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Identification of TMEM45A binding partners: TMEM45A, a protein involved in the chemoresistance of cancer cells

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IDENTIFICATION OF TMEM45A BINDING PARTNERS: TMEM45A, A PROTEIN INVOLVED IN THE CHEMORESISTANCE OF CANCER CELLS

Mémoire présenté pour l'obtention

du grade académique de master en sciences biomédicales

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Identification des protéines partenaires de TMEM45A, une protéine impliquée dans la chimiorésistance des cellules cancéreuses

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Résumé

Des études réalisées sur TMEM45A ont montré que cette protéine protégeait les cellules cancéreuses de l'apoptose induite par la chimiothérapie et jouait un rôle dans la chimiorésistance des tumeurs solides en hypoxie. Cependant, rien n'est connu sur la fonction de cette protéine.

L'objectif de cette étude est d'identifier les protéines interagissant avec TMEM45A dans le but de comprendre par quels mécanismes TMEM45A est impliqué dans la chimiorésistance des cellules cancéreuses.

Des études précédemment réalisées en URBC ont utilisé la technique du double hybride afin d'identifier les protéines candidates pouvant interagir avec TMEM45A: VKORC1, HAMP, SERP1 et TMEM230. Des cellules HEK293 ont été transfectées avec les vecteurs d'expression de ces protéines d'intérêt. Les interactions entre TMEM45A et ces protéines candidates ont été étudiées par marquage en immunofluorescence et par co-immunoprecipitation.

Les résultats du marquage en immunofluorescence ont montré des co-localisations partielles entre TMEM45A et les quatre candidats. Néanmoins, une étude préliminaire par co-immunoprecipitation entre TMEM45A et SERP1 n'a pas confirmé d'interaction.

Des expérimentations futures devront être réalisées afin de valider les interactions entre TMEM45A et les autres protéines candidates.

Mémoire de master en sciences biomédicales

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Previous studies performed in URBC, using yeast two-hybrid system, have identified proteins interacting with TMEM45A: VKORC1, HAMP, SERP1 and TEM230. HEK293 cells were transfected with vectors expressing TMEM45A and these proteins of interest. Interactions were studied by immunofluorescence staining and co-immunoprecipitation.

Results of immunofluorescence staining showed partial co-localization of TMEM45A with all four candidates. However, the preliminary study of the interaction between TMEM45A and SERP1, by co-immunoprecipitation, did not evidence an interaction. Further experiments will be performed to validate the interacting candidates.

Mémoire de master en sciences biomédicales

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Abstract

TMEM45A has been shown to protect cancer cells against drug-induced apoptosis and to play a role in the chemoresistance of solid tumors under hypoxia. However, the function of this protein is unknown. The purpose of this study is to identify the binding partners of TMEM45A in order to understand by which mechanisms TMEM45A could be involved in the chemoresistance of cancer cells.
Previous studies performed in URBC, using yeast two-hybrid system, have identified proteins interacting with TMEM45A: VKORC1, HAMP, SERP1 and TMEM230. HEK293 cells were transfected with vectors expressing TMEM45A and these proteins of interest. Interactions were studied by immunofluorescence staining and co-immunoprecipitation.
Results of immunofluorescence staining showed partial co-localization of TMEM45A with all four candidates. However, the preliminary study of the interaction between TMEM45A and SERP1, by co-immunoprecipitation, did not evidence an interaction. Further experiments will be performed to validate the interacting candidates.

Keywords: Chemoresistance, cancer, TMEM45A, interaction, co-immunoprecipitation

Introduction

Approximately 12.4 millions of new cancer cases appeared in the world in 2008 and among these cases, 7.6 millions of deaths were reported [1]. These numbers will increase in further years due to an increase in life expectancy and changes in people life style including altered nutrition and tobacco.

Cancer treatment is based on three main approaches: surgery, radiotherapy and chemotherapy. Despite the development of effective chemotherapy, many patients show no response to the drug or relapse few years later [2]. The consequences are non-effective treatment against cancer but also a risk of worsening patient's health due to the side effects of the chemotherapeutic agents. One explanation for this situation is the development of cancer cell resistance to chemotherapeutic agents.

There exist two types of resistance: firstly, resistance could already be present before the treatment. In this way, the cancer cells show a lack of response to drug therapy caused by the patient genetic background that provides heredity resistance. Furthermore, few tumor cells could express resistance genotype and become selected by chemotherapy, causing the repopulation of the tumor and relapse. Secondly, resistance could be acquired. In this case, patients first seem to respond though relapse appearing later due to appearance of new genetic changes induced by the drug.

In addition, the tumor micro-environment also seems to play a role in the appearance of resistance to chemotherapy. Indeed, hypoxia is observed

in a majority of solid tumors due to oxygen delivery deficiency caused by tumor growth. This environment leads to the reduction of the effectiveness of chemotherapy by increasing the diffusion distance thus decreasing the distribution of chemotherapeutic agents [3]. Hypoxia also induces modification in gene expression of cancer cells through the activation of hypoxia-inductible factor 1 (HIF-1) [4]. This transcription factor is involved in tumor cell survival mechanisms by increasing the expression of vascular endothelial growth factor (VEGF), allowing angiogenesis and neovascularization, as well as the expression of enzymes involved in glycolysis, in glucose transport (GLUT) in cell mobility and several membrane transporters (MDR-1) [4, 5, 6].

Furthermore, hypoxia and HIF-1 have an anti-apoptotic effect in tumor cells by the inhibition of p53 activity, decreasing BAX and BAK1 expression, but other yet unidentified mechanisms are also involved. Hypoxia also protects against drug-induced apoptosis and in this way, increases the chemoresistance of cancer cells [5, 7].

In the aim to identify new mechanisms responsible for hypoxia protection against drug-induced apoptosis, Flamant et al. discovered that TMEM45A overexpression is associated with cancer cell resistance to taxol and etoposide under hypoxia [8]. Indeed, when the TMEM45A gene was silenced, they observed an increased apoptosis in MDA-MB-231 or HepG2 cells exposed to taxol or etoposide under hypoxia.

