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ORIGINAL PAPER

Nuclear localization of the mitochondrial ncRNAs in normal and cancer cells

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Abstract
Background We have previously shown a differential expression of a family of mitochondrial ncRNAs in normal and cancer cells. Normal proliferating cells and cancer cells express the sense mitochondrial ncRNA (SncmtRNA). In addition, while normal proliferating cells express two antisense mitochondrial ncRNAs (ASncmtRNAs-1 and −2), these transcripts seem to be universally down-regulated in cancer cells. *In situ* hybridization (ISH) of some normal and cancer tissues reveals nuclear localization of these transcripts suggesting that they are exported from mitochondria.
Methods FISH and confocal microscopy, *in situ* digestion with RNase previous to ISH and electron microscopy ISH was employed to confirm the extra-mitochondrial localization of the SncmtRNA and the ASncmtRNAs in normal proliferating and cancer cells of human and mouse.
Results In normal human kidney and mouse testis the SncmtRNA and the ASncmtRNAs were found outside the organelle and especially localized in the nucleus associated to heterochromatin. In cancer cells, only the SncmtRNA was expressed and was found associated to heterochromatin and nucleoli.
Conclusion The ubiquitous localization of these mitochondrial transcripts in the nucleus suggests that they are new players in the mitochondrial-nuclear communication pathway or retrograde signaling. Down regulation of the ASncmtRNAs seems to be an important step on neoplastic transformation and cancer progression.

Keywords Mitochondria · Cancer · ncRNAs · Nuclear localization · Retrograde signaling

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1 Introduction

Since the reports by Warburg, mitochondrial defects have been suspected to play an important role in the development and progression of cancer [1]. In recent years, the focus of attention has been the correlation found between mutations and deletions of mtDNA and cancer, especially in solid tumors [reviewed in 2, 3]. The hallmark of cancer cells is deregulation of the cell cycle resulting in uncontrolled cell proliferation. Therefore, if mitochondrial dysfunction is involved in the development and progression of cancer, an anomalous mitochondrial-nuclear communication should be involved. Indeed, there are results that support the idea that stress or mitochondrial dysfunction induce a tumorogenic phenotype.Partial depletion of mtDNA or treatment with mitochondrial inhibitors that induce disruption of the mitochondrial membrane potential ($\Delta\Psi m$) transform the non-tumor C2C12 myoblast cell line and the non-invasive human lung carcinoma A549 cells into highly tumorigenic cells, with an invasive phenotype

and resistance to chemically induced apoptosis [4–7]. Mitochondrial-nuclear communication or retrograde signaling, initially described in yeast [8], is a communication pathway from the mitochondria to the nucleus that is triggered by a mitochondrial stress signaling pathway induced by mitochondrial inhibitors, partial mtDNA depletion, inhibition of mitochondrial transcription and mtDNA mutations [8–10]. In mammalian cells, mitochondrial stress triggered by an altered mitochondrial membrane potential ($\Delta\Psi m$), causes an increase in cytoplasmic free Ca^{2+} [7, 11]. The elevated concentration of cytosolic Ca^{2+} activates calcineurin, a calcium regulated phosphatase, and Ca^{2+}-dependent kinases [11, 12], which in turn activate other regulators of transcription [13, 14]. Whether other mitochondrial molecules, besides the increase of cytoplasmic Ca^{2+} are involved as messengers of the retrograde signaling remains to be investigated.

We reported that human cells express a unique family of non-coding mitochondrial RNAs (ncmtRNAs) containing large stem-loop structures. One of these transcripts named sense ncmtRNA or SncmtRNA, contains an inverted repeat (IR) of 820 nt joined at the 5′ terminus of the 16 S mitochondrial ribosomal RNA (mtrRNA), This transcript is expressed in normal proliferating cells and in tumor cells but not in resting cells [15, 16]. Besides the close correlation between the expression of the SncmtRNA and cell proliferation, other experimental evidences strongly suggests that this transcript might play a role in the cell cycle [15, 16]. On the other hand, normal proliferating cells express two antisense transcripts named ASncmtRNA-1 and ASncmtRNA-2 which also contain IRs linked to the 5′ end of the antisense 16 S mtRNA transcribed from the L-strand of the mtDNA [16–18]. In striking contrast, the ASncmtRNAs are down regulated in several tumor cell lines as well as in tumor cells present in different types of human cancer and patients, suggesting that down regulation of these transcripts is a common and necessary event in carcinogenesis [16].

In situ hybridization (ISH) of the SncmtRNA and the ASncmtRNAs in cultured cells revealed mainly cytoplasmic localization, although some nuclear staining is also observed. However, in biopsies of normal and cancer tissues, nuclear localization of these transcripts was frequently observed [16]. Here we confirm the extra-mitochondrial localization of these transcripts. To gain more information on the ultrastructural localization of the ncmtRNAs, electron microscopy ISH was carried out on human and mouse normal and tumor cells and tissues. In normal cells, the SncmtRNA and the ASncmtRNAs were found "crossing" the nuclear envelope and in the nucleus associated to chromatin. In tumor cells the SncmtRNA shows similar localization plus association with nucleoli, while the ASncmtRNAs are down-regulated. The ubiquitous localization in the nucleus suggests that these transcripts might be new components of the mitochondrial-nuclear communication pathway or retrograde signaling [8–10].

