UNIVERSITAT DE BARCELONA

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Role of CD44 in clear cell renal cell carcinoma invasiveness after antiangiogenic treatment

Main Field: Biochemistry and Molecular Biology

Secondary Fields: Cellular Biology

Physiology and Pathophysiology

Pharmacology and Therapeutics

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Abstract

During last century, big effort to understand the biochemical basis of cancer was carried out. One of the principal branches of these cancer investigations used drugs to prevent the formation of new blood vessels –process called angiogenesis– responsible for the nutrients supply of the tumour. These drugs are generally called antiangiogenics. It was discovered that some types of tumour have or develop resistance to these drugs when treatment was long enough. For that reason, mechanisms of resistance, aggressiveness, invasion and/or metastasis after the treatment are nowadays relevant to study. Recently, a protein that could be involved in the increased invasiveness of tumour cells after the antiangiogenic treatment appeared.

This project collects some evidence that indicates that this protein, called CD44, might play a role in the increased invasion after antiangiogenic treatment in mouse models of renal carcinoma.

Resum

Durant l'últim segle, s'ha fet un gran esforç per aprofundir en la basant bioquímica de la investigació contra el càncer. Una de les branques principals d'aquesta investigació utilitza fàrmacs que prevenen la formació de nous vasos sanguinis –procés anomenat angiogènesis- encarregats de nodrir el tumor. Aquests fàrmacs es diuen generalment antiangiogènics. S'ha descobert que alguns tipus de tumor tenen o desenvolupen resistència a aquests fàrmacs quan el tractament és prou llarg. Per aquesta raó, actualment s'està investigant profundament quins són els mecanismes pels quals apareix aquesta resistència, així com també perquè els tumors es tornen més agressius, invasius i/o metastàtics després del tractament. Recentment s'ha descobert una proteïna que podria estar involucrada en l'augment de la invasivitat de les cèl·lules tumorals després del tractament antiangiogènic.

Aquest treball recull algunes de les evidències que apunten cap al paper de la proteïna CD44 en l'increment de la invasió tumoral post-tractament amb fàrmacs antiangiogènics en models ratolins de càncer renal.

Integration of the different scopes

This experimental work integrated different educational fields. The main one was Biochemistry and Molecular Biology. Since the principal issue of this project was cancer, it was very important to study and understand the molecular biology hidden behind this pathology and the mutation mechanisms of cells to become malignant. It was also important to know why tumours respond or resist to therapies or why and how they develop resistance.

As secondary educational fields, Cellular Biology, Physiology and Pathophysiology, and Pharmacology and Therapeutics were included. Cellular Biology was important to understand the tumour cellular cycle, how malignant cells proliferate and invade other tissues; Physiology and Pathophysiology was used to understand the manifestation of the disease and it was useful to differentiate a tumour cell from a healthy one in order to determinate the invasiveness of the cancer into other tissues. Pharmacology and Therapeutics was important to understand the effect of the therapy to the cells, and how they responded to the antiangiogenic treatment.

Introduction

More than one million new cases of cancer were diagnosed in 2016 in the United States, and nearly 600.000 people are going to die due to the disease (1). Every year in Europe, cancer has been responsible of 20% of deaths (2). Specifically in Spain, the incidence of cancer in 2012 was nearly 216.000 new cases according to the Sociedad Española de Oncología Médica (SEOM) (3).

Kidney and renal cancer can be found in the list of the most common cancers in 2016 published by the National Cancer Institute (1). Renal cell carcinoma affects over 30.000 individuals in the United States every year (4) and is responsible of 2-3% of cancer in Spain (5). If it is detected in the early stages and is localized in the kidney, the survival rate of the patients is approximately 95% in 5 years; nevertheless, if it is not detected in early stages the survival rate decreases significantly (4).

Clear cell renal cell carcinoma (ccRCC) is the most common subtype of renal cancer (6). Its name is attributed to the high accumulation of lipids and glycogen into the cells, giving a white colour to the tumour after histological examination (6). This type of cancer is mainly characterized by a loss of renal function, a down-regulation of some metabolic pathways and the activation of the immune system (6). It was found that patients had more risk to develop ccRCC if they had the von Hippel Lindau syndrome (VHL). This syndrome is characterised by a loss-of-function mutation in the VHL gene. In normoxic conditions, the VHL protein forms a complex which targets HIF1α (the hypoxia-inducible

factor) for its degradation, but not in a hypoxic situation. Mutant VHL protein is unable to bind HIF1α so it cannot be destructed and its accumulation promotes increased transcription of some growth factors as VEGF, EGFR, TGFα and PDGF (4). Tumours need nutrients and oxygen to grow and survive and these overexpressed growth factors contribute to new blood vessels formation. The appearance of new vessels is known as angiogenesis –or angiogenic process. Angiogenesis is a physiological process seen in other circumstances such as pregnancy or menstruation (7). The main drawback appears when transcription of pro-angiogenic factors is increased to feed a tumour. The vascular endothelial growth factor (VEGF) is one of the pro-angiogenic signalling factors involved in that process, and is also the most studied in angiogenesis. Its receptor is called VEGF receptor (VEGFR) and there are 3 types: VEGFR1, VEGFR2 and VEGFR3. They all are transmembrane tyrosine kinase receptors found in endothelial cells from blood vessels (7).

When this mechanism of tumour survival was discovered, most investigators though that tumours would not progress and tumour cells would be destructed if the formation of new blood vessels was inhibited. For that reason the antiangiogenic therapy appeared (8). Some drugs like sunitinib or bevacizumab are nowadays used as an antiangiogenic treatment for renal cell carcinoma and other types of cancer. The target of those drugs is different: sunitinib is a small molecule that inhibits VEGF and VEGF receptor (VEGFR) among other targets; bevacizumab is a monoclonal antibody that blocks the human ligand VEGF (9). DC101 is another antiangiogenic drug used in animal experimentation which is a monoclonal antibody that inhibits murine VEGFR, concretely the receptor 2 (10). There is also a human monoclonal antibody capable of inhibit the human VEGFR2 called ramucirumab or IMC-1121B, and it has been tested as an antiangiogenic drug in several tumour types (11).

In the first initial pre-clinical experiments, animals were treated with DC101 for a short period of time (10 days) showing a significant decrease of tumour growth. The problem appeared when researchers kept the treatment longer (4 weeks) and drug resistance appeared. It was observed that after a period of reduction and stabilization, tumour regrew and its phenotype was more invasive and malignant than the initial tumour (7).

Later, two different type of resistance mechanisms to antiangiogenic treatments were discovered: the adaptive -or acquired- resistance and the intrinsic resistance. While in the adaptive resistance the treatment was effective at first but tumour regrew, in the intrinsic resistance, tumours never responded to the treatment (12). Nowadays these mechanisms of resistance are being deeply studied.

Nevertheless, unpublished results from the Institut d'Investigació Biomèdica de Bellvitge (IDIBELL) laboratory, showed that there were two types of tumour behaviour after the

antiangiogenic treatment. One tumour cell type demonstrated an increase of the invasion in the kidney after the antiangiogenic treatment. On the other hand, the second type of tumour cells turned out not to increase invasion after the treatment.

To continue investigating tumour invasiveness and possible implicated molecules, it was developed a massive RNA sequencing from tumour samples and it was found CD44 protein, among others, as a candidate gene involved in the resistance and the increased malignancy after the antiangiogenic treatment (unpublished results from the laboratory).

CD44 is a cell-surface glycoprotein expressed on the surface of different cells including endothelial cells, epithelial cells, fibroblasts, keratinocytes and leucocytes (13). It is encoded on chromosome 11, concretely in its short arm (14). Its molecular weight is around 85-200KDa (15). Due to an extensive splicing phenomenon, there are different isoforms of the protein, called variant isoforms (CD44v) (14). The shortest isoform of the protein is the standard CD44 (CD44s or CD44) and it is also the most abundantly one (13). Figure 1 represents the structure of CD44s and its isoforms.

