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AGR2, Senescence, and Breast cancer

GUILBARD Marianne

Host Laboratory – RyTME team, ARTiSt group, Inserm U1312,
Université de Bordeaux, Institut Bergonié

Internship directors – Dr. Frédéric Delom

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INTRODUCTION

I) CONTEXT

1) The dual role of AGR2 in adenocarcinomas

In physiological conditions, AGR2 is exclusively located in the Endoplasmic Reticulum thanks to the presence of an NH2-terminal ER signal sequence and an ER retention motif (KTEL domain) in C-terminal (**Figure 1**). As an ER-localized molecular chaperon, it mediates the formation of disulfide bonds and assists to the quality control of protein via its pseudo thioredoxin domain (CXXS) [1] (**Figure 1**). However, studies carried out by the host laboratory have demonstrated that, in cancer, AGR2 is not only overexpressed in the ER but is also secreted in the tumor microenvironment since it can be detected in the blood of different cancers such as breast, colon, lung of patients [2]. Indeed, under physiological conditions the protein-folding demand does not exceed the cell's folding capacity thus AGR2 is restricted to the ER (intracellular AGR2 (iAGR2)). Whereas, in cancer cells increase ER stress could be the mediator of AGR2 secretion in the tumor microenvironment (extracellular AGR2 (eAGR2)) [3]. The mechanisms by which eAGR2 accesses to the extracellular environment still remain unclear.

Overall, AGR2 plays a dual role in cancer cells with an iAGR2 (intracellular AGR2) which contributes to tumor development by enhancing ER proteostasis and thus modulating the secretome, and an eAGR2 (extracellular AGR2) which has been shown to confer to tumor specific and evolutive features as it plays extracellular roles independent of its ER function (**Figure 2**).

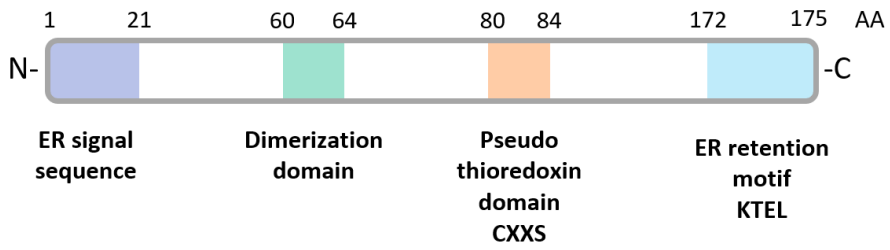

Figure 1 – Schematic representation of anterior gradient-2 (AGR2) protein primary structure
Colored boxes represent the function domain involved in the regulation of AGR2 function.
AA: amino acid, ER: Endoplasmic reticulum, CXXS : KTEL: [4]

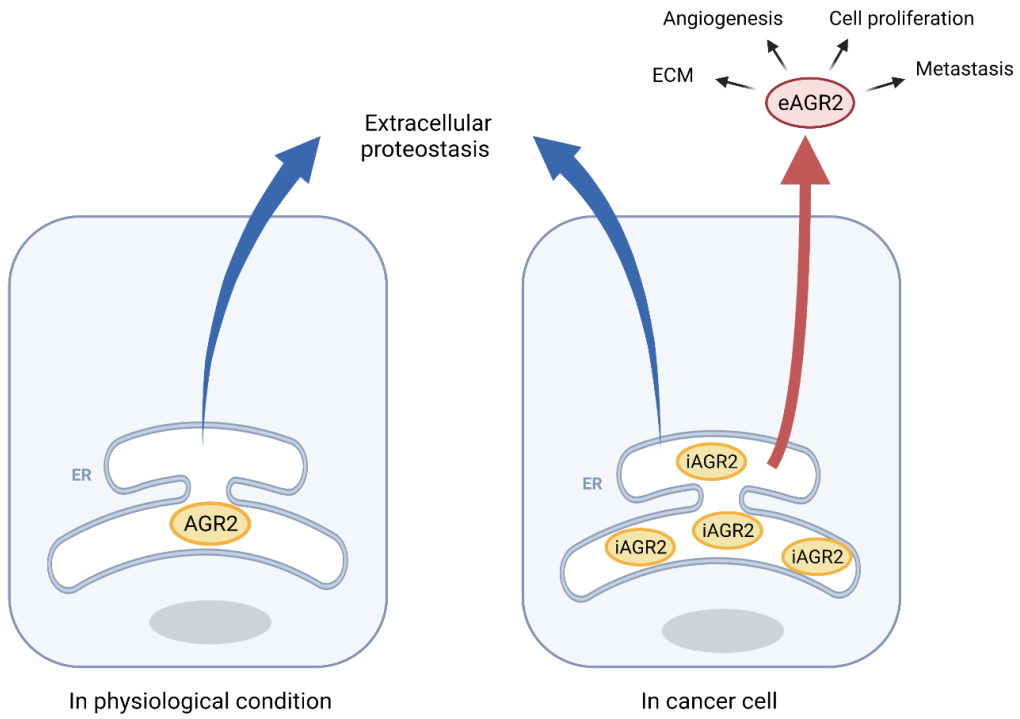

Figure 2 – The dual role of AGR2 in cancer cell
In physiological conditions AGR2 is exclusively located in the ER (left panel), whereas in cancer cells (right panel) this protein is not only present in the ER (iAGR2) but also secreted (eAGR2) in the tumor microenvironment where it plays a pro-oncogene role.

2) eAGR2: a secreted pro-oncogene factor

eAGR2 has been demonstrated to be involved in various tumor-associated processes such as proliferation, migration, invasion, and metastasis in different cancer (breast, colon, lung…) [4]. Indeed, secretion of eAGR2 in lung cancer models promotes the acquisition of invasive and metastatic features via the disruption of basal laminin as well as cell-cell contact due to the loss of E-cadherin, beta-catenin, and Laminin-V at the cell membrane [3]. Overall, the upregulation of AGR2 reduced cell adhesion and increase metastases number which suggests a potential role of eAGR2 in the regulation of the epithelial-mesenchymal transition (EMT). eAGR2 sustains tumorigenesis via its direct interaction with vascular endothelial growth factor A (VEGFA) which stimulates angiogenesis through the enhancement of vascular endothelial growth factor/ Vascular endothelial growth factor receptor 2 (VEGFR2) [4].