2 Materials and methods

2.1 Cell culture

Human umbilical vein endothelial cells (HUVEC) and the human melanoma cell line 42/95 were cultured as reported before [16]. The murine myoblast cell line C2C12 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin and 50 µg/ml streptomycin (pen/strep). The murine melanoma cell line B16F10 was cultured in RPMI 1,640 plus 10% FCS and pen/strep. Cultures were maintained in a humidified incubator at 37°C and 5% CO_2.

2.2 Human and mouse tissues

Ten percent formalin-fixed, paraffin-embedded human tissue samples were obtained from archival biopsies from patients at the Barros Luco Trudeau Hospital (Santiago, Chile). A 67 year-old female patient, diagnosed with a left renal tumour, was subjected to radical nephrectomy by laparoscopy. Immediately after the surgical piece was removed, a pathologist prepared 3 tumour pieces of about 1 cm^3 and 3 pieces of normal tissue of about 1 cm^3. To generate a mouse melanoma tumor, B16F10 cells were cultured to confluence as described, after which they were harvested, washed and resuspended in culture medium without FCS. Two male C57/BL6 mice were injected in their quadriceps with 200 µl of the suspension containing 150.000 cells. Three weeks after injection, the mice developed tumours of about 1 cm in diameter. The mice were sacrificed by cervical dislocation, under the rules of the Animal Bioethics Committee. The tumour was dissected and pieces of about 1 cm^3 were fixed. From the same animals, samples of the brain and testis were also obtained. The human and mouse tissues were used in agreement with the ethical guidelines approved by the ethical committee of the Hospitals and our Institutions.

2.3 RNase A digestion

The human melanoma cell line 42/95 and C2C12 cells were cultured for 48 h in 8-well chamber slides (Lab-Tek, NUNC), after which the medium was removed, cells were washed three times with cold PBS and fixed in 4% *p*-formalde-hyde in PBS for 10 min at room temperature. After fixation, cells were washed three times with PBS and then incubated for 5 min at room temperature with PBS

containing 0.2% Triton X-100. After one PBS wash, cells were incubated for 3 h at 37°C in PBS containing 100 mg/ml of RNase A (Sigma). The cells were again washed twice with PBS and subjected to FISH (42/95 cells) or chromogenic ISH (C2C12 cells) as described before [15, 16].

2.4 Fluorescent in situ hybridization (FISH)

FISH was carried out as described before [16] with few modifications. Briefly, probe 1 complementary to the SncmtRNA (5′ ACCGTGCAAAGGTAGCATAATCA 3′) or probe 2 complementary to the ASncmtRNAs (5′ TGATTATGCTACCTTTGCA-CGGT 3′) was labeled at the 3′ end with digoxigenin-11-dUTP (Villegas et al., 2007). These probes are also complementary to the mouse SncmtRNA (Genbank Access N° AF089815) and the mouse ASncmtRNAs (Genbank Access N° GU345834, GU332589, GU332588), respectively. Probe 3 for 18 S rRNA (5′ AGTGGACTCATTCCAATTA 3′), probe 4 for small nucleolar RNA U3 (5′ CGCGTTCTCTCCCTCTCACT 3′) and probe 5 for the RNA genome of ISA virus [19] were also labeled with digoxigenin. Cells cultured in 8 well chamber slides were fixed with 4% *p*-formaldehyde for 10 min. The cells were then hybridized with probe 1, probe 2, probe 3 or probe 4 and the hybrids were detected with anti-digoxenin antibody conjugated to Alexa Fluor 488 (Molecular Probes), diluted 1:250. After washing 3 times the cells were mounted in fluorescent mounting media (DAKO). For co-localization studies, previous to FISH the cells were incubated with 75 nM MitoTracker® Red CMXRos (Molecular Probes) in the culture medium and then fixed with 4% p-formaldehide in PBS. The cells were then subjected to FISH as described before and analyzed with a laser confocal Olympus FV-1000 microscope.

2.5 Electron microscopy ISH

We used protocols described before [20–22] with few modifications. Normal human kidney, kidney carcinoma, mouse brain and testis and the freshly obtained mouse melanoma tumor were fixed in 4% *p*-formaldehyde and 0.5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4. After 30 min, tissues were washed twice with cacodylate buffer and then with TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20). Tissues were then incubated in 50 mM ammonium chloride for 10 min and washed three more times in TBST. Dehydration of tissues was performed by serial incubation for 15 min at 4°C in an ethanol series of 70, 80, 90 and 95%, twice for 30 min at 4°C in 100% ethanol and finally in pure acetone at 4°C for 15 min and twice in 100% acetone at −20°C for 1 h each. To embed in Lowicryl HM20 (Electron Microscopy Sciences, US) tissues were incubated for 1 h at −50°C in 3:1, 1:1 and 1:3 mixtures of acetone/Lowicryl and then twice in Lowicryl for 1 h at −50°C and a final embedding overnight. The samples were transferred to gelatin capsules filled with Lowicryl and then polymerized under UV light for 48 h at −50°C. The embedded tissues were brought to room temperature and 600 Å sections were obtained and applied on Ni grids (150 mesh) previously coated with xylane. The grids were previously incubated for 60 min with the hybridization solution containing 50% formamide. 5x SSC, 150 ug/ml salmon sperm DNA. 50 ug/ml heparin and 0.1% Tween 20. Hybridization was carried out on drops containing probe 1, probe 2 or probe 4 labeled with digoxigenin as described before in the same hybridization solution and incubated overnight at 37°C in a humid chamber.