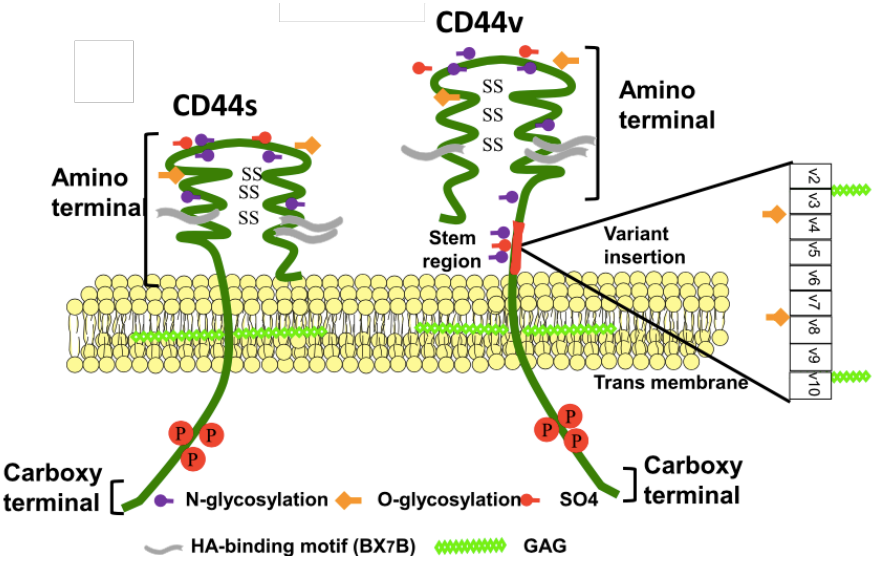

Figure 1. Structure representation of standard CD44 (CD44s) and its variant isoforms (CD44v) (16).

The protein consists of an extracellular part, a transmembrane part and a cytoplasmic part, containing different number of amino acids: 270, 23 and 72 respectively (14).

The principal ligand of CD44 is hyaluronic acid, a high molecular weight linear polysaccharide (13). That binding takes place in the extracellular domain of CD44, concretely in the N-terminal part, and is responsible of cellular signalling and regulation of other cell biologic processes (15). Nevertheless, CD44 can bind to other extracellular matrix molecules like fibronectin, collagen, cytokines… (16).

CD44 takes part of some physiological processes like cell proliferation, adhesion, migration, hematopoiesis and lymphocyte activation (13).

It was seen that CD44 is a protein involved in metastasis and tumour growth, also after antiangiogenic treatment, and that there is a relation between CD44, primary tumour stages, distant metastasis and poor prognosis (13,17). Moreover, CD44 is used as a marker of cancer stem cell in several types of cancer (17).

High levels of CD44 protein are found in some malignant tumours, chronic inflammatory reactions and autoimmune alterations (15). It was described that some variants of CD44, specially CD44v6, can induce tumour progression and metastasis in breast, lung and colon cancer (16).

In this project, we studied the role of CD44 in tumour invasiveness using a renal carcinoma mice model.

Hypothesis and objectives

Hypothesis

After unpublished results obtained in the laboratory, we thought that CD44 could be a possible protein involved in the increased tumour invasiveness after the antiangiogenic treatment.

Objectives

- Determine the expression of CD44 in treated and non-treated samples with antiangiogenic drug in a cellular type of ccRCC induced in a mice model.
- Determine the distribution of CD44 in the tumour in treated and non-treated animals with antiangiogenic drug.
- Determine the invasiveness of the tumour and correlate it with the expression of CD44.

Materials and methods

Bibliographic research

The information needed to write this work was searched in different scientific articles. To get those articles some database like PubMed and Google Scholar were used. Their main topic was about renal cell carcinoma, focusing in the anti-angiogenic therapy and the potential mechanisms that tumours use to develop resistance to this therapy.

Bioinformatics analysis

Several programs were used for gene expression analysis, like cBioPortal, to find out the relationship between various proteins or genes expressed in patients with renal cell carcinoma and compare them with healthy patients.

The experimental part of the project included the execution of some techniques. The main ones were Western blotting and Immunohistochemistry, explained below.
All the acquired data was statistically analysed using RStudio and basic lineal model fitting.

Cell lines

Renal tumour cells used in the experiments were SN12C and Ren99. These cell lines are pVHL-positive, their VHL gene is not mutated. They were grown at 37ºC in RPMI 1640 medium supplemented with foetal bovine serum (FBS), penicillin, streptomycin sulphate and L-glutamine. Two type of cells were used, SN12C, an established cell line, and Ren 13, that were primary cells from tumour and needed, apart from the supplemented medium, insulin, EGF (epithelial growth factor) and fungizone to grow.

pTRIPZ-shCD44 lentiviral production and transduction of SNC12 cells

To develop some experiments, it was necessary to silence CD44 protein. To do that, a pTRIPZ lentiviral inducible sh-RNA vector was used. pTRIPZ is a plasmid that was used to produce lentiviral particles. This plasmid (Figure 2) contains a sh-RNA that can bind to a specific target mRNA and destruct it. It also contains a tetracycline inducible element that allows the sh-RNA expression only in the presence of tetracycline or doxycycline.

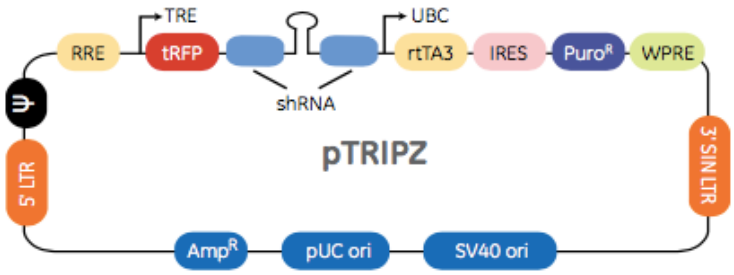

Figure 2. Representation of the plasmid and its parts used to generate the lentivirus for cells transduction (18).

By cotransfection of pTRIPZ along packaging plasmids, lentiviral particles are produced. This lentivirus can be used to infect tumour cells, leading to integration of the plasmid into the genome of the cells (18). Transduced cells are selected using puromycin, which kills cells not expressing the vector.

In our experiments, the target of the sh-RNA was CD44. It was also performed a sh-RNA with no target (non-silencing or NS), as a control to evaluate the effectiveness of the CD44 silencing and verify that doxycycline and the incorporation of the vector itself did not have effect.

So, two vectors were developed, the sh-CD44-1, which inhibits the expression of CD44 protein, and the sh-CD44-NS, as a control.

Generating tumour samples and in vivo experiments in mice

To obtain tumour samples, an established cellular line (SN12C tumour cells) and a series of animals were needed. Mice were injected with 50μL of culture media containing one million and a half cells. The injection was done intrarenally, in the left kidney of athymic nude mice. These cells proliferated leading to a renal tumour. When tumour was 1500-1750mm^3, mice were sacrificed and tumours were extracted and divided in pieces. These pieces were re-implanted into other mice in order to amplify the number of subjects of the experiment and to perpetuate the tumour model.

For our experiment 24 animals were studied. They were randomly divided in 4 groups depending on the treatment received. The first group was non-treated, the second one received only DC101 -administrated via intraperitoneal-, the third one was treated only with doxycycline -dissolved in the drinking water of mice-, and the fourth group received both DC101 and doxycycline. Animals were treated during three weeks. After that time, mice were sacrificed and the tumour and some organs (liver, lung, diaphragm, right kidney and spleen) were taken off. Tumour weight and volume were measure. Then, the tumour was divided in 2 parts allowing the observation of the front between tumour and kidney (invasive front). One part was embedded with optimal cutting temperature (OCT) and frozen (-80ºC) and the other piece was fixed with formol and later treated to be embedded in paraffin. Additionally, a small portion of each part was cut and divided in little pieces, then frozen (-80ºC) for molecular analysis.

Western Blot

Western blot is a technique used to separate and identify proteins by means of an electrophoresis in a polyacrylamide gel. The gel is divided in a top and a bottom part. The applied voltage difference between the two parts of the gel separates the different proteins as a function of their molecular weight. Then, the proteins from the studied samples are identified comparing their displacements in the gel with the calibration ones from a known set of proteins previously selected (19).

Going into more detail, polyacrylamide gel is composed of two gels with different pH and polyacrylamide proportion. When more percentage of polyacrylamide, the pores of the

gels are smaller and the smallest proteins can be separated better, for this reason the upper gel has lower percentage of polyacrylamide, because we do not want to separate proteins, we just want to pack them. However, in the lower gel the percentage of polyacrylamide is higher, because we are searching for the separation of the proteins. The lower gel is called resolving gel and it is where the separation of the proteins takes place. The upper gel is called stacking gel and helps to pack the proteins into a very narrow band.

First of all, solutions of both gels were prepared separately. Then, the solution of the resolving gel was introduced between two glasses hold by a support. When it became solid, the stacking gel solution was loaded. Before solidification of the upper gel, we used a comb to generate 10/15 wells. Once solid, the comb was taken off and gels were placed into the electrophorator (Bio-Rad Laboratories Inc, California) filled with running buffer. Samples and a reference marker of molecular weight were loaded into the wells (~30μL). Then, electrophoresis was initiated.

Once finished, proteins were transferred to a Polyvinylidene difluoride (PVDF) membrane. To do that, a sandwich made of one sponge, two Watman papers, the gel, the membrane, two more Watman papers and another sponge was prepared. The sandwich was introduced again in the electrophorator, this time filled with transfer buffer. The transfer was carried on under cold conditions (4ºC) during 90 minutes.

