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ALOX 5 as a CSC-specific target in Breast Cancer

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Grau en BIOTECNOLOGIA

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Abbreviations

ALOX5: Arachidonate 5-lipoxygenase

CSCs: Cancer stem cells

PTX: Paclitaxel

FBS: Fetal Bovine Serum

LTs: Leukotrienes

AA: Arachidonic acid

Resum

El càncer de mama és el segon més comú en la població mundial, però els tractaments quimioterapèutics actuals han mostrat reaccions adverses i fenòmens de multiresistència, essent aquest últim principalment degut a l'existència d'una població de *Cancer Stem Cells* (CSCs). Per tant, l'objectiu d'aquest projecte ha estat testar un inhibidor especific (siRNA) del gen ALOX5 per veure l'efecte que té en les CSCs. Per tal d'obtenir candidats adequats, es va utilitzar *high-throughput screening* comparant CSC amb no-CSC. Diversos candidats van ser escollits per a ser validats per PCR quantitativa, dels quals l'araquidonat 5-lipoxigenasa (ALOX5) va ser escollit com la diana més prometedora per a dissenyar una teràpia contra les CSCs.

El mètode utiltizat per identificar les CSCs es mitjançant la separació cel·lular per un reporter gene (tdTomato) sota l'expressió d'un marcador establert de CSCs. D'aquesta manera es podia evaluar si després dels tractaments hi havia canvis significatius en el percentatge de CSCs. Un dels punts de partida va ser testar un quimioterapèutic com el paclitaxel (PTX) per veure l'efecte que produïa en les CSCs. Seguidament es va testar l'efecte combinat del paclitaxel amb el siRNA del gen ALOX5 comprovant els percentages de CSCs al final de l'experiment. L'assaig de mamosferes permet testar si el silenciament té un efecte sobre la supervivència de les CSCs. També es va testar l'eficiència d'internalització de les nanoparticles per mitjà del microscòpi confocal.

Els resultats d'aquest treball mostren com els tractaments de quimioteràpia convencionals tenen un efecte en les non-CSCs però no en les CSC. Després de tractar les cèl·lules de càncer de mama amb paclitaxel sempre hi ha un augment relatiu del percentatge de CSCs. L'experiment que combina el PTX amb el siALOX5 mostra que, la presència del siRNA evita l'augment relatiu del nombre de CSCs observat quan només hi ha l'efecte del PTX. Un cop normalitzats els replicats, no hi ha diferències significatives entre els percentatges de CSCs. No és pot descartar ALOX5 com a diana per les CSCs en l'assaig de mamosferes el silenciament d'aquest gen té un efecte en la supervivència de les CSCs en dues línes de càncer de mama. L'assaig d'internalització de les nanoparticles mostra un alta eficència d'internalització al citoplasma, però també hi ha agreggació en els lisosomes. Aquesta agregació és un problema atès que les condicions d'aquest orgànul poden degradar el siRNA.

Abstract

Breast cancer is the second most common cancer in the overall world population, but its actual chemotherapeutic treatment has shown adverse effects and multi-resistance phenomena, being this last due mainly to the existence of a Cancer Stem Cells (CSC) population within the tumor. Thus, the aim of this project has been to test an ALOX5 inhibitor to test the impact on CSCs behaviour, as well as the internalitzation of a potencial nanocarriers for drug delivery, Several candidates were chosen to be validated through quantitative PCR, from which arachidonate 5-lypoxigenase (ALOX5) was chosen as the most promising candidate to target CSC.

The methodology used to identify CSCs is based on the detection of a reporter gene (tdTomato) under the expression of a well-established CSC promotor, by cell sorting. This model allows to detect changes in CSCs percentatge. The first step was to test quimiotherapeutic agent (paclitaxel) in order to determine the effect in CSCs population. The second step was to design an experiment that combines paclitaxel and siALOX5 at different point times and analyze the CSCs percentatge. Mammospehere assay was another experiment to check survival of CSCs. To design a new specific nanomedicine with an inhibitor of ALOX5 Internalitzation assays with nanoparticles was carry on to monotorize the ubication of it.

The results showed that conventional therapeutic agents did not affect CSCs population but it affects non-CSCs. According to this result, paclitaxel is a good choice to reduce bulk tumor. After treating cells with paclitaxel the percentatge there is a relative increase of CSCs, this is because paclitaxel only affects non-CSCs. The results from assay that combines PTX and siALOX5 showed that the presence of siALOX5 impairs the relative increased observed only with PTX. Normalized data did not show significant differences between siC and untreated samples but this results are not enough descart ALOX5 as a CSCs target because mammospehre assay showed that silencing of ALOX5 have an effect on CSCs survival in a two diferent breast cancer cell lines. Internalitzation assays of nanoparticles showed high internalitzation efficency in citoplasm, as well as an aggregation in lisosomes. This aggregation is a probem because siRNA is degradate due tu de conditions of lisosomes, such us acid pH.

Introduction

For decades, tumour initiation and development have been regarded as a multistep process, reflected by the multiple genetic alterations found in the tumour mass cells that drive the transformation of normal human cells into highly malignant derivatives, and by the absence of animal models able to reproduce the human cancer with only one genetic mutation. Currently, increasing evidences indicate that the events leading to tumour initiation are orchestrated by cancer stem cells (CSCs) [1]. Despite advances in cancer treatment, many patients still fail therapy, resulting in disease progression, recurrence, and reduced overall survival. Historically, much focus has been on the genetic and biochemical mechanisms that cause drug resistance. However, cancer is widely understood to be a heterogeneous disease and there is increasing awareness that intratumoral heterogeneity contributes to therapy failure and disease progression [2].

There are two models that explain how tumor heterogeneity arises and contributes to disease progression. First model is the clonal evolution model, which suggest that cancer cells evolve progressively during multistep tumorigenesis and that tumor cell heterogeneity, which is created by heritable genetic and epigenetic changes, creates the raw material for the selection and clonal outgrowth of novel cell populations. A second model is CSCs theory, which proposes the residence of cells with different states of stemness and differentation within the tumor that generate tumor heterogeneity

It is believed that CSCs are not only the source of the tumor, but also may be responsible for tumor progression, metastasis, resistance to therapy, and subsequent tumor recurrence. Therefore, a better understanding of the biology of CSCs in each tumor may be critical step toward the development of treatments to eventual cure of cancer.

1. BREAST CANCER:

Breast cancer is the second most common cancer in the overall world population, but its actual chemotherapeutic treatment has shown adverse effects and multi-resistance phenomena, being this last due mainly to the existence of CSCs population within the tumor.

Previous work in my group finds a set of target genes via high-throughtput screening comparing CSCs with non-CSCs from breast cancer cell lines (data not published yet). To do so, samples were collected from luminal (MCF7) and basal (MDA MB 231) breast cancer cell lines, in a way to include a broad range of breast cancer types. Suitable candidates, were chosen to be validated by through quantitative PCR. Arachidonic 5-lipoxygenase (ALOX 5) was chosen as the most promising candidate to target CSCs ***(fig.1).***

Gene Name	log2ratio MCF7tdT+ vs MCF7tdT- / log2ratio MDAtdT+ vs MDAtdT- / log2ratio tdT+ vs tdT-	FC MCF7tdT+ vs MCF7tdT-	FC MDAtdT+ vs MDAtdT-	FC tdT+ vs tdT-	P-Value tdT+ vs tdT-	Validated primers	Confirmed expression in MCF7	Confirmed expression in MDA MB 231
PGC		3.76	3.62	3.69	1.21E-02	—	—	—
ALOX5		5.15	2.50	3.59	4.67E-03	Yes	Yes	Yes
EGR4		3.33	2.80	3.05	3.83E-02	Yes	Yes	No*
BST2		2.60	2.62	2.61	2.55E-04	Yes	No	Yes
TGFB2		3.38	1.49	2.25	1.99E-02	Yes	No*	No
HLA-DRB5		2.34	2.15	2.24	1.32E-02	—	—	—
PTPRE		2.08	2.02	2.05	8.67E-04	Yes	No*	No
SNAI2		1.63	2.57	2.05	1.77E-02	Yes	Yes	No
IL10RA		1.23	3.21	1.99	1.01E-01	—	—	—
GRM5		1.52	1.96	1.73	3.63E-02	—	—	—
EPHA4		1.83	1.06	1.40	4.48E-01	—	—	—
LIPA		1.27	1.22	1.24	6.13E-02	Yes	Yes	No
TLR2		1.55	-1.09	1.19	4.51E-01	—	—	—
CMKLR1		-1.02	1.28	1.12	3.63E-01	Yes	No	Yes
GATA4		1.04	1.17	1.11	2.87E-01	—	—	—
SNAI1		-1.02	-1.11	-1.06	5.91E-01	Yes	Yes	Yes
SPARC		-1.03	-1.28	-1.15	3.91E-01	Yes	Yes	No

***Figure* 1 Expression microarray analysis showing the chosen candidates**. The three right columns summarize the validation steps for quantitative PCR. Genes that don't show significant difference between CSC and non-CSC, even though there is a clear divergence are marked with *, since this was attributed to disparity between the analyzed samples. FC=fold-change. P-value > 0,05 showed in light red, P-value < 0,05 showed in light green.