Moreover, colony formation assay demonstrated that eAGR2 overexpression promotes breast cancer cell survival [2]. Investigation of the cellular mechanisms by which eAGR2 promotes tumor survival and development have shown that eAGR2 can act by repressing the tumor

suppressor p21, causing an increase of cells in S phase thus enhancing proliferation [5]. Once secreted in the tumor microenvironment eAGR2 acts on surrounding cells in a paracrine fashion. Indeed, the ARTIST team have demonstrated that addition of eAGR2 in the medium of non-tumorigenic human bronchial epithelial cells stimulates the formation of non-tumorigenic organoids [3].

Overall, eAGR2 can be defined as a pro-oncogene as it takes part in many processes that characterize tumorigenesis.

3) Involvement of AGR2 in the process of senescence

Hu Z *et al.* have demonstrated that strong depletion of AGR2 in prostate cancer cells had a negative impact on cell proliferation as it induces a cell cycle arrest that could lead to cellular senescence [6]. Similarly, the ARTIST team by knocking out AGR2 using the CRISPR/Cas9 system led to the observation of a senescence phenotype (proliferative arrest and induction of SA-β-Galactosidase activity) in lung cancer cells (**Figure 3**).

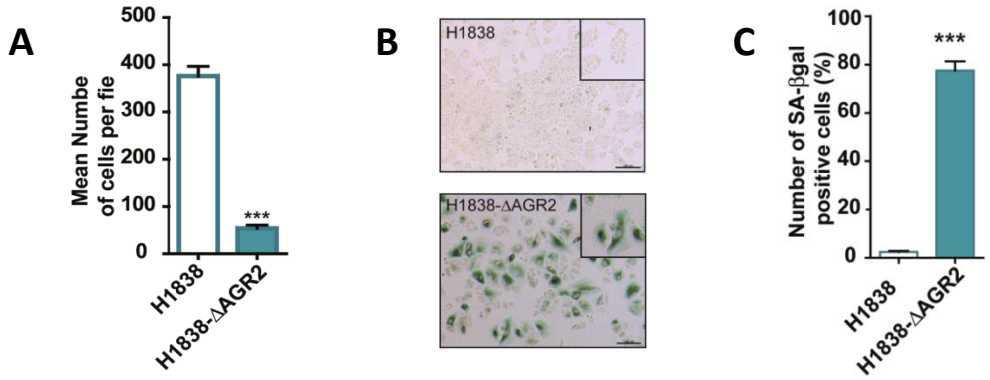

Figure 3 - Preliminary data: CRISPR/Cas9-mediated knockout of AGR2 in lung cancer cells (H1838) induces senescence

(A) Cell growth was assessed and compared in CRISPR/Cas9-mediated AGR2 knockout cells as well as control cells (H1838). The results are representative of 3 independent experiments. Data are mean ± SD. ***p<0.0001, (B) SA-β Galactosidase assay was performed to measure the senescence rate in H1838-ΔAGR2 and control cells (H1838). Scale bars, 100µm, (C) Results obtained following the SA-β-Galactosidase assay were quantified in both cell lines (three independent experiments). Data are mean ± SD. ***p<0.0001. Patent n° WO2018130518

4) Evolution of the concept of senescence over time

Senescence was first described by Hayflick in 1961 as a phenomenon of cellular aging which results in the permanent state of cell cycle arrest [7]. Indeed, he demonstrated that cultured human fibroblast undergoes a finite number of cell divisions, once this limit has been reached

the aged cells escape the pool of cells that contribute to tissues homeostasis and stop dividing irreversibly entering a replicative senescence [8]. It is only in 1990 that a correlation was made between telomere attrition and the Hayflick limit. Indeed, through experimental assays it was shown that in cells that do not express telomerase, the telomeres ends were lost during cell division, and once a certain length was reached it triggered the entry of the cell into senescence [9].

However, over the last decade, the notion of senescence evolved and became relevant beyond the aging context. Senescence has emerged as a critical cellular barrier against abnormal physiological processes in various diseases, especially cancer. Indeed, it was shown that senescence is not only caused by telomere attrition but also triggered by genetic stresses such as DNA damages, pro-oncogenes activation, oxidative stress as well microenvironmental stresses [10]. Thus, in this context senescence is qualified as induced.

Despite the nature of senescence - replicative or induced - it will inescapably result in a stable arrest of the cell cycle between the phase G1 and S. This arrest is mediated through two main pathways, p53/p21CIP1 and p16INK4a/Rb. p53 and p16 are endogenous cyclin-dependent kinase (CDK) which act by repressing the protein cyclin-dependent kinase 4/6 (CDK4/6) resulting in hypophosphorylated Rb [7]. p53/p21CIP1 pathway is activated downstream of the DNA damage response. Indeed, accumulation of DNA damages can occur through: uncoating of telomere end, hyper-replication of oncogene, increase ROS production may elicit the activation of the ATM and ATR signaling pathway which in turn activate *CHK1 or CHK2*, respectively, resulting in the sustained activation of p53 via its phosphorylation at the level of its N-terminal domain [7]. Whereas, p16 is encoded by the INK4A–ARF locus which is epigenetically silenced by the Polycomb repressive complexes (PRCs) in normally growing cells but is activated in response to cellular stresses which triggers the expression of both $p16^{INK4A}$ and $p19^{ARF}$ [11] (**Figure 4**).

Overall, cells harboring oncogenic mutations are able to detect these potential threats for their integrity by activating the INKa/ARK gene and sustaining p53 activity which triggers their entry into senescence allowing them to avoid their further transformation into a malignant cell. However, by definition premalignant cells may progress into cancer thus there must be strategies to resist or overcome this critical anti-proliferative barrier which is senescence. This cellular process is now widely recognized as a tumor-suppressor response that acts to inhibit the development of pre-tumorigenic cells due to its suppression effect on proliferation [12].