Next, membranes were incubated with blocking buffer (Tris-HCl buffered saline, TBS with 0,1% Tween-20 and 5% non-fat milk) for 1 hour at room temperature, followed by incubation with a primary antibody that recognized the protein of interest, in a cold chamber (4ºC) overnight.

After that, membranes were washed and incubated during an hour with a secondary antibody that recognized the primary. Membranes were then washed again and put in contact for 2 minutes with ECL detection reagent. The light from the reaction between the secondary antibody and the ECL reagent was measured from the membranes with ChemidocTouch (Bio-Rad Laboratories Inc, California) after removing the liquid excess Images were taken and analysed with ImageLab software.

In our experiment, we used a specific primary mouse anti-human antibody to detect CD44 protein and another primary mouse anti-human antibody to detect β-actin. As a secondary antibody in both cases a goat anti-mouse antibody was used.

β-actin is a protein expressed in all tissues and was quantified to normalize the results.

Immunohistochemistry

This is a technique to determine the localization of a protein or an antigen of interest using specific antibodies (20).

To develop this technique, we used embedded tumour samples in paraffin blocks. These blocks were sectioned into slides. Once samples were prepared we could start the immunohistochemistry.

First, slides were put in a cuvette and it was introduced in a series of alcohols and xylenes: 10 minutes in 4 different xylenes, 5 minutes in 3 absolute ethanol, 5 minutes in 3 96% ethanol, 5 minutes in a 70% ethanol and 5 minutes more in a 50% ethanol. This treatment helped to take off the paraffin of the sample.

Then, slides were washed during 5 minutes in distilled water. After that, using sodium citrate and under pressure and temperature, the antigen we would like to detect was unmasked.

After that, samples were incubated with hydrogen peroxide and during that, peroxidases became inactivated. It is important to avoid oxidative reactions. Next, samples were washed again and then, permeated by a solution of PBS and triton (a detergent). Permeation of the samples is important to let the antibody cross the tissue and all membrane and detect the antigen of interest.

Then, samples were incubated in a wet chamber during 1 hour with goat serum to block unspecific interactions that could occur, and after that they were incubated overnight with primary antibody at 4ºC.

Next day, samples were washed 5 minutes with PBS and triton (0,1%), it was repeated 3 times.

Then, slides were incubated during 1 hour with the secondary antibody that recognises the primary one, at room temperature.

Once finished the incubation, samples were washed again. Next, samples were treated with DAB which is a substrate chromogen system, during 6 minutes. This treatment stained samples. Then, a washing was needed again, but this time with current water. After that, samples were stained with hematoxylin, to contrast the staining nuclei/cells, during 1 minute and immediately washed. Then, samples were rapidly passed through a cuvette with HCl, the water, then to another cuvette with NH_3 and finally washed with more water.

Next, samples were dehydrated. To do that the same alcohol and xylene series was needed but this time backwards.

Once finished, tissue samples were covered with a slide cover using DPX as a mounting medium. Slides were analysed under a microscope.

Data analysis

The graphical and statistical results obtained after experiments were analysed with RStudio (Boston, USA) analysis program. For statistical significance study, the Mann-Whitney U test was used since the number of individuals could not be considered to follow a normal distribution.

The graphic model used is a boxplot, schematically explained bellow (Figure 3).

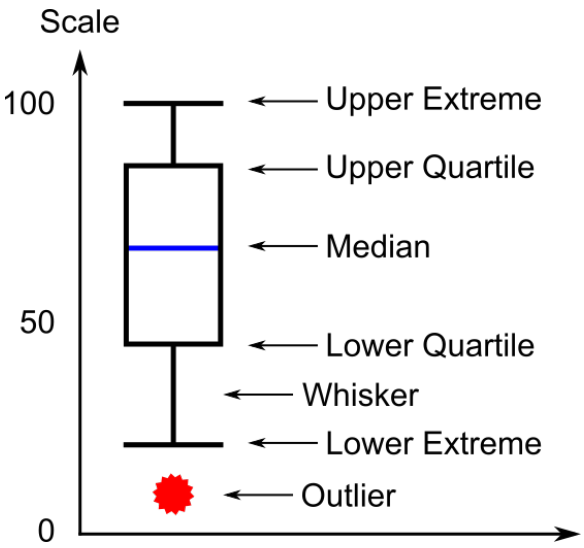

Figure 3. Representation of a Boxplot and its different parts.

Statistic difference between groups is represented by asterisks:

- One asterisk (*): p <0,05
- Two asterisks (**): p<0,01
- Three asterisks (***): p< 0,001
- Four asterisks (****): p< 0,0001

Evaluation of tumour local invasiveness

Evaluation of tumour invasion was studied with ImageJ software.

Two methods to determine invasion were developed. One method consisted in obtain 4X zoomed images from a microscope after staining with an immunohistochemistry assay using anti-vimentin antibody; the other method used images taken from a loupe after haematoxylin-eosin (H&E) staining by immunohistochemistry assay.

Then, independently of the method used to take the pictures, in the images tumour-kidney interface was traced and distance of the deepest protrusion of tumour into kidney was evaluated.

Due to an existing correlation between depth and tumour weight, invasion was calculated as the average of invasion depth (μm or pixels) normalized for tumour weight.

Results and discussion

Expression of CD44 in SN12C renal tumour cells

Our aim in this study was to determine the expression of CD44 protein in SN12C ccRCC cells and then correlate that expression with the invasiveness of the tumour. In that way, we could see if after the antiangiogenic treatment there is an increase of that protein and if it is responsible of the increase invasion of the tumour when resistance to the treatment appears.

First, we decided to develop a Western Blot using SN12C sh-CD44-1 and SN12C sh-CD44-NS. On one hand, we wanted to demonstrate the sh-RNA efficiency in silencing the expression of CD44 protein and in the other hand, to observe if there were differences in the CD44 expression in samples treated or non-treated with the antiangiogenic drug.

In Figure 4 is shown the results of the two Western Blots. Black band represent the detected protein, and its intensity depends on the quantity of protein.

Each lane represents a piece of tumour from individual mice.

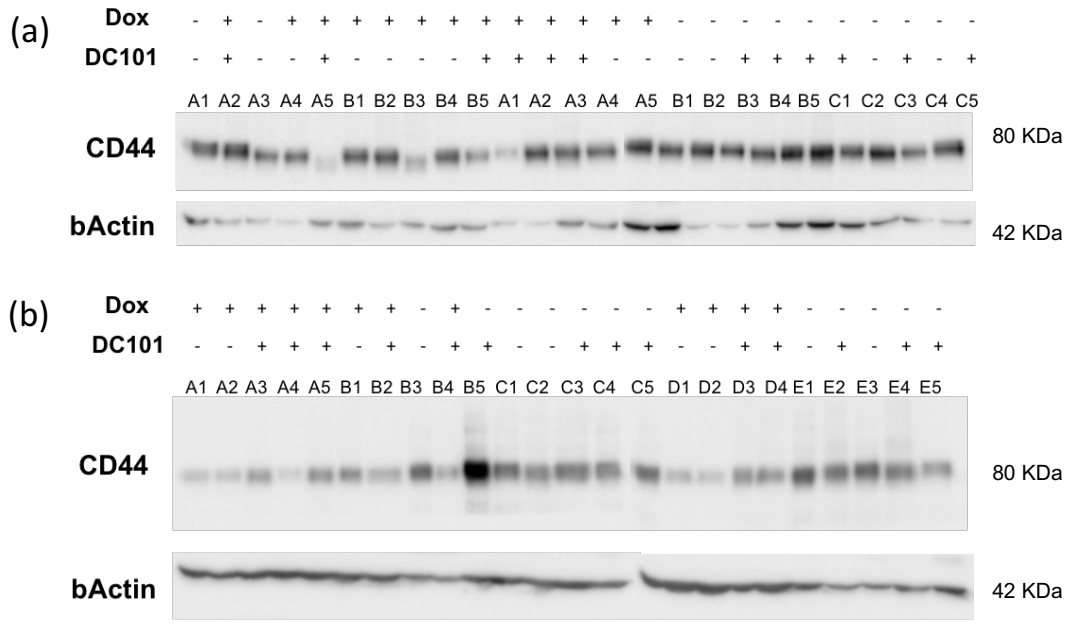

Figure 4. Western Blot results of the expression of CD44 protein from the silencing and non-silencing SN12C cells from a tumour sample. Each letter and number is the name of a different renal tumour of a mouse (e.g. A1, A2, A3, …). The treatment that mice received is represented at the top of the image (Dox and/or DC101). The expression of β-Actin was used to normalize the results and avoid variability due to differences when samples are loaded into the gel. (a) Western Blot results of the non-silencing SN12C cells (sh-NS). (b) Western Blot results of the silencing SN12 cells (sh-1).