TMEM45A gene, also called DERP7 or FLJ10134, is located in the region 12.2 of the short arm of chromosome 3. This gene codes for the transmembrane protein 45A. This protein has a molecular weight of 31 kDa or 33 kDa (due to alternative splicing), is overexpressed in skin, adipocytes and smooth muscle cells but nothing is known about its function (BioGPS, at 5th December 2013).

In order to learn more about TMEM45A function, it is interesting to identify TMEM45A binding partners. That could help for the characterization of this protein and of its role in the chemoresistance of cancer cells.

The binding partners of TMEM45A were identified in previous studies performed in URBC, using yeast two-hybrid system adapted for transmembrane protein: split-ubiquitin system [9]. This method uses ubiquitin, expressed in the form of two fragments: NubG (N-terminal) and Cub (C-terminal). Protein of interest or the "bait", TMEM45A, binds with Cub and with the reporter gene: LexA-VP16. "Prey" proteins, coming from a cDNA bank of HepG2 cells, bind with NubG. If "bait" and "prey" have affinity, they bind each other and form the split-ubiquitin, recognized by the ubiquitin protease and splited. The reporter protein is released and goes into the nucleus to induce gene expression (LacZ in this case). Several proteins were found to interact with TMEM45A and considered as the most interesting candidates: VKORC1, HAMP, SERP1 and TMEM230.

VKORC1, vitamin K epoxide reductase complex subunit 1, is involved in vitamin K cycle by the reducing of vitamin K which is essential for blood clotting by the production of vitamin K-dependent coagulation factors (factors II, VII, IX and X) [9, 10].

HAMP gene, hepcidin antimicrobial peptide, codes for hepcidin, a hormone secreted by the liver for regulation of iron homeostasis. Hepcidin regulates ferroportin at cell surface of enterocytes, macrophages, hepatocytes and placenta cells by its internalization and its degradation through the proteasome [11, 12].

SERP1, stress-associated endoplasmic reticulum protein 1, also called ribosome-

associated membrane protein 4 (RAMP4), is present in the endoplasmic reticulum (ER) and plays a role in the stabilization of membrane proteins under stress such as hypoxia [13]. It interacts with calnexin, a membrane chaperone in ER that plays a role in the quality control of membrane proteins [13, 14].

TMEM230 is a transmembrane protein but nothing is known about its function.

The purpose of this study is to confirm the interaction between TMEM45A and its binding partners. HEK293 cells were transfected with vectors expressing the proteins of interest (TMEM45A, VKORC1, HAMP, SERP1 and TMEM230). Transfected cells were analysis for co-localization. Furthermore, co-immunoprecipitation experiments were performed. This work aims to understand by which mechanisms TMEM45A could be involved in the chemoresistance of cancer cells.

Materials and methods

Cell culture of HEK293

HEK293 (Human Embryonic Kidney) cells grown in 75 cm^2 flasks with medium composed by Dulbecco's Modified Eagle's Medium (DMEM, 4.5 g/l glucose, Gibco) with 10 % of fetal calf serum (FBS, Gibco) and incubated at 37°C in a humidified atmosphere containing 5 % CO_2.

DNA Transfection

HEK293 cells were seeded in 75 cm^2 flasks, in 6-well plates or in 24-well plates at a density of 4,000,000, 200,000 and 60,000 cells/well respectively. 24 hours after seeding the cells, cells were transfected with different plasmids, following the Lipofectamine® 2000 DNA Transfection Reagent Protocol (Invitrogen). 18.75 µg of plasmidic DNA were transfected in each 75 cm^2 flaks, 2.5 µg were added in 6-well plates and 500 ng were added in 24-well plates. Plasmids pCMVHA, pcDNA-3.1Myc/hisA, pDest-475, pDest475TMEM45A, pcDNA3.1-Myc/HisVKORC1, pcDNA3.1Myc/his-HAMP, pcDNA3.1Myc/hisSERP1 and pcDNA-3.1Myc/hisTMEM230 were used to transfect cells.

Protein Extraction and Western blot analysis

HEK293 cells were seeded in 6-well plates at a density of 200,000 cells/well. 24 hours after seeding the cells, they were transfected with the plasmids of interest. 24 hours after transfection, cells were washed with PBS and incubated 30 min with lysis buffer composed of MES 20 mM (Invitrogen #NP002), Tris 30 mM (Merck #108387), NaCl 100 mM (Merck #109945), NEM 20 mM (Sigma-Aldrich #112448), Triton X-100 1 % (Merck #648462) and protease inhibitor cocktail. Cells were recovered and centrifuged for 30 min at 12,000 g and 4°C. The supernatant was collected for western blot analysis. 20 µg of proteins/well were separated by electrophoresis on 10 % or 4-20 % Tris-Glycine gels (Bio-Rad) and transferred on PVDF membranes after migration. These membranes were blocked thanks to 1 hour incubation in Odissey® blocking buffer (Li-Cor Biosciences, 1/2 dilution with PBS) at room temperature or overnight at 4°C. Primary antibodies and secondary antibodies were diluted with Odyssey® blocking buffer. Membranes were incubated 1 hour at room temperature with primary antibodies: mouse anti-HA (Cell Signaling, #23675, 1/1000 dilution) or mouse anti-MYC (Calbiochem, #OP10L, 1/100 dilution). After four washes during 5 min with PBS/Tween 0.1 % (Tween-20, Bio-Rad), membranes were incubated 1 hour at room temperature with secondary antibodies: goat anti-mouse IRDye® 680RD (Li-Cor Biosciences, #926-68070, 1/10,000 dilution) or goat anti-mouse IRDye® 800 (Li-Cor Biosciences, #926-32210). Membranes were washed four times during 5 min with PBS/Tween 0.1 % and two times with PBS before the

revelation of proteins using an Odyssey® scanner (Li-Cor Biosciences).

