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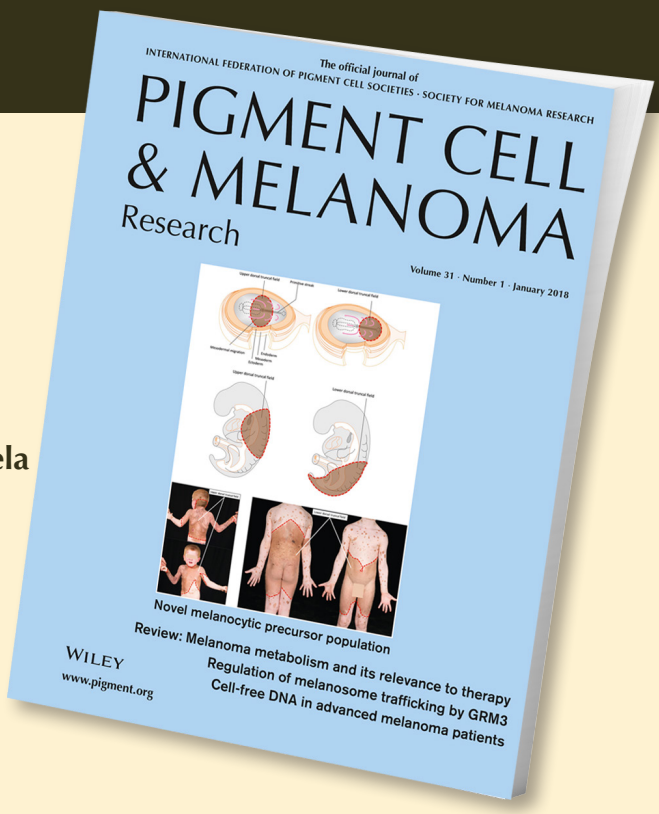

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DOI: 10.1111/pcmr.12615
Volume 31, Issue 1, Pages 64–72

Received: 8 February 2017 | Accepted: 7 July 2017

DOI: 10.1111/pcmr.12615

ORIGINAL ARTICLE

WILEY

In vivo knockdown of antisense non-coding mitochondrial RNAs by a lentiviral-encoded shRNA inhibits melanoma tumor growth and lung colonization

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Funding information
Universidad Andrés Bello, Grant/Award Number: DI11/11; Comisión Nacional de Investigación Científica y Tecnológica, Grant/Award Number: Fondecyt 1110845, Fondecyt 11150624, Fondecyt 1140345, Fondecyt 1160889 and PFB16

Summary

The family of non-coding mitochondrial RNAs (ncmtRNA) is differentially expressed according to proliferative status. Normal proliferating cells express sense (SncmtRNA) and antisense ncmtRNAs (ASncmtRNAs), whereas tumor cells express SncmtRNA and downregulate ASncmtRNAs. Knockdown of ASncmtRNAs with oligonucleotides induces apoptotic cell death of tumor cells, leaving normal cells unaffected, suggesting a potential application for developing a novel cancer therapy. In this study, we knocked down the ASncmtRNAs in melanoma cell lines with a lentiviral-encoded shRNA approach. Transduction with lentiviral constructs targeted to the ASncmtRNAs induced apoptosis in murine B16F10 and human A375 melanoma cells in vitro and significantly retarded B16F10 primary tumor growth in vivo. Moreover, the treatment drastically reduced the number of lung metastatic foci in a tail vein injection assay, compared to controls. These results provide additional proof of concept to the knockdown of ncmtRNAs for cancer therapy and validate lentiviral–shRNA vectors for gene therapy.

KEYWORDS
lentivirus, melanoma, metastasis, mitochondria, mouse, ncRNA

1 | INTRODUCTION

We reported a family of novel non-coding RNAs in human and mouse cells, derived from the mitochondrial genome (mtDNA) (Burzio et al., 2009; Lobos-Gonzalez et al., 2016; Villegas et al., 2000, 2007). These transcripts, termed non-coding mitochondrial RNAs (ncmtRNAs), originate from the bidirectional transcription of the mtDNA. Specifically, segments from the 16S rRNA gene combine by an as yet unknown mechanism to give rise to transcripts that contain long inverted repeats (IRs) and, as a result, double-stranded (ds) structures. The human and mouse sense ncmtRNA (SncmtRNA) is comprised of the H-strand transcript of the 16S gene, joined at its 5′ terminus to a segment from the L-strand transcript of the same gene, forming a molecule that contains a long ds structure (Lobos-Gonzalez et al., 2016; Villegas et al., 2000, 2007). Conversely, the antisense ncmtRNAs (ASncmtRNAs) are composed of the complete L-strand 16S transcript, with an IR of variable length derived from the H-strand transcript (Burzio et al., 2009; Lobos-Gonzalez et al., 2016). We showed by in situ hybridization that the different types of ncmtRNAs are differentially expressed according to proliferative status in human and mouse cells: While SncmtRNA is expressed in all proliferating cells, ASncmtRNAs are readily detectable in normal proliferating cells but downregulated in human and