We could see different levels of expression of the CD44 protein from each tumour in the Western Blot figures. In order to understand how the expression of this protein depended on the therapy, results were statistically analysed and expressed as boxplots. We wanted to observe if doxycycline affected the expression of CD44 independently of the antiangiogenic drug, and we also wanted to know if the treatment with DC101 lead to an increase of CD44 independently of doxycycline. For that reason, we developed different boxplots that could help us to understand a bit more of the protein and the cell tumour type.

Boxplot analysis of CD44 expression in the non-silencing samples under the different treatments is shown in Figure 5. No statistical difference was found between any treatment condition ($p>0.05$).

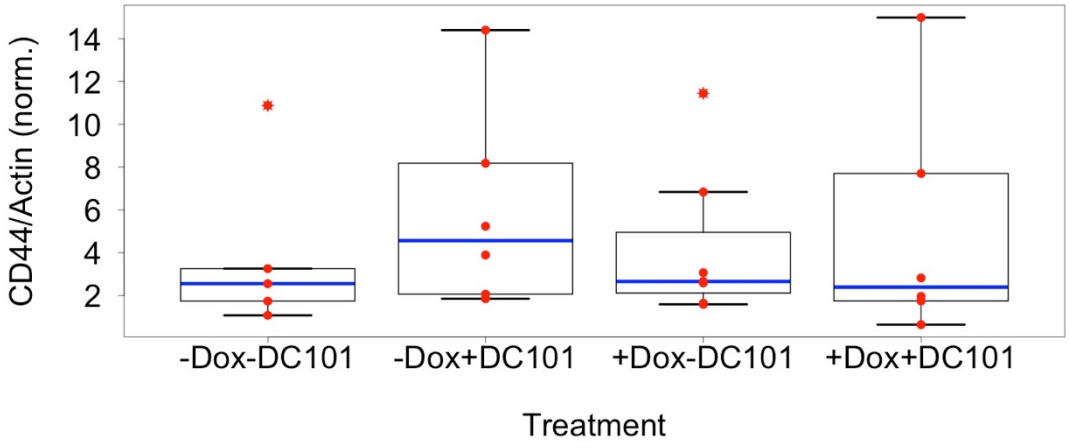

Figure 5. Boxplot representation of CD44 expression depending on the treatment of SN12C sh-CD44-NS tumour cells (Figure 4a). Samples are grouped depending on the treatment that mice received. Each red spot represents a determinate mouse and blue line indicates the median of the samples belonging to a specific treatment group. β-Actin is a protein expressed in all tissues and was used to normalize the results.

These results were interesting to compare because they show the different combinations of drug treatments that we administrated to the animals, and how the expression of the CD44 protein varied in each group. For that reason, these results were merged together to analyse the effect of a single condition, with or without doxycycline (Figure 6) and with or without DC101 (Figure 7).

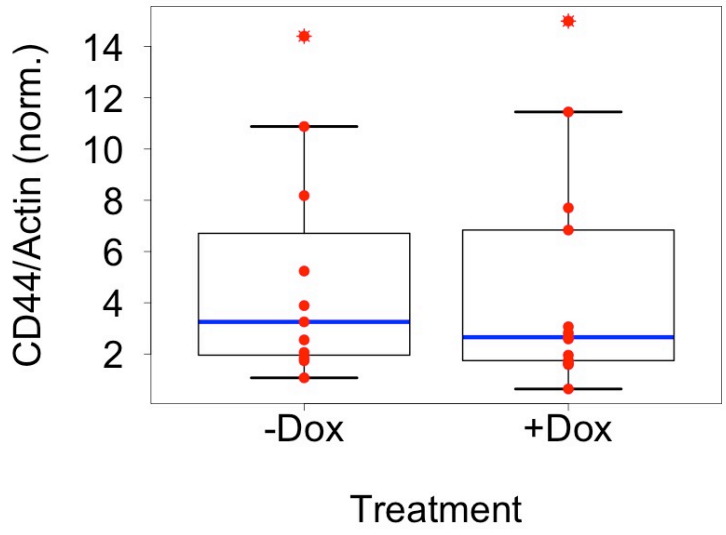

Figure 6. Boxplot representing the expression of CD44 depending on the administration of doxycycline in SN12C sh-CD44-NS tumour cell (Figure 4a). Each red spot represents a tumour mouse and the blue line indicates the median of the group samples. β-Actin is a protein expressed in all tissues and was used to normalize the results.

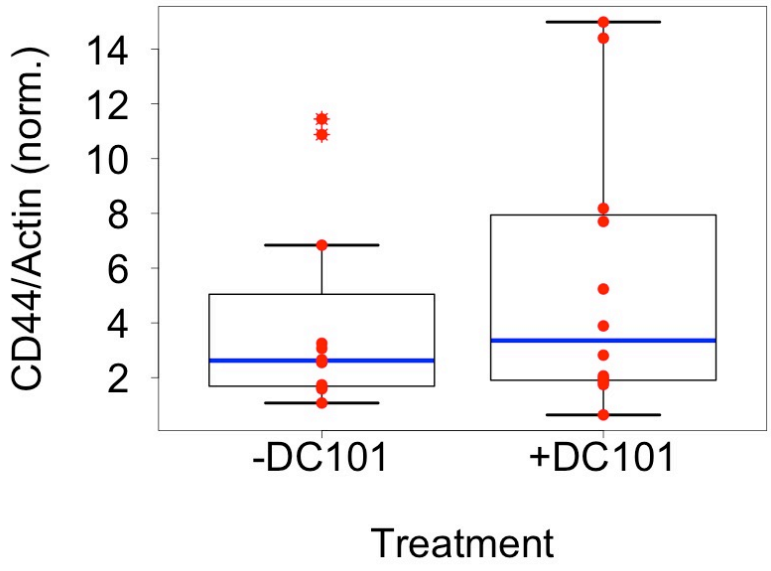

Figure 7. Boxplot representing the expression of CD44 of mice treated without or with DC101 antiangiogenic drug in SN12C sh-CD44-NS tumour cells (Figure 4a). Each red spot represents a tumour mouse and the blue line indicates the median of the group samples. β-Actin is a protein expressed in all tissues and was used to normalize the results.

As we can see in the results, doxycycline treatment to the SN12C clear cell renal cell carcinoma cells without silencing of CD44 (Figure 6) does not reduce the expression of CD44 ($p>0.05$), indicating that the vector does not interfere in the transcription of the protein. Furthermore, the antiangiogenic treatment with DC101 to the tumour (Figure 7) does not increase the expression of CD44 ($p>0.05$), contrary to expectations. It could indicate that in this type of cells, the hypothesis we thought is not valid. Nevertheless, these are the results of one experiment and to confirm the rejection of the hypothesis,

more experiments should be done with the same type of cells. Moreover, other cell types should be used to test that experiment, because maybe it works with other type of cells.

On the other hand, if we take a look to the samples transduced with the sh-CD44-1, statistic differences can be observed between –Dox -DC101 and +Dox -DC101 groups (p=0,014) and also between -Dox +DC101 and +Dox +DC101 groups (p=0,007) (Figure 8).

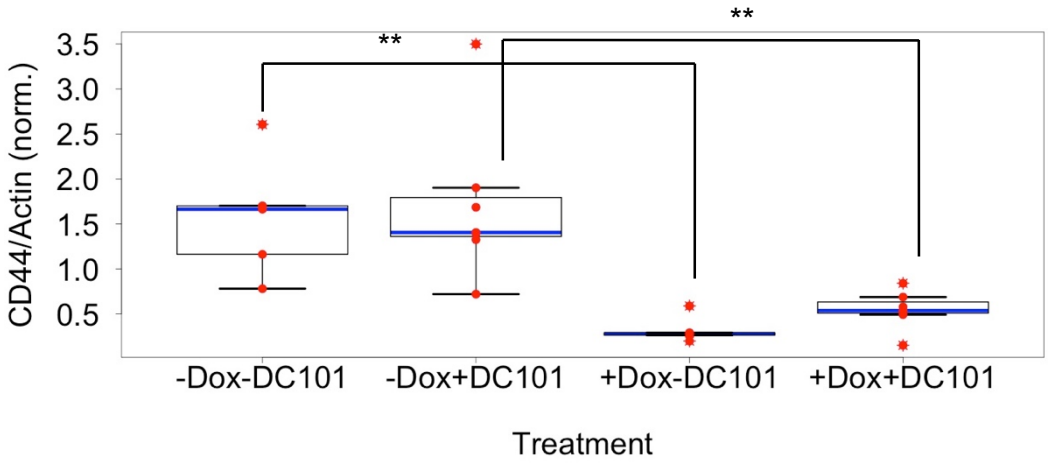

Figure 8. Boxplot representation of CD44 expression depending on the treatment of SN12C sh-CD44-1 tumour cells (Figure 4b). Samples are grouped depending on the treatment that mice received. Each red spot represents a determinate mice and blue line indicates the median of the samples belonging to a specific treatment group. β-Actin is a protein expressed in all tissues and was used to normalize the results. (**) p<0,01.