Immunofluorescence staining

HEK293 cells were seeded at a density of 60,000 cells/ well, in 24-well plates on coverslips coated with gelatin 0.1 %. Cells were transfected with plasmidic DNA and medium was changed 4 hours after transfection. 24 hours after transfection, cells were fixed with PFA 4 % (paraformaldehyde) in PBS during 10 minutes at room temperature. The coverslips were washed twice with PBS before addition of PBS-Triton 1 % (Triton X-100, Sigma-Aldrich) during 5 min and three washes with PBS-BSA 2 % (bovine serum albumin, ChemCruz).

Each coverslip was incubated with 30 µl of each primary antibody diluted in PBS-BSA 2 % and incubated 2 hours at room temperature or overnight at 4°C. Primary antibodies were rabbit anti-TMEM45A (Sigma, #HPA024082, 1/100 dilution), rabbit anti-MYC (Calbiochem #OP10L, 1/100 dilution), mouse anti-Golgin-97 (Abcam #A21270, 1/100 dilution) and mouse anti-Calnexin (BD Calbiochem, 1/500 dilution). Cells were washed three times with PBS/BSA 2 % and incubated 1 hour with secondary antibodies: Alexa-Fluor®-488 conjugated anti-rabbit IgG antibody (Invitrogen #A11008, 1/1000 dilution), Alexa-Fluor®-488 conjugated anti-mouse IgG antibody (Invitrogen, #A11001, 1/1000 dilution) and Alexa-Fluor®-568 conjugated anti-rabbit IgG antibody (Invitrogen, #A11011, 1/1,000 dilution). Cells were washed three times with PBS-BAS 2 % and staining nuclei, TO-PRO®-3 (Life Technologies, #T3605) was diluted 1/80 with RNase (2 mg/ml). This reagent was incubated 30 min in moist chamber at room temperature and protected from light.

The coverslips were mounted on slides with Mowiol (Sigma) and observed with a confocal fluorescence microscope (Leica SP5 Microscope).

Co-immunoprecipitation

HEK293 cells were seeded at a density of 4,000,000 cells in 75 cm^2 flasks. To express the two necessary proteins for co-immunoprecipitation experiments (co-IP), cells were co-transfected with two plasmidic DNA. As negative control, cells were co-transfected with pcDNA3.1Myc/ hisA and pCMVHA empty plasmids (HA-MYC). Two positive controls were used: one for SERP1 where cells were co-transfected with pcDNA3.1Myc/hisSERP1 and pCMVHA plasmids (SERP1-HA) and one for TMEM45A where cells were co-transfected with pDest475TMEM45A and pcDNA3.1Myc/hisA plasmids (TMEM45A-HA). In order to check for the interaction between TMEM45A and SERP1, cells were co-transfected with pDest475-TMEM45A and pcDNA3.1Myc/hisSERP1 plasmids (TMEM45A-SERP1).

Medium was changed 4 hours after transfection. Cells were lysed 24h after transfection according to the protocol described by Wajih et al. [15]. Co-immunoprecipitation was performed according to the protocol described by Schaafhausen et al. [9]. Extracted proteins were analyzed by Western blot and using primary antibodies: mouse anti-HA (Cell Signaling, #23675, 1/1000 dilution) or mouse anti-MYC (Calbiochem, #OP10L, 1/1000 dilution). Secondary antibodies were goat anti-mouse IRDye® 680RD (Li-Cor Bio-sciences, #926-68070, 1/10,000 dilution) or goat anti-mouse IRDye® 800 (Li-Cor Biosciences, #926-32210). Proteins were revealed with Odyssey® scanner (Li-Cor Biosciences).

Results

Transfected cells expressed the proteins of interest

To understand by which mechanisms TMEM45A is involved in the chemo-resistance of cancer cells, it is interesting to know its normal function, hypothesized by identifying binding partners. In previous studies performed in URBC,

VKORC1, HAMP, SERP1 and TMEM230 proteins were identified by split-ubiquitin system as the most interesting candidates for interacting with TMEM45A. To confirm these results, we studied interaction by co-localization and co-immunoprecipitation. HEK293 cells were transfected with vectors expressing candidate proteins by using Lipofectamine®. This reagent contained lipids allowing the formation of liposome (positive charged) in which plasmidic DNA (negative charged) is trapped and then form DNA-lipid complex. This complex was formed by incubation of Lipofectamine® and plasmidic DNA, diluted in Opti-MEM® (medium without serum) during 5 min. After formation of the complex, the mixture was added to normal medium for cell culture and was placed in flasks or plates where cells were seeded. To increase the efficiency of the transfection, medium was changed 4h after transfection because Lipofectamine® shows toxicity for cells and after this time, the DNA-lipid complexes are separated. The first step of the study was the verification of the transfection efficacy.

HEK293 cells were seeded in 6-well plates and transfected with each expressing vector of interest. 24h after transfection, cells were lysed and the lysates were analyzed by Western blot by using anti-HA or anti-MYC antibodies.

We observed that VKORC1, HAMP, SERP1 and TMEM230 proteins, recognized by anti-MYC antibody, were expressed after transfection at expected molecular weight: 18 kDa for VKORC1, 9 kDa for HAMP, 7 kDa for SERP1 and 13 kDa for TMEM230 (Figure 1a, c). The two molecular weights of TMEM45A at 31 and 33 kDa, due to alternative splicing and recognized by anti-HA antibody, were also observed (Figure 1b). The signal must be saturated to observe HAMP because this protein is secreted and hence, very few of the protein remains in the cells (Figure 1c).

These results suggest that HEK293 cells were correctly transfected according to the protocol and that proteins were correctly expressed. We thus can use this transfection technique for further immunofluorescence and co-immunoprecipitation experiments that need HEK293 transfected cells.

