Knockdown of Antisense Noncoding Mitochondrial RNA Reduces Tumorigenicity of Patient-Derived Clear Cell Renal Carcinoma Cells in an Orthotopic Xenograft Mouse Model

Mariela Araya , Francisca Guevara , Jaime Villegas , Luis Alarcón , Luis O. Burzio , Verónica A. Burzio * , Vincenzo Borgna *

Posted Date: 3 January 2024

doi: 10.20944/preprints202401.0225.v1

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Article

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Mariela Araya [1], Francisca Guevara [1], Jaime Villegas [2], Luis Alarcón [3], Luis O. Burzio [4], Verónica A. Burzio [4,5,*] and Vincenzo Borgna [1,6,7,*]

1 Centro Cientifico & Tecnologico de Excelencia Ciencia & Vida, Santiago 8580702, Chile; mariela.araya.ortega@gmail.com (M.A.); franciisca.g@gmail.com (F.G.)
2 School of Veterinary Medicine, Faculty of Life Sciences, Universidad Andrés Bello, Santiago 8370251, Chile; jaime.villegas@unab.cl
3 Urology Service, Hospital Barros Luco-Trudeau, Santiago, Chile; luisalarconmd@gmail.com
4 Department of Biological Sciences, Faculty of Life Sciences, Universidad Andrés Bello, Santiago 8370251, Chile; lburzio@gmail.com (L.O.B.)
5 Institute of Biomedical Sciences, Faculty of Medicine, Universidad Andrés Bello, Santiago 8370134, Chile; vburzio@unab.cl
6 School of Medicine, Faculty of Medical Sciences, Universidad de Santiago de Chile, Santiago, Chile; vborgna@cienciavida.org
7 Faculty of Medicine and Science, Universidad San Sebastián, Santiago, Chile; vborgna@cienciavida.org
* Correspondence: vburzio@unab.cl (V.A.B.); vborgna@cienciavida.org (V.B.)

Simple Summary: The most common form of kidney cancer is clear cell renal cell carcinoma, but its treatment is challenging due to acquisition of resistance to existing therapies. We propose an alternative potential treatment based on targeting a non-coding RNA of mitochondrial origin, termed antisense non-coding mitochondrial RNA. Supporting this strategy is the data we present in this work, using cell cultures obtained directly from primary and metastatic patient tumors, showing that the treatment causes a stall in cell proliferation both *in vitro* and in *in vivo* mouse models, and significantly improves survival of animals. The results from this study strongly support the use of this strategy for the development of an alternative therapeutic approach for ccRCC patients and potentially other types of renal cancer.

Abstract: Clear cell renal cell carcinoma (ccRCC) is the most prevalent form of renal cancer and its treatment is hindered by resistance to targeted and immunotherapies and combinations ob both. We have reported that knockdown of the antisense noncoding mitochondrial RNAs (ASncmtRNAs) with chemically-modified antisense oligonucleotides induces proliferative arrest and apoptotic death in tumor cells from many human and mouse cancer types. These studies have been mostly performed *in vitro* and *in vivo* on commercially-available cancer cell lines and have shown that, in mouse models, tumor growth is stunted by the treatment. The present work was performed on cells derived from primary and metastatic ccRCC tumors. We established primary cultures fom primary and metastatic ccRCC tumors, which were subjected to knockdown of ASncmtRNAs *in vitro* and *in vivo* in an orthotopic xenograft model in NOD/SCID mice. We found that these primary ccRCC cells are affected in the same way as tumor cell lines and, in the orthotopic model, tumor growth was significantly reduced by the treatment. This study on patient-derived ccRCC tumor cells represents a model closer to actual patient ccRCC tumors and shows that knockdown of ASncmtRNAs poses a potential treatment option for these patients.