It seems to be statistical differences between +Dox-DC101 and +Dox+DC101 groups. For that reason, we also gather results by doxycycline treatment and DC101 treatment as before.

If we compare the +Dox and -Dox groups (Figure 9), a statistic difference appears (p=0,0001), indicating that doxycycline activates the sh-CD44-1 and consequently the transcription and so the expression of the CD44 protein is avoided.

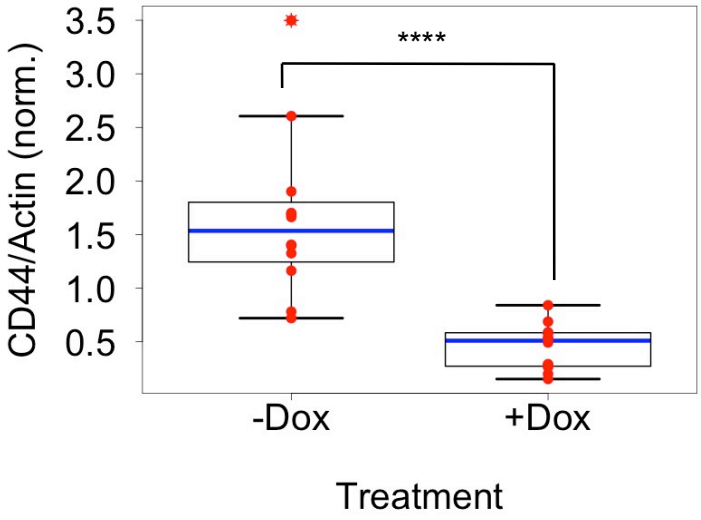

Figure 9. Boxplot representation of CD44 expression depending on the treatment with doxycycline in SN12C sh-CD44-1 tumour cells (Figure 4b). Each red spot represents a specific tumour of a mice and blue line indicates the median of the samples of a group. β-Actin is a protein expressed in all tissues and was used to normalize the results. (****) p<0,0001.

When the SN12C cells sh-CD44-1 cells are treated with or without DC101 antiangiogenic drug, there was no significant differences between the groups (p>0.05), consistent with results from NS tumours (Figure 10).

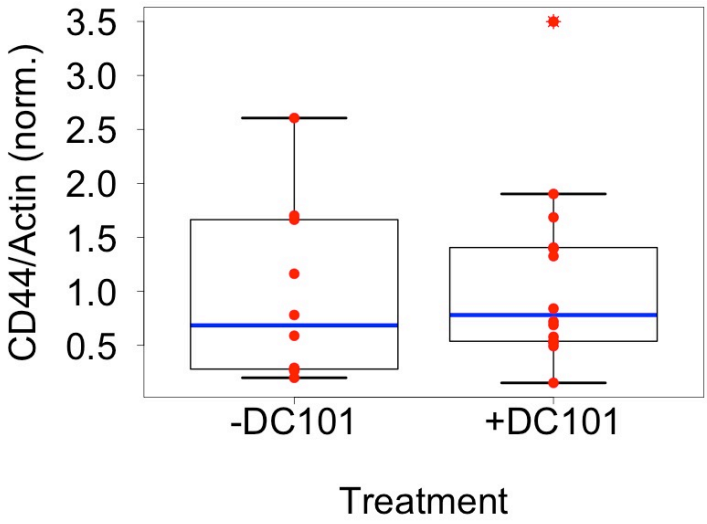

Figure 10. Boxplot representation of CD44 expression depending on the treatment with the antiangiogenic drug DC101 in SN12C sh-CD44-1 tumour cells (Figure 4b). Each red spot represents a specific tumour of a mice and blue line indicates the median of the samples of a group. β-Actin is a protein expressed in all tissues and was used to normalize the results.

With all these results, we can suspect that our hypothesis is not valid. But, as said before, more analysis should be done with this type of cells, and also with other ones. Tumours are very heterogeneous, and a mechanism that works for one type of cell tumour is not true for another cell type. For that is important to still investigate if this hypothesis is true in other cell types. Next step would be to investigate this hypothetic mechanism of invasion in 786O$^-$ tumour cell, that has been implanted in mice a month ago. It would be also important to discover the mechanism that produces the increase invasiveness after the treatment in SN12C cells, and why this type of cells acts different as others.

Invasiveness of the tumour in SN12C renal tumour cells

One of our objectives was to determine the invasiveness of the tumours and then correlate that with the expression of CD44. We wanted to investigate if CD44 had a role in invasiveness and malignancy acquired after an antiangiogenic treatment.

For that reason, we used an immunohistochemistry assay to determine the part of the kidney that was invaded by the tumour. Samples of SN12C sh-CD44-1 cells were used in this assay. We used a primary antibody that could recognise vimentin, a protein used as a marker because is always expressed in clear cell renal cell carcinoma cells (6). This type of staining let distinguish the tumour, the kidney and also if there is a necrotic part in the tumour. Here we compare the different conditions to which the animals were subjected (Figure 11a, b, c, d).

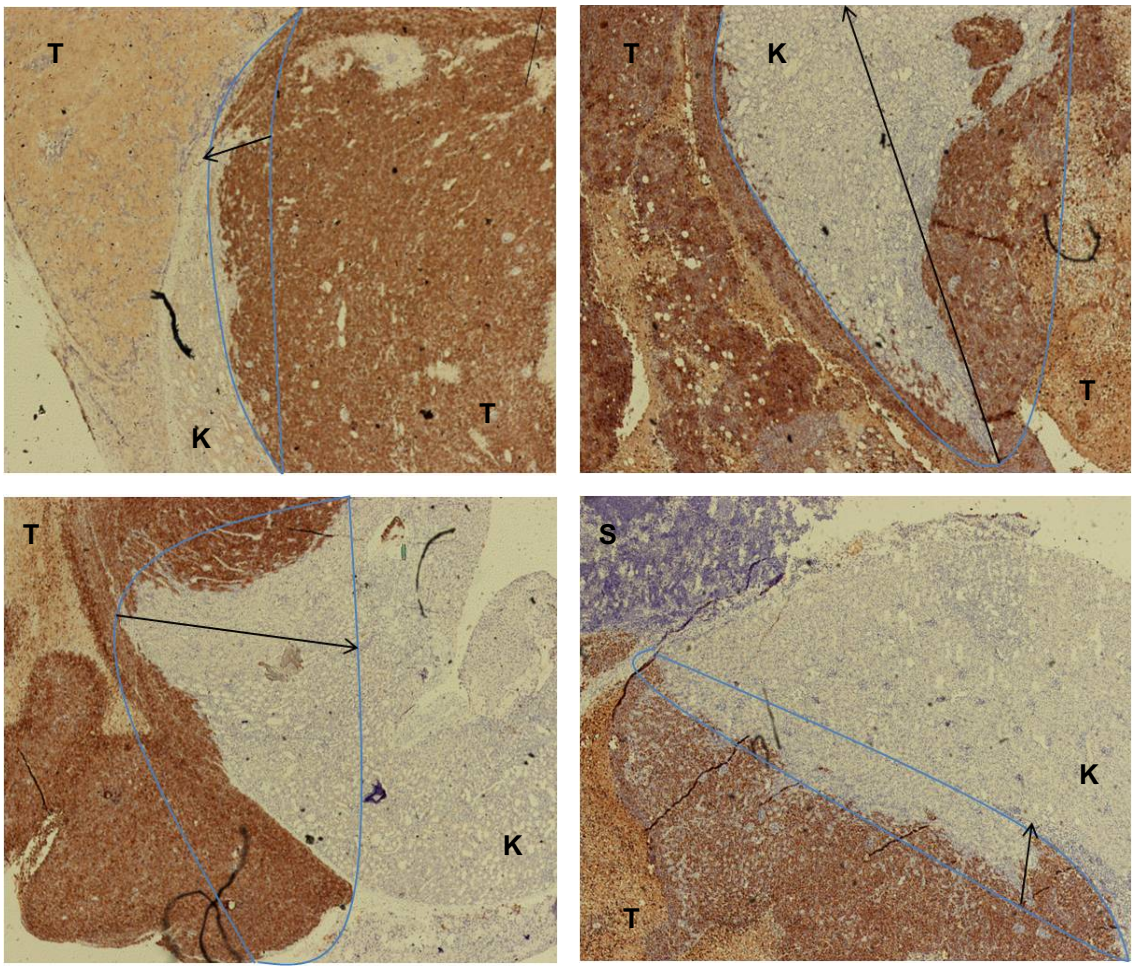

Figure 11. Immunohistochemistry of SN12C tumour cells samples under different conditions of doxycycline and DC101 antiangiogenic drug. Tumour gets brown after the immuno-treatment and kidney cells gets clear purple. Orange parts represent necrosis parts of the tumour. Blue curves define kidney-tumour interphase and black arrow, tumour introduction into the kidney. In the picture, T is tumour; K is kidney and S is spleen. (a) Interphase kidney-tumour of SN12C sh-DC44-1 tumour cells not treated with doxycycline nor DC101. (b) Interphase kidney-tumour of SN12C sh-CD44-1 tumour cells treated with DC101 but not doxycycline. (c) Interphase kidney-tumour of SN12C sh-1 tumour cells treated with doxycycline but not with DC101. (d) Interphase kidney-tumour of SN12C sh-1 tumour cells treated with doxycycline and DC101. Strong purple part represents the splint.