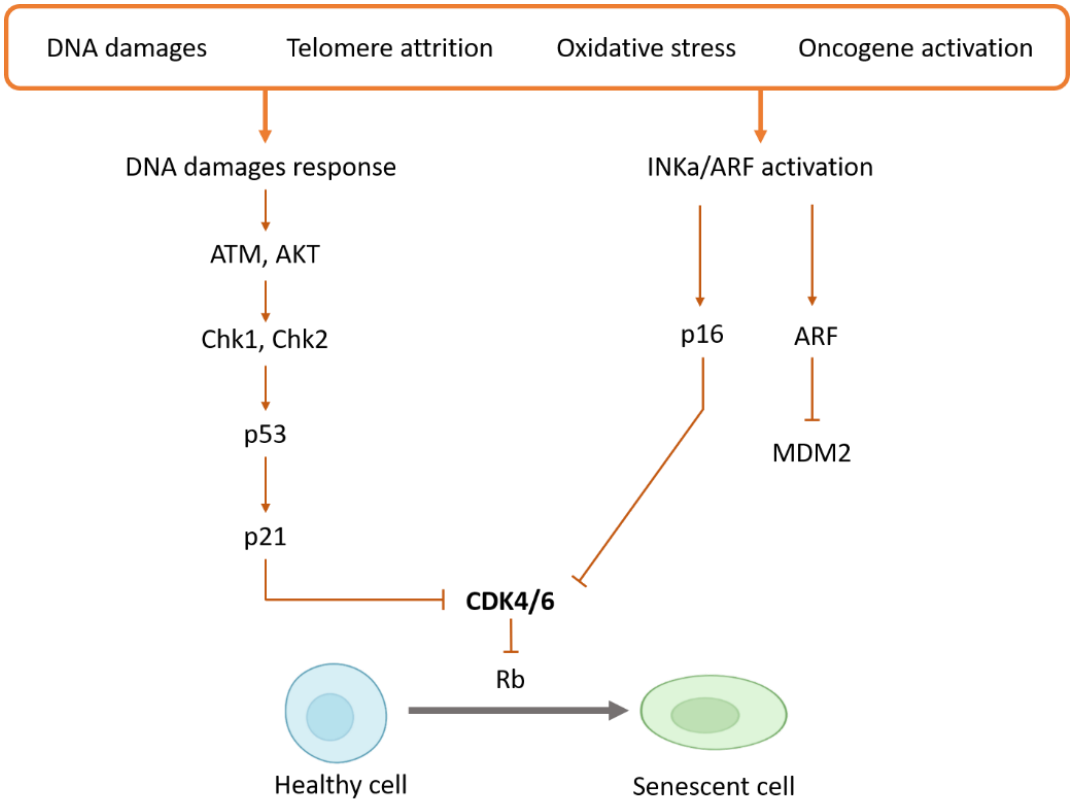

Figure 4 – **Pathways regulating senescence**
Different types of stimuli can provoke cellular senescence such as DNA damages, Telomeres attrition, oxidative stress, or oncogene activation. It results in the activation of 2 main pathways: p16INK4a/Rb and p53/p21CIP1, both of which converge on the repression of CDK4/6. The INK4A/ ARF locus is normally silenced by the PRCs but becomes activated during senescence whereas p53/p21CIP1 is activated downstream of the DNA damages response (DDR) triggered by the accumulation of DNA damages.

II) PROBLEMATIC

Breast cancer remains the leading cause of cancer-related death among women worldwide. While strategies targeting the primary tumor at early stages have improved significantly, preventing or treating maligned breast cancer and metastasis have been less successful. Indeed, spread of the tumor is one of the underlying causes of death for many patients suffering from breast cancer. Thus, the search of new anticancer drugs remains in the heart of concern. Studies are currently focusing on the induction of senescence in cancer cells as a potent therapeutic strategy. Indeed, researches have shown that cancer cells retain the ability to undergo senescence in response to treatment with various therapeutic agents [13], [14]. Therefore, a

better understanding of the proteins and mechanisms that prevent senescence might allow the development of new approaches to target tumorigenesis.

Our project will tend to bring light on the role of AGR2 in the senescence process in breast cancer cell. Indeed, based on the three following finding:

1/ AGR2 is overexpressed and secreted (eAGR2) in the tumor microenvironment of cancer cells,

2/ KO of AGR2 in lung cancer cells leads to a senescence phenotype,

3/ eAGR2 is involved in various pro-tumoral mechanisms (angiogenesis, proliferation, metastasis),

thus, we hypothesize that overexpression of AGR2 could allow breast cancer cells to escape senescence via its secretion (eAGR2) in the tumor microenvironment.

III) MAIN OBJECTIVES

In order to answer our hypothesis, our research project will be divided into three aims. First, we will tend to demonstrate the link between AGR2 and senescence in breast cancer cells by knocking out AGR2 in MCF7 using the CRISPR-Cas9 system and observe if it results in a senescent phenotype. While our second aim, we will investigate if therapy-induce- senescence (TIS) has an impact on AGR2 expression and localization. Finally, we will demonstrate that breast cancer cells overexpressing AGR2 overcome the senescence barrier thought its secretion in the tumor environment. To do so, we will assess if the addition of eAGR2 in the culture medium rescues cells from TIS.

MATERIAL AND METHODS

Cell culture

MCF-7 human breast cancer cell lines were obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), and 1% streptomycin/penicillin (Pen/Strep) at 37°C in a humidified atmosphere with 5% CO_2.

Design of pX462-AGR2-sgRNA plasmid

The pX462 plasmid (Addgene plasmid # 62987 - Feng Zhang Lab) was digested with BbsI and purified by spin column using the PCR purification Kit (Qiagen). A pair of oligos for each targeting site were annealed and ligated into a linearized pX462 vector to generated the pX462-AGR2-sgRNA plasmid.

Plasmidic DNA amplification and purification

pX462-AGR2-sgRNA or peGFP-N3 plasmids were transformed into competent bacteria (DH5-α) via heat shock (42 secs at 42°C). Bacteria were then plated on an LB agar dish containing ampicillin (20µg/mL) and incubated overnight at 37°C. One colony was picked up and put in LB Broth medium containing ampicillin (20µg/mL) and incubated overnight at 37°C under agitation. DNA purification was performed using the maxiprep kit from Zymo Research. The concentration of plasmid DNA were determined by spectrophotometry.