On the next day, the grids were washed with 0.5x SSC for 15 min at 37°C, 0,5x SSC for 5 min and three times with TBST at room temperature. The grids were then incubated on drops with anti-digoxigenin antibody conjugated to colloidal gold (10 or 20 nm in diameter) (Electron Microscopy Sciences, US) and diluted 1:20 in TBST containing 0.1% BSA followed by six 10 min washes in TBST. Finally, the grids were incubated for 10 min with 0.5% glutaraldehyde and 4% *p*-formaldehyde, washed with water and stained with 2% uranyl acetate for 10 min and lead citrate for 5 min [9, 10]. The grids were analyzed in a Phillips Tecnai 12 Biotwin electron microscope (Phillips Electron Optics, Holland).

3 Results

Chromogenic ISH of normal human tissues including pancreas, spleen and normal kidney shows nuclear localization of the SncmtRNA and the ASncmtRNA (Fig. 1, arrows). Notice that in some nuclei the staining shows some degree of texture. In human biopsies of lymphoma, cervix and kidney carcinoma, the SncmtRNA also shows nuclear localization (Fig. 1, arrows), while the ASncmtRNAs were down-regulated in cancer tissues as described before (Fig. 1) [16].

The above results prompted us to re-evaluate the co-localization of these transcripts with mitochondria. Previously we showed extensive co-localization of the SncmtRNA with the mitochondrial markers cytochrome c and endonuclease G [15]. In that study FISH was carried out before the immune-detection of the mitochondrial markers and therefore the cells were subjected to extensive permeabilization that probably includes permeabilization of the organelle membranes. Here we repeated this study with human umbilical vein endothelial cells (HUVEC) using Mitotracker Red CMXRos that passively diffuses into the cell, concentrates in mitochondria and fixes to mitochondrial

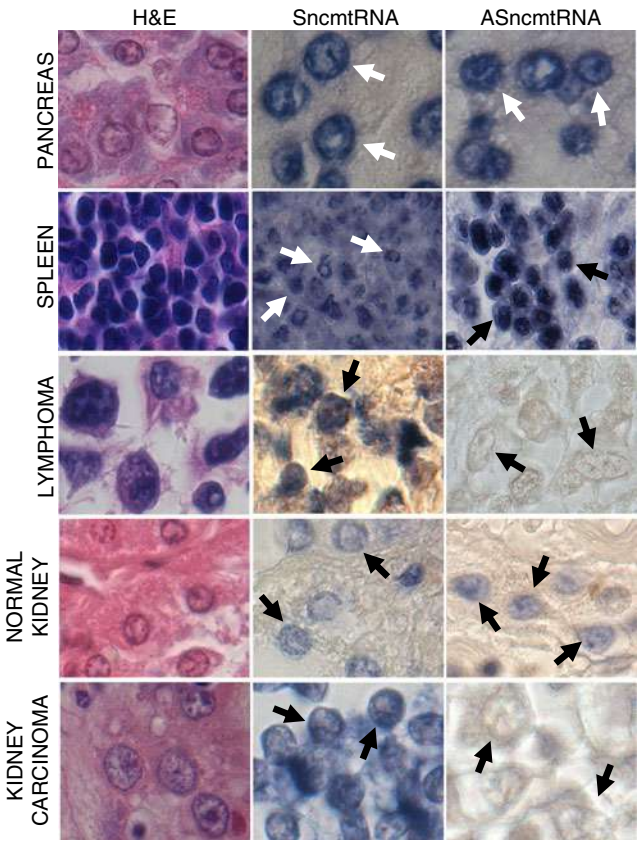

Fig. 1 Nuclear localization of the SncmtRNA and the ASncmtRNAs in human tissues. Parallel biopsy section of normal pancreas, spleen and kidney together with samples of lymphoma and kidney carcinoma were subjected to ISH to detect the mitochondrial transcripts. Another section was stained with hematoxylin-eosin (H&E). The hybridization reveals nuclear localization of the SncmtRNA and the ASncmtRNAs (*arrows*). As reported before, the ASncmtRNAs are down-regulated in tumor tissues (magnification 100×)

proteins by the thiol-reactive chloromethyl moiety (Molecular Probes). After incubation with the dye, the cells were fixed and subjected to FISH as described before [16]. Confocal analysis revealed only partial co-localization of the SncmtRNA or the ASncmtRNAs to mitochondria (Fig. 2a). Although the hybridization signal was mainly cytoplasmic, small nuclear structures were also labeled (Fig. 2a, white arrows). A probe directed to the small nucleolar RNA U3 shows nucleolar staining that do not co-localize with mitochondria (Fig. 2a, white arrows). Hybrization was negative with a control probe targeted to the salmon virus ISAV [19] (Fig. 2a, Control).