These images were taken using the first method described in materials and methods. Once stained and traced depth line, measurements were statistically analysed (Figure 12).

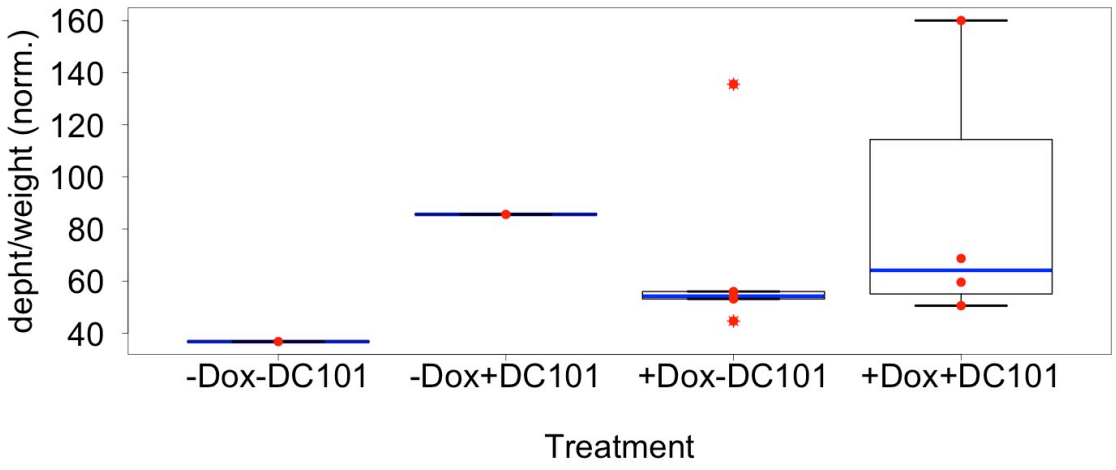

Figure 12. Boxplot representation of tumour depth into kidney depending on the treatment of SN12C sh-CD44-1 tumour cells. Samples are grouped depending on the treatment that mice received. Each red spot represents a determinate mice and blue line indicates the median of the samples belonging to a specific treatment group. Results were normalized by tumour weight.

Comparing all groups between them, no statically differences can be observed. Even so, samples were grouped, as before, depending on doxycycline (Figure 13) and DC101 (Figure 14) treatment.

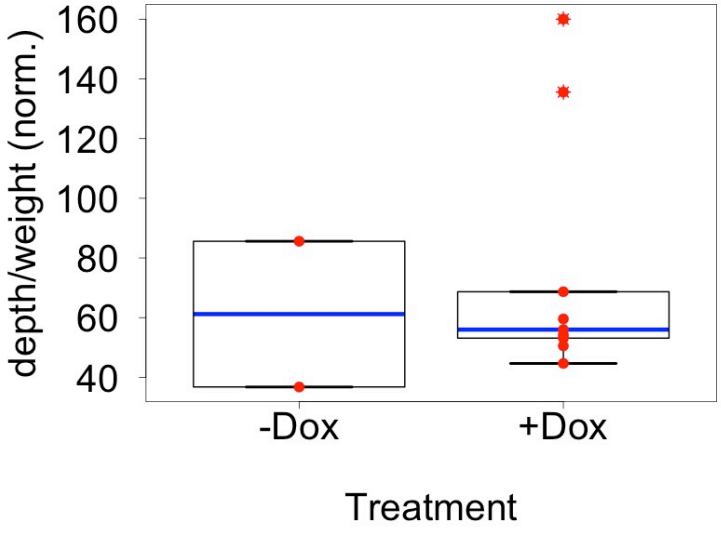

Figure 13. Boxplot representation of tumour depth into kidney depending on the treatment with doxycycline in SN12C sh-CD44-1 tumour cells. Each red spot represents a specific tumour of a mice and blue line indicates the median of the samples of a group. Results were normalized by tumour weight.

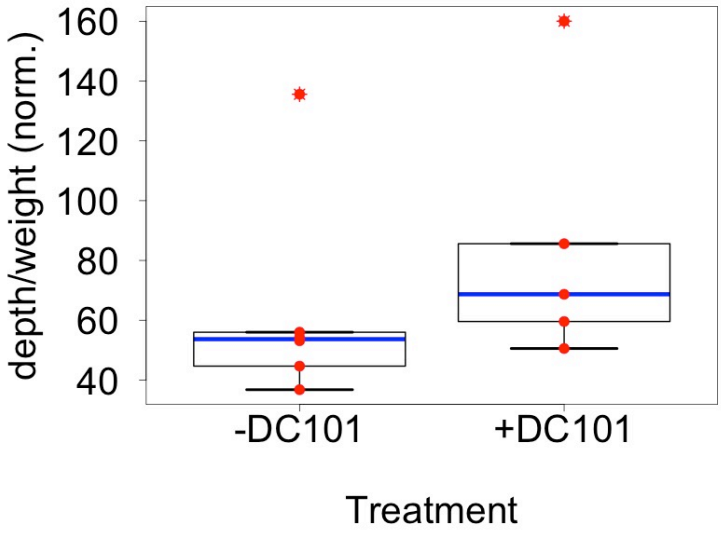

Figure 14. Boxplot representation of tumour depth into kidney depending on the treatment with DC101 antiangiogenic drug in SN12C sh-CD44-1 tumour cells. Each red spot represents a specific tumour of a mice and blue line indicates the median of the samples of a group. Results were normalized by tumour weight.

Neither doxycycline nor antiangiogenic treatment produce an increase of the invasiveness of the tumour.

The main issue of determining tumour invasiveness using that method was that in some samples, kidney could not be identified due to the aggressiveness of this tumour type. That was the reason why in some treatment groups only one sample was analysed.

To overcome that problem, same samples were analysed using another method. In this case, images were obtained through a loupe after haematoxylin-eosin staining by an immunohistochemistry assay. This method allowed having an image the entire tumour, and consequently leading to a more representative analysis of its invasiveness.

Results of the immunostaining are shown in Figure 15.