Lipid-mediated cell transfection

One day prior to transfection, MCF-7 were seeded in a 6-well plate at a cell density of 4×10^5 cells per well in DMEM with 10% FBS, 1% Pen/Strep and incubated overnight at 37°C in 5% CO_2. The expression of eGFP reporter gene encoded by the peGFP-N3 plasmid allowed us to identify the optimal DNA/ Lipofectamine 3000 to be used for transfection. Fluorescent detection was performed using the DMi8 inverted microscope (Leica). Different DNA/lipofectamine ratios were tested, the best ratio was then used to transfect pX462-AGR2-sgRNA in MCF-7. The protocol was followed as specified by the manufacturer. Plasmid DNA was mixed with OPTI-MEM and P3000 reagent whereas Lipofectamine 3000 reagent was diluted in OPTI-MEM. The two solutions were then gently mixed together and incubated at room temperature for 15 minutes, allowing DNA/lipid complexes to form. The transfection mixture was then added to the cultured cells. After 16h, the transfected medium was removed and replaced with fresh culture medium for 72h with medium change every 48h.

Lysis and protein dosage

MCF-7 were harvested and lysed in RIPA lysis buffer (Tris HCl pH 7.4 50mM, NaCl 150mM, $CaCl_2$ 1mM, NP40 1%, Deoxycholate 0.1%, 100X protease inhibitor mix). Then the samples were placed on a stirring wheel for 30 min. The total protein fraction was recovered after centrifugation at high speed (15 000 rpm, 4°C, 30 mins). The total protein crude extract obtained from cell lysis was determined according to the Bradford Assay against a standard range of BSA (from 1 up to 20mg/mL), and the absorbance was read at 595nm.

Western blotting (WB)

Lysates were resuspended in laemmli 5X (Tris HCl pH 6.8 250 mM, 50% Glycerol, 2% SDS, 0.01% Bromophenol blue) and loaded in a 12% polyacrylamide gel. Migration was performed at 200V for 1 hour in migration buffer (Tris base 25mM, Glycine 2.5M, SDS 20%). SDS-PAGE gel was transferred to Polyvinylidene Difluoride membrane (PVDF) and incubated for 1h at

100V. After incubation, the PVDF membrane was washed 3 times (PBS 1%, 0.05% Tween20) and placed in a blocking solution (PBS 1X, 0.05% Tween20, 10% Milk) for 1 hour. After 3 washes, transfer membrane was incubated with the HRP-conjugated secondary antibodies for 1 hour at room temperature. The signals were detected using an enhanced chemiluminescence detection kit (Millipore, #P90720). The sources of the primary antibodies used on this study are as follow: anti-AGR2 (17kDa) (Abnova, #H00010551-M03), anti-GAPDH (36kDa) (Merk, #MAB-374), anti-Transferrin (71kDa) (Ozyme, #13113S), anti-Calnexin (90kDa) (Cell signaling, #2679). The secondary antibodies used were as follows: anti-Mouse (Cytiva, #NA031), anti-Rabbit (VWR, # NA934-1ML).

Senescence associated β-Galactosidase staining

MCF7 cell were plated in a 12 well plate at a density of 40 000 cells per well. After treatment with 100nM of doxorubicin, β-Galactosidase activity was detected using the Senescence β-Galactosidase Staining Kit (Cell signaling Technology, #9860). Briefly, culture medium was removed and cell were washed once with PBS 1X then place in fixative solution at room temperature for 15min. Cells were rinsed twice in 1X PBS before being stained with Galactosidase Staining Solution for 20 hours at 37°C in a dry incubator with no CO_2. The presence of a blue precipitate, which indicates a positive result for β-Galactosidase staining, was detected using a light microscope.

Cell proliferation

Cells were seeded into 96-well plate at a density of 7500 cells per well using the Perkin Elmer Janus Mini liquid handling platform and incubated overnight. The following day fresh medium containing doxorubicin (100nM) was added. Subsequently, Hoechst solution (8µg/mL) was added overtime to each well and incubated for 1h at 37°C. High-content images were acquired with the Cytation 3 automated microscope (Biotek) at 4× magnification (Threshold: 3000, Min object size: 10µm, Max object size: 100µm), and analysis was performed using the Analysis software (BioTek Gen5).

Collection of culture media

MCF7 cell were plated in a 6 well plate at a density of 200 000 cells per well and treated for 96h with 100nM of doxorubicin. One-day prior medium collection, cells were incubated for 24h with DMEM without FBS. Medium was collected and centrifuged at 12 000 rpm for 5min. Protein precipitation was performed as follow, 400mL of culture medium were incubated of 1h at -20°C with 1.6 mL of cold-acetone. Subsequently, samples were centrifuged for 10min at

15 000g and 4°C. These steps were repeated until culture medium has been all precipitated. The pellet was air-dried and resuspend in 30µL of laemmli 5X for further analysis by western blot.

Production and purification of recombinant AGR2 protein

pET-60-DEST containing the recombinant GST fusion AGR2 proteins was transformed into competent bacteria (BL21) via heat shock (42 secs at 42°C). Bacteria were then plated on an LB agar dish containing ampicillin (20µg/mL) and incubated overnight at 37°C. One colony was picked up and put in LB Broth medium containing ampicillin (20µg/mL) and incubated overnight at 37°C under agitation. The following day, the pre-culture was placed in a larger flask and the DO was measure at 600nm every 30min until it reaches 0.8. Once the exponential phase reached, IPTG (100nM) was induced. After 2h of incubation at 37°C under agitation, bacterial culture was centrifuged at 3500g, 20min, 4°C. Subsequently, the pellet was purified as follow: cells were lysed in buffer containing Tris pH 7.5 50mM, NaCl 15mM, and 1X protease inhibitor mix (#4693124001 Roche), then AGR2-GST fusion protein were incubated for 2h at 4°C with Glutathione Sepharose 4B. Beads were then centrifuged (500g, 4°C, 5min) and submitted to several rounds of wash (Tris ph7.5 50mM, NaCl 15mM, NP40 0.05%, protease inhibitor mix 1X). Then, Glutathione Sepharose 4B beads coupled with AGR2-GST were digested with 5µL of thrombin for 20h at 22°C. The supernatant was collected and incubated with Thrombin Sepharose Beads for 30 min at room temperature. The purity and appropriate size of AGR2 protein was analyzed by Coomassie blue staining of 12% SDS-PAGE gel.