murine tumor cells from varied tissue origins (Borgna et al., 2017; Burzio et al., 2009; Lobos-Gonzalez et al., 2016). Based on this differential expression profile, ASncmtRNAs appear to act as tumor suppressors because of their downregulation in tumor cells in comparison to normal proliferating cells. Nevertheless, they are detectable by more sensitive techniques, such as RT-PCR and northern blot (Lobos-Gonzalez et al., 2016; Vidaurre et al., 2014), which would suggest that they are playing an essential role in cell viability and are necessary at a minimal intracellular level. This notion is supported by the fact that in vitro knockdown of the ASncmtRNAs (ASK) with chemically modified antisense oligonucleotides (ASOs) induces a massive degree of apoptotic death in tumor cells from both species but does not affect viability of normal cells (Borgna et al., 2017; Lobos-Gonzalez et al., 2016; Vidaurre et al., 2014). In addition, invasive properties of tumor cells are hindered by the in vitro treatment. At the molecular level, the observed effects on viability and invasiveness are mediated by a strong downregulation of survivin and factors associated with epithelial–mesenchymal transition, such as N-cadherin and metallopeptidase-9, among others (Borgna et al., 2017; Lobos-Gonzalez et al., 2016; Vidaurre et al., 2014). These global effects on tumor cell viability and invasiveness exerted by ASK strongly suggest the potential of this approach for the development of an effective and safe therapeutic strategy against different types of cancer.

We translated these results to a preclinical scenario, using the highly aggressive B16F10 murine melanoma cell line in syngeneic C57BL6/J mice. Through different experimental approaches, we observed that injection of an ASO targeted to the murine ASncmtRNAs caused a significant delay in tumor growth and metastasis to the lungs and liver (Lobos-Gonzalez et al., 2016). These results are very promising in light of a potential therapeutic strategy for cancer. However, the half-life of oligonucleotides in the bloodstream and organs is short, making it necessary to administer doses every 2–3 days, in the case of mice (Borgna et al., 2017; Dean & Bennett, 2003; Geary et al., 1997; Iversen, 1991; Lobos-Gonzalez et al., 2016). For this reason, we explored the knockdown strategy using a different delivery method, consisting of lentiviral particles encoding shRNAs directed to the mouse ASncmtRNAs, in the same B16F10 melanoma model, both in vitro and in vivo. Lentivirus-mediated gene therapy possesses the advantage of stability, has been used efficiently in vivo (Gusella, Fedorova, Marras, Klotman, & Klotman, 2002; Naldini, Blomer, Gage, Trono, & Verma, 1996; Naldini, Blomer, Gallay, et al., 1996; Ohmori et al., 2007; Yang, Bailey, Baltimore, & Wang, 2006; Zufferey et al., 1998), and has been shown to be free from obvious side effects in clinical trials (Alton et al., 2017; Connolly, 2002; Escors & Breckpot, 2010; Flight, 2013; Levine et al., 2006; Mcgarrity et al., 2013).

In the present work, we report the efficient knockdown of ASncmtRNAs in B16F10 melanoma cells, both in vitro and in vivo, through the use of an shRNA-encoding lentiviral vector. This approach induced apoptosis, evidenced by DNA fragmentation and phosphatidylserine translocation to the outer layer of the plasma membrane. In vivo, the specific shRNA delayed tumor growth and, more importantly, significantly reduced the degree of lung colonization in a tail vein injection assay, enhancing overall survival in the experimental animals.

Significance

Melanoma is the most aggressive form of skin cancer and continues on the rise worldwide. Once the primary tumor surpasses the skin boundary, the disease develops into the highly metastatic malignant melanoma, with very poor prognosis. We reported that treatment with chemically modified oligonucleotides directed against the antisense non-coding mitochondrial RNAs (ASncmtRNAs) induces apoptotic death and reduction in metastatic properties of human melanoma cells. Our present findings show that lentiviral–shRNA vectors directed against ASncmtRNAs in a mouse melanoma model retard tumor growth and reduce lung colonization, confirming ASncmtRNAs as potent targets for melanoma therapy.

These results offer an additional proof of concept on the ASncmtRNAs as efficient molecular targets in malignant melanoma and open an alternative avenue for the interference of these transcripts in light of the development of a new treatment for this disease.

2 | RESULTS

2.1 | Lentiviral-encoded shRNA targeted to ncmtRNAs induces apoptotic death of B16F10 cells