Keywords: clear cell renal cell carcinoma; non-coding RNA; cancer therapy; orthotopic mouse renal model; patient-derived primary cultures

 doi:10.20944/preprints202401.0225.v1

1. Introduction

Renal carcinoma (RCC) is a type of cancer located in the renal parenchyma. It is the 12th most prevalent cancer, with over 1.2 million patients worldwide and a mortality of 1.8 per 100.000 patients [1] and represents the second highest mortality of urogenital cancers in the USA [1]. RCC is classified in subtypes [2], of which Clear Cell Renal Carcinoma (ccRCC) is the most prevalent, corresponding to 75% of all renal carcinomas. This subtype is characterized by the microscopic observation of a clear cytoplasm due to the accumulation of glycogen and lipids which dissolve during histological treatment [3]. One of the main problems of RCC in advanced stages is its resistance to chemo- and radiotherapy [4,5]. The development of targeted therapies [6], immunotherapies [6,7] and combined therapies [8] have improved the survival rate of these patients, however, only a small subgroup is capable of responding and the results remain poor, thus the development of new therapeutic alternatives is of the essence.

We described a family of mitochondrial non-coding RNAs (ncmtRNAs) derived from the 16S rRNA gene of mitochondria, comprised of sense [9,10] and antisense (ASncmtRNA) members [10,11]. These transcripts are differentially expressed in human and mouse cells according to proliferative status, where SncmtRNA is expressed in all proliferating cells and ASncmtRNAs, on the other hand, are highly expressed only in normal proliferating cells, whilst tumor cells display downregulation of these transcripts [10–14]. We have shown that knockdown of ASncmtRNAs with a chemically-modified antisense oligonucleotide (ASO-1537) exerts detrimental effects on tumor cells from varying tissues origins, including proliferative arrest, inhibition of metastatic potential and, ultimately, massive apoptotic death [10,12–15], while the same treatment on normal cells is essentially innocuous [10,12–14]. Moreover, *in vivo* syngeneic and xenograft murine models inoculated with several different tumor cell lines have shown a reduction in tumor growth and metastasis upon ASncmtRNA knockdown [10,12,14–16].

A major part of our work has been focused on tumor cell lines, with similar outcomes in each case. In this work, we present the study of ASO-1537 treatment on primary cultures of patient-derived ccRCC cells, both *in vitro* and *in vivo*. We show that these cells are affected in the same manner as tumor cell lines, including an orthotopic xenograft pre-clinical model of patient-derived ccRCC cells.

2. Materials and Methods

2.1. Primary cultures

The study was conducted in accordance with the Declaration of Helsinki and approved by the Scientific Ethics Committee of the "Servicio de Salud Metropolitano Sur (SSMS)" from Santiago, Chile. Four primary and two metastatic ccRCC tumor samples were obtained from patients at the Barros Luco-Trudeau Hospital in Santiago, Chile, under informed consent. Tissues were minced and washed in PBS, followed by enzymatic digestion (1 mg/ml collagenase I, 1 mg/ml collagenase II, 2 mg/ml collagenase IV, 1mg/ml dispase, 20 µg/ml hyaluronidase, 2000 U/ml DNase I and 2 mM $CaCl_2$ in DMEM (Thermo Fisher Scientific, Waltham, MA) at 37ºC for 45 min under constant mixing. Cell suspensions were centrifuged at 1200 rpm for 5 min and the pellet was washed in PBS, followed by another centrifugation step at 200 x *g* for 5 min. Suspensions were then seeded into collagen I-coated T75 culture flasks (Thermo Fisher Scientific) in complete medium: RPMI (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 50 µg/ml hydrocortisone and 1X Insuline-Selenium-Transferrin supplement (Thermo Fisher Scientific) and cultured at 37°C under a 5% CO_2 atmosphere.

2.2. Transfection

Antisense oligonucleotides used in this study contained 100% phosphorothioate internucleosidic bonds (LGC Biosearch Technology Inc., Novato, CA). Cells were seeded into 12-well plates (Thermo Fisher Scientific) at 40,000 cells/well and transfected on the next day with 200 nM ASO-1537 (5′ CACCCACCCAAGAACAGG) or control ASO (ASO-C: 5′

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ATATACGTACACCCTCTAACCTA) and 2 µl/well LipofectAmine2000 (Thermo Fisher Scientific), following manufacturer's recommendations, or left untreated. Cells were then cultured for 24, 48, 72 or 96 h. Cell viability was assessed through the Trypan blue exclusion method.