Stimulation with eAGR2

Cells were seeded into 12 well plate at a density of 40 000 cells per well. 24h after seeding, cells were treated with 100nM of doxorubicin with or without eAGR2 at 5ng/mL and 10ng/mL as used in the lab [3] [15]. The medium was challenged with eAGR2 every 24h. After 48h, cells were stained for SA-β-Galactosidase activity.

Statistics

Statistical analyses were performed using GraphPad Prism software. All results are presented as means ± SD, additionally two-tailed unpaired t-test were used to determine statistical significance between 2 experimental groups. $p<0.05$ was considered statistically significant.

RESULTS

Optimization of MCF7 transfection with Lipofectamine 3000

In order to optimize the protocol to transfect MCF7, a plasmid containing a GFP tag (peGFP-N3) was used to determine the most efficient DNA: Lipofectamine 3000 ratio to achieve optimal transfection. Several ratios were tested and their efficacy was measured via fluorescence detection of the GFP tag and compared to the total amount of cells using DAPI staining. The 1:4 ratio showed the highest amount of transfection (around 80%) and a low cell death rate (less than 10%) as depicted in **Figure 5**. Thus, this ratio was selected for our further experiments.

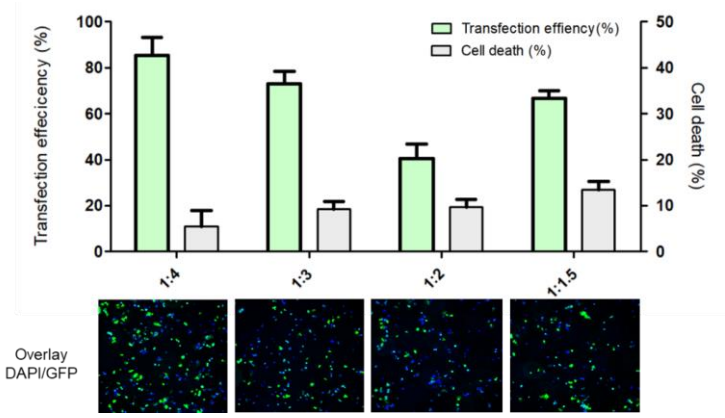

Figure 5 – Optimization of MCF7 transfection with lipofectamine 3000

Different ratio of peGFP-N3/Lipofectamine 3000 were tested to optimize transfection in MCF7 cell line. Transfection efficiency was determined as the percentage of the number of fluorescent cell (GFP) over the total number of cell (DAPI staining), and compared to cell death.

CRISPR/Cas9-mediated knockout of AGR2 in MCF7

To investigate the relationship between AGR2 and senescence in breast cancer cells, we based ourselves on previous results obtained by the ARTIST team where they demonstrated that KO of AGR2 in lung cancer cells led to a senescence process. A similar approach was used in the breast cancer cell line: MCF7. To do so, sgRNA sequences targeting exon 1 of AGR2 were previously designed using the CRISPOR software (**Figure 6.A**) and cloned in the pX462 plasmid which contains a sequencing coding for the Cas9 (**Figure 6.B**). Results of the sequencing showed that the sgRNA cloning was successful (**Figure 6.C**). Therefore, pX462-AGR2-sgRNA was transfected in MCF using the ratio (1:4) decipher in our preliminary experiment. We first tested if increasing the amount of DNA transfected would have an impact on the transfection efficiency, western blot analysis revealed that transfection with 0.5µg of DNA and 2µg of Lipofectamine 3000 decreased moderately AGR2 expression compared to the

other ratio tested (**Figure 6.D**). Using the same ratio (0.5:2) MCF7 were transfected with the empty plasmid and the pX462-AGR2-sgRNA, western blot analysis showed that AGR2 expression was slightly decreased when transfected with the plasmid containing the sgRNA targeting AGR2 (**Figure 6.E**). In addition, to investigate if this slight decrease in AGR2 expression had an impact on senescence we performed a SA-β-Galactosidase assay, a marker for senescent cells. We found that the proportion of SA-β-Galactosidase positive cells, when transfected with pX462-AGR2-sgRNA, was similar to the control (cell transfected with lipid vector) (**Figure 6.F**). Together, these results indicate that KO of AGR2 was unsuccessful as it only resulted in a slight decrease of AGR2 expression and no entry of the cells in senescence was observed.

It led us to conclude that more optimization of the efficiency of the CRIPSR-Cas9 system is required to obtain a total knock out of AGR2.