2. ALOX5 gene

Human ALOX 5 is a non-heme iron containing dioxygenase. It's gene spans > 82kb and consists of 14 exons. This enzyme is also known as arachidonate: oxygen 5-oxido-reductase that catalyzes the formation of leukotriene (LT) or ecosatetracnoic acid from arachidonic acid ***(fig.2)***

These derived substances implicated in the pathogenesis o variety of human diseases, including cancer, are now believed to play greater role in tumor progression, metastasis and angiogenesis among others. Inhibition of ALOX5 delays tumorigenesis in animals and humans, which in turn can stop tumor progression in various tissues.

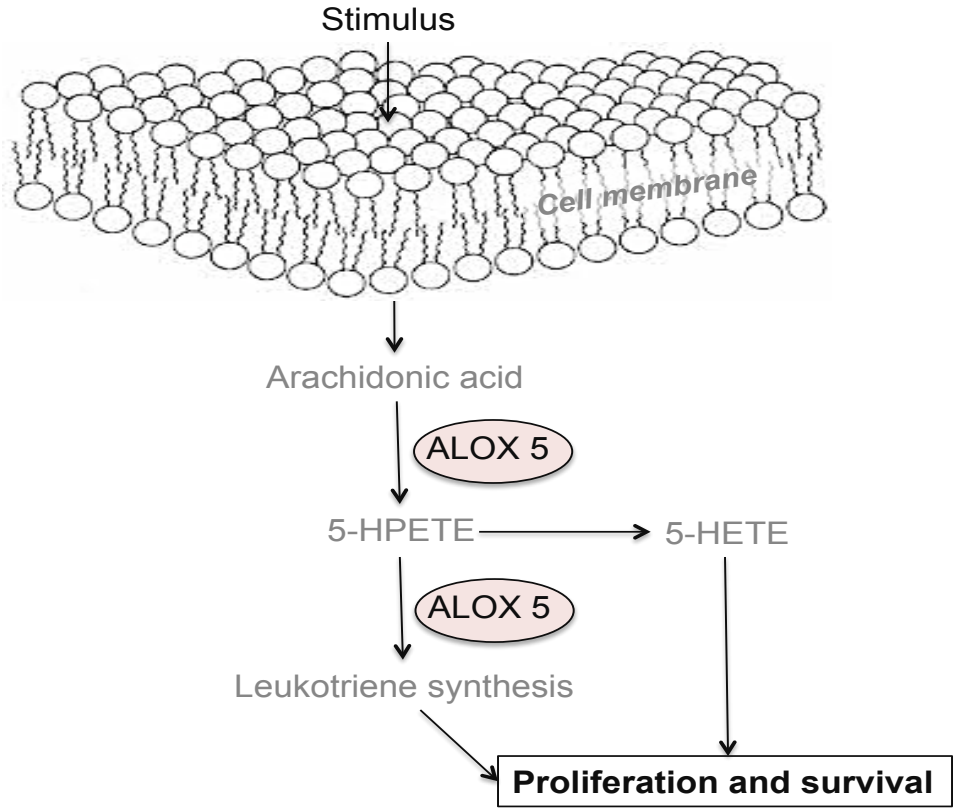


Figure 2 Schematic representation of LT biosynthesis: ALOX 5 catalyzes the first two steps in LTs formation, and the reaction starts with the intracellular release of AA. ALOX 5 in the presence of FLAP (ALOX 5 Activating Protein) catalyzes the oxidation of AA in to 5-HEPTE, followed by a second reaction in which 5-HEPTE is dehydrated to from the epoxide LTA4. Once formed, LTA4 is further metabolized to various pathways.

3. Breast cancer model

To precisely evaluate the therapeutic effect of proposed treatment on CSCs, cells with CSC properties need to be properly identified and characterized. Previously, an original, stable and cost effective *in vitro* breast cancer model was developed in our laboratory. We were able to identify, separate and sort the CSCs, using pLenti6-vector containing the tdTomato reporter gene (red fluorescence) under the control of CSC-specific promoter. Permanent CSC tagging permits identification and separation of CSC from heterogeneous populations, as well as allows monitoring of CSC biological performance within cell population.

Just cells expressing the CSC-specific promoter are detected by red fluorescence (tdTomato+) while the non-CSCs are not red in the cell culture (tdTomato-) (Fig. 3). This CSC model was established in two well-known breast cancer cell lines: MCF7 and MDA MB 231. MCF7 represents the luminal type, while MDA MB 231 represents the basal, Triple negative breast cancer (TNBC). When a pathology report says that the breast cancer cells tested negative for estrogen receptors, progesterone receptors, and HER2 this means the cancer is triple-negative. About 10-20% of breast cancers — more than one out of every 10 — are found to be triple-negative.

4. Chemotherapeutic agent: Paclitaxel

Paclitaxel (PTX) is one of several cytoskeletal drugs that target tubulin that causes defects in mitotic spindle assembly, chromosome segregation, and cell division. It is used as a chemotherapeutic agent in breast cancer that has spread.

The mecanisme of PTX consists on stabilization of microtubules polymer that protects it from disassembly. Chromosomes are thus unable to achieve a metaphase spindle configuration. This blocks progression of mitosis, and prolonged activation of the mitotic checkpoint triggers apoptosis or reversion to

G-phase of the cycle without cell division. The ability of paclitaxel to inhibit spindle function is generally attributed to its suppression of microtubule dynamics.

5. Nanoparticles as Drug Delivery Systems in Cancer Medicine

Nanomedicine is a growing research field dealing with the creation and manipulation of materials at a nanometer scale for the better treatment, diagnosis and imaging of diseases. In cancer medicine, the use of nanoparticles as drug delivery systems has advanced the bioavailability, in vivo stability, intestinal absorption, solubility, sustained and targeted delivery, and therapeutic effectiveness of several anticancer agents.

In cancer medicine, nanotechnology has become a potential application for the development of nanoparticles as drug delivery systems. Classical very potent chemotherapeutic agents, including camptothecin, taxenes, platinating agents, doxorobucin, and nucleoside and nucleotide analogs have been used against several tumor types for several decades. However, they have the disadvantage of affecting both tumor cells and normal cells, with the concomitant secondary effects including, cardiotoxicity, cytotoxicity, neurotoxicity, nephrotoxicity, and ototoxicity. Some of these chemotherapeutic-associated problems have been solved by the use of nanoparticle formulations of these drugs.

The expansion of novel nanoparticles for drug delivery is an exciting and challenging research filed, in particular for the delivery of emerging cancer therapies, including small interference RNA (siRNA) and microRNA (miRNAs)-based molecules.

There are a various nanotechnology-based drug delivery systems including, carbon nanotubes, dendrimers, micelles, quantum dots, fullerenes, nanofibers, metal-based nanoparticles, and nanoliposomes. However, in this project we emphasize on the application of polymeric micelles for the delivery of siRNA of ALOX5 gene.

5.1 Polymeric micelles

Polymeric micelles, self-assembling nano-constructs of amphiphilic copolymers with a core-shell structure have been used as versatile carriers for delivery of drugs as well as nucleic acids. They have gained immense popularity owing to a host of favorable properties including their capacity to effectively solubilize a variety of poorly soluble pharmaceutical agents such as siRNA, biocompatibility, longevity, high stability *in vitro* and *in vivo* and the ability to accumulate in pathological areas with compromised vasculature.

Since the discovery of RNAi, there has been an increased interest in developing siRNA based therapies to achieve sequence-specific post-transcriptional silencing of aberrant genes in diseases such as cancer.