2.3. Determination of apoptosis

After *in vitro* treatments, apoptosis was detected using the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis kit (Thermo Fisher Scientific), following manufacturer's instructions. Dead cells were stained with propidium iodide (Sigma-Aldrich, St. Louis, MO) and the % of apoptotic cells was measured by flow cytometry on a BD FACS Canto-II instrument (Centro Científico y Tecnológico de Excelencia Ciencia & Vida, Santiago, Chile). Apoptosis was also measured using the Dead-End Fluorometric TUNEL System (Promega, Madison, MI) and results were analyzed under an Olympus BX53 epifluorescence microscope (Centro Científico y Tecnológico de Excelencia Ciencia & Vida).

2.4. Western blot

For determination of changes in protein levels, treated cells were lysed in RIPA buffer supplemented with protease inhibitors (Promega), followed by protein quantification with Bradford reagent (Bio-Rad, Hercules, CA). Total proteins from each sample were separated on a 12% SDS-PAGE gel and transferred to PVDF membranes (Bio-Rad) with a Transblot system (Bio-Rad). Proteins were detected with rabbit primary antibodies direct to surviving (Abcam, Cambridge, UK), PARP1 (Cell Signaling Technology, Danvers, MA), N-cadherin (Thermo Fisher Scientific), cyclin B1 (Abcam), cyclin D1 (BD Pharmigen, Franklin Lakes, NJ) or β-actin (Abcam) and secondary peroxidase-labeled anti-rabbit IgG. Blots were revealed with the EZ-ECL chemoluminiscent substrate (Beit-Haemek, Israel) and detected in a C-Digit Blot Scanner (LI-COR Biosciences, Lincoln, NE). Quantification of protein bands was performed with the imageJ software (National Institutes of Health, Bethesda, MD).

2.5. Orthotopic xenograft model

Animal studies were conducted according to the Declaration of Helsinki, following the principle of "3Rs". Protocols were performed following the guidelines of the National Agency for Research and Development (ANID), Chile and approved by the Research and Ethics Committee of the "Centro Científico y Tecnológico de Excelencia Ciencia & Vida", Santiago, Chile. NOD/SCID mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained at the animal facility of the "Centro Científico y Tecnológico de Excelencia Ciencia & Vida" under a 12/12 h light/dark regimen with sterile water and food *ad libitum*. For each experiment, ten 6-8 week-old mice (20-25 g) were inoculated with 2 x 10^6 PT2 or MT1 cells in the subcapsular area of the left kidney. At 8-10 weeks post-cell inoculation (pci), treatments were started by intraperitoneal (ip) injections of 100 µg ASO-1537 or 100 µl saline, spaced 48 h apart. After 28 doses (17 weeks pci for PT2 and 19 weeks pci for MT2), mice were euthanized and kidneys were collected, weighed and fixed in 4% formalin for obtention microscopy sections at the Anatomopathology Unit of the Luis Tisné Hospital (Santiago, Chile). Sections were stained in Hematoxilin/Eosin (H&E) and photographed under a BX53 microscope.

2.6. Graphs and statistical analysis

Data was plotted and analyzed using the Graphpad Prism 6 software. *In vitro* experiments were performed in triplicate with 3 independent biological replicates and analyzed by non-parametric *t*-test. For *in vivo* experiments, comparisons between groups were performed with non-parametric one-sided Mann Whitney test. Survival analysis was performed using the Log-Rank (Mantel-Cox) test. Statistical significance was set at the nominal level of $p<0.05$ or less.

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3. Results

3.1. Primary Cultures of human ccRCC

In order to explore the effects of ASncmtRNA knockdown on patient-derived ccRCC cells, we first established primary cultures from four primary tumors (named as PT1-4) and two metastatic tumors (MT1 and 2), collected at the Urology Service of the Barros Luco-Trudeau Hospital in Santiago, Chile. All six cultures displayed the characteristic clear cytoplasm typical of ccRCC cells (Figure 1).