To determine the optimum in vitro conditions for lentiviral particles assembled with the pLL3.7 vector used in this study, B16F10 cells were transduced with the empty vector, encoding EGFP (Lv-EGFP), under multiplicity of infection (MOI) ranging from 1 to 150 viral particles/cell. Maximal transduction was reached at a MOI of 50 (Figure 1a,b); therefore, we used this MOI for further analysis with shRNA-encoding pLL3.7 vectors. We previously reported that ASO-1537S, targeted to the single-stranded loop region of the human ASncmtRNAs, induced a massive degree of apoptotic cell death, along with a strong reduction of tumorigenic and invasive properties of human tumor cells in vitro (Vidaurre et al., 2014). Therefore, we designed a lentiviral vector encoding a shRNA based on the analogous sequence of the murine ASncmtRNAs (Lv-sh-1560). A second vector was designed encoding another sequence directed to the mouse ASncmtRNAs (Lv-sh-912). As control, we used lentiviral particles (Lv-sh-C) encoding an shRNA directed against luciferase. Transduction of cells with Lv-sh-912 and Lv-sh-1560 induced a significant degree of cell death, evidenced by massive surface detachment (Figure 1c) and increase in Trypan blue (Tb) staining (Figure 1d), compared to controls (NT and Lv-sh-C). As expected, transduction efficiency was similar for all lentiviral vectors used in B16F10 cells (Figure S1). Transduction efficiency essentially depends on the cell line due to differences in sialic acid composition of the plasma membrane among cell types. The higher the sialic acid content, the more the static the repulsion between the cell and the lentiviral particles, which will negatively

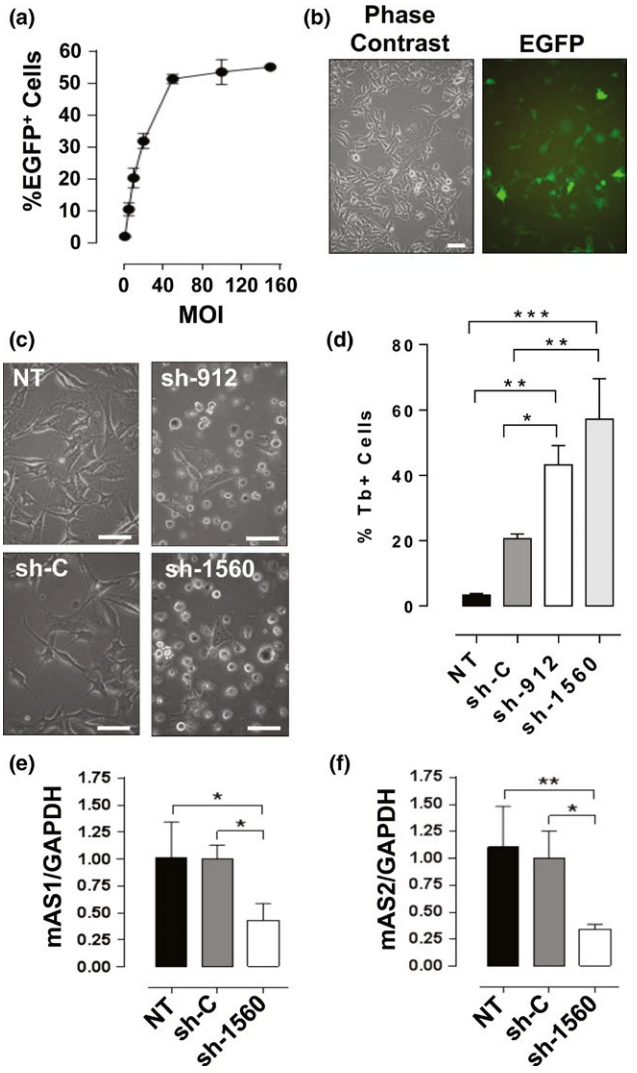

FIGURE 1 In vitro transduction of lentiviral particles in B16F10 cells. (a) B16F10 cells were transduced with Lv-EGFP at multiplicity of infection (MOI) 1–150. At 72 hr, transduction level was determined by flow cytometry. An optimum % of transduced cells was achieved with MOI of 50. (b) Phase contrast and fluorescence image of B16F10 cells transduced with Lv-EGFP at MOI of 50 (bar = 50 μm). (c) Lv-sh-1560 and Lv-sh-912 induced massive detachment from the substrate, compared to controls (bars = 50 μm). (d) Trypan blue exclusion showed significant death of B16F10 cells by transduction of Lv-sh-1560 (55%–60%) and Lv-sh-912 (40%–45%), compared to controls (**$p < 0.01$; ***$p < 0.005$). (e) B16F10 cells were transduced with Lv-sh-1560 or Lv-sh-C or left untreated for 48 hr. Total RNA was extracted, and levels of mouse ASncmtRNA-1 (mAS1) and ASncmtRNA-2 (mAS2) were determined by RT-qPCR. Both ASncmtRNAs were significantly knocked down by transduction with the Lv-sh-1560 (*$p < 0.05$; **$p < 0.01$)

influence transduction efficiency (Davis, Rosinski, Morgan, & Yarmush, 2004; Shimamura, Shibuya, Ito, & Yamagata, 1994; Wallach & Kamat, 1966). The 50% transduction efficiency we obtained for B16F10 cells was not improved by a higher MOI and, moreover, a MOI of 200 or higher induced significant cytotoxicity. Furthermore, 50% transduction efficiency achieved a 60% death rate in comparison with a basal toxicity of 20% induced by sh-C, resulting in a 40% specific death induction by sh-1560, which ultimately translates into an 80% death induction by knockdown of the ASncmtRNAs. For these reasons