So, to access and activate the RNAi machinery, the siRNA must be delivered to the cytoplasm of the cell. However, this “delivery of siRNA” poses one of the most formidable challenges to realizing the potential and utility of siRNA therapeutics. Whereas localized targets are accessed directly, the main hurdle is encountered when siRNA is delivered to tissues which are accessed only through systemic administration of agents via the blood [13].

Polymeric micelles are a good option to delivery siRNA and opens a wide range of nanomedicine aplications in cancer.

Objectives

- Test chemotherapeutic agent (paclitaxel) in breast cancer cell lines to determine its effect in CSCs population.
- Test specific inhibitor of ALOX 5 gene, Zileuton.
- Test siALOX5 as a possible CSCs therapy.
- Test polymeric micelles as a potential siRNA nano-carrier.

Materials and methods

1 Cell culture

Breast cancer cell lines MDA MB 231 were obtained from American Type Culture Collection (ATCC). Both cell lines were cultured in DMEM/F12 medium (Life Technologies) supplemented with a 10% of Foetal Bovine Serum (FBS) (Lonza) and 5% Anti-anti. Cells expressing tdTomato+ were treated with 0.1 mg/ml of blasticidin as a selective antibiotic for the CSC-specific promoter. The cell line was maintained in an atmosphere with 5% of CO_2 at 37ºC.

1.1 Thawing cells

For long storage cells were placed into liquid N_2 tank, and need to be unfrozen in order to start working with them. To thaw the cells from liquid N2, cells were unfrozen at 37ºC and then quickly added to plate (10 cm) with 9 mL of antibiotic free medium described above. The plates were incubated overnight. Next day, cells were cleaned with 10 mL PBS (Lonza) and the medium was changed in order to remove the remaining Dimethyl sulfoxide (DMSO) (Sigma) from freezing medium.

1.2 Cell passaging

In order to maintain continuous cell growth, cells were passed periodically. Old medium was removed, and then the cells were cleaned with 10 mL PBS and detached with 2 mL of Tripsin (1X) (Life Technologies). Within minutes, Trypsin was inactivated with 4 mL of PBS-FBS (10%), and cells were collected and centrifuged at 1200 rpm for 5 minutes. After, the supernatant was removed, cells were counted by automatic Cell Counter Countess (Invitrogen), and the required amount of cells was resuspended in a new plate with 9 mL of complete medium.

2 Paclitaxel-siALOX5 assay

0 h

2.1 Seed cells

MDA MB 231 cells were seed in 6-well plates. $3 \cdot 10^5$ cells/well were seeded in paclitaxel conditions and $1 \cdot 10^5$ cells/well in NON-paclitaxel condition. Cells were cultured with complete medium and incubated overnight.

24 h

2.2 Paclitaxel

Medium was removed and 2mL of DMEM/F12 +AA + FBS +blasticidine was added. The concentration of paclitaxel stock used is 1000 µM and the required for this experiment was 0.1 µM/well.

72 h

2.3 Transfection

The sense anti-ALOX5 siRNA sequence used was *5'-CUGAGCGCAACAAGAAGAATT-3'*. A control siRNA also designed by Shanghai gene Pharma with the sequence *5'-UUCUCCGAACGUGUCACGUTT-3'* was used as negative control in each experiment. The transfection was done in 6-well plate and the required final concentration in each well of siRNA is 50nM in medium without antibiotics. Medium was changed after transfection for fresh complete medium with antibiotics.

72 h

2.4 FACS sample preparation and analysis

Medium was removed, and then the cells were washed with 10 mL of PBS and detached with 500 µL of trypsin (1X) (Life Technologies). Within minutes, trypsin was inactivated with 1 mL of PBS-FBS 10%, and cells were collected and centrifuged at 1200 rpm for 5 minutes. Finally, the supernatant was removed and pellets were resuspended with PBS-DAPI 1%. After, 20 µL were collected to count the number of viable cells. Finally, the % tdTomato was analyzed with FACS Fortessa.

3 ALOX5 silencing efficacy

siRNA were designed by Shanghai Gene Pharma. The sense anti-ALOX5 siRNA sequence used was 5'-CUGAGCGCAACAAGAAGAATT-3'. A control siRNA also designed by Shanghai Gene Pharma with the sequence 5'-UUCUCCGAACGUGUCACGUTT-3' was used as negative control in each experiment. The siRNA were transected into cells with lipofectamine 3000 (Invitrogen), a transfection reagent that efficiently forms complex with siRNA, allowing its delivery into the cells. Transfection was done in 6 well plates with at least 70% confluence, and left overnight. Final concentration of 50 nM siRNA in medium without antibiotics was used. The medium was changed after the transfection for fresh complete medium and the cells were subsequently processed as required for qPCR

4 Mammosphere assay

Breast cancer cell lines *MDA MB 231* and *MCF7* were previously transfected with siALOX5 and siC designed by *Shanghai Gene Pharma* for 72 hours. After, cells were transferred into very low attachment 6-well plates, in 2 mL/well of Ruth Media (composition). Cells were incubated 4 days at 5% of CO_2 at 37ºC. The mamospheres were formed in low attachment conditions. Then, medium was collected and centrifuged at 1000 rpm. Cells were transferred to conventional 6 well plates using 2 mL of complete medium. When visible colonies were detectable at naked eye they were stained with crystal violet.

Briefly, aspirate the medium, wash with 3 mL PBS. Fix the cells with 3 mL methanol: acetic acid (3:1) solution for 5 minutes. Add 0.5% crystal violet solution in methanol for 15 minutes (first 5 minutes shaking on low speed). Wash extensively with tap water. Dry the plate, scan and count the colonies.

5 Polymeric micelles preparation

4.1 Internalization assay

Parental MDA MB 231 were seed in 24 well plate ($5 \cdot 10^3$ cells/well) and were cultured with DMEM/F12 medium (Life Technologies) supplemented with a 10% of FBS (Lonza) and 5% Anti-Anti.

4.2 NPs preparation

siRNA with fluorescent tag and cationic polymer were mixed and vortex 5 minutes and incubate at room temperature 30 minutes. Finally, amphiphilic polymer was added and the mixature was vortexed 5 minutes.

4.3 Confocal preparation

Cells were incubated 24 hours with nanoparticles and then lysotracker was added. Cells were whashed with PBS and fixed with PFA 4% in PBS 30 minutes at 4 ºC. PFA were removed and triton-x100 in PBS was added to permeate cells. Finally, cells were staining with DAPI and mounted a coverslip.

Results

Effect of PTX on MDA MB 231:

The assay was realized in 12 samples, comparing 3 technical replicates in each condition. The percentage of CSCs from MDA-MB-231 was obtained with FACS Fortessa at 72 hours and 72 hours + 72 hours after adding PTX ***(fig.3).***