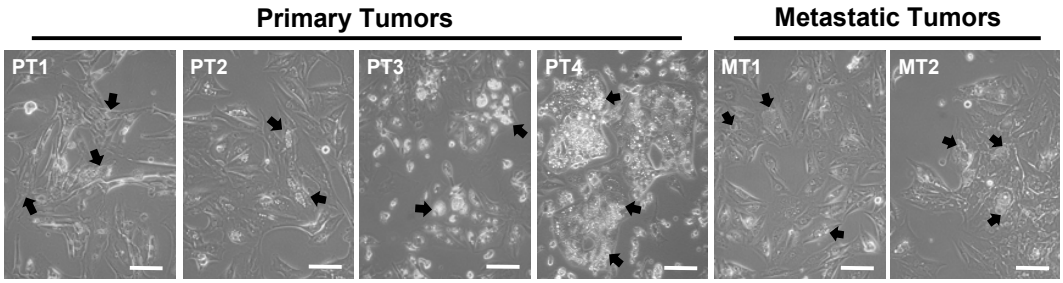

Figure 1. Phase contrast images of primary cultures derived from ccRCC primary tumors (PT1-4) and metastatic tumors (MT1 and 2), showing the characteristic clear cytoplasm of ccRCC cells (arrows). Bars = 100 µm.

3.2. Knockdown of ASncmtRNA induces apoptotic death of primary ccRCC cultures

We studied the effect of transfection of ASO-1537 (vs ASO-C and non-treated cells) on the six primary ccRCC cultures (Figure 2a). We found that the treatment induces between 70 and 80% cell death in all cultures, compared to 8-20% for control cells (Figure 2a), which is in line with our previous results on a wide array of tumor cell lines (REFS). For further studies, we selected one primary tumor culture (PT2) and one metastatic tumor culture (MT1). Transfection of both cultures with ASO-1537 induced a high degree of apoptosis as determined by Annexin V binding assay, compared to basal levels in controls. These levels were comparable to those obtained in cells treated with the pan-kinase inhibitor staurosporin (STP) (Figure 2b). We further demonstrated apoptotic death in the MT1 culture by DNA fragmentation (TUNEL) assay (Figure 2c), reduction in survivin levels and PARP1 cleavage (Figure 2d).

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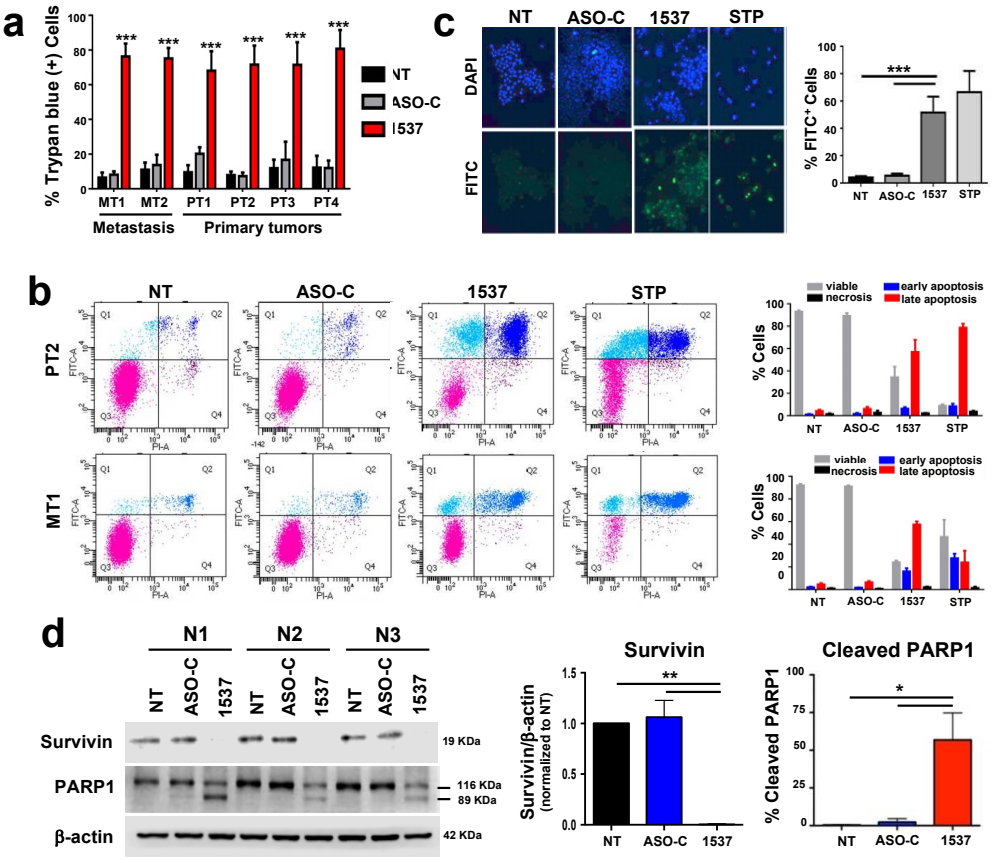

