

SHORT REPORT

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# Antisense noncoding mitochondrial RNA-2 gives rise to miR-4485-3p by Dicer processing in vitro

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## Abstract

**Background:** The antisense noncoding mitochondrial RNAs (ASncmtRNAs) derive from the mitochondrial 16S gene. Knockdown of these transcripts with chemically-modified antisense oligonucleotides induces proliferative arrest, apoptosis and invasiveness reduction in tumor but not normal cells. One of these transcripts, ASncmtRNA-2, contains the complete and identical sequence of hsa-miR-4485-3p and, upon knockdown of this transcript, there is a strong increase in levels of this miRNA, suggesting ASncmtRNA-2 as a source for miR-4485-3p, which is supported by several evidences from our group and others, in the ex vivo setting.

**Results:** Here we show that incubation of in vitro-transcribed ASncmtRNA-2 with recombinant Dicer produces RNA fragments corresponding to hsa-miR-4485-3p, showing that Dicer binds to and processes ASncmtRNA-2, strongly supporting the hypothesis that ASncmtRNA-2 acts as a precursor for miR-4485-3p.

**Conclusion:** The in vitro results presented here strengthen the hypothesis that miR-4485-3p is derived from ASncmtRNA-2 by Dicer processing. Since miR-4485-3p is classified as a tumor suppressor miRNA, this evidence strengthens the application of ASncmtRNA knockdown for cancer therapy.

**Keywords:** ncRNA, miRNA, Cancer, lncRNA, Dicer, Cloning

## Background

The mitochondrial noncoding RNAs (ncmtRNAs), comprised of Sense (SncmtRNA) and Antisense (ASncmtRNA-1 and ASncmtRNA-2) members, arise from bidirectional transcription of the 16S rRNA gene of the mitochondrial genome (mtDNA) [1, 2]. SncmtRNA contains the complete sense 16S sequence, joined at its 5' end to a segment from the antisense transcript [1] and the antisense versions are composed of the antisense 16S transcript, with different segments from the sense transcript at its 5' end [2] (Fig. 1A). Consequentially, these RNAs contain inverted repeats (IRs) and therefore share a distinct structural feature consisting of long

double-stranded regions, flanked by a single-stranded "loop" and a single-stranded 3' region (see Fig. 1B for ASncmtRNA-2 secondary structure, predicted using the ViennaRNA package 2.0 [5]). These transcripts are synthesized inside mitochondria and are exported to the cytosol and nucleus [1, 3].

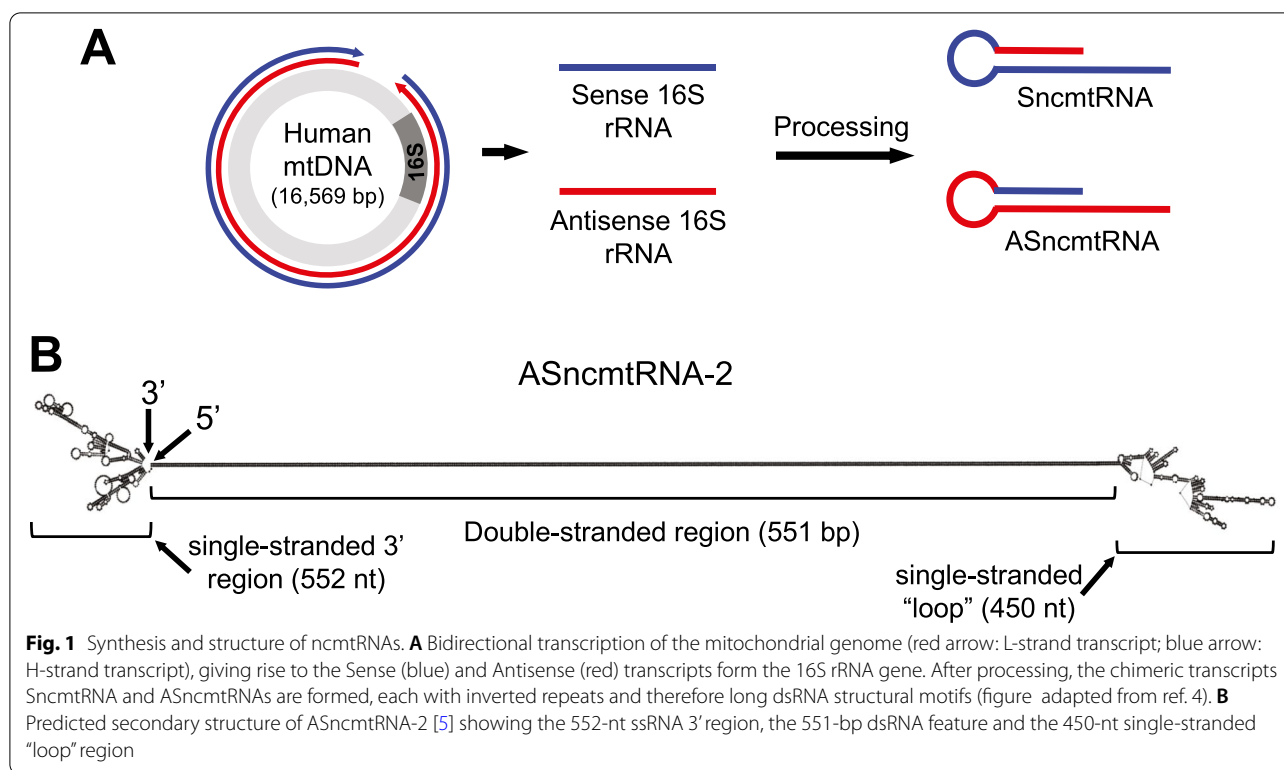
Knockdown of ASncmtRNAs with chemically-modified antisense oligonucleotides (ASOs) triggers massive apoptotic death in several tumor cell types, preceded by cell cycle arrest, and surviving cells exhibit greatly reduced invasive capacity ex vivo and in vivo [4, 6–9].