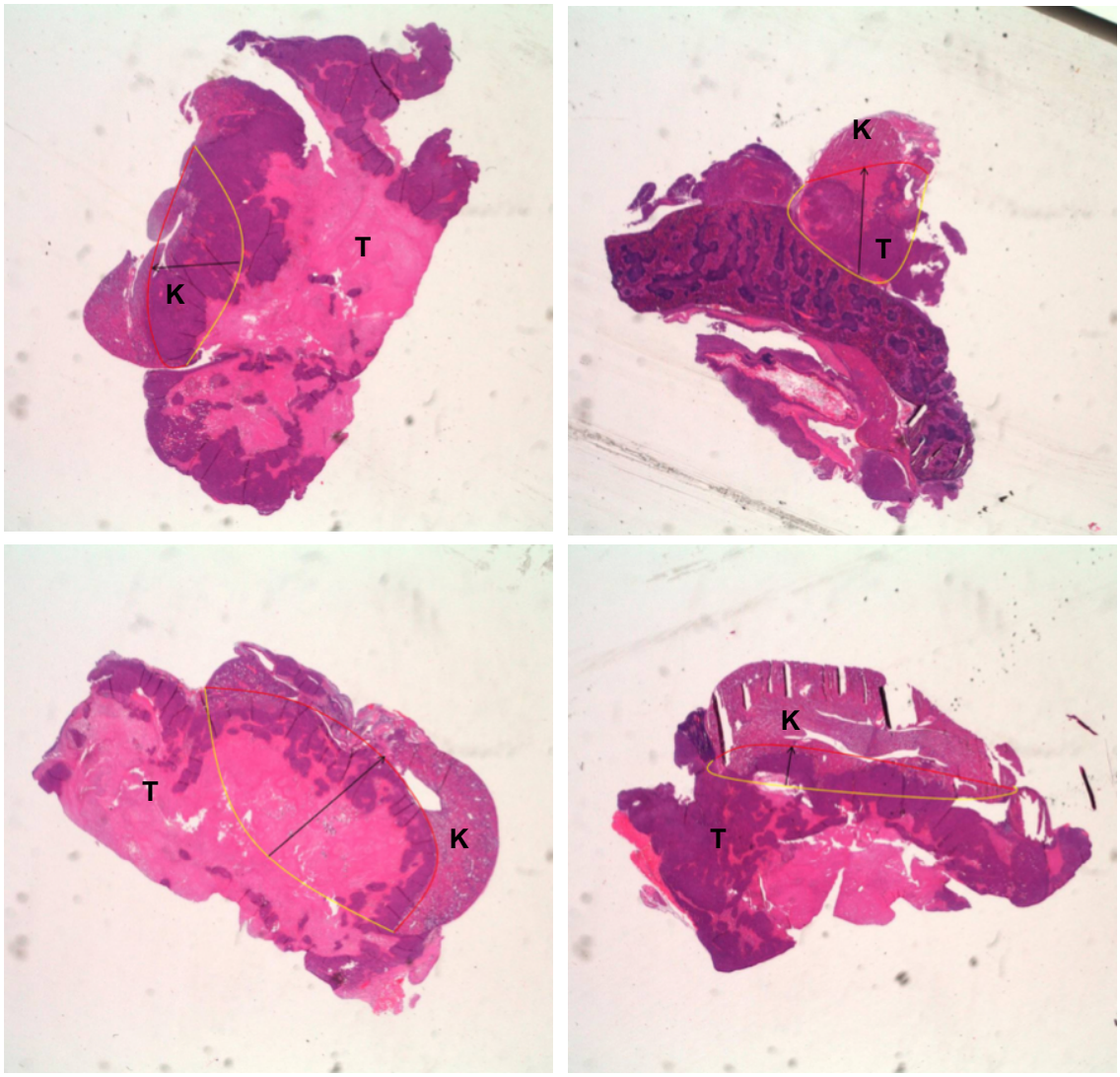

Figure 15. **Immunohistochemistry of SN12C tumour cells samples under different conditions of doxycycline and DC101 antiangiogenic drug.** Tumour gets clear pink after the immuno-treatment and kidney cells gets purple. Red and yellow curves define kidney-tumour interphase and black arrow, tumour introduction into the kidney. In the picture, T is tumour and K is kidney. (a) Interphase kidney-tumour of SN12C sh-DC44-1 tumour cells not treated with doxycycline nor DC101. (b) Interphase kidney-tumour of SN12C sh-CD44-1 tumour cells treated with DC101 but not doxycycline. (c) Interphase kidney-tumour of SN12C sh-1 tumour cells treated with doxycycline but not with DC101. (d) Interphase kidney-tumour of SN12C sh-1 tumour cells treated with doxycycline and DC101.

Invasion was measured using the same software as before and results were statistically studied and represented as boxplots (Figure 16).

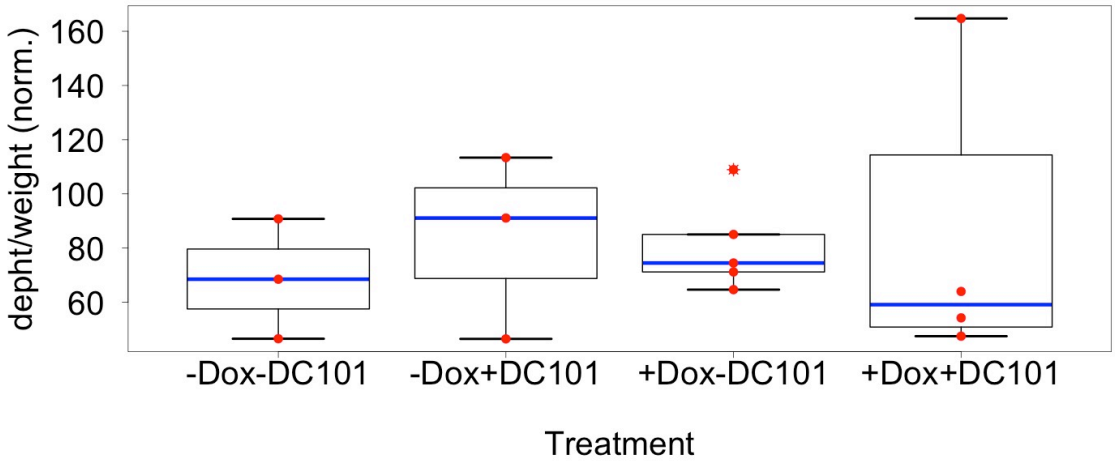

Figure 16. Boxplot representation of tumour depth into kidney depending on the treatment of SN12C sh-CD44-1 tumour cells. Samples are grouped depending on the treatment that mice received. Each red spot represents a determinate mice and blue line indicates the median of the samples belonging to a specific treatment group. Results were normalized by tumour weight.

All groups compared do not show statistically differences between them, and when compared depending on the treatment (doxycycline or DC101) statistical differences were not observed either (Figure 17 and Figure 18).

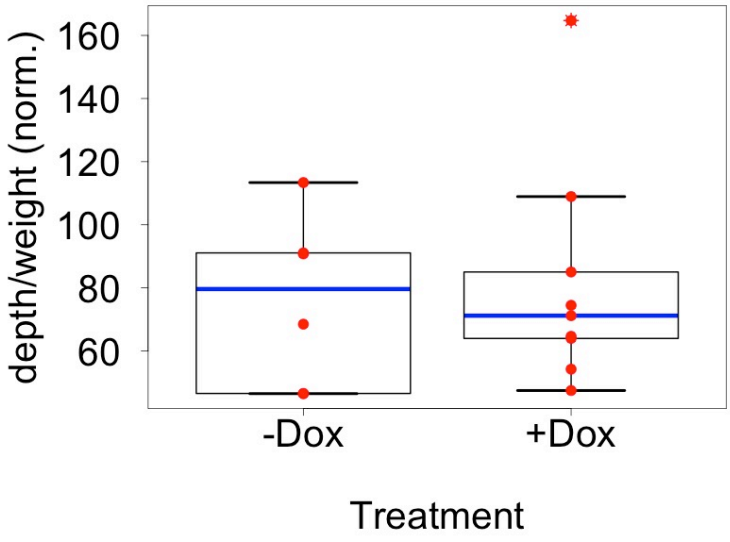

Figure 17. Boxplot representation of tumour depth into kidney depending on the treatment with doxycycline in SN12C sh-CD44-1 tumour cells. Each red spot represents a specific tumour of a mice and blue line indicates the median of the samples of a group. Results were normalized by tumour weight.

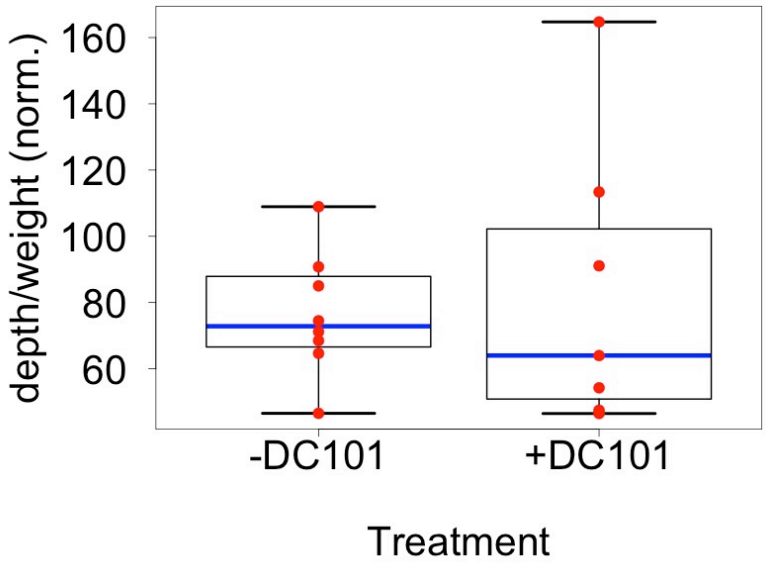

Figure 18. Boxplot representation of tumour depth into kidney depending on the treatment with DC101 antiangiogenic drug in SN12C sh-CD44-1 tumour cells. Each red spot represents a specific tumour of a mice and blue line indicates the median of the samples of a group. Results were normalized by tumour weight.

Both methods to determine the invasiveness were used to avoid method errors, because it was not easy to find a reliable method. As shown in boxplots, both methods presented same results, what was positive.

Nevertheless, our hypothesis of an increased invasiveness after an antiangiogenic treatment was not validated in SN12C tumour model.

We though that, probably this tumour behaviour was not as we thought and it was a type that, after the antiangiogenic treatment, invasiveness was not increased, as was reported in other not published studies in the laboratory.