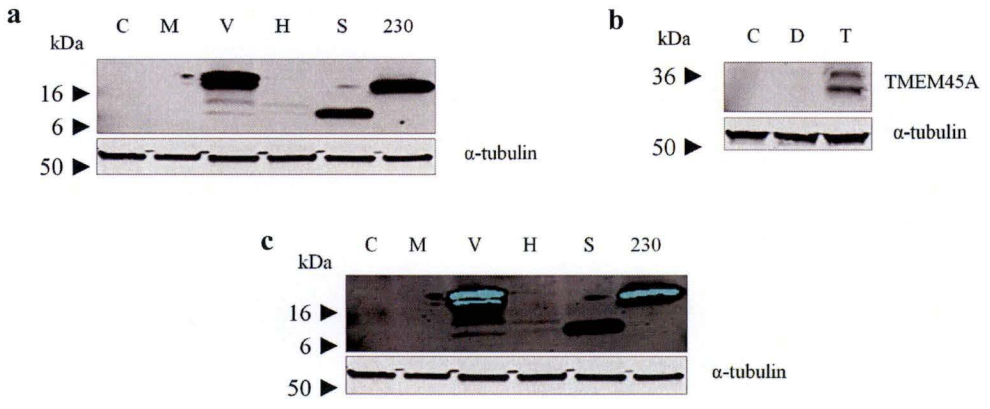


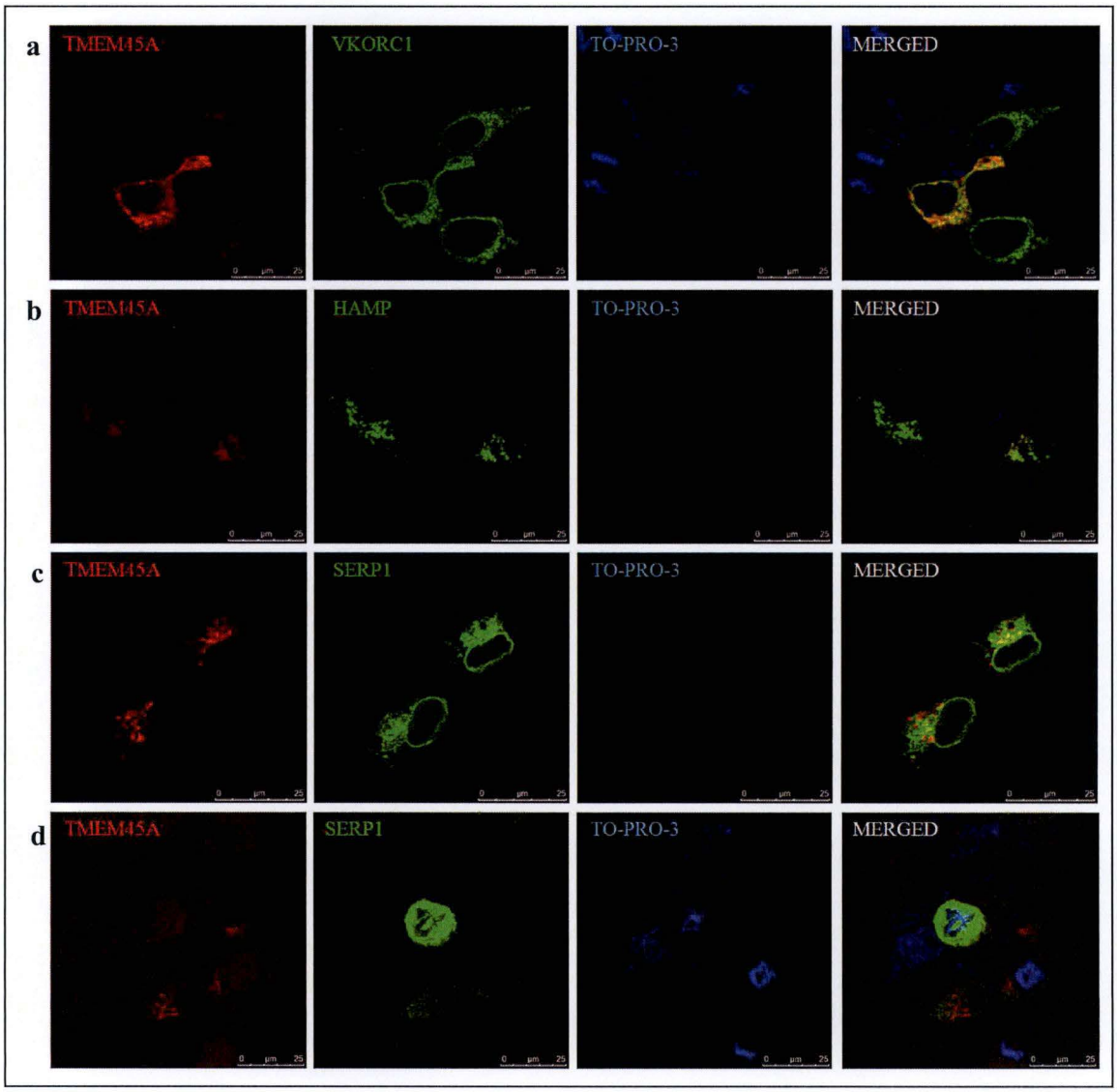
Figure 1: HEK293 cells transfected with different plasmids expressed protein of interest. Control (C) was untransfected cells, empty plasmid control were cells transfected with pcDNA3.1Myc/hisA (M) and pDest475 (D). To express protein, cells were transfected with pcDNA3.1Myc/HisVKORC1 (V), pcDNA3.1Myc/hisHAMP (H), pcDNA3.1Myc/hisSERP1 (S), pcDNA3.Myc/hisTMEM230 (230), and pDest475TMEM45A (T). 24 h after transfection, proteins were detected by Western blot analysis, using specific antibodies. Anti- α-tubulin antibody was used to assess the total amount of proteins loaded on the gel. (**a**) Proteins were loaded on a gel of 4-20% and detected with anti-MYC and anti-mouse 680RD antibodies. (**b**) Proteins were loaded on a gel 10% and detected with anti-HA and anti-mouse 680RD antibodies. (**c**) Signal was saturated in order to see the band corresponding to HAMP.

TMEM45A is localized at the same place as its binding partners

To confirm the interaction between TMEM45A and its binding partners, HEK293 cells were co-transfected with TMEM45A and each candidate expressing vectors. Co-localizations were observed by immunofluorescence staining, by using anti-TMEM45A or anti-MYC antibodies and anti-rabbit 568 or anti-mouse 800 in confocal fluorescence microscopy.