An emerging topic in cancer biology is the alteration in levels of many microRNAs (miRNAs) in tumor tissue. In this scenario, some miRNAs are deemed as oncogenic and others as tumor suppressors [10–12]. Several miRNAs derived from the mitochondrial genome have been described [13–15] and one of these, miR-4485-3p,

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is completely and identically contained in the sequence of the IR of ASncmtRNA-2 [6, 8, 16], suggesting that this lncRNA could constitute a precursor for miR-4485-3p. Indeed, transfection with the ASO Andes-1537, directed to the loop region of the ASncmtRNAs, triggers a nine-fold increase in miR-4485-3p levels [8]. Moreover, transfection of MDA-MB-231 breast cancer cells with miR-4485-3p mimics reduces proliferation [8], suggesting that the effects of the ASO treatment are induced in part by increased expression of miR-4485-3p. We and others have presented strong evidence of the mitochondrial origin of miR-4485 [8, 16] and we showed evidence of an interaction of ASncmtRNA-2 with Dicer by RNA immunoprecipitation followed by RT-PCR [6]. However, whether this interaction translates into Dicer-mediated processing of ASncmtRNA-2 to yield miRNAs had not been determined. Since in vitro interaction and processing studies require in vitro-transcribed RNA from a plasmid containing the complete sequence under study, our research on ncmtRNAs was until recently hindered by cloning difficulties through RT-PCR amplification due to replication slippage of thermostable DNA polymerases on templates with extensive double-stranded structures [17]. Here we present a novel stepwise strategy for cloning this type of transcript with long double-stranded structures. From the resulting clone, we produced in vitro-transcribed ASncmtRNA-2 in order to explore

the generation of miR-4485-3p from this transcript. We show that incubation of the transcript with recombinant Dicer in vitro generates a fragment corresponding to this miRNA. This work is an extension of the results we have published previously in an ex vivo setting [8], and strengthens the concept of ASncmtRNA-2 as a precursor for miR-4485-3p.