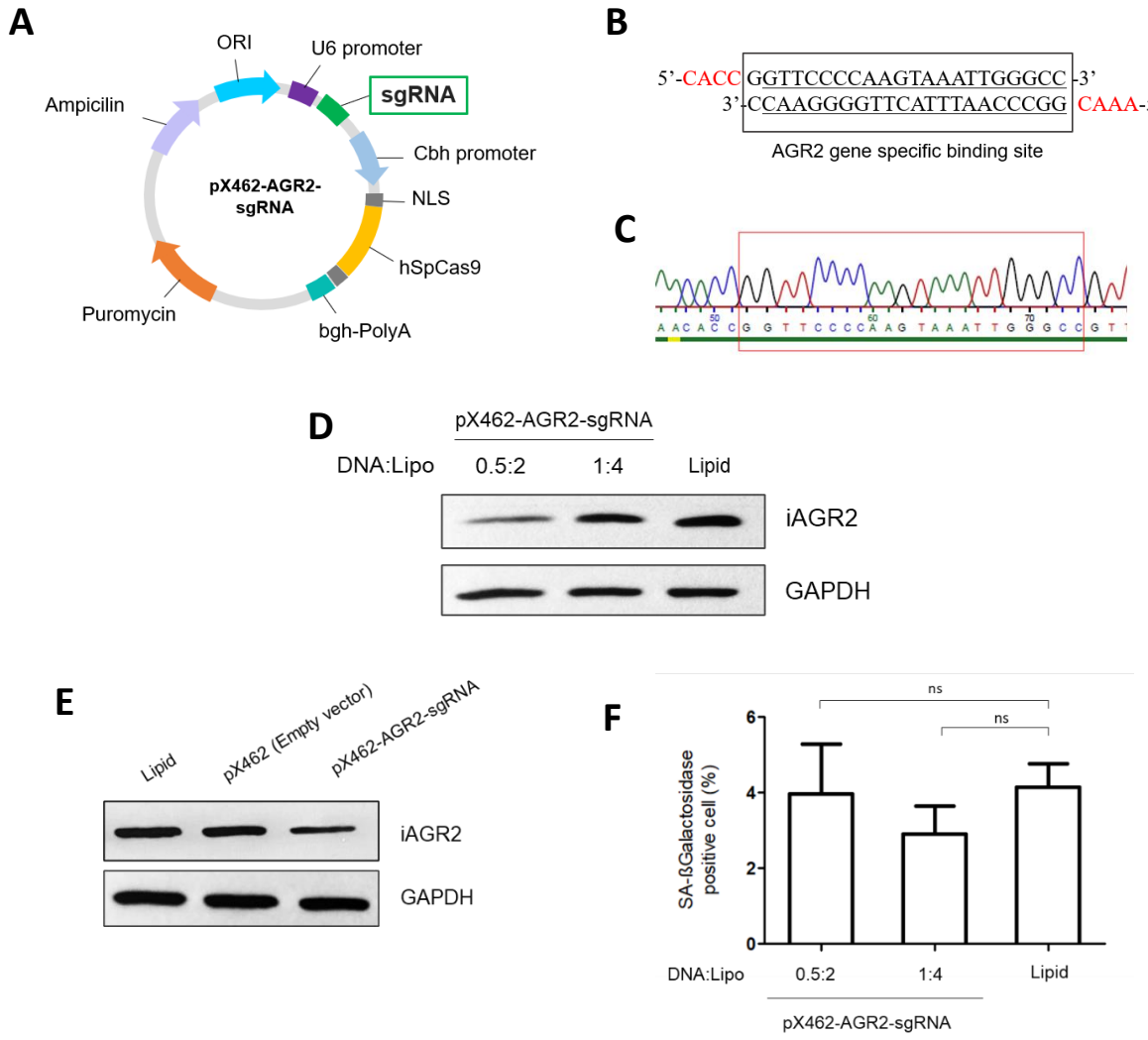

Figure 6 – CRISPR/Cas9-mediated knockout of AGR2 in MCF7 decreases slightly AGR2 expression but did not induce a senescence phenotype

A - Map of the pX462-AGR2-sgRNA plasmid.
B - Doubled stranded sequences targeting exon 1 of AGR2 with Bsb I cohesive ends for its integration in the pX462 plasmid.
C - Gene sequencing of the pX462-AGR2-sgRNA recombinant plasmid.
D - Representative Western blot for iAGR2 and GAPDH protein expression in control cell (MCF7 transfected with lipid vector), in MCF7 transfected with the empty vector (pX462) and after CRISPR/Cas9-mediated AGR2 knockout.
E - iAGR2 and GAPDH protein expression was assessed by Western blot in control cell (MCF7 transfected with lipid only), and compared to MCF7 transfected with either 0.5µg/µL of DNA and 2µL of lipofectamine 3000 or with 1µg/µL of DNA and 4µL of lipofectamine 3000.
F - Quantitation of SA-β-Galactosidase positive cells in control cells (MCF7 transfected with lipid vector) and after CRISPR/Cas9-mediated AGR2 knockout AGR2. The results are representative of 3 independent experiments. Data are mean ± SD, ns: not significant.

Doxorubicin-induced MCF7 senescence decreases AGR2 expression

In this second approach, we took the problem the other way around by investigating if the induction of senescence using a chemotherapeutic agent would have an impact on AGR2 expression and localization. Studies have demonstrated that doxorubicin a commonly used chemotherapeutic drug can not only induce apoptosis but also promotes the senescence of cancer cells [13]. Additionally, it has been reported that cancer cell senescence can be observed after treatment with 100nM of doxorubicin.

Basing ourselves on these results, we first assessed the effect of doxorubicin (100nM) on MCF7 proliferation over a time course of 96h. As shown in **Figure 7.A**, cells treated with doxorubicin entered a proliferative arrest whereas the non-treated group kept proliferating.

We subsequently performed a SA-β-Galactosidase staining to detect the presence of senescence cells **(Figure 7.B & C)**. This experiment revealed that treatment with 100nM of doxorubicin increased significantly β-Galactosidase activity over time. At 96h more than 50% of the cell population exerts an increased β-Galactosidase activity while the control remained around 4% which correspond to the basal senescence (**Figure 7.C**).

Following these observations, we investigated whether Doxorubicin-induced senescence had an impact on AGR2 expression and localization. To do so, MCF7 were treated with 100nM of doxorubicin for 96h, subsequently cell lysates were analyzed for intracellular level of iAGR2 whereas culture mediums were analyzed for extracellular level of eAGR2.

As seen in **Figure 7.D**, the addition of doxorubicin led to a sharp decline of iAGR2 by a 3-fold-decrease (**Figure 7.E**), which was correlated with a 4-fold-decrease of eAGR2 (**Figure 7.F**). Both iAGR2 and eAGR2 expression were decreased after 96h of treatment with 100nM of doxorubicin and the localization of AGR2 is decreased both in the intra and extracellular.

Taken together, these data demonstrate that doxorubicin-induce-senescence results in proliferation arrest, which was correlated with the entry of cells in senescence and a decrease

of endogenous iAGR2 and secreted eAGR2, and thus both in the intra- and extracellular localizations.