The previous results indicate that these mitochondrial transcripts were outside the organelle and therefore should be readily digested by nucleases. Mitochondrial transcripts inside the organelle are resistant to nuclease treatment [15, 16, 23–25]. Hence, cells were treated with RNase A previous to the detection of the transcripts. Human melanoma cells (42/95) express the 18 S rRNA and the SncmtRNA, while the ASncmtRNAs are down-regulated (Fig. 2b). When cells were treated with RNase A previous to FISH, the hybridization revealed total digestion of the 18 S rRNA and the SncmtRNA (Fig. 2b, RNase). Similarly, the mouse myoblast C2C12 cell line, as normal proliferating cells, express the SncmtRNA and the ASncmtRNAs (Fig. 2c). When cells were previously treated with RNase A, the chromogenic staining was negative (Fig. 2c, RNase).

To confirm those results and to gain information on the ultrastructural localization of the SncmtRNA and the ASncmtRNAs, electron microcopy ISH was carried out on ultrathin sections (~600 Å) of normal human kidney and renal cell carcinoma (clear cells) obtained from a female patient (see Methods). Sections were hybridized with digoxigenin-labelled probes and the hybrid was then revealed with an anti-digoxigenin antibody coupled to 10 nm gold particles. Hybridization of the SncmtRNA in normal kidney cells reveals groups of gold particles on the perinuclear area, on the nuclear membrane and inside the nucleus associated to chromatin (Fig. 3a, plates 1 and 2, arrows and arrowheads). Gold particles are also associated with a large mass of heterochromatin attach to the nuclear membrane that seems to correspond to the Barr body (Fig. 3a, plate 1, Bb). Occasionally, clusters of gold particles were seen on nuclear pore-like structures. Gold particles were also seen in the cytoplasm and outside the organelle (Fig. 3a, plate 2, arrows). Similarly, hybridization of the ASncmtRNAs reveals gold particles in the nucleus associated to chromatin, on the nuclear membrane and in the cytoplasm (Fig. 3a, plates 3 and 4, arrows and arrowhead).

In renal cell carcinoma, the SncmtRNA was also found outside the mitochondria. Fig. 3b (plates 1) shows gold particles on the nucleolus (arrowhead) in association to structures that seem to correspond to the granular components and associated to heterochromatin (Chr) close to the nuclear membrane (Fig. 3b, plates 1 and 2, arrows). As reported before [16], the ASncmtRNAs were practically absent in kidney carcinoma cells. Only a few sections show small groups of gold particles on the nucleus and in the cytoplasm (Fig. 3b, plates 3 and 4, arrows). As expected, gold particles were also found inside mitochondria (Fig. 3b, plate 4, arrowhead).

In mouse tissues, the SncmtRNA and the AsncmtRNAs are also outside the organelle. The probes were targeted to the mouse SncmtRNA and to the ASncmtRNAs (see Methods). Hybridization of the SncmtRNA in mouse testis revealed gold particles in the nucleus associated to chromatin and on the nuclear membrane (Fig. 4a, plate 1, arrow and arrowhead, respectively) and also in the cytoplasm close to mitochondria (Fig. 4a, plate 2, arrows). Similarly, the ASncmtRNA is also localized in the cytoplasm close to mitochondria (Fig. 4a, plate 3, arrow) and in the nucleus associated to perinuclear chromatin (Fig. 4a, plate 4, arrowhead).