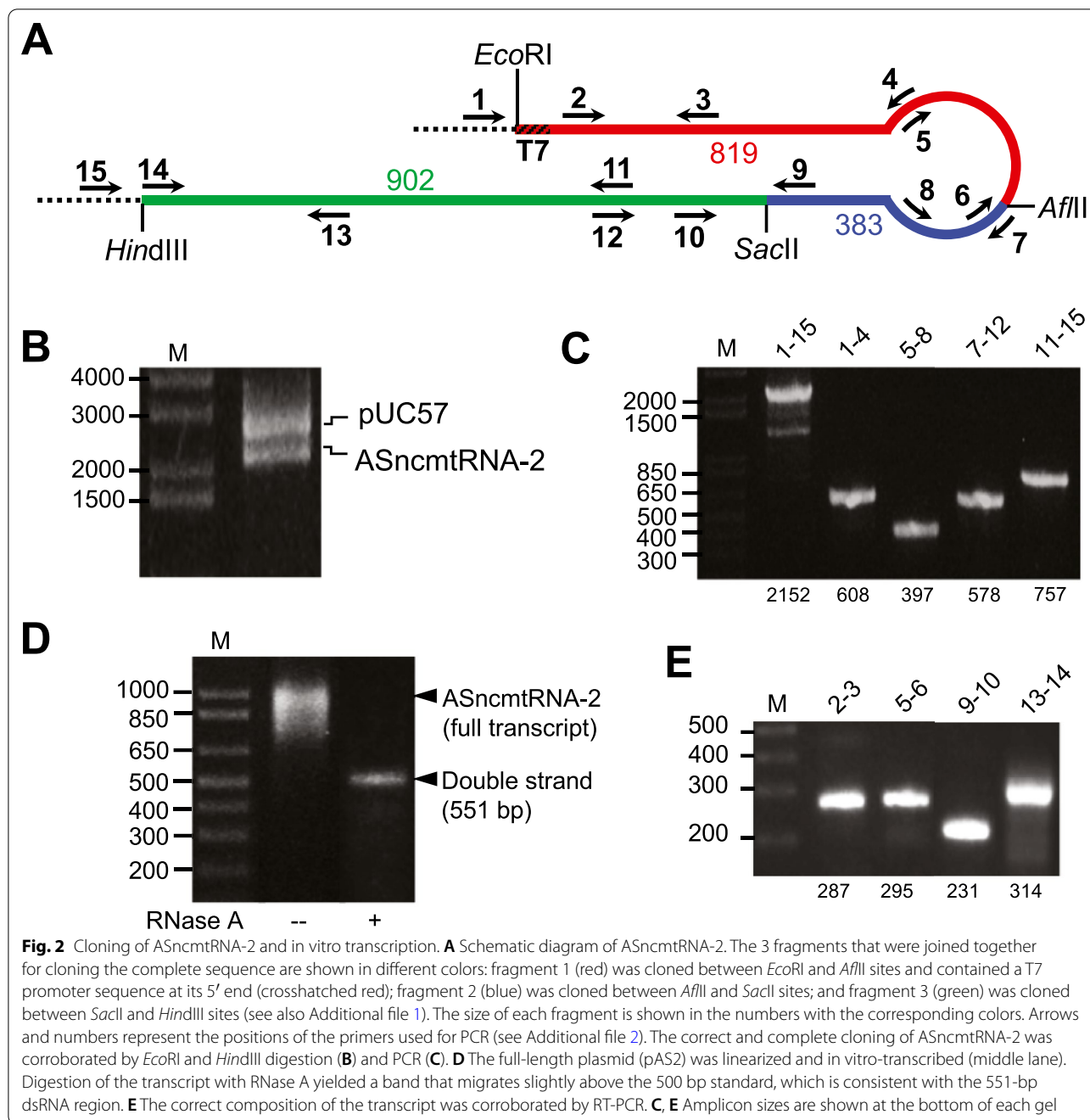
## Methods

### Cell culture

HEK293T cells (ATCC CRL-3216) were cultured in DMEM (Life Technologies) supplemented with 10% FCS and maintained at 37 °C under a 5% CO<sub>2</sub>-containing atmosphere. Transfections were performed in OptiMEM (Life Technologies).

### ASncmtRNA-2 cloning

The complete sequence of ASncmtRNA-2 (GenBank: EU863790.1) was divided into 3 fragments, cloned into the pUC57 vector, obtained by gene synthesis (GenScript; Hong Kong). Figure 2A and Additional file 1 depict the 3 fragments in different colors, starting from the 5' fragment (red), followed by the middle (blue) and 3' (green) fragments. Each of these fragments was contained in plasmids pAS2-1, pAS2-2 and pAS2-3, respectively. The insert in pAS2-1 was cloned between the *EcoRI* and *AflII* restriction sites, and contained a T7 promoter



sequence at the 5' end (Fig. 2A; Additional file 1); pAS2-2 contained the second fragment cloned between *AflIII* and *SacII* (Fig. 2A; Additional file 1) and pAS2-3 contained the 3' fragment cloned between *SacII* and *HindIII* (Fig. 2A; Additional file 1). To obtain a plasmid encompassing the complete ASncmtRNA-2 sequence, the cloning procedure was performed in a stepwise manner. First, 2 µg pAS2-3 and 1 µg of pAS2-2 (Additional file 1) were digested with 20 U each of *SacII* and *HindIII*,