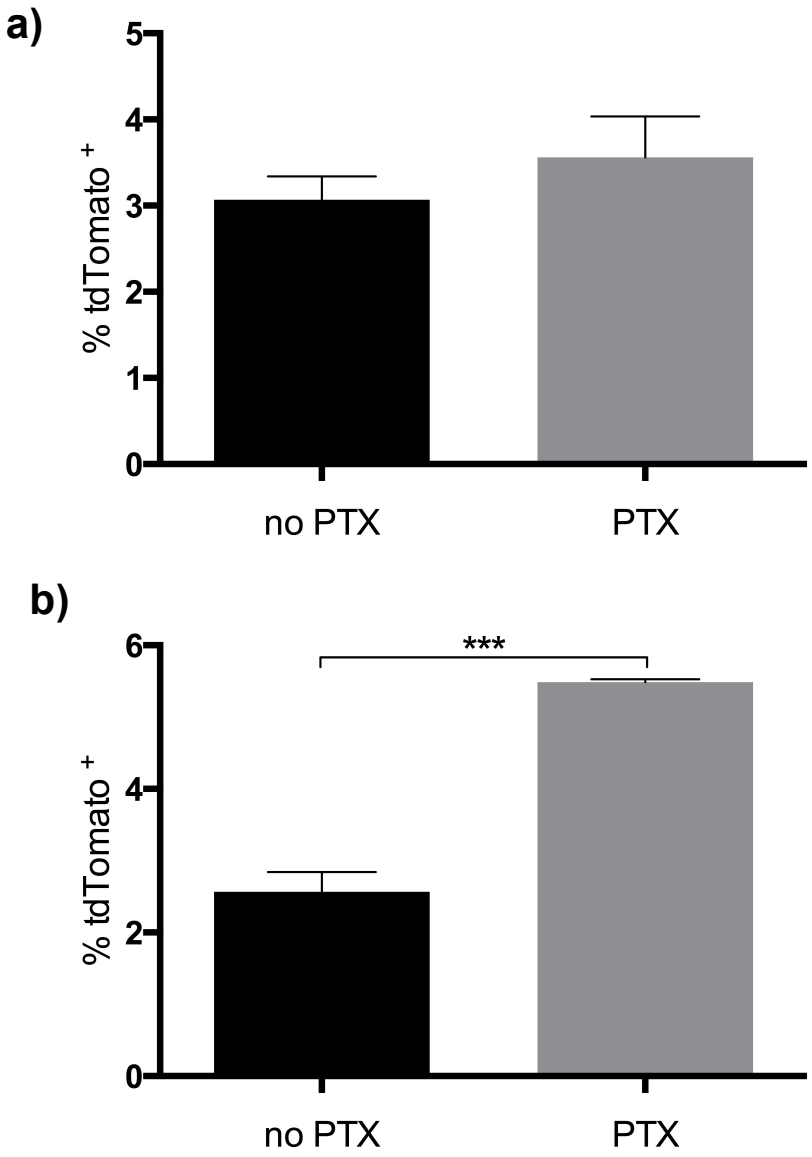


Figure 3 Effect of PTX on CSCs: a) 72 hours b) 72 + 72 hours. Data were expressed as the mean + SD (Standard desviation). Data were evaluated statiscally by Student's t-test. Significance levels: P<0.05, *: P<0.01, **: P<0.001, ***.

There are no significant differences in the percentatge of CSCs percentage 72 hours after adding PTX (fig.3a). However, we observed a significant increase of CSCs increase in PTX condition after 72h + 72h (fig.3b). This relative increase of CSCs on MDA MB 231 could be a relative increase of % CSCs because paclitaxel target non-CSCs but don't affect CSCs. Another hypotesis is that the presence of paclitaxel estimulates phenotype reversion (non-CSCs pass to CSCs). Phenotype reversion is a hypotesis that is currently tested in our group.

Effect of ALOX5 silencing:

Anti-ALOX5 siRNA was purchased from Shanghai Gene Pharma, in order to observe ALOX5 silencing. MDA MB-231 were transfected with siALOX5 and mRNA expression for ALOX5 gene was quantified by qPCR ***(fig.4)***

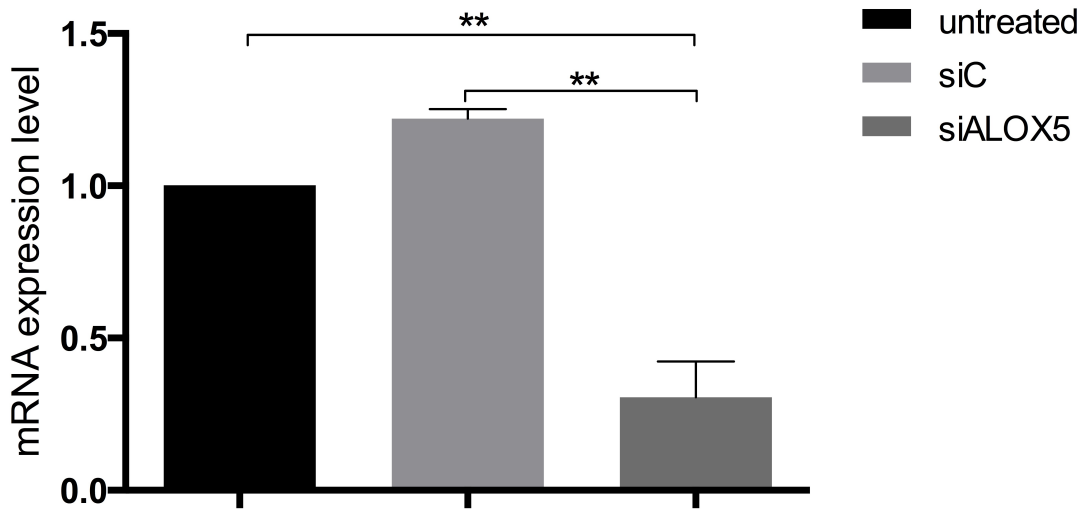


Figure 4 silencing ALOX5: Data were expressed as the mean + SD (Standard desviation). Data were evaluated statiscally by Student's t-test. Significance levels: P<0.05, *: P<0.01, **: P<0.001, ***.

Results showed a significant decrease in ALOX5 mRNA expression after anti-ALOX5 siRNA transfection when compared to control siRNA or untreated samples. The tested siRNA is a good option to silence specifically the ALOX5 gene.

Paclitaxel-siALOX5 assay:

MDA MB-231 with low percentage of tdTomato+ (3%) was used in this assay to reproduce realistic conditions in a tumor.

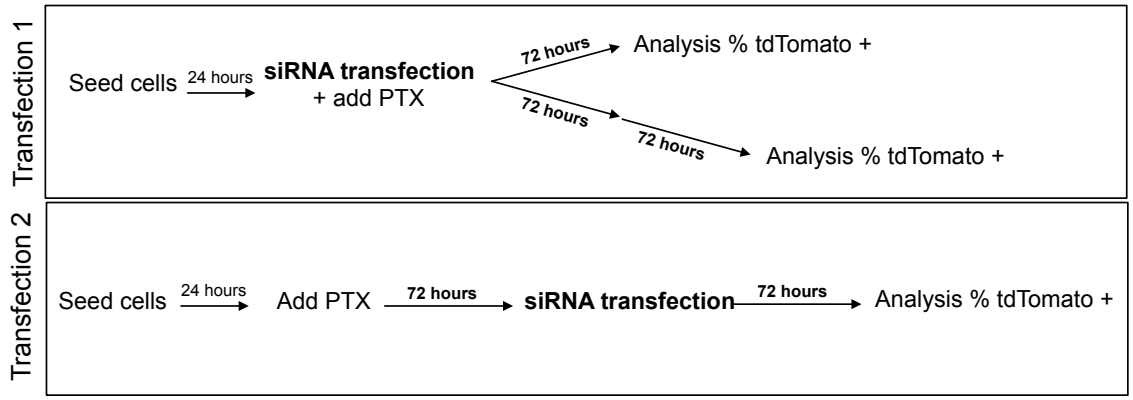
This assay was set up to analyze the effect of chemotherapeutic agent combined with siALOX5, with special attention to CSCs population ***(fig.5)***

Paclitaxel was added at 72 hours after seeding cells in both cases but siRNA was transfected in two diferent times (fig.5):

- Transfection 1: siC and siALOX5 were transfected the same day we add PTX.
- Transfection 2: siC and siALOX5 were transfected 72 after adding PTX.

Analysis of % tdTomato+ was made in 3 different times after transfection with FACS fortessa:

- Transfection 1: after 72 hours and 72 hours + 72 hours after transfection.
- Transfection 2: After 72 hours of transfection.