VKORC1, HAMP and TMEM230 proteins showed partial overlap with TMEM45A (Figure 2a, b, e). We also observed partial overlap with SERP1 although this overlap was not present in all cells (Figure 2c, d). A weak signal in cells can be explain by a lower rate of transfection in co-transfected cells than in cells only transfected with TMEM45A. We have suggested a competition between the plasmidic DNA during the lipofection or between the antibodies during the incubation.

These results thus confirm the possible interaction between TMEM45A and VKORC1, HAMP, TMEM230 and SERP1.



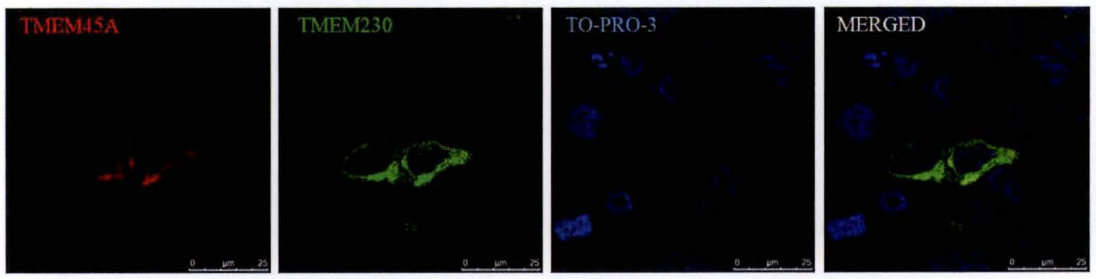


Figure 2: Binding partners co-localize with TMEM45A. HEK293 cells were co-transfected with plasmid of TMEM45A and binding partners. They were observed with confocal fluorescent microscopy by using anti-TMEM45A or anti-MYC antibodies and by using anti-rabbit 568 or anti-mouse 800 antibodies. (**a**) Cells were co-transfected with pDest475TMEM45A and pcDNA3.1Myc /HisVKORC1. (**b**) Cells were co-transfected with pDest475TMEM45A and pcDNA3.1Myc/hisHAMP. (**c**) Cells were co-transfected with pDest475TMEM45A and pcDNA3.1Myc/hisSERP1. (**d**) Cells were co-transfected with pDest475TMEM45A and pcDNA3.1Myc/hisSERP1. (**e**) Cells were co-transfected with pDest475TMEM45A and pcDNA3.Myc/hisTMEM230.

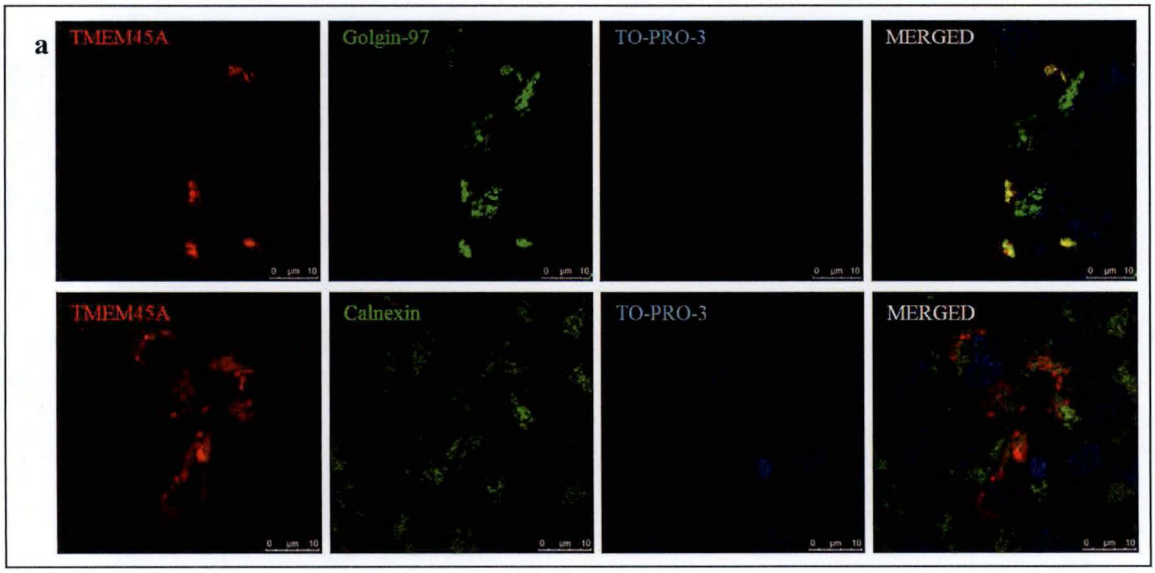
Subcellular localization of TMEM45A and its binding partners