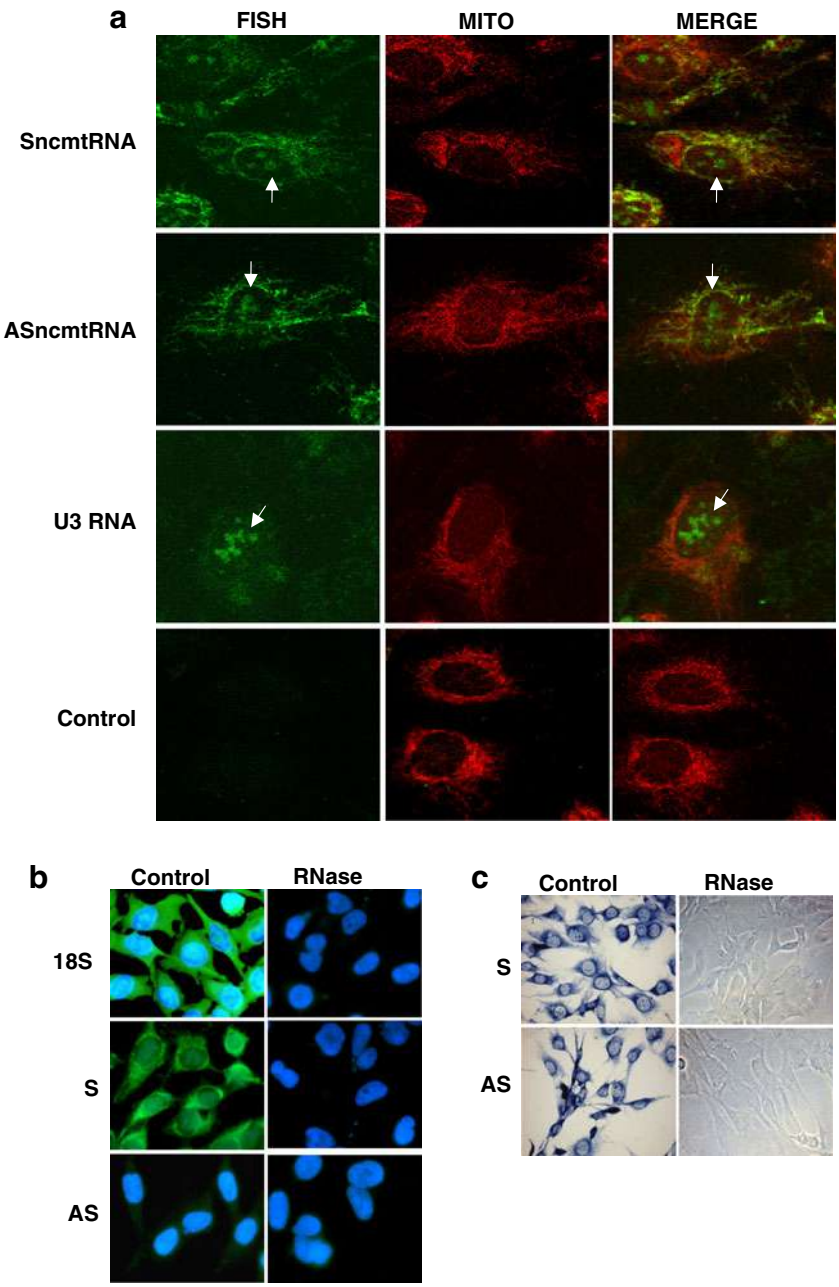

Fig. 2 Extra-mitochondrial localization of the SncmtRNA and the ASncmtRNAs. **a** HUVEC were stained with MitoTracker®Red CMXRos and then subjected to FISH to detect SncmtRNA and the ASncmtRNAs. Probes 1 or 2, labeled at the 3′ end with digoxigenin were revealed with anti-digoxigenin conjugated to AlexaFluor 488. The merged images show that the green fluorescence of the SncmtRNA or the ASncmtRNA partially co-localize with the mitochondrial probe. FISH reveals association of the mitochondrial transcripts with intra-nuclear structures (*white arrows*). No co-localization of the U3 snorRNA with mitochondria was found. No hybridization signal was obtained with an unrelated probe (Control). (magnification 60×). **b** FISH of the human melanoma cell line 42/95 reveals expression of the 18 SrRNA and the SncmtRNA (18 S and S, respectively) while the ASncmtRNAs were down regulated (AS). Nuclei were stained with DAPI (*Control*). After *in situ* digestion with RNase A, hybridization signals were abolished (RNase). **c** The hybridization signal of the SncmtRNA and the ASncmtRNAs on the mouse myoblast cell line C2C12 (*Control*) was abolished after *in situ* digestion with RNase A. (magnification 60×)

Then we studied a murine melanoma tumor generated after intramuscular injection of B16F10 cells into C57BL6/J mice (see Methods). The SncmtRNA was found associated to electron-dense structures that could correspond to the granular or fibrillar components of the nucleolus (Fig. 4b, plate 1, arroheads) and in the nucleus associated to chromatin (Fig. 4b, plate 2, arrows) and in structures that might correspond to nuclear pores (Fig. 4b, plate 2, arrowheads). After hybridization with the probe complementary to the ASncmtRNA only few gold particles were observed (Fig. 4b, plates 3 and 4, arrows).

Confirming previous results [16], in mouse brain, gold particles were practically absent after hybridization of the SncmtRNA or the ASncmtRNA. Few gold particles were found only inside mitochondria (Fig. 5a, and b, arrows). Mitochondrial localization was also found in normal human kidney (Fig. 5c, plate 1 and 2, arrows). On the other hand, hybridization of sections of normal human kidney with an unrelated probe (Fig. 6a) or without probe (Fig. 6b) was negative.

4 Discussion

The mitochondrial origin of the SncmtRNA and the ASncmtRNA transcripts is supported by several evidences.