in a final volume of 100 µl, for 2 h at 37 °C. Reaction mixes were then separated by agarose gel electrophoresis and the pAS2-3 insert and the pAS2-2 plasmid were purified from the corresponding bands. Ligation was performed in a mix containing 200 ng pAS2-3 insert, 200 ng pAS2-2 plasmid (Additional file 1) and 2 U T4 DNA ligase (Promega) in a total volume of 20 µl, at 4 °C for 12 h. Ligation mix was used to transform One shot Max Efficiency DH5α *E. coli* cells (Invitrogen), according

to manufacturer's directions. Plasmid DNA was purified from colonies using the GeneJET Plasmid Miniprep Kit (Thermo Scientific), following manufacturer's instructions and positive clones were selected by PCR. The resulting plasmid, pAS2-2/3 (1 µg), and pAS2-1 (2 µg) were digested in the manner described above, with *Afl*III and *Eco*RI (Additional file 1). The pAS2-2/3 plasmid and the pAS2-1 insert (Additional file 1) were gel-purified, ligated as above and the ligation mix was used to transform bacteria. The correct insertion and structure of the complete ASncmtRNA2 construction (pAS2; Additional file 1) was corroborated by PCR, restriction analysis and sequencing (Macrogen, South Korea).

#### Plasmid PCR

Plasmid pAS2 (50 ng) was amplified by PCR in 25 µl using GoTaq polymerase (Promega). The amplification protocol consisted of an initial 95°C denaturing step for 10 min, 25 cycles of 95 °C for 30 s, annealing temperature depending on primer T<sub>m</sub> (Additional file 2) for 45 s and 72 °C elongation for the time determined by amplicon size (1 min/kbp), and finalizing with a 10 min extension step at 72 °C.

#### In vitro transcription

Complete plasmid pAS2 (2 µg) was linearized with HindIII (3' end of the full transcript) and incubated in a mix containing 10 mM DTT, 80 U RNase-Out, 0,5 mM each rNTP (Promega) and 40 U T7 RNA polymerase (Promega) in the buffer supplied with the enzyme, in a final volume of 100 µl. Polymerization proceeded for 3–6 h at 37 °C. Afterwards, the plasmid was digested with DNA-Turbo (Ambion), according to manufacturer's directions, and RNA was quantified by UV spectrometry.

#### RNase A treatment

Two µg in vitro-transcribed ASncmtRNA-2 was subjected to RNase A digestion using the RNase A kit (Ambion), according to manufacturer's instructions.

#### RT-PCR of in vitro-transcribed RNA

One µg in vitro-transcribed RNA was used for cDNA synthesis in a mix containing 20 pmol specific reverse primer, 3 mM MgCl<sub>2</sub>, 0,5 mM each dNTP, 1 U/µl RNase-Out and 160 U ImProm-II Reverse Transcriptase (Promega) in the buffer supplied with the enzyme, in a final volume of 15 µl. Synthesis was carried out for 5 min at 25 °C, 42 °C for 1 h and 70 °C for 15 min. PCR was performed as described under "Plasmid PCR", using the primers (IDT) listed in Additional file 2.

#### Expression of recombinant Dicer (rDicer)

HEK293T cells ( $2 \times 10^6$ ) were seeded into 100-mm petri dishes and transfected the following day with 24 µl LipofectAmine 2000 (Life Technologies) and 12 µg pCMV-DDK-C (Transomic Technologies), which contains the coding sequence for C-terminal FLAG-Tagged wild-type human Dicer1 (pDicer-FLAG). At 24 h, media was switched to DMEM/10% FCS and culture proceeded for another 24 h. Cells were then lysed in 100 µl RIPA (Thermo Scientific), 2 µl PMSF 100X (Sigma-Aldrich) and 2 µl 50X protease inhibitor cocktail (Promega). Mix was incubated on ice with constant pipetting and then centrifuged at 14,000×g for 15 min at 4 °C. Dicer-FLAG was immunoprecipitated from the lysate with FLAG Immunoprecipitation kit (Sigma Aldrich), according to manufacturer's directions. Detection of Dicer-FLAG in the lysate and the immunoprecipitate was confirmed by Western blot as described before [8] (Additional file 3).