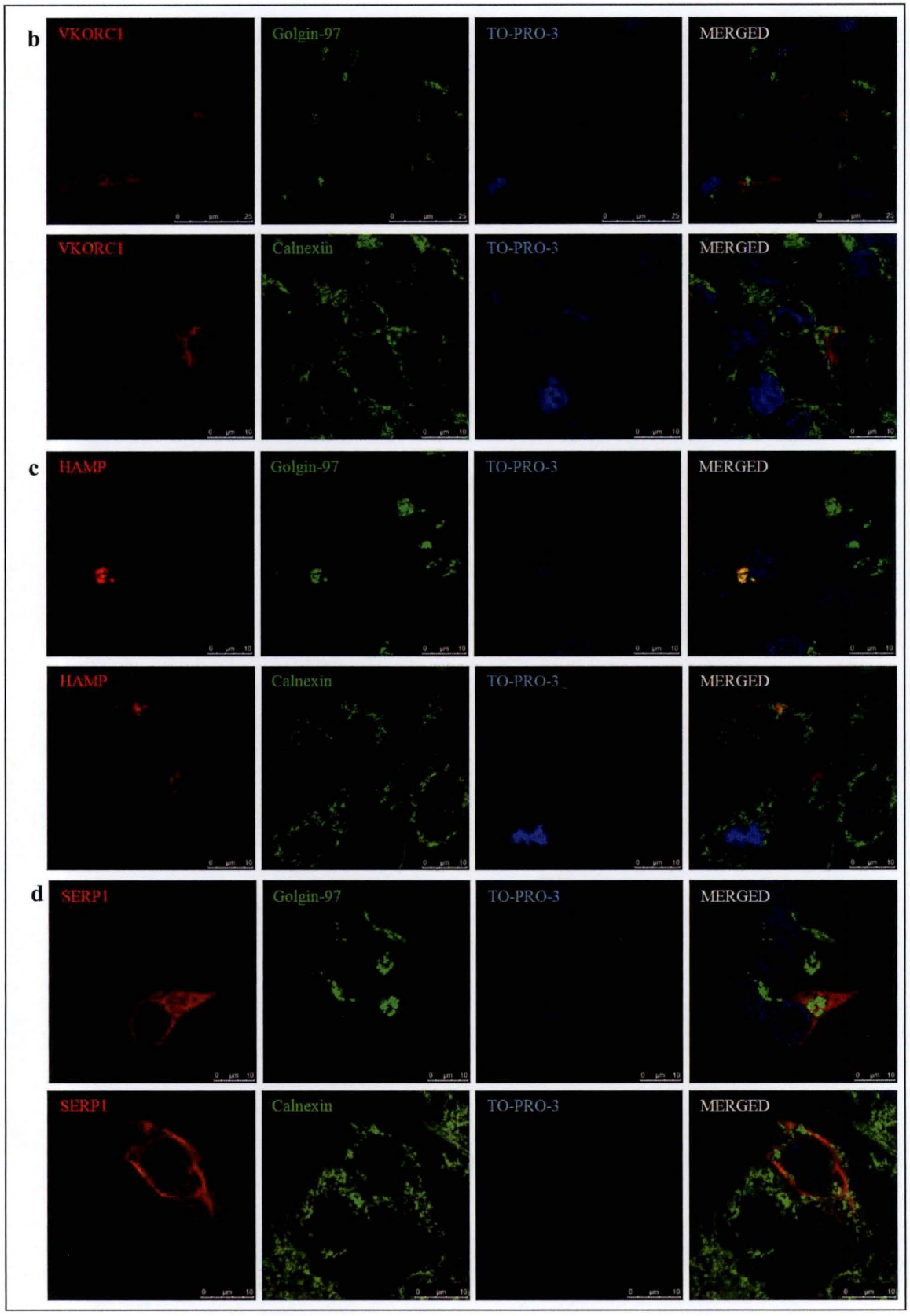
We further studied the subcellular localization of each protein by immunofluorescence staining in HEK293 transfected cells. We compared the different localization of each protein with a marker for two organelles: Golgi apparatus and endoplasmic reticulum.

HEK293 cells were transfected with TMEM45, VKORC1, HAMP, SERP1 and TMEM230 expressing vectors. Localization was observed by confocal fluorescence microscopy by the labeling of the proteins of interest with anti-TMEM or anti-MYC antibodies and by the labeling of the Golgi apparatus and ER with anti-Golgin-97 antibody that specifically recognizes the Golgi apparatus and anti-calnexin antibody that specifically recognizes the ER. Secondary antibodies were anti-rabbit 568 and anti-mouse 800 antibodies.

TMEM45A is located in the Golgi apparatus (Figure 3a) as well as HAMP and TMEM230 (Figure 3c, e). We could not show location of VKORC1 and SERP1 neither in the Golgi apparatus nor in the ER (Figure 3b, d).

These results suggest a more possible interaction between TMEM45A and HAMP or TMEM230 than with VKORC1 or SERP1 since the latter are not localized at the same place than TMEM45A.




b
VKORC1
Golgin-97
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c
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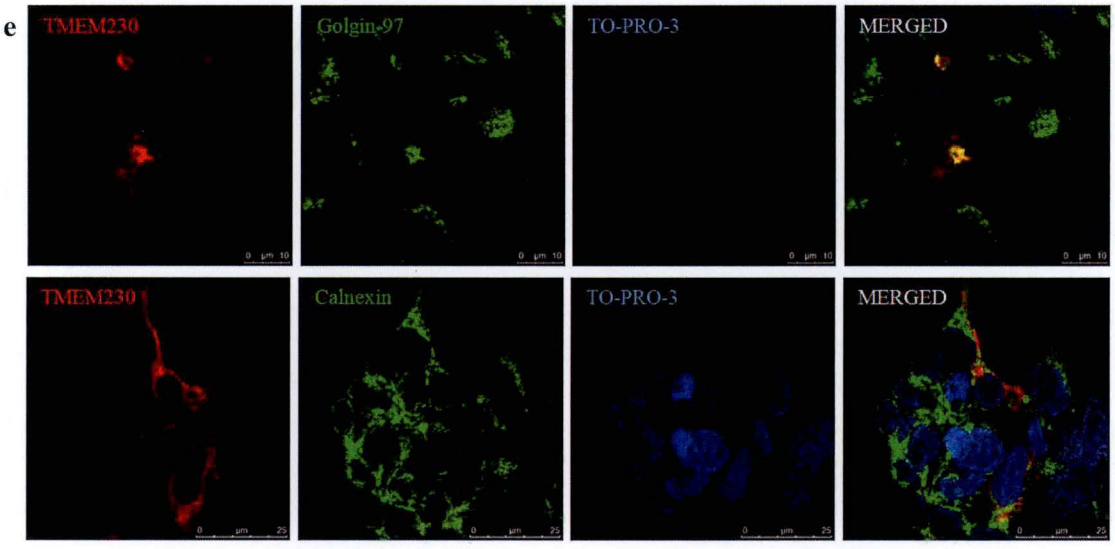

Figure 3: TMEM45A, HAMP and TMEM230 colocalize with marker of the Golgi apparatus in HEK293 cells. Cells were transfected with plasmids encoding TMEM45A and binding partners. They were observed by confocal fluorescence microscopy. (**a**) Cells were transfected with pDest475TMEM45A and observed after labeling with anti-TMEM45A and anti-Golgin-97 or anti-Calnexin antibodies. (**b**) Cells were transfected with pcDNA3.1Myc/hisVKORC1 and observed after labeling with anti-MYC and anti-Golgin-97 or anti-Calnexin antibodies. (**c**) Cells were transfected with pcDNA3.1Myc/hisHAMP and observed after labeling with anti-MYC and anti-Golgin-97 or anti-Calnexin antibodies. (**d**) Cells were transfected with pcDNA3.1Myc/hisSERP1 and observed after labeling with anti-MYC and and-Golgin-97 or anti-Calnexin antibodies. (**e**) Cells were transfected with pcDNA3.Myc/hisTMEM230 and observed through anti-MYC and anti-Golgin97 or anti-Calnexin antibodies.