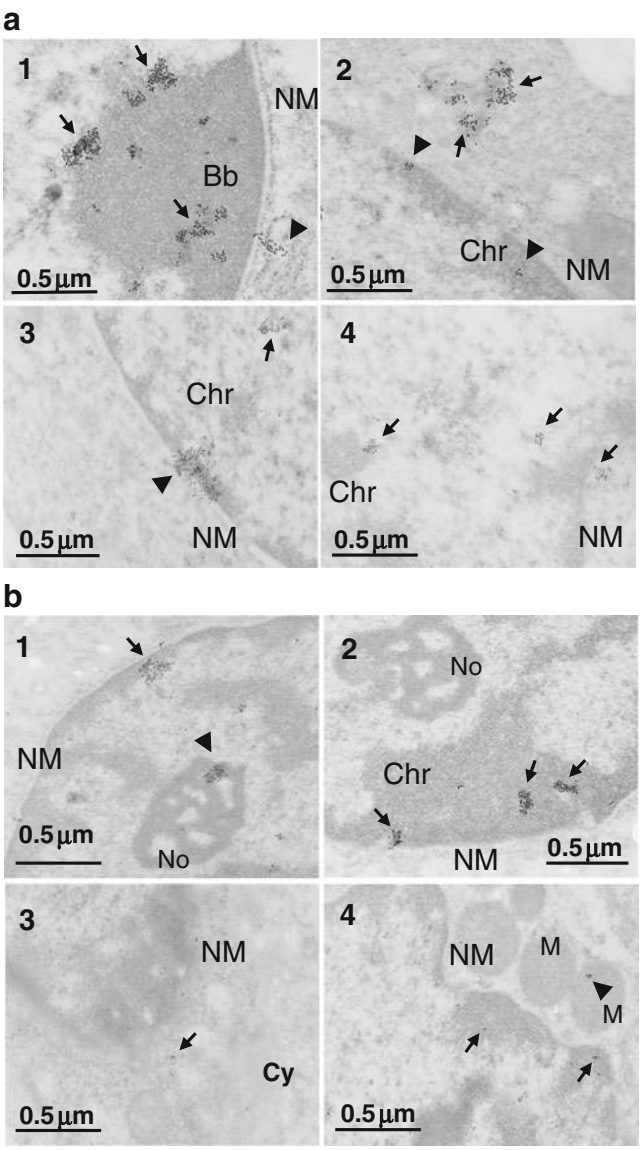

Fig. 3 Nuclear localization of the SncmtRNA and the ASncmtRNAs in normal human kidney and renal cell carcinoma. **a** Electron microscopy ISH of normal human kidney sections were carried out with digoxigenin-labeled probes and anti-digoxigenin conjugated to colloidal gold (10 nm). Plate 1 and 2 show hybridization of the SncmtRNA. In plate 1 a large perinuclear heterochromatic mass that correspond to the Barr body is decorated with gold particles (*arrows*, Bb). Gold particles are also associated to the nuclear membrane (*arrowhead*). Plate 2 shows gold particles in the cytoplasm (*arrows*) and associated to heterochromatin (Chr, *arrowhead*). Plates 3 and 4 correspond to hybridization of the ASncmtRNAs. Both plates show gold particles associated to heterochromatin (*arrows*) and close to the nuclear membrane (*arrowhead*). **b** Plates 1 and 2 show hybridization of the SncmtRNA on sections of renal cell carcinoma. Both plates show gold particles in close proximity of the nuclear membrane (NM) (arrows) and on the nucleolus (No) (Plate 1, *arrowhead*). Plate 2 also gold particles are associated to heterochromatin (*arrows*) and in the proximity of the nucleolus (No) (*arrowhead*). Plates 3 and 4 correspond to hybridization of the ASncmtRNAs. Only few gold particles are present in the nucleus, in the cytoplasm (Cy) and inside mitochondria (arrowhead) (Bar: 0.5 µm)