Figure 2. ASncmtRNA knockdown induces apoptotic death of patient-derived ccRCC cells. Cultures were transfected with ASO-1537 or ASO-C, or left untreated (NT), for 48 h. (**a**) Cell death was evaluated in all six primary cultures by Trypan blue exclusion, asterisks indicate significant difference of ASO-1537-treated cells compared to both controls (NT and ASO-C); Apoptosis was determined by Annexin V binding in PT2 and MT1 cells (**b**) and TUNEL assay in MT1 cells (**c**), using staurosporin (STP) as a positive control; (**d**) survivin levels and % of cleaved PARP1 were determined by Western blot. *p<0.05; **p<0.01; ***p<0.001.

3.3. Knockdown of ASncmtRNA reduces proliferative capacity of primary ccRCC cultures

We reported that tumor cell lines from a wide array of human tissues undergo a proliferative block upon knockdown of ASncmtRNAs (REFS). Therefore, we studied whether the patient-derived primary culture of metastatic ccRCC (MT1) would follow the same tendency. As depicted in Figure 3a, MT1 cells transfected with ASO-1537 suffer a sudden blockage of proliferation, similar to STP-treated cells, while control cells continue to proliferate. This cell cycle arrest is evidenced at the molecular level by a loss in cyclins B1 and D1 (Figure 3b-d). In addition, this metastasis-derived primary culture also displays a strong reduction in N-cadherin, representing a loss in EMT potential (Figure 3b and e).

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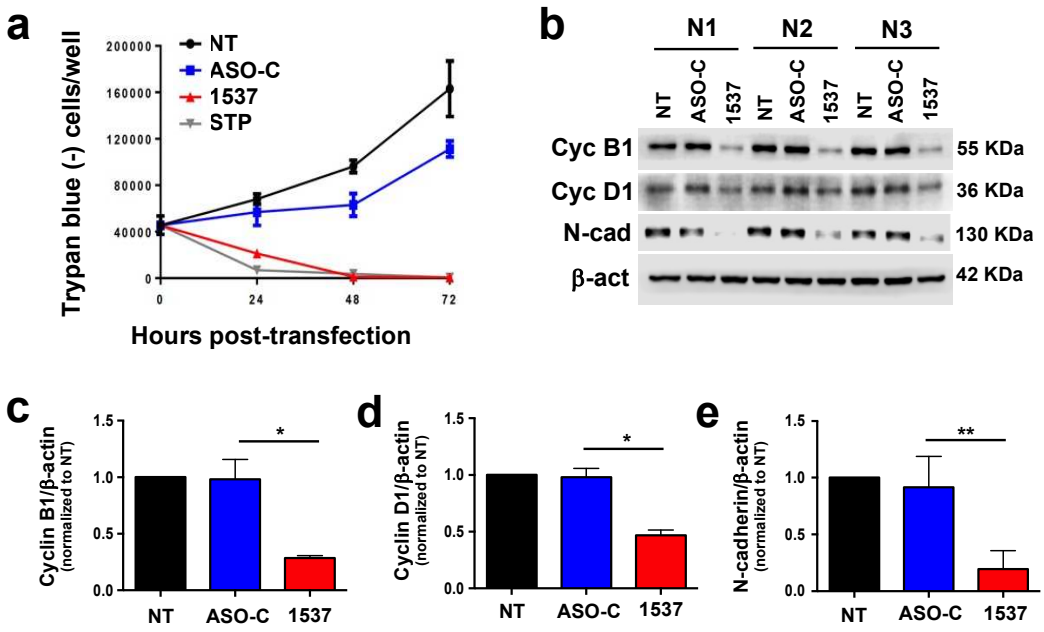