#### In vitro Dicer processing of ASncmtRNA-2

In vitro-transcribed-ASncmtRNA-2 (3 pmol) was incubated for 15, 30, 45 and 60 min at 37 °C, in a reaction mix containing 30 mM Tris-HCl pH 7.4, 3 mM MgCl<sub>2</sub>, 5 mM DTT, 100 mM KCl, 10% glycerol and 3 pmol rDicer. One mix was prepared without rDicer (T<sub>0</sub> control). Samples were resolved on a biphasic (top 5% and bottom 15%) denaturing polyacrylamide/8 M Urea electrophoresis alongside microRNA marker and Low Range ssRNA Ladder (New England BioLabs). The gel was transferred to a Nylon membrane (Hybond), which was then UV-crosslinked and pre-hybridized for 30 min at 40°C in hybridization buffer (HB; 50% Formamide, 5X SSC, 0,1% SDS, 0,2 mg/ml Salmon Sperm DNA and 3X Denhardt's solution). Membranes were probed with 50 pmol/ml 5'-biotin-labeled probes (IDT) (Additional file 4) in HB for 16 h at 40 °C under constant mixing. Membranes were then washed for 15 min in 2X SSC/0.1% SDS and 15 min in 0.5X SSC, at the same temperature. Biotin label was revealed with the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific), using a C-DiGit Blot Scanner (Li-Cor Biosciences).

#### Results

We previously showed that ASncmtRNA-2 co-immunoprecipitates with Dicer [6], suggesting that this transcript could constitute a substrate for Dicer-mediated processing as a precursor for miR-4485-3p, which is encompassed in the IR of ASncmtRNA-2. In support of this hypothesis, Bianchessi et al. [16] reported that cells treated with ethidium bromide, which precludes mitochondrial transcription [18], induces downregulation of both ASncmtRNA-2 and miR-4485 and, conversely,

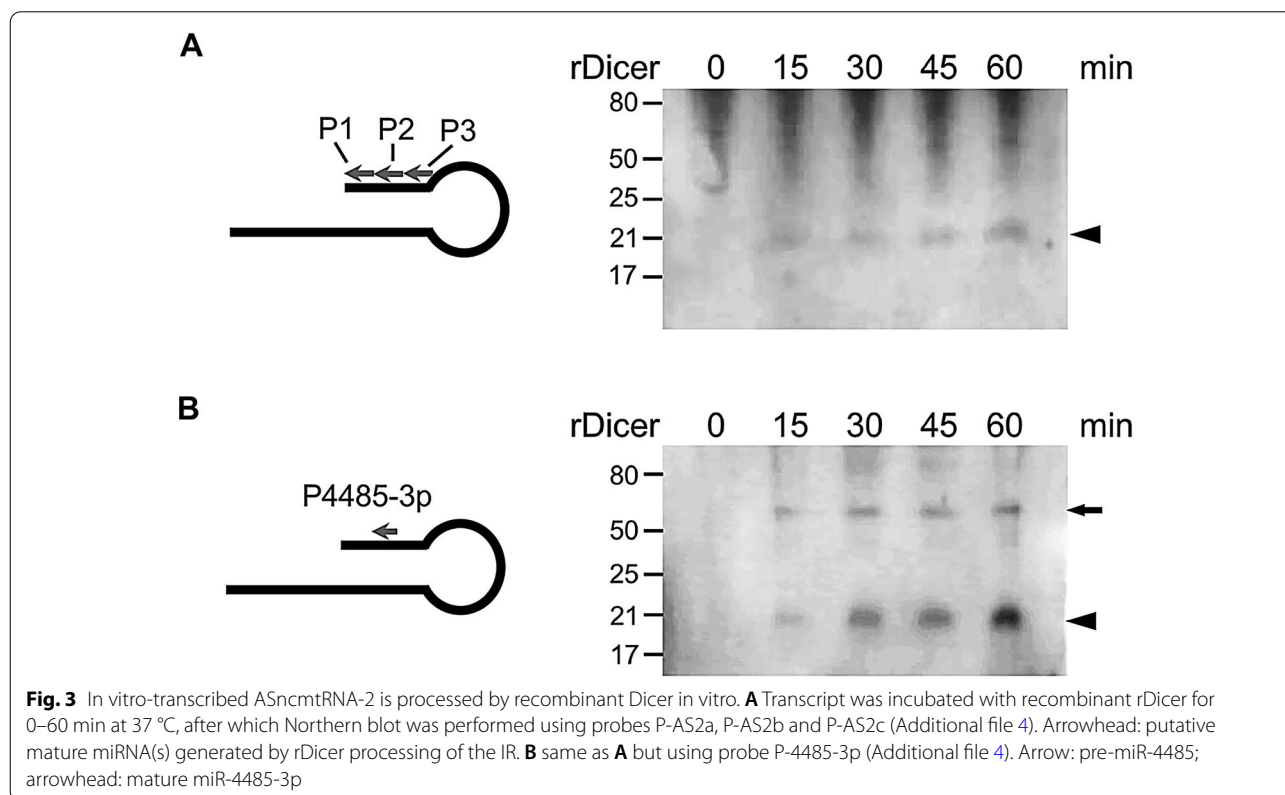
upregulation of ASncmtRNA-2 brings about an increase in miR-4485 [16].