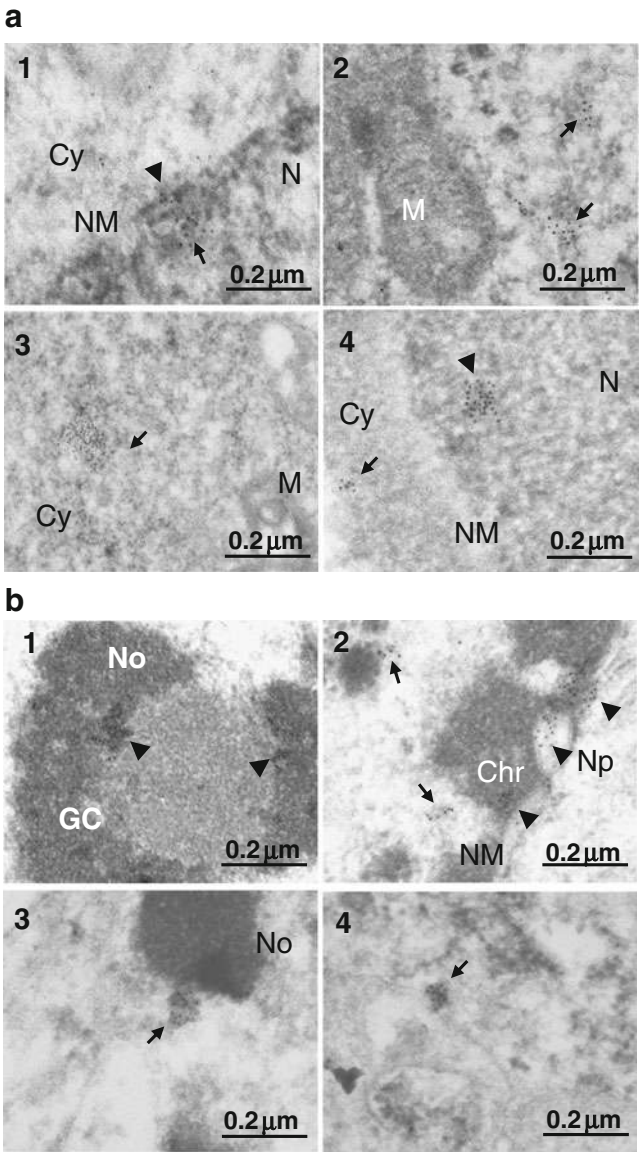

Fig. 4 Nuclear and cytoplasmic localization of the SncmtRNA and ASncmtRNAs in mouse testis and mouse melanoma. **a** Electron microscopy ISH was carried out as in Fig. 3 using anti-digoxigenin conjugated to colloidal gold (20 nm). Plates 1 and 2 show hybridization of the mouse SncmtRNA. Gold particles are present on the nuclear membrane (NM) (*arrowheads*) and associated to chromatin (plate 1) and in the cytoplasm close to mitochondria (M, plate 2, *arrows*). Hybridization of the ASncmtRNAs shows gold particles in the cytoplasm (Cy) close to mitochondria (plate 3, M) and inside the nucleus associated to chromatin (Chr) and close to the nuclear membrane (plate 4, *arrowhead*). **b** Plates 1 and 2 show hybridization of the SncmtRNA on mouse melanoma sections. Gold particles are found on the granular component (GC) of nucleolus (No) (plate 1) and on the nuclear membrane (NM) in the proximity of a nuclear pore (Np) and in association with chromatin (Chr) (plate 2). Hybridization of the ASncmtRNAs in mouse melanoma reveals only few gold particles on the nucleus and on the cytoplasm (plates 3 and 4, *arrows*) (Bar: 0.2 µm)

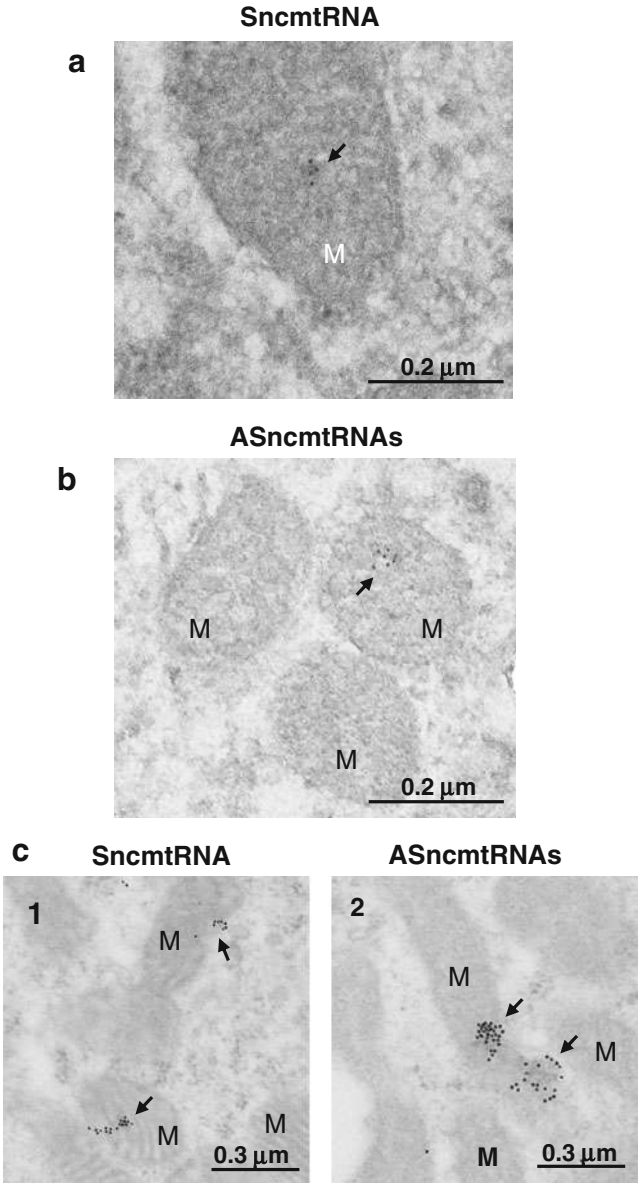

Fig. 5 Down regulation of the SncmtRNA and the ASncmtRNAs in mouse brain. Hybridization of the SncmtRNA (**a**) or the ASncmtRNAs (**b**) revelead only gold particles inside mitochondria (M, *arrows*) (Bar: 0.2 μm). **c** These transcripts are also found inside the organelle of normal human kidney cells (plates 1 and 2, *arrows*) (Bar: 0.3 μm)