Figure 3. ASncmtRNA knockdown induces proliferative blockage of patient-derived ccRCC cells. MT1 cultures were transfected with ASO-1537 or ASO-C, or left untreated (NT), for 48 h. (**a**) growth curves determined by Trypan blue exclusion; STP was used as a positive control; (**b**) cell cycle (cyclin B1 and D1) and EMT (N-cadherin) protein levels were determined by Western blot; the image shows the results of three independent experiments, which were used for quantification of cyclin B1 (**c**), cyclin D1 (**d**) and N-cadherin (**e**); **p*<0.05; ***p*<0.01.

3.4. Knockdown of ASncmtRNA reduced tumor growth in vivo of orthotopic tumors generated with ccRCC primary cultures

We established an *in vivo* orthotopic model of ccRCC primary cultures by injecting PT2 cells into the subcapsular region of the left kidney of NOD/SCID mice (Figure 4a). At 6 weeks post-cell inoculation (pci), tumors were already visible and displayed the characteristic clear cytoplasm of ccRCC (Figure S1, left panel). For the analysis of tumor growth, treatment was initiated at 8 weeks pci by ip injection every 48 h, of 100 µg ASO-1537 or 100 µl saline, with a total of 28 doses at 17 weeks, where mice were euthanized and kidneys were collected, weighed and fixed. The inoculated (left) kidneys displayed larger tumors in saline-injected mice, compared to ASO-1537-treated animals (Figure 4b), as evidenced by tumor weight, which was slightly over twice as higher in the saline control group (Figure 4c).

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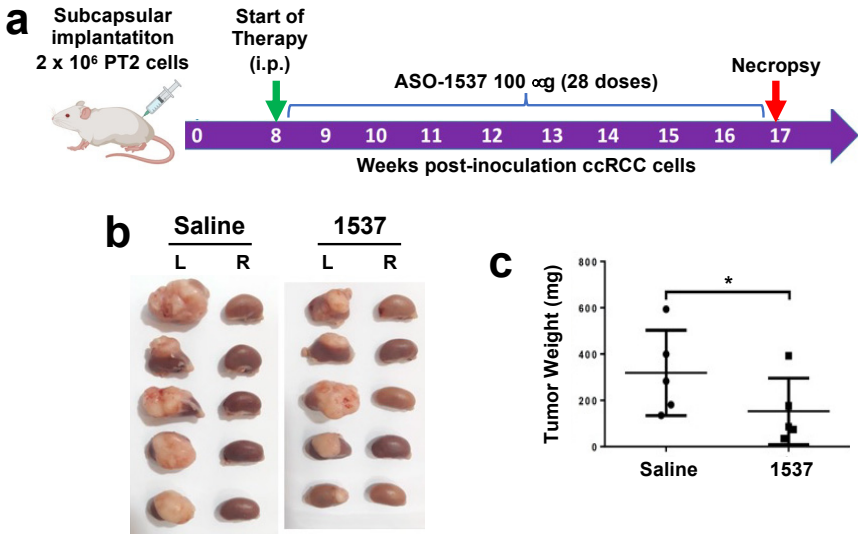

Figure 4. ASO-1537 reduces growth of PT2 subcapsular tumors. (**a**) Scheme depicting the protocol used for the orthotopic xenograft model; injections were performed every 48 h and saline was used as control; (**b**) images showing the left (inoculated) and right (non-inoculated) kidneys of the five mice in each experimental group at 17 weeks post-cell inoculation; (c) tumor weight of the five mice in each group; *p<0.05. Mouse and syringe image in (**a**) were created using Biorender (biorender.com).