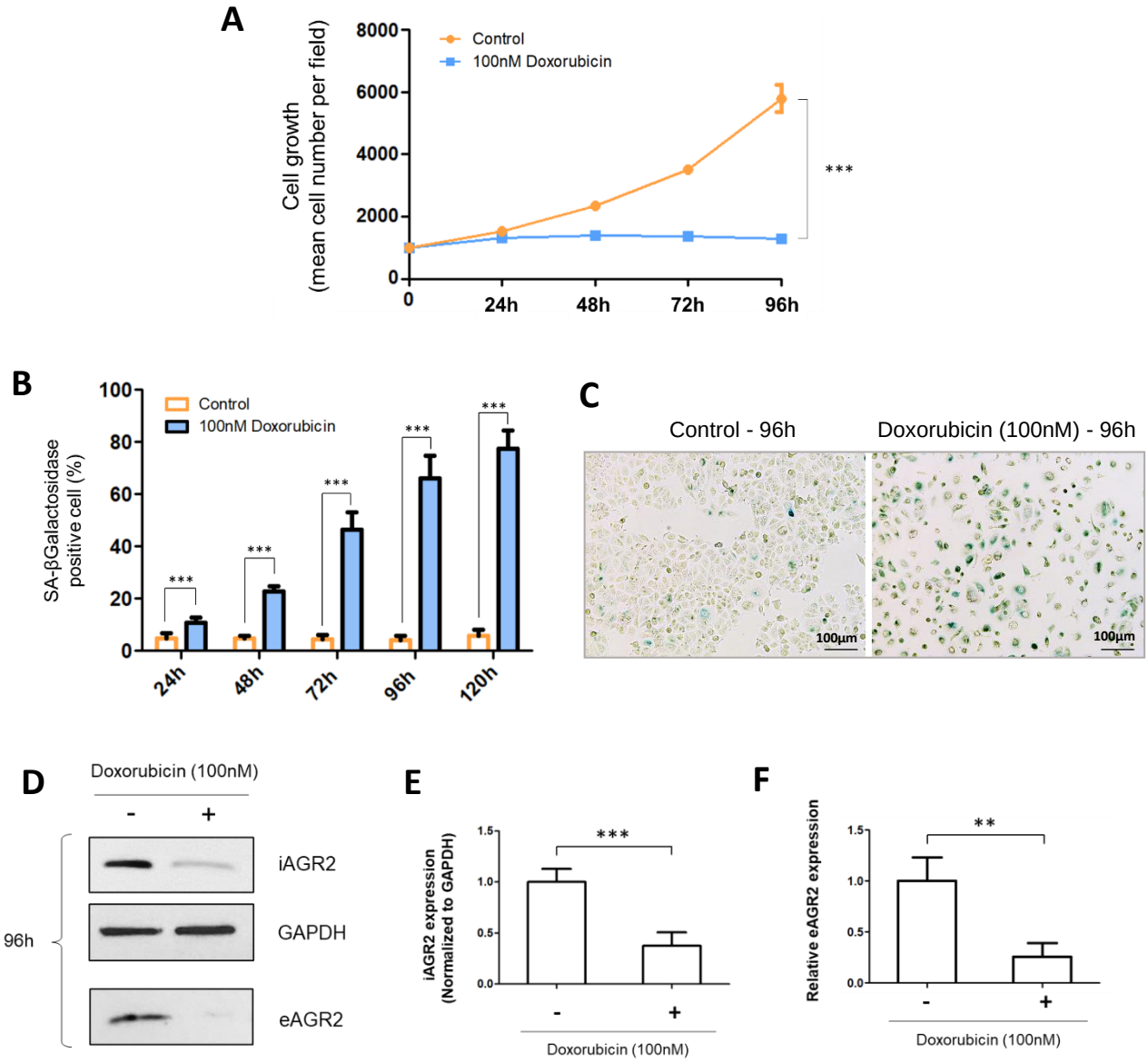

Figure 7 – Addition of doxorubicin on MCF7 cells induces senescence & decreases AGR2 expression and secretion

A - Assessment of cell growth at different time point using Hoechst dye in control cell (MCF7) as compared to MCF7 treated with 100nM of doxorubicin. The results are representative of three independent experiments. Data are mean ± SD. *** = $p < 0.001$.

B - Quantification of SA-β-Galactosidase positive cells in control cells (MCF7) as compared to MCF7 treated with 100nM of doxorubicin. Results are representative of three independent experiments. Data are mean ± SD. *** = $p < 0.001$.

C - Microscopic observation of SA-β-Galactosidase activity in control cell (MCF7) after 96h of incubation as compared to MCF7 treated over 96h with 100nM of doxorubicin. Scale bars, 100 µm.

D - Representative Western blot for iAGR2, eAGR2 and GAPDH protein expression in control cell (MCF7) after 96h of incubation as compared to MCF7 treated over 96h with 100nM of doxorubicin.

E - Quantification of the relative signal intensity of iAGR2 normalized to GAPDH levels from MCF7 treated over 96h with or without 100nM of doxorubicin. Data are mean ± SD. *** = $p < 0.001$.
F - Quantification of the relative signal intensity of eAGR2 level from MCF7 treated over 96h with or without 100nM of doxorubicin. Data are mean ± SD. ** = $p < 0.01$.

Extracellular AGR2 rescues breast cancer cell from Therapy-induced senescence

Given that there is strong correlation between AGR2 downregulation and entry in senescence, we hypothesized that AGR2 when overexpressed and thus secreted in the tumor microenvironment (eAGR2) might allow the tumoral cells to overcome senescence. To investigate if eAGR2 play a role in senescence, we assessed whether the addition of eAGR2 in the culture medium of Doxorubicin-induced MCF7 senescence would have any impact on the β-Galactosidase activity.

As shown in **Figure 8.A & B**, addition of recombinant human AGR2 (5ng/mL or 10ng/mL) to the medium of Doxorubicin-induced MCF7 senescence reversed the effect of doxorubicin by decreasing significantly the number of SA-β-Galactosidase positive cells.

These results indicate that AGR2 plays a role in the senescence pathway as its secretion allow breast cancer cell to escape form TIS.