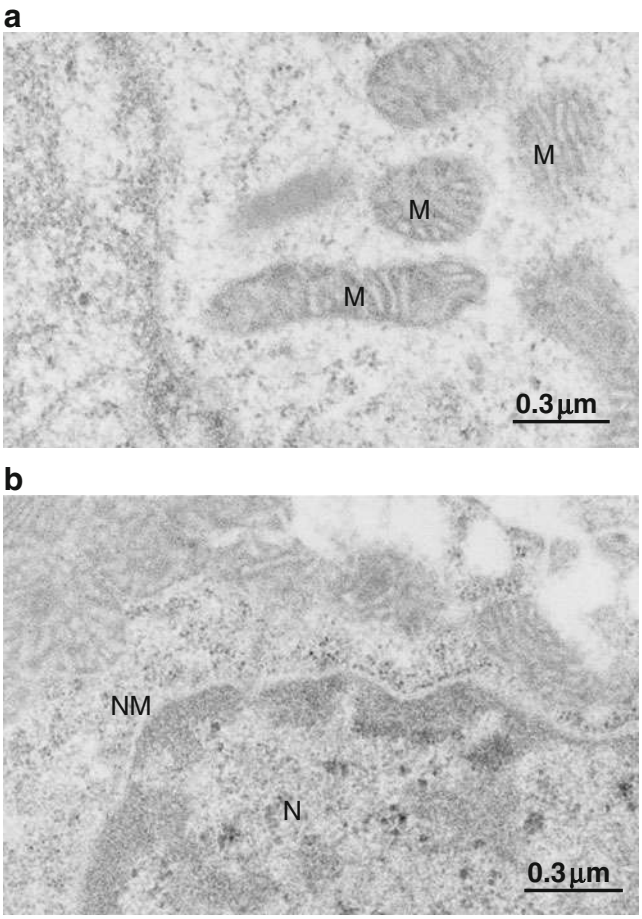

Fig. 6 Control hybridization. Sections of normal human kidney were hybridized with a probe labeled with digoxigenin complementary to the genomic RNA of the ISAV salmon virus and revealed with anti-digoxigenin conjugated to colloidal gold (**a**) or without probe but reveals with anti-digoxigrnin conjugated to colloidal gold (**b**). No gold particles were observed (Bar: 0.3 μm)

They display over 99% identity with the mtDNA sequence and are present in isolated mitochondria previously treated with RNase to eliminate contamination with cytoplasmic transcripts [15, 16, 23–25]. Moreover, the expression of the SncmtRNA and the ASncmtRNAs is blocked by ethidium bromide and rhodamine 6 G, two drugs that interfere with mitochondrial transcription and DNA replication [15, 16, 26–29]. However, and in spite of the mitochondrial origin, here we present unequivocal evidence that these transcripts exit the organelle to localize to other cellular structures in human and mouse cells. Electron microscopy ISH revealed the same pattern of expression of these transcripts as reported before using FISH or chromogenic ISH [15, 16]. Normal proliferating cells express the SncmtRNA and the ASncmtRNAs while tumor cells down-regulate the expression of the ASncmtRNAs. Probes targeted to other regions of these transcripts including the linker region between the IR and the sense or antisense 16 SmtRNA yielded the same results. The ubiquitous nuclear localization of these transcripts is interesting. In normal cells (human kidney and mouse testis) the SncmtRNA and the ASncmtRNAs are associated to chromatin and nucleoli and a similar localization of the SncmtRNA is observed in tumor cells (renal cell carcinoma and mouse melanoma). In general, a preferential association of these transcripts to heterochromatin was observed. An illustrative example is the close association of the SncmtRNA with the inactive X chromosome or Barr body in a female normal kidney cell (see Fig. 4). The gold particles were mainly on the periphery of the Barr body. A similar distribution of the heterochromatin-

associated trimethylated histone H3 (Lys 27) on the Barr body was reported [30]. Whether the association of the SncmtRNA with heterochromatin indicates a hypothetical epigenetic role of this transcript warrants future work. However, it is important to mention that the nuclear localization in tumor cells is not universal. Chromogenic ISH of tumor biopsies of breast, prostate, lung, uterine cervix and thyroid revealed that the SncmtRNA shows either mainly nuclear or mainly cytoplasmic localization when analyzing biopsies obtained from different patients. The meaning of this differential distribution of the SncmtRNA is unclear but it is plausible to speculate that it might have a prognostic value for patients. Prospective and retrospective studies on the nuclear or cytoplasmic localization of the SncmtRNA in different types of tumors and its relation with the outcome and survival of the patient is underway.

Although the meaning of the nuclear localization of the SncmtRNA and the ASncmtRNAs is unclear, the present results suggest that these transcripts might play a role in retrograde signaling [8–10]. Retrograde signaling is opposite to anterograde signaling, which consists of control of destination of proteins [31, 32], RNAs [25, 33, 34] and microRNAs [35] from the nucleus to the organelle. The ubiquitous nuclear localization of the SncmtRNA and the ASncmtRNAs suggests that these molecules might be new mitochondrial messengers to the nucleus, forming part of the retrograde signaling pathway. Some findings seem to support this hypothesis. As discussed before, retrograde signaling is recognized as a pathway closely implicated in cancer progression and the invasive phenotype of tumor cells [4, 6, 7], which somehow correlates with the universal down-regulation of the ASncmtRNAs in tumor cells [16]. On the other hand, we have described a close correlation between the expression of the SncmtRNA and cell proliferation suggesting that this transcript plays a role in the progression of the cell cycle [15, 16]. For example, phytohemagglutinin-stimulated lymphocytes express the SncmtRNA, in contrast to resting cells, in which neither transcript is detectable [15, 16]. If the stimulated cells are treated with rhodamine 6 G, a drug that inhibits mitochondrial transcription and mtDNA replication [16, 29, 35], the expression of the SncmtRNA is inhibited concomitantly with inhibition of DNA synthesis and PCNA expression. This effect is reversible, since after removing the drug lymphocytes resume proliferation and expression of the SncmtRNA and the ASncmtRNAs [16]. Furthermore, recent observations indicate that knocking down the SncmtRNA induces inhibition of cell proliferation. Treatment of HeLa and the myoblast cell line C2C12 with antisense oligonucleotides targeted against the SncmtRNA induces inhibition of DNA synthesis and cell proliferation in parallel with knocking-down of the transcript (V. Burzio and S. Vidaurre, unpublished results). Altogether, these results suggest that the SncmtRNA is a component of the retrograde signaling involved in cell proliferation.

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