We then performed the same analysis with the MT1 metastasis-derived primary culture (Figure 5a), which also generated tumors at 6 weeks pci (Figure S1, right panel). The same treatment regimen was applied, except for the commencement of treatment (10 weeks pci) and mice were euthanized at week 19 (Figure 5a). After necropsy, the tumors in the left kidneys of saline-treated mice were again larger than tumors from the ASO-1537 group (Figure 5b), with an average weight around 2.5 higher in the control group (Figure 5c). In a separate experiment, we determined that the survival rate of ASO-1537-treated mice was significantly higher than the saline group (Figure 5d).

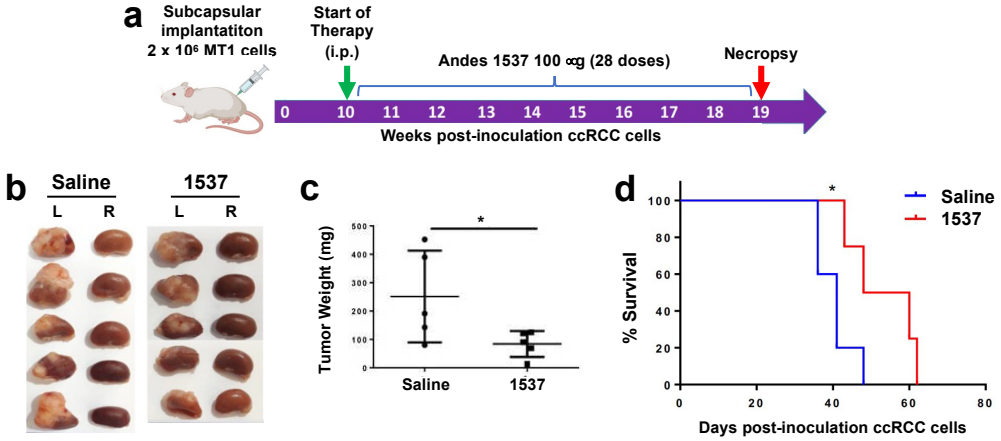

Figure 5. ASO-1537 reduces growth of MT1 subcapsular tumors and increases survival. (**a**) Scheme depicting the protocol used for the orthotopic xenograft model; injections were performed every 48 h and saline was used as control; (**b**) images showing the left (inoculated) and right (non-inoculated) kidneys of the five mice in each experimental group at 19 weeks post-cell inoculation; (**c**) tumor weight of the five mice in each group; (**d**) independent experiment showing survival curves of saline and ASO-1537-treated mice; *p<0.05. Mouse and syringe image in (**a**) were created using Biorender (biorender.com).

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4. Discussion

As stated above, treatment for ccRCC has proven challenging, mainly due to the development of resistance to existing therapies. Therefore, new alternative treatments are urgently needed. In this work we show that, similar to our previous work in a number of cell lines from different types of cancer, primary cultures obtained from ccRCC patient tumors respond to ASncmtRNA knockdown by ceasing to proliferate and dying through apoptosis. The novelty of the present study is that primary tumor cultures reflect more accurately the physiological reality of real tumors. This is especially true for the *in vivo* model utilized in our study, in which we inoculated the primary cells into their tissue of origin, reflecting further similarity to a clinical scenario.

As depicted in Figure 2a, all six primary cultures, derived from primary and metastatic sites, show a massive degree of cell death induced by the treatment. This result is important, since metastatic tumors tend to be more refractory than primary tumors with conventional treatments, again, due to acquisition of resistance. And it is essential that cancer treatments kill tumor cells through apoptosis, as opposed to necrosis, which is also the case in our study, as shown for the metastasis-derived MT1 cells (Figure 2b-d) and a wide variety of tumor cell lines [10,12–15]. Although not included in this study, it is important to note that primary cultures of normal cells are only marginally affected by the knockdown treatment, as observed in several normal human and mouse cell types [10,12–14], which include normal kidney epithelia [12–14]. Thus, a therapy for ccRCC based on this strategy should have minor side effects, in contrast to first-line chemotherapy, and should therefore ensure a better quality of life for patients undergoing treatment.