Distribution of CD44 in Ren99 renal tumour cells

Another feature of the renal tumour cells we wanted to investigate was the distribution of the CD44 in the tumour. We suspected that CD44 was mostly expressed in the front between the tumour and the kidney and in the periphery of the tumour, because these are the principal invasive zones. For that reason, we stained samples by an immunohistochemistry assay.

For this experiment, we used Ren99 ccRCC tumour samples, that come from a cerebral metastasis of renal tumour Ren13. Importantly, this tumour type has been shown to increase local invasion and metastasis upon antiangiogenic treatment in mice (unpublished data from the laboratory).

Since our principal aim was to detect CD44, we used a mouse anti-human CD44 antibody as the primary antibody and we used an anti-mouse antibody as secondary antibody. The result of this series of incubations become in a particular staining where

cellular nucleus, tumoral and renal, gets bluish purple, and protein CD44 gets brown. Furthermore, murine stroma is not stained by the assay.

We analysed tumours of mice that did not receive treatment and tumours of mice treated with DC101. As we can see in the following images of the front between the tumour and the kidney (Figure 19), in the treated samples the staining is more intense than in the control ones.

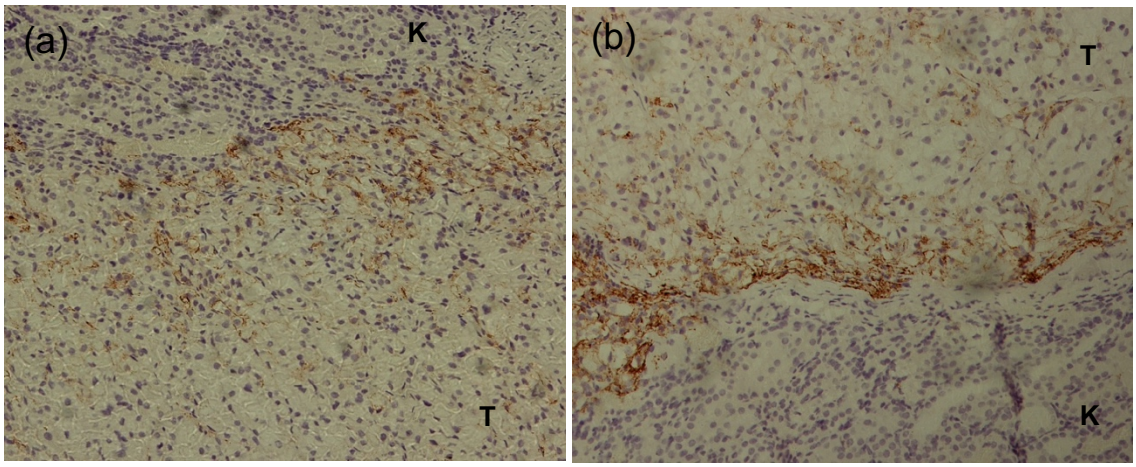

Figure 19. Immunohistochemistry staining of Ren99 cell tumour samples. The nucleous of the cells, tumoural and kidney ones, gets purple after the immunohistochemistry treatment and CD44 protein became brown. Tumour cells and kidney cell can be differentiate because of its shape. Tumour cells are more irregular and they grow more separate between them. Renal cells are more circular and grow more joined. In the picture, T is tumour and K is kidney. (a) Front between kidney and tumour cells of Ren99 control tumour sample (b) Front between kidney and tumour cells of Ren99 tumour cells treated with CD101 antiangiogenic drug.

If we take a look to the periphery of the tumour (Figure 20a, b), we can observe the same pattern than before. The expression of CD44 in the treated sample of Ren99 tumour cells with DC101 (Figure 20b) is notably more intense than in the control samples (Figure 20a), and again our hypothesis was confirmed.

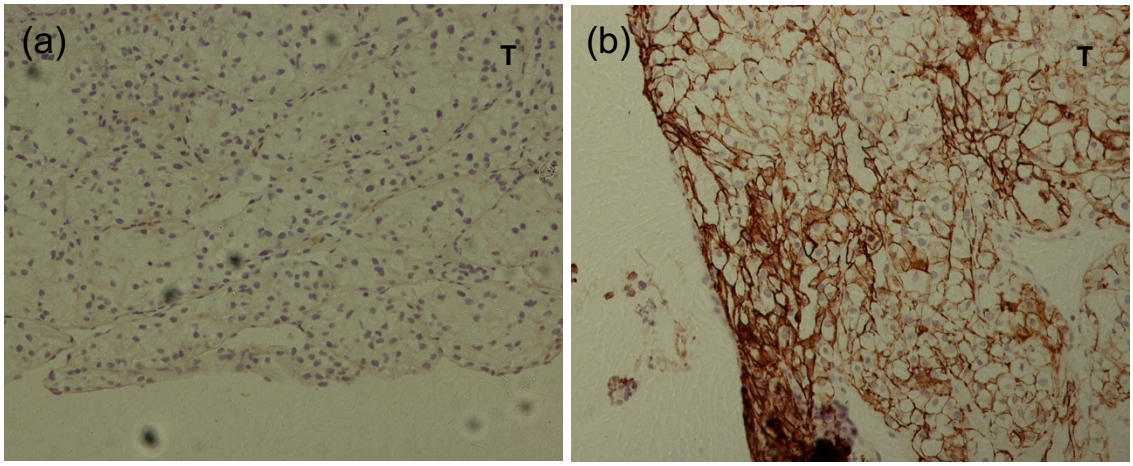

Figure 20. Immunohistochemistry staining of Ren99 cell tumour samples. The nucleous of the tumoral cells, gets purple after the immunohistochemistry treatment and CD44 protein became brown. In the picture, T is tumour. (a) Periphery of tumour cells of Ren99 control tumour sample (b) Periphery of tumour cells of Ren99 tumour cells treated with CD101 antiangiogenic drug.

Observing the middle of the tumour (Figure 21), the staining is less intense in both cases, control and treated samples. As we expected, middle of the tumour is not an invasive area, and that is why the expression of CD44 is not as intens as in the other samples.

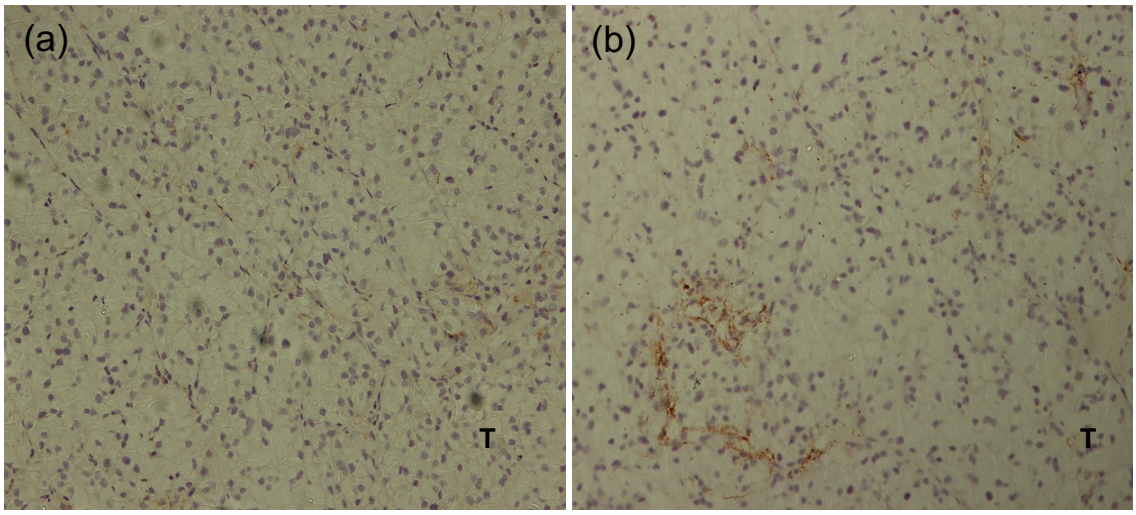

Figure 21. Immunohistochemistry staining of Ren99 cell tumour samples. The nucleous of the tumoral cells, gets purple after the immunohistochemistry treatment and CD44 protein became brown. In the picture, T is tumour. (a) Middle of tumour cells of Ren99 control tumour sample (b) Middle of tumour cells of Ren99 tumour cells treated with CD101 antiangiogenic drug.

Conclusions

After finishing all the experiments and according to our objectives, our conclusions are:

- In silencing and non-silencing SN12C tumour cells, the expression of CD44 protein in antiangiogenic treated mice with DC101 is not higher than in the non-treated ones.
- Silencing vector of CD44 protein is an effective method to induce the reduction of that protein expression by doxycycline.
- There is no relation between tumour invasiveness and expression of CD44 protein in SN12C tumour cells.
- SN12C tumour cells does not increase malignancy after antiangiogenic treatment.
- In Ren 99 tumour cells, CD44 protein is mainly observed in the front kidney-tumour and in the periphery.

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