, but not in $ALDH^{+}$ CSCs.
2. Wnt/β-catenin signaling activity is low in MCF-7 cells and this is related to the low expression levels of TCF/LEF family proteins in this cell line.
3. TCF1 not only restored a canonical Wnt response to MCF-7TamR cells, but also activated signaling independently of β-catenin and this could be blocked by ΔATF2.
4. Wnt-1 activates ATF-2-dependent signaling in MCF-7TamR cells.
5. *WNT1* expression in tumors is heterogeneous with respect to the different subtypes, but correlates with high expression of ER and PR but not with HER2 levels. Moreover, *WNT1* expression correlates with poorer OS and DDFS in ER+ breast cancer patients after tamoxifen treatment. In addition, Wnt-1 levels are lower in tumor metastases.
6. *WNT1* expression does not affect to tamoxifen resistance but it may play a role in proliferation of CSCs population.
7. Wnt-1 has different effects on tamoxifen sensitive and tamoxifen resistant cells, enhancing expression of EMT markers and enriching for $CD44^{+}CD24^{-/low}$ cells, respectively.
8. Wnt-1 binding to FZD_5 enhances ATF2-mediated signaling and this may be regulated by USP6. FZD_5 and USP6 may be targets to block Wnt-1-mediated signaling in breast cancer.



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