Prior to this massive cell death, as also observed before for tumor cell lines [10,12–15], there is an immediate arrest in proliferation (Figure 3a), which is also reflected in the lower growth rate of tumors in the *in vivo* models (Figure 4 and 5). This decreased proliferation is underpinned by a strong reduction in the cell cycle factors cyclin B1 (Figure 3b and c), cyclin D1 (Figure 3b and d) and also survivin (Figure 2d), which is not only an anti-apoptotic protein but is also involved in cell cycle progression during the M-phase [17]. These results are in line with that reported previously by our group, in which we have also shown in other cell models that additional cell cycle progression factors are also downregulated by ASncmtRNA knockdown, such as CDK1, CDK4 [15], Aurora Kinase A and Topoisomerase IIα, the latter two involved in ensuring genomic integrity throughout the cell cycle [19]. In addition, the metastasis-related protein, N-cadherin, an adhesion protein also involved in proliferation [20,21], is also strongly downregulated by the treatment (Figure 3b and e). N-cadherin plays a role in epithelial-mesenchymal transition, an essential step in metastasis, suggesting a reduction in invasive potential, as shown in previous reports [10,12,14]. Indeed, one of these studies was performed in the RenCa murine renal carcinoma model, in which ASncmtRNA knockdown with the mouse equivalent of ASO-1537 (ASO-1560) reduced significantly tumor growth and metastasis to the lungs in a syngeneic orthotopic model [12]. Also, as mentioned above, normal cell cultures are not damaged by the treatment, but we have also observed in *in vivo* models an absence of damage to healthy tissue and of inflammatory response [10].

Together with our prior body of evidence in tumor cell lines, the results presented here on a cell model representing a closer similarity to a clinical scenario, support the application of ASncmtRNA knockdown as a treatment strategy for ccRCC and likely other types of renal cancer.

5. Conclusions

The work presented here shows that primary cultures obtained from both primary and metastatic ccRCC patient tumors suffer a proliferative blockage and apoptotic death after knockdown of ASncmtRNAs, as we had observed before in tumor cell lines. In addition, the treatment precludes the growth of orthotopic xenograft tumors in mice and increases survival. These results are very promising, since they reflect a closer reality to actual patient tumors, as opposed to established tumor cell lines, and potentiates the use of this technology for a treatment alternative against ccRCC, which commonly presents resistance to other therapeutic strategies.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org, Figure S1: H&E-stained sections of subcapsular patient-derived ccRCC tumors in NOD-SCID mice.

Author Contributions: Conceptualization, V.B, V.A.B and L.O.B; formal analysis, M.A. and V.B.; investigation, M.A., F.G. and J.V.; resources, L.A.; writing—original draft preparation, V.A.B; writing—review and editing, M.A. and V.B.; visualization, M.A. and V.A.B.; supervision, V.B., V.A.B, J.V. and L.O.B.; funding acquisition, V.B. and V.A.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by ANID, Chile, grants number Fondecyt 1230760 to V.A.B, Fondecyt 11190897 to V.B. and Basal AFB-170004 to Centro Científico y Tecnológico de Excelencia Ciencia & Vida. Funding was also provided by Universidad Andrés Bello, grant number DI-05-20/REG to V.A.B. The APC was funded by ANID, Chile, grant number Fondecyt 1230760 to V.A.B.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Scientific Ethics Committee of the "Servicio de Salud Metropolitano Sur (SSMS)" from Santiago, Chile. ccRCC tumors were obtained from patients through written informed consent. The animal study protocol was performed according to the National Agency for Research and Development (ANID), Chile and approved by the Research and Ethics Committee of the "Centro Científico y Tecnológico de Excelencia Ciencia & Vida", Santiago, Chile

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest: The authors declare no conflicts of interest.

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