To test this hypothesis *in vitro*, we cloned the complete sequence of ASncmtRNA-2 under the control of a T7 promoter. Because of the cloning difficulty presented by the long dsRNA segment (551 bp, see Fig. 1B), we carried out this cloning process in a stepwise manner; 3 successive segments of ASncmtRNA-2 (Fig. 2A; Additional file 1) were cloned into pUC57 (GenScript) and fragments were joined sequentially into a single plasmid (pAS2) encompassing the complete sequence of ASncmtRNA-2. The correct sequence and orientation of the 3 fragments in the complete clone was corroborated by *EcoRI* and *HindIII* digestion (Fig. 2B), PCR (Fig. 2C) and sequencing (Macrogen). Full-insert PCR yielded the ASncmtRNA-2 size, but also additional smaller amplicons, probably due to the formation of secondary structures in the template during amplification, which can cause slippage of thermostable DNA polymerases as mentioned [17]. The results show that we successfully cloned the complete sequence of ASncmtRNA-2 using this stepwise strategy, which would be very difficult otherwise. This is especially true for ASncmtRNA-1 which contains a smaller (96-nt) loop region [2], better favoring the fold-back of the IR onto the complementary region in the AS

16S sequence and, in consequence, further hindering a one-step full-sequence cloning strategy. Incidentally, we also managed to successfully clone the full-length ASncmtRNA-1 sequence using the same strategy (N. Farfán, unpublished).

The pAS2 plasmid was used for *in vitro* transcription with T7 RNA polymerase (Fig. 2D, middle lane). In the native gel, *in vitro*-transcribed ASncmtRNA-2 migrates close to the 1000 bp dsDNA standard, due to the extensive dsRNA region. The presence of the 551-bp dsRNA motif was corroborated by treatment of the transcript with RNase A (Fig. 2D, right lane). The completeness of the *in vitro*-generated transcript was verified by RT-PCR (Fig. 2E).

In order to study the ability of Dicer to process ASncmtRNA-2 *in vitro*, we expressed wild-type rDicer in HEK293 cells (Additional file 3). We chose not to purchase commercial Dicer enzymes for these experiments since they contain engineered mutations aimed to enhance activity for miRNA functional studies, which could alter the real activity and/or specificity in our experimental setting. The *in vitro* transcript was incubated with rDicer for 0–60 min and products were analyzed by Northern blot, probed with a mix of 3 biotin-labeled probes (Additional File 4) spanning the complete IR of ASncmtRNA-2 (Fig. 3A). This approach



yielded a band that migrated close to the 21-nt RNA standard (Fig. 3A, arrowhead), consistent with the average size of miRNAs. Since this band could hypothetically correspond to or include miR-4485-3p, we then used a probe complementary to this miRNA (see Additional file 4 for sequence and estimated T<sub>m</sub> [19]) and we found a defined and more intense band just below the 21-nt standard, consistent with the 20-nt length of miR-4485-3p (miRbase.org). Interestingly, another band was observed between the 50 and 80 standards, which is consistent with the 57-nt length of pre-miR-4485 (miRbase.org). Both bands increased in a time-dependent manner (Fig. 3B). This data shows that rDicer is capable of generating short RNAs of around 20 nt, and that miR-4485-3p and its precursor, pre-miR-4485, are specifically detected.