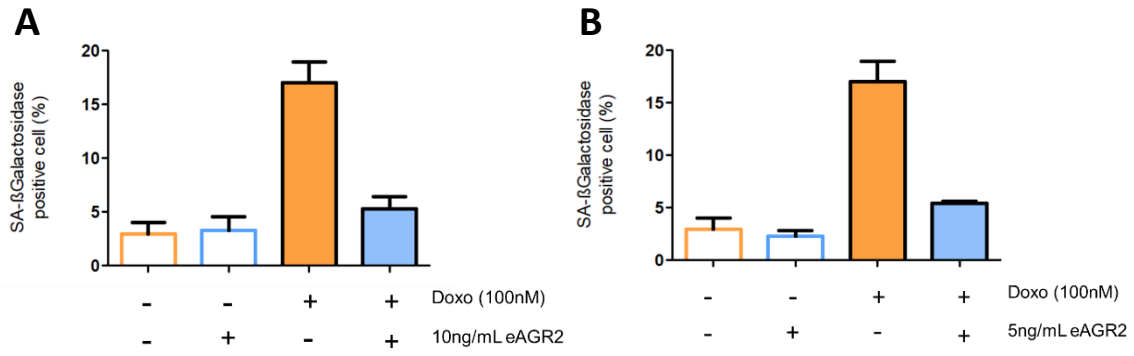

Figure 8 – Addition of eAGR2 decrease doxorubicin-induced MCF7 senescence

Quantification of SA-β-Galactosidase positive cells after 48h of treatment with + or – doxorubicin (100nM) and in + or – eAGR2 at a dose 10ng/mL(A) or 5ng/mL (B). Results (A) are representative of two independent experiments. Results (B) are representative of only one experiment.

DISCUSSION

Anterior gradient-2 (AGR2) protein has been reported to be overexpressed and associated with an unfavorable prognosis in breast cancer. In addition, in cancer AGR2 is not only overexpressed in the Endoplasmic Reticulum (iAGR2) but also secreted in the tumor microenvironment (eAGR2) where it acts as an extracellular regulator in several tumor-associated processes such as proliferation, migration, invasion and metastasis. Studies carried out by the ARTIST team have shed the light on a new potential tumorigenic role of AGR2 in senescence (**Figure 3**), as a key tumor-suppressor mechanism that acts to inhibit development of pre-tumorigenic cells thought proliferative arrest. Thus, in the current study, we proposed to investigate the role that AGR2 could play in the senescence process in breast cancer cells.

We first investigated the relationship between AGR2 expression and senescence in MCF7 cell line by performing a KO of AGR2 using the CRISPR/Cas9 system. Sequencing analysis of the pX462-AGR2-sgRNA revealed that it contained the sgRNA targeting AGR2 however its transfection did not result in a senescence phenotype. Thus, this approach did not allow us to decipher if there is any correlation between AGR2 expression and senescence. Further optimization of the CRISPR-Cas9 system is required to obtain total KO of AGR2. Several parameters could be optimized such as the addition of a GFP tag in the pX462-AGR2-sgRNA which would allow to sort by FACS cells that have integrated the plasmid. In addition, our guided RNA sequence targeting AGR2 might not be specific enough, thus new sgRNA sequences could be design to increase KO efficiency. Optimization of the transfection condition could also be done to increase the transfection efficiency by playing on several parameters such as the cell density at the time of lipid-DNA complex addition, the incubation time, or the ratio transfection reagent to DNA.

Secondly, to rule out the relationship between expression and localization of AGR2 and senescence, we induced senescence, using a chemotherapeutic agent (doxorubicin) and measure if it had an impact on AGR2 expression and localization. Herein, we observed that treatment with 100nM of doxorubicin led to a proliferative arrest, senescence entry and decreased the expression of endogenous AGR2 localizations (intra- and extracellular). These findings indicated that there is a correlation between a decrease AGR2 expression (both i- & eAGR2) and the process of senescence. These results support the one obtained by the ARTIST team showing that KO of AGR2 led to a senescent phenotype.

In cancer, overexpression of AGR2 is correlated with the secretion of eAGR2, which had been demonstrated to play a role in cancer cell proliferation and cell cycle. Thus, we do the hypothesis that eAGR2 could be involved in the bypass of senescence, and play a role as an anti-senescence factor. To investigate this potential role played by eAGR2 we cultured MC7, in presence of doxorubicin (TIS), in a medium enriched in eAGR2. We show that addition of eAGR2 decreased significantly entry of the cell in senescence, thus reverting the effect of TIS. Zhang Ying *et al.* have shown that up-regulation of AGR2 decreased doxorubicin-sensitivity whereas its down regulation increased doxorubicin-sensitivity [16]. Thus, secretion of eAGR2 can mediate TIS resistance by blocking the entry of breast tumor cell in senescence. Therefore, blocking eAGR2 secreted thought the use of antibodies might counteract TIS resistance in breast cancer cell, particularly in those with high level of eAGR2 secreted.

CONCLUSION

The present work demonstrates that secretion of eAGR2 allows breast cancer cell to escape to TIS. Overall, our results led us to propose that eAGR2 could mediate resistance to TIS enabling cancer cell to escape senescence. The proliferative ability of such cells was not investigated in this project however Fessart *et al.* have shown that addition of eAGR2 on Human normal bronchial epithelial cells led to a significant increase of their proliferative activity (3). These results strongly suggest that tumor cells escape senescence by re-gaining their proliferative activity allowing them subsequently to promote tumorigenesis. In the light of these results, it could be interesting to investigate further the cellular mechanisms by which eAGR2 impede the senescence pathway to development new approaches to target tumorigenesis.

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ABSTRACT

Breast cancer remains the leading cause of cancer-related death among women worldwide. An endoplasmic reticulum resident's protein AGR2 (anterior gradient 2) which belong to the protein disulfide isomerase (PDI), has been shown to be overexpressed in breast cancer and involved in various tumor associated processes such as tumor growth, invasion and metastasis. In addition to be overexpressed, our team has demonstrated that AGR2 is also secreted the tumor microenvironment (extracellular AGR2 (eAGR2)) where it plays a role of pro-oncogenic factor. Recently, a new role of AGR2 has been unveiled by the team has they shown that KO of AGR2 in lung cancer cell led to a senescent phenotype. Therefore, the aim of this project was to investigate the role of AGR2 in the process of senescence in breast cancer cell. Herein, we show that therapy-induced-senescence (TIS) led a proliferative arrest, senescence entry and decrease expression of AGR2 both intra- and extracellular localizations. We demonstrated, also, that addition of eAGR2 in the culture medium of MCF7 in presence of doxorubicin (TIS) reversed the effect of TIS by decreasing significantly the entry of the cell in senescent. In conclusion, our findings indicate that AGR2 can contribute to TIS resistance in breast cancer cell, by escaping senescence, thought its secretion in the tumor microenvironment.

Keywords: AGR2, Breast cancer cell, therapy-induced-senescence, Drug resistance.