TMEM45A does not show interaction with SERP1

Experiments of localization and co-localization suggested that TMEM45A and SERP1 may not interact. We try to confirm these results in a preliminary study by co-immunoprecipitation (co-IP).

For each condition, two proteins must be expressed for co-IP experiments, HEK293 cells were then co-transfected with two different plasmidic DNA: with HA-MYC, SERP1-HA, TMEM45A-MYC or TMEM45A-SERP1. This last co-transfection will permit to evaluate the interaction of interest between the two proteins. Medium was changed 4 hours after transfection. Proteins were extracted 24h after transfection, using lysis buffer, and incubated with C4B-agarose beads to clear the cell lysates. After washes with PBS and centrifugation, the supernatant was collected and incubated with the agarose beads coupled to the anti-MYC antibody. In each condition, there are a protein expressing the MYC tag and another protein containing the HA tag.

SERP1 contains a MYC tag that recognizes the beads and bind to them. If TMEM45A (containing the HA tag) interacts with SERP1, it is expected that these two proteins would be detected in the Western blot analysis, after washes and elution of the beads.

The negative controls were cells untransfected and cells co-transfected with the two empty plasmids HA-MYC. The positive controls were cells co-transfected with SERP1-HA and TMEM45A-MYC.

Western blot analysis of the proteins from eluted IP-MYC beads contain SERP1 as expected band of 7 kDa but not TMEM45A (Figure 4a, b). TMEM45A was detected in the input post-IP (first wash of beads) as expected bands of 31 and 33 kDa but not SERP1 (Figure 4c, d).

The results suggest that TMEM45A and SERP1 were expressed by the cells after co-transfection but that there was no interaction between TMEM45A and SERP1.

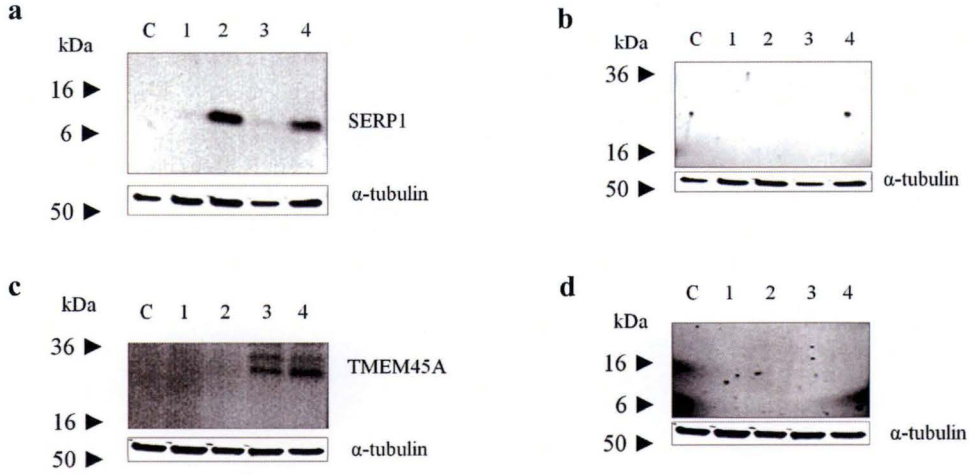

Figure 4: SERP1 does not co-immunoprecipitate with TMEM45A. HEK293 cells were co-transfected with protein of interest expressing plasmids. Control (C) was untransfected cells. To co-express the two proteins, cells were co-transfected with two plasmidic DNA: pcDNA3.1Myc/hisA and pCMVHA (1), pcDNA3.1Myc/hisSERP1 and pCMVHA (2), pDest475TMEM45A and pcDNA3.1Myc/hisA (3) and pDest475TMEM45A and pcDNA3.1Myc/hisSERP1 (4). The first input post-IP and eluted proteins of beads were analyzed by Western blot analysis on 4-20% gels, using specific antibodies. α-tubulin was used to assess the total amount of proteins loaded on the gel. (**a**) Proteins from eluted beads were detected with anti-MYC antibody. (**b**) Proteins from eluted beads were detected with anti-HA antibody. (**c**) Proteins from input of beads were detected with anti-MYC antibody. (**d**) Proteins from input of beads were detected with anti-HA antibody.

Discussion

A major cause of failure in the cancer treatment is the appearance of resistance to chemotherapy. One explanation for this resistance is the tumor microenvironment. Indeed, hypoxia, appearing in a majority of solid tumors, has been shown to reduce the effectiveness of chemotherapeutic agents and to protect cancer cells against apoptosis [3, 5, 7]. However, how hypoxia provides resistance is still largely unknown.

In the purpose to identify the mechanisms responsible for hypoxia protection against drug-induced apoptosis, Flamant et al. demonstrated that TMEM45A is involved in chemoresistance of cancer cells under hypoxia although its functions are unknown [8]. To learn more about TMEM45A function and its role in the chemoresistance of cancer cells, we performed immunofluorescence staining and co-immunoprecipitation in HEK293 cells. These cells were transfected with plasmids encoding TMEM45A and the four candidate proteins, previously identified in URBC: VKORC1, HAMP, SERP1 and TMEM230.