## Discussion

As mentioned before, knockdown of ASncmtRNAs by Andes-1537 triggers an increase in miR-4485, especially miR-4485-3p [8]. The widely-acknowledged mechanism of ASO treatment relies upon the action of cellular RNase H to cleave the target RNA in the hybrid RNA:DNA duplex segment by hydrolysis of a phosphodiester bond. According to our hypothesis, this cleavage could give rise to an RNA strand, including the IR and part of the ssRNA loop region, that could fold into a prior pre-miRNA, which could constitute a substrate for Dicer. So why do we observe processing in vitro without prior RNase H-mediated cleavage? The answer is elusive at the moment and warrants further studies, but we could hypothesize that there is a basal mechanism in the cell that relies on Dicer-mediated processing of the long dsRNA region, in a process similar to the endo-siRNA synthesis mechanism [20]. This first processing step could liberate a ssRNA fragment from the IR containing the pre-miR-4485 which could then be further cleaved by Dicer following the canonical miRNA synthesis pathway, and giving rise to miR-4485-3p. Based on our results, ASncmtRNA-2 could constitute a *bona fide* substrate for Dicer processing in the cell which could account for a basal level of miR-4485-3p, while the knockdown treatment of ASncmtRNA-2 by Andes-1537 could spur a boost in this process. Another possible explanation could involve proteins bound to the ASncmtRNA-2 in the cellular context, which are absent from the in vitro scenario. These are speculations that require further research. Of note, to our knowledge there is no *bona fide* evidence to date on the presence of Dicer in the mitochondrion, so this process would probably occur in the cytosol where the ncmtRNAs are also located [3].

The biological significance of miR-4485 is found as a tumor suppressor in breast and gastric cancer [21, 22]. We showed that this miRNA was upregulated in breast

cancer cells after knockdown of ASncmtRNAs [8], which could partly explain the anti-tumoral effect of Andes-1537. We also presented sequence evidence strongly suggesting the mitochondrial origin of pre-miR-4485 [8]. This is not to discard a nuclear origin for this miRNA but it could possibly have a dual origin. Of note, the full mature sequence of miR-4485-5p is also present and in proximity (upstream) of the 3p sequence, but this miRNA increases only modestly after ASncmtRNA knockdown, which led us to hypothesize that it could constitute the passenger strand of the miR-4485 duplex, at least in the mitochondrial-encoded context [8]. The IR also contains the sequence corresponding to miR-1973, which is also upregulated upon ASncmtRNA knockdown. Studies of the possible roles of these mitochondrial-encoded miRNAs are under way. As mentioned above, the ncmtRNAs are synthesized inside mitochondria and exported to the cytosol [3]. Since no hard evidence has been found to date for the presence of Dicer inside mitochondria, the putative miRNA(s) derived from ASncmtRNA-2 are probably Dicer-processed outside the organelle.

## Conclusions

This study provides the first direct evidence of Dicer processing on ASncmtRNA-2, complementing our previous research on the association between ASncmtRNA knockdown and an increase in levels of the tumor suppressor miR-4485-3p [8]. These results shed light upon the molecular mechanisms underlying the selective and widespread effects of ASncmtRNA knockdown on tumor cells.

## Abbreviations

ASncmtRNA: Antisense ncmtRNA; ASO: Antisense oligonucleotide; DMEM: Dulbecco's modified Eagle's medium; dNTP: Deoxyribonucleotide triphosphate; dsRNA: Double-stranded RNA; dsDNA: Double-stranded DNA; DTT: Dithiothreitol; FCS: Fetal calf serum; HB: Hybridization buffer; IR: Inverted repeat; lncRNA: Long non-coding RNA; miRNA: MicroRNA; mtDNA: Mitochondrial DNA; ncmtRNAs: Non-coding mitochondrial RNAs; pDicer-FLAG: Plasmid expressing FLAG-tagged human Dicer; rDicer: Recombinant FLAG-tagged human Dicer; RIPA: Radioimmunoprecipitation assay buffer; RNase A: Ribonuclease A; RNase H: Ribonuclease H; rNTP: Ribonucleoside triphosphate; RT-PCR: Reverse transcription coupled to polymerase chain reaction; SncmtRNA: Sense ncmtRNA; ssRNA: Single-stranded RNA.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40659-021-00356-0>.

**Additional file 1:** Stepwise cloning scheme for ASncmtRNA-2.

**Additional file 2:** Sequence of primers (sequence and T<sub>m</sub> of primers used for RT and PCR).

**Additional file 3:** Expression of FLAG-tagged recombinant Dicer.

**Additional file 4:** Sequence of probes used for Northern blot.

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**Authors' contributions**

NF contributed to experimental design and acquisition, analysis and interpretation of results. NS and MB contributed to acquisition and analysis of results. LOB participated in the conception and design of the study and critical review of the MS. VAB participated in the conception and design of the study, and wrote the MS. All authors have read and approved the final manuscript.

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**Availability of data and materials**

The complete sequence of ASncmtRNA-2 can be found at ncbi.nlm.nih.gov (GenBank: EU863790.1).

**Declarations****Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

LOB holds stocks of Andes Biotechnologies. Remaining authors declare no competing interests.