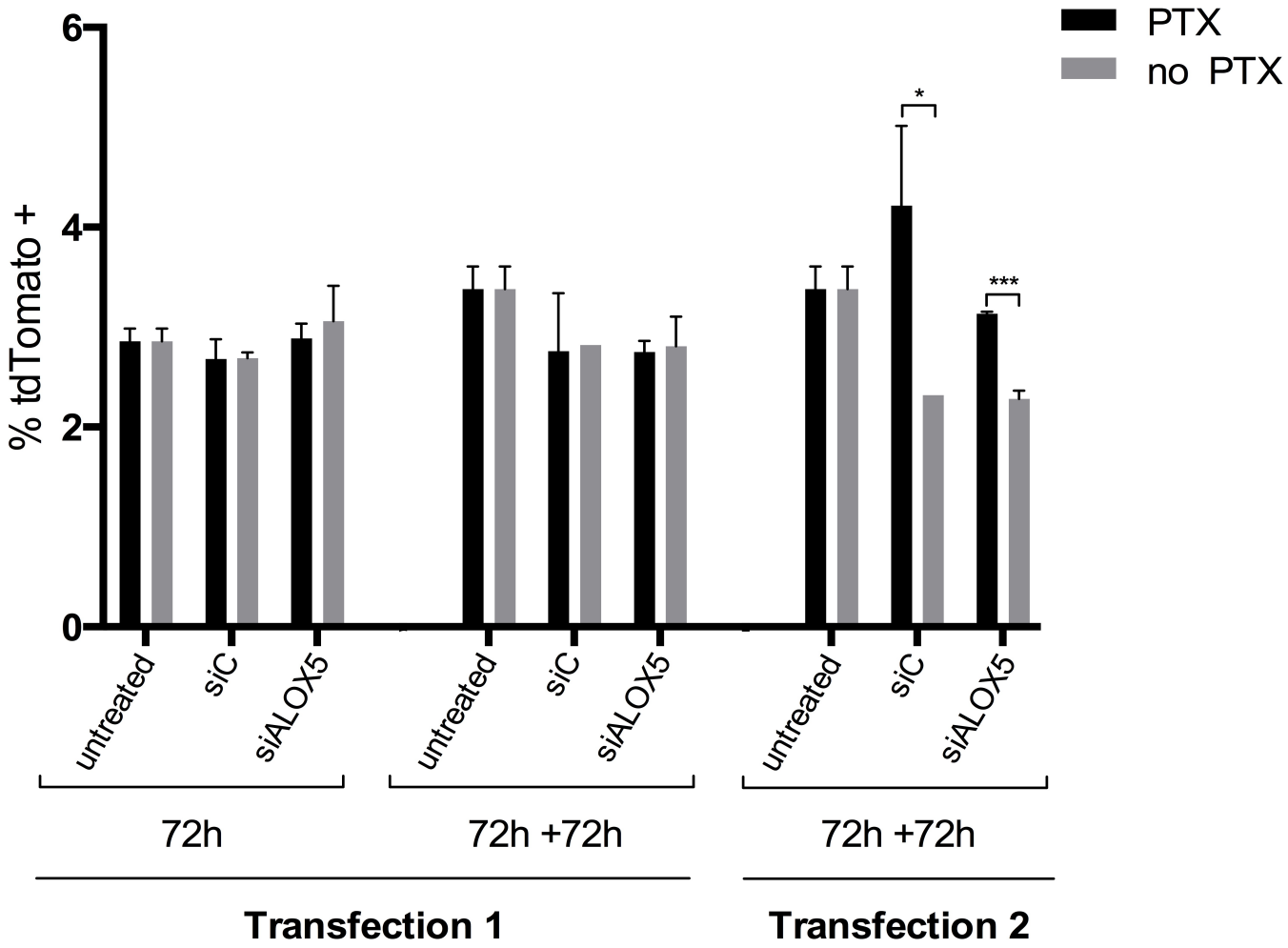


Figure 5: Paclitaxel-siALOX5: Data were expressed as the mean + SD (Standard desviation). Data were evaluated statiscally by Student's t-test. Significance levels: P<0.05, *: P<0.01, **: P<0.001, ***.

There are no significant in the percentatge of CSCs on transfection 1. However, in transfection 2 samples that were treated with PTX showed an increase in the percentatge of CSCs compared with samples that were not treated with PTX.

There are differences between the relative ammount of CSCs in siC-PTX and siALOX5-PTX suggesting that ALOX5 silencing have an effect on CSCs.

Paclitaxel-siALOX5 assay tdTomato+ high percentage:

Taking into account previous results this assay was replicated 3 times with 2 technical replicates for each condition.

This assay was set up following these conditions:

Seed cells $\xrightarrow{24\text{ hours}}$ Add PTX $\xrightarrow{72\text{ hours}}$ **siRNA transfection** $\xrightarrow{72\text{ hours}}$ Analysis % tdTomato +

The only difference between previous assays was the percentage of tdTomato+ which was higer (60%). The reason is to see whether the differences between siC and siALOX5 become more pronounced.

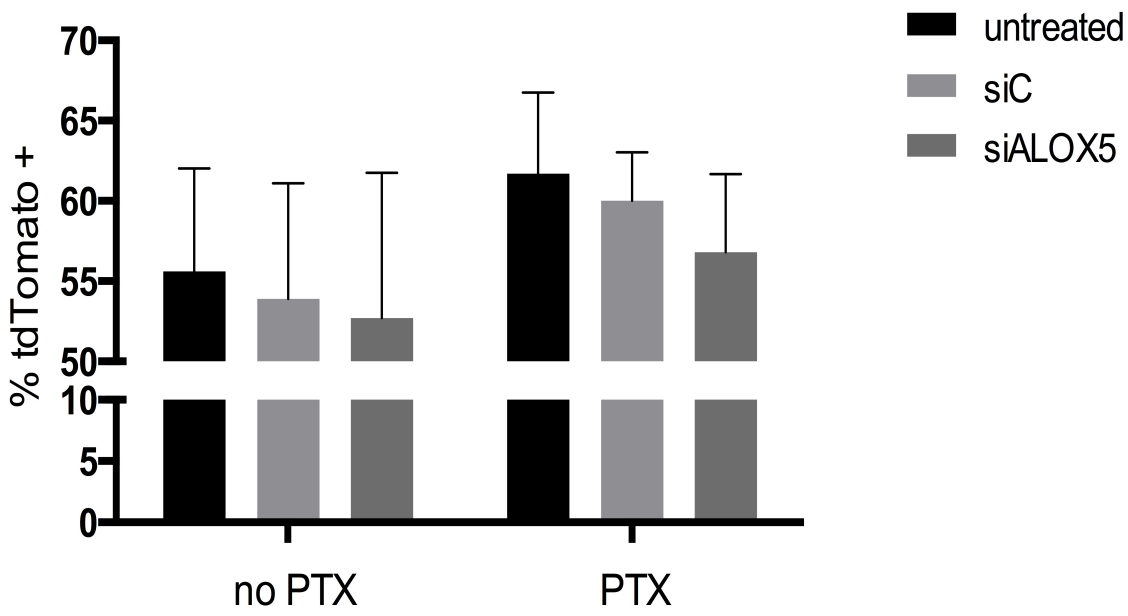


Figure 6 Paclitaxel-siALOX5 assay tdTomato+ high percentatge: Data were expressed as the mean + SD (Standard desviation). Data were evaluated statiscally by Student's t-test. Significance levels: P<0.05, *: P<0.01, **: P<0.001, ***.

There were no significant differences between the different experimental setups. A decrease of CSCs was observed in siALOX5-PTX as compared to siC and untreated samples in all replicates.

However if we only llok at the last replicate of this assay, here there were significant differences between siALOX5 and siC and untreated conditions ***(Fig.7)***

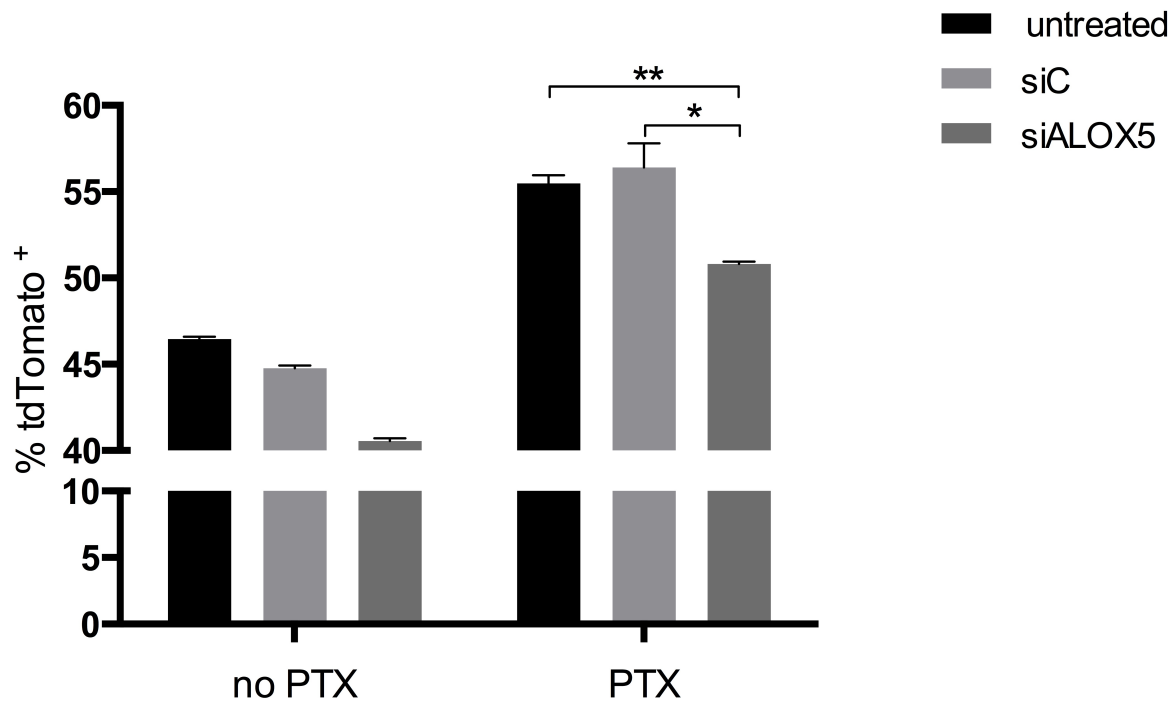


Figure 7 Paclitaxel and no PTX one replicate. Data were expressed as the mean + SD (Standard desviation). Data were evaluated statiscally by Student's t-test. Significance levels: P<0.05, *: P<0.01, **: P<0.001, ***.

Paclitaxel data was normalized to no PTX data (fig.8)

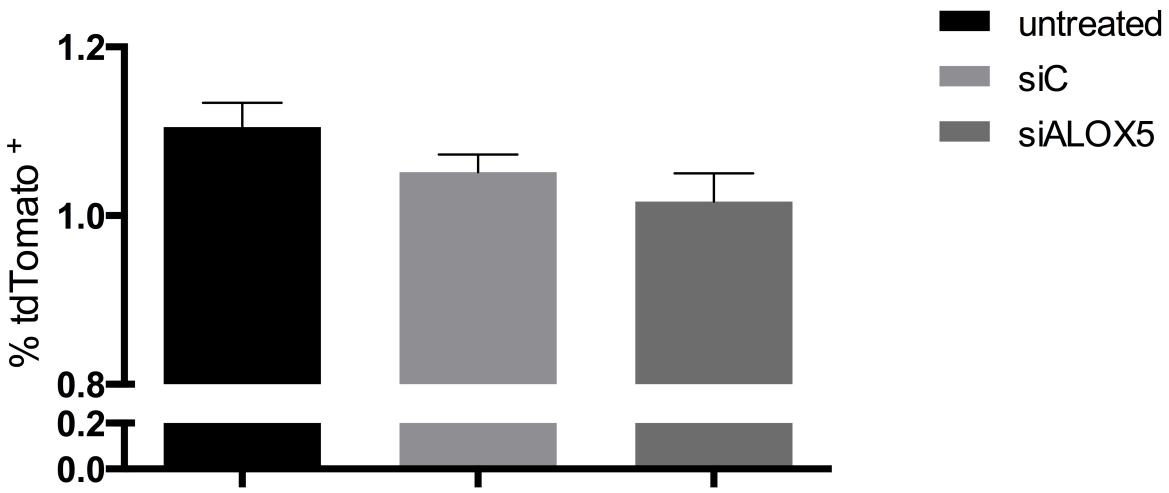


Figure 8 Paclitaxel normalized data: 3 assays with 2 technical replicates were normalized to NO PTX data. Data were expressed as the mean + SD (Standard desviation). Data were evaluated statiscally by Student's t-test. Significance levels: P<0.05, *: P<0.01, **: P<0.001, ***.

At 72h and 72 hours + 72 hours cells were counted for each condition:

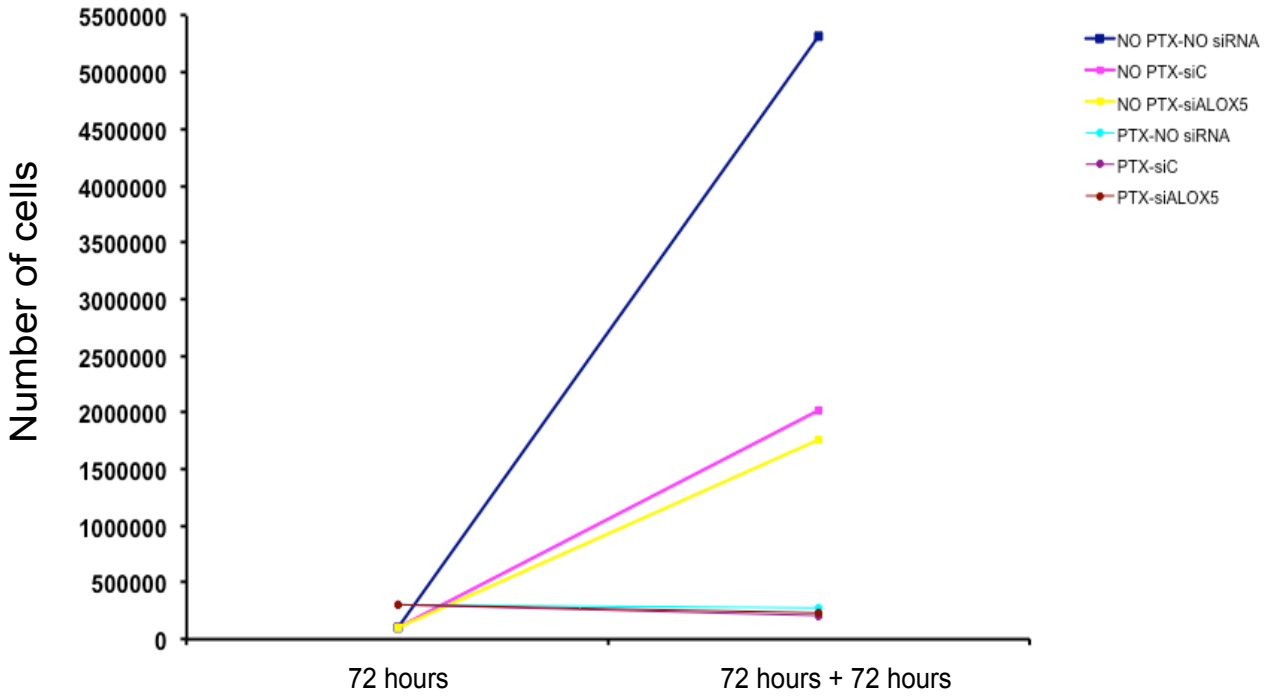


Figure 8 ***MDA MB-231 cell counting***. 3 replicates of paclitaxel-siALOX5 with 2 technical replicates are represented.

Samples that were treated with pactlitaxel did not growth. On the other hand, no paclitaxel condition showed a normal growth. However, samples that were transfected with siRNA showed a lower number of cells because lipofectaimine is a toxic substance used to transfect siRNA.

Mammosphere assay:

Mammosphere assay allows to access whether a single cell harbors the potential to both initiate and maintain tumors in the absence of cellular interaction and adhesion. Stem cells are capable of unlimited self-renewal ability, and unique subsets of cancer cells that acquire stem cell properties have the theoretical ability to form de novo tumors when grown under low-attachment conditions within minimal growth factor supplementation.

siRNA were transfected in low attachment condition to check if ALOX5 silencing have any effect on CSCs survival. Two different breast cancer cell lines (MDA MB-231 and MCF7) were used.

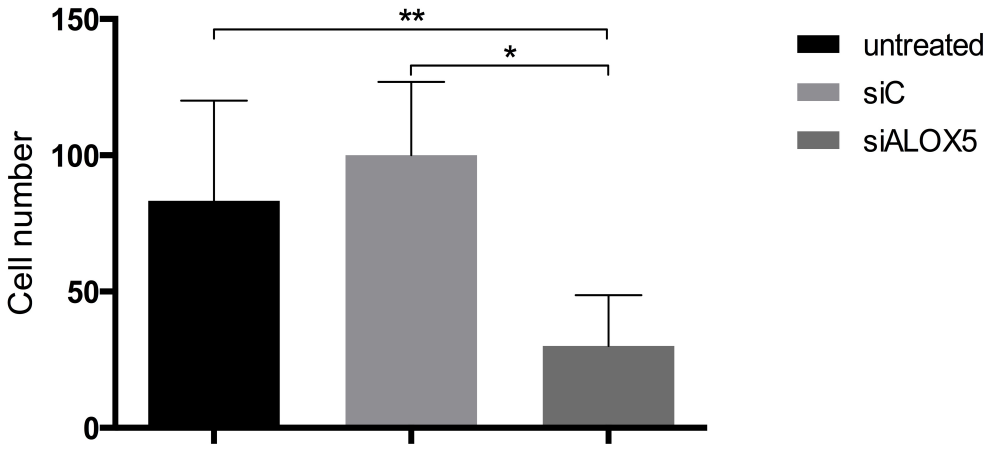


Figure 10: ***MDA MB-231 mamosphere counting.*** Data were expressed as the mean + SD (Standard desviation). Data were evaluated statiscally by Student's t-test. Significance levels: P<0.05, *: P<0.01, **: P<0.001, ***.

siALOX5 silencing was showed significant differences between siC ($p<0.05$) and untreated ($p<0.01$). However, mamospheres were not able to count, and then cells were tripsinized and counted it.

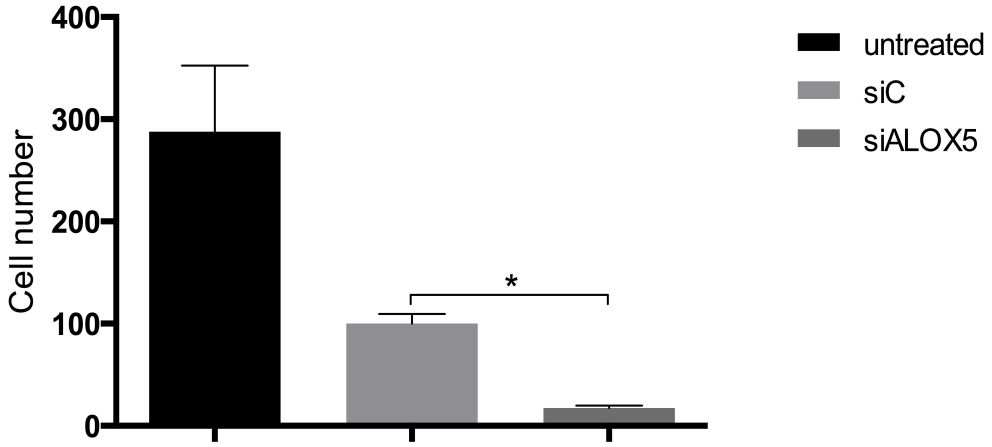


Figure 11: ***MCF 7 mammosphere counting.*** Data were expressed as the mean + SD (Standard desviation). Data were evaluated statiscally by Student's t-test. Significance levels: P<0.05, *: P<0.01, **: P<0.001, ***.

There are also significant differences between siALOX5 to siC ($p<0.05$) in MCF7 cell line.

Nanoparticles siRNA delivery:

In order to administrate siRNA that targets a specific gene as a therapy, a polymeric micelles was choosen as a promising candidate to deliver siALOX5.

Before in vivo experiments iare performed, it is essential to check some technical points to ensure that potential nanocarrier is good for delivery a specific type of drug, in our case siRNA. In particular, to use siRNA as a potential therapy it is important to check the internalization of nanoparticles. siRNA molecules are unstable molecules and it is necessary to check whether nanoparticles are in lysosomes or not. The pH of lysosomes is acid (4.8) as compared to cytoplasm that has neutral. siRNA would be degradated in the lysosomes. The escape from the early endosome is thus necessary for efficient siRNA delivery to the cell.

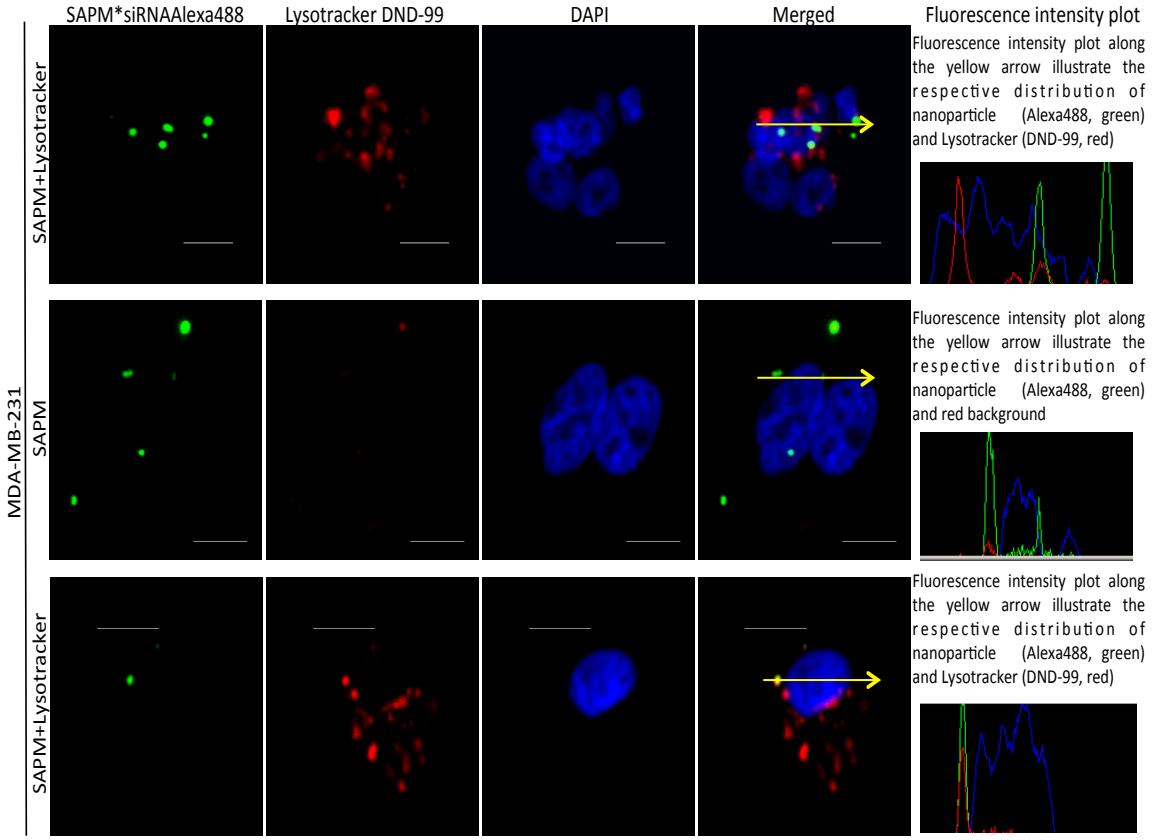


Figure 12: ***Polymeric micelles internalitzation.*** siRNA Alexa488 green fluorescence, lysotracker DND-99 red fluorescence stains lysosomes and DAPI blue fluorescence stains nucleus. Fluorescence intensity plot ilustrate fluorescence curves, if red and green curves are overlap it means that polymeric micelle is in lysosome. However, there is a red background when there is not staining with lysotracker.

The Internalization assay was repeated but lysotracker was substituted for cell mask to stain cytoplasm.

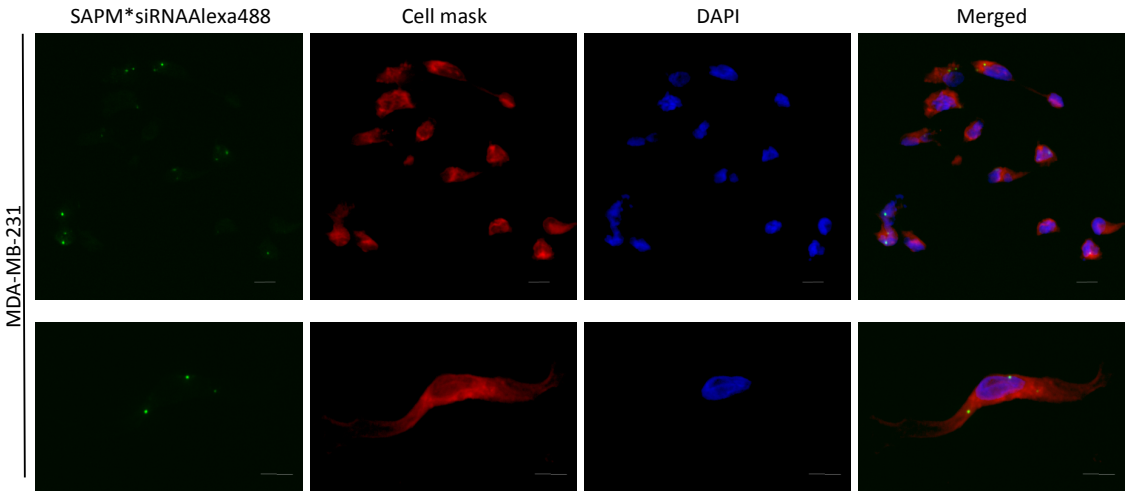


Figure 13: polymeric micelles internalitzation. siRNA Alexa488 green fluorescence, cell mask red fluorescence stains citoplasm and DAPI blue fluorescence stains nucleus.

Internalitzation assays of nanoparticles showed high internalitzation efficency in citoplasm ***(fig.13)*** as well as an aggregation in lisosomes ***(fig.12)***. This aggregation is a probem because siRNA is degradate due tu de conditions of lisosomes, such acid pH.

Discussion

In this study we test CSCs as specific target to design new effective nanomedicine against breast cancer. We choosed the gene ALOX5 gene according to previous work in our research team as a specific CSCs pathway inhibitor.

Pactlitaxel was tested on MDA MB-231 breast cancer cell line to determine the effect on CSCs and non-CSCs. As expected, the chemotherapeutic agent did not affect the CSCs population but it affected non-CSCs. According to this result, paclitaxel is a good choice to reduce bulk tumor. However, the percentage of cancer stem cells increase as compared to samples that were not treated with paclitaxel. This is because paclitaxel only affects non-CSCs and there is a relative increase in the CSCs number ***(fig.14).*** CSCs resistance to chemotherapeutic agents it may explain relapses in many cancers due to CSCs characteristics.

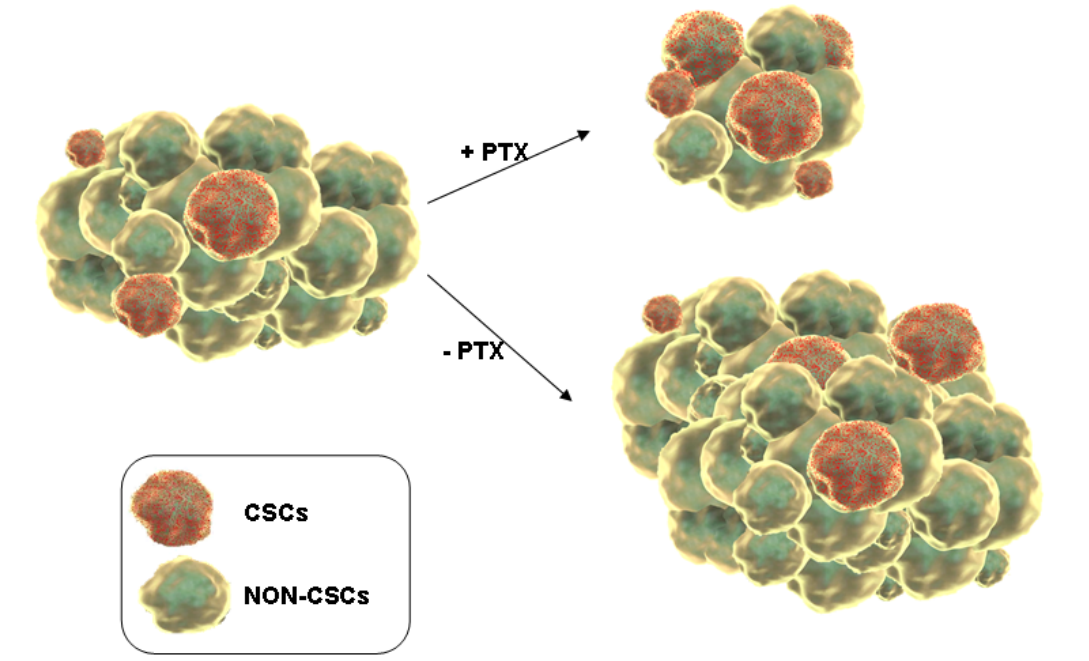


Figure 14: Schematic representation of Paclitaxel effect on breast cancer cell line (MDA-MB 231).

Based on these results, Paclitaxel-ALOX5 assay was set up to fix the optimal conditions to evaluate the effect of silencing ALOX5 in CSCs. Pronounced differences between PTX and no-PTX were observed in these conditions:

Seed cells $\xrightarrow{24\ hours}$ Add PTX $\xrightarrow{72\ hours}$ **siRNA transfection** $\xrightarrow{72\ hours}$ Analysis % tdTomato +

Paclitaxel-siALOX5 assay was replicated 3 times with MDA-MB-231 with higher percentage of tdTomato+ (60%). Our results suggested that ALOX5 silencing have an effect on CSCs but it was not statistically significant when results were normalized. However, in 3 assays the percentatge of CSCs in siALOX5 samples were lower than the control and the untreated. Moreover, in one replicate the percentatge of CSCs decreased significantly in siALOX5 as compared to siC and untreated.

The results from the assay that combines PTX and siALOX5 showed that the presence of siALOX5 impairs the relative increased observed only with PTX effect **(fig.15).**

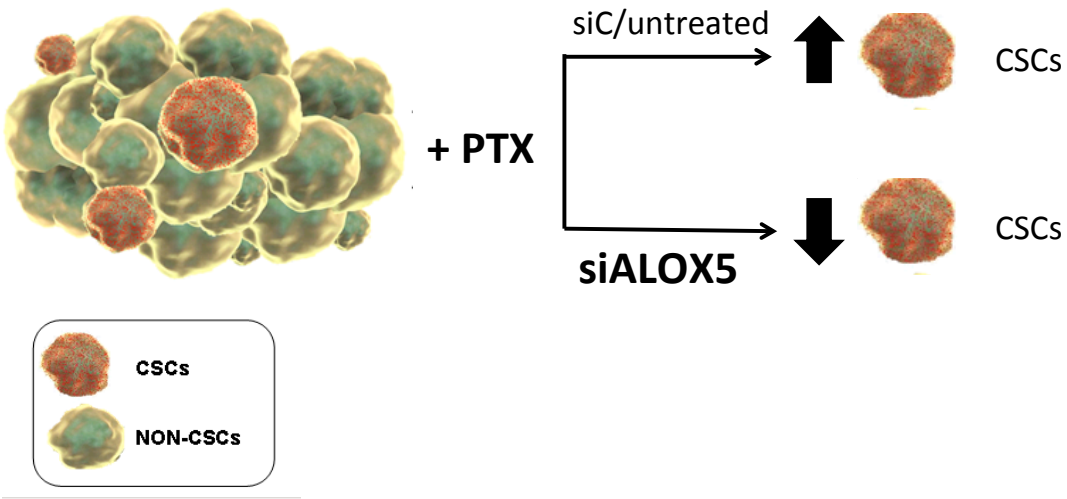


Figure 15: Schematic representation of Paclitaxel effect plus siALOX5 in CSCs.

The cell counting results showed the cytotoxic effect of PTX. It suggests that chemotherapy is a good option to reduce bulk tumor but not to eliminate CSCs.

The Mammosphere assay was performed to check the effect of ALOX5 silencing in the survival of CSCs. The siRNA was transfected in low attachment because CSCs have the ability to form mammospheres in this conditions.
Results showed significant differences in mammospheres total number. So, this suggests that ALOX5 have a role in CSCs survival.

The results of theses assays show that of ALOX5 is still a promising candidate for a novel siRNA based therapies against CSCs.

In siRNA based therapies there is a limitation because of the difficulty to access and activate the RNAi machinery, the siRNA must be delivered to he cytoplasm of the cells. However, this “delivery of siRNA” poses one of the most formidable challenges to realizing the potential and utility of siRNA therapeutic.

In this project, we used polymeric micelles as candidate to delivery siALOX5. To design new effective nanomedicine against breast cancer siRNA is entrapped inside of polymeric micelles to produce the desired toxic effect. The next step will be the targeting of CSCs using a specific receptor on the surface of these cells.

The performed Internalitzation assay performed suggests that polymeric micelles are a good option because they stabilize siRNA. However, polymeric micelles were observed in the cytoplasm of cells but some were also inside lysosmes, this is a problem due to the low pH that could degrade siRNA. These nanoparticles are still in development but are promasing carriers to design anti-CSCs therapies.

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Conclusions

1- Paclitaxel is a chemotherapeutic agent that eliminate non-CSCs (bulk tumor).

2- Paclitaxel increases the relative number of CSCs.

3- Silencing ALOX5 had an effect on CSCs survival, so

4- Polymeric micelles could be used to encapsulate siALOX5 as a nanocarrier of CSCs inhibitor therapy.

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