Some links between these proteins and cancer and hypoxia have already been described. Indeed, HAMP (hepcidin) is involved in iron absorption and homeostasis, an important mechanism for cell survival, by the regulation of the level of ferroportin at cell surface via its internalization and its degradation through the proteasome [11]. A study on different aggressive breast cancer cell lines have observed that all these lines show a high level of hepcidin gene expression and a low level of ferroportin gene expression [16]. Moreover, anemia and inflammation state are currently observed in patients

with cancer. Inflammation that develops within tumors leads to IL-6 expression and leads to hepcidin production [17]. Increase hepcidin involves a low level of ferroportin on the cell surface and then less absorption of iron by enterocytes that lead to anemia, a poor survival prognosis for cancer [18]. These results suggest that the ferroportin and hepcidin genes could be prognosis biomarkers for patient with cancer.

Hypoxia and HIF are present in solid tumors and induce the expression of several gens including the stimulation of erythropoiesis by EPO expression [4]. A study has observed that hepcidin was indirectly regulated by HIF and needed to EPO-induced erythropoiesis to decrease its expression. And then when HIF and EPO are simultaneously induced by the hypoxic environment of the tumor, hepcidin level decreases in the cells, leading to the decrease in iron export and to intracellular iron accumulation that permits the tumor growth [19]. This could explain how tumor cells survive in hypoxic environment.

HIF and EPO not only induce HAMP expression inhibition. Indeed, less tumor vascularization induces the switch from aerobic glycolysis to anaerobic glycolysis and leads to production of lactic acid that decreases the pH in tumor environment. This acidic environment leads to ROS formation by the macrophages [20] and it is observed that ROS also inhibit hepcidin expression [21].

Finally, HAMP promoter contains a p53-response element (p53RE) that leads to increased hepcidin expression when p53 is activated [22]. Moreover, p53 is mutated in half of human cancer, leading to the downregulation of HAMP and the increase in iron rate and cell proliferation. HAMP could thus be another pathway of the tumor suppressor activity of p53.

These different observations show several links between HAMP and its implication in the cancer. Further experiments will be performed to confirm interaction between TMEM45A and HAMP and understand by which mechanisms these ones could be link with the chemoresistance of cancer cells.

SERP1 is involved in the stabilization of membrane proteins after a cell stress, by its interaction with the chaperone calnexin [14]. Its expression is induced during cell stress, as hypoxia [13, 14]. As previously shown, less tumor vascularization and hypoxia induce ROS formation by macrophages [20]. The ROS production is a cellular stress that could also induce an overexpression of SERP1. A possible hypothesis is that, under hypoxia, SERP1 permits to keep the quality of the membrane proteins and then seems contributing to cancer cell survival.

Further experiments on interaction with TMEM45A and SERP1 may permit to find other functions of this protein.

VKORC1 is involved in vitamin K cycle playing an important role for blood coagulation. This protein reduces the vitamin K epoxide to the hydroquinone. This one is reoxidized by the electron transfer chain and then could be considered as an antioxidant [10] and playing a role in cancer cell survival by providing a protection against ROS production.

Nothing is known about the function of TMEM230, thus this protein has no described role in cancer or hypoxia.

According to what is described here above, HAMP appears as the more likely to show a link between TMEM45A and cancer. However, there is a lack of information on the other candidates and there is a need for extensive research on each other to find which protein and by which pathway it is involved in the chemoresistance of cancer cells.

In the aim to identify binding partners of TMEM45A, immunofluorescence staining, using co-transfection of TMEM45A and candidate plasmids in HEK293 was performed. This experiment has shown partial co-localization between TMEM45A and VKORC1, HAMP, SERP1 and TMEM230. However, partial overlap has

been shown in cells co-transfected with TMEM45A and SERP1 but not in all cells.

To determine the subcellular localization of the different proteins of interest, HEK293 cells were transfected with vectors expressing these proteins. By immunofluorescence staining, we have found that TMEM45A, HAMP and TMEM230 were located in the Golgi apparatus. Several studies confirmed the localization of HAMP in the Golgi apparatus [19, 23] but the subcellular localization of TMEM230 is unknown. According to these results, HAMP and TMEM230 have a more likely interaction with TMEM45A. For VKORC1 and SERP1, we did not observe the presence of these proteins in the Golgi apparatus or in the ER even though it is know that VKORC1 forms a lipid-protein complex in the ER membrane [9, 10, 15, 25] and SERP1 is found in the luminal side of the ER membrane [13, 26]. We suggest that a weak rate of transfection in cells transfected with VKORC1 and SERP1 expressing vectors could explain the results obtained for these proteins. VKORC1 and SERP1 proteins appear diffuse and with a filament-like distribution while the proteins appear as spots in other cells. That suggests a problem with the anti-MYC antibody or during the transfection.

Finally, we have performed a preliminary study of the interaction between TMEM45A and SERP1 by co-immunoprecipitation. Indeed, the two previous experiments show that SERP1 is the less likely binding partner of TMEM45A. The co-Ip did not evidence an interaction between TMEM45A and SERP1. This result suggests that SERP1 is not a binding partner of TMEM45A.

Further in vitro co-immunoprecipitation experiments will be performed with the other binding partners (VKORC1, HAMP and TMEM230) to confirm their interaction with TMEM45A.

Assessment of the role of binding partners in the expression or in the function of TMEM45A would be confirmed by inactivation the expression of these binding partners by siRNA in cell lines that expressed TMEM45A. Indeed, this transmembrane protein is overexpressed in cancer cell lines showing resistance to drug-induced apoptosis under hypoxia. It should be interesting to compare the TMEM45A expression in the cancer cells, under hypoxia or normoxia, with or without chemotherapeutic agents, when its binding proteins expression is inhibited. And conversely by the inactivation of TMEM45A by siRNA in the cancer cells and then compare its binding partner expression under different oxygen levels.

In vivo experiments can be performed on knock-out mouse for TMEM45A. Indeed, the lack of this protein will permit to understand in which mechanisms it is involved in a living animal. Moreover, tumors could be induced in this model and then may contributed to understand the role of TMEM45A in cancer cells as well as in the chemoresistance of cancer cells.

Characterization of TMEM45A will permit the understanding of its function and of its implication in the chemoresistance of cancer cells. In the long term, a future clinical application could be performed to target this protein as treatment to overcome the chemoresistance.

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