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**References**

- Villegas J, Burzio V, Villota C, et al. Expression of a novel non-coding mitochondrial RNA in human proliferating cells. *Nucleic Acids Res*. 2007;35:7336–47.
- Burzio VA, Villota C, Villegas J, et al. Expression of a family of noncoding mitochondrial RNAs distinguishes normal from cancer cells. *Proc Natl Acad Sci USA*. 2009;106:9430–4.
- Landerer E, Villegas J, Burzio VA, Oliveira L, Villota C, Lopez C, Restovic F, Martinez R, Castillo O, Burzio LO. Nuclear localization of the mitochondrial ncRNAs in normal and cancer cells. *Cell Oncol*. 2011;34:297–305.
- Borgna V, Villegas J, Burzio VA, et al. Mitochondrial ASncmtRNA-1 and ASncmtRNA-2 as potent targets to inhibit tumor growth and metastasis in the RenCa murine renal adenocarcinoma model. *Oncotarget*. 2017;8:43692–708.
- Lorenz R, Bernhart SH, Siederdisen HZC, et al. ViennaRNA package 2.0. *Algorithms Mol Biol*. 2011;6:26.
- Vidaurre S, Fitzpatrick C, Burzio VA, et al. Down-regulation of the antisense mitochondrial non-coding RNAs (ncRNAs) is a unique vulnerability of cancer cells and a potential target for cancer therapy. *J Biol Chem*. 2014;289:27182–98.
- Lobos-González L, Silva V, Araya M, et al. Targeting antisense mitochondrial ncRNAs inhibits murine melanoma tumor growth and metastasis through reduction in survival and invasion factors. *Oncotarget*. 2016;7:58331–50.
- Fitzpatrick C, Bendek MF, Briones M, et al. Mitochondrial ncRNA targeting induces cell cycle arrest and tumor growth inhibition of MDA-MB-231 breast cancer cells through reduction of key cell cycle progression factors. *Cell Death Dis*. 2019;10:423.
- Borgna V, Lobos-González L, Guevara F, et al. Targeting antisense mitochondrial noncoding RNAs induces bladder cancer cell death and inhibition of tumor growth through reduction of survival and invasion factors. *J Cancer*. 2020;11:1780–91.
- Adams BD, Kasinski AL, Slack FJ. Aberrant regulation and function of microRNAs in cancer. *Curr Biol*. 2014;24:R762–76.
- Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. *Trends Mol Med*. 2014;20:460–9.
- Acunzo M, Romano G, Wernicke D, Croce CM. MicroRNA and cancer—a brief overview. *Adv Biol Regul*. 2015;57:1–9.
- Barrey E, Saint-Auret G, Bonnamy B, Damas D, Boyer O, Gidrol X. Pre-microRNA and mature microRNA in human mitochondria. *PLoS ONE*. 2011;6:e20220.
- Bandiera S, Rüberg S, Girard M, Cagnard N, Hanein S, Chrétien D, Munnich A, Lyonnet S, Henrion-Caude A. Nuclear outsourcing of RNA interference components to human mitochondria. *PLoS ONE*. 2011;6:e20746.
- Sripada L, Tomar D, Prajapati P, Singh R, Singh AK, Singh R. Systematic analysis of small RNAs associated with human mitochondria by deep sequencing: detailed analysis of mitochondrial associated miRNA. *PLoS ONE*. 2012;7:e44873.
- Bianchessi V, Badi I, Bertolotti M, et al. The mitochondrial lncRNA ASncmtRNA-2 is induced in aging and replicative senescence in endothelial cells. *J Mol Cell Cardiol*. 2015;81:62–70.
- Viguera E, Canceill D, Ehrlich SD. In vitro replication slippage by DNA polymerases from thermophilic organisms. *J Mol Biol*. 2001;312:323–33.
- King MP, Attardi G. Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science*. 1989;246:500–3.
- Blake RD, Delcourt SG. Thermodynamic effects of formamide on DNA stability. *Nucleic Acids Res*. 1996;24:2095–103.
- Song MS, Rossi JJ. Molecular mechanisms of Dicer: endonuclease and enzymatic activity. *Biochem J*. 2017;474:1603–18.
- Sripada L, Singh K, Lipatova AV, et al. hsa-miR-4485 regulates mitochondrial functions and inhibits the tumorigenicity of breast cancer cells. *J Mol Med*. 2017;95:641–51.
- Yang B, Jing C, Wang J, et al. Identification of microRNAs associated with lymphangiogenesis in human gastric cancer. *Clin Transl Oncol*. 2014;16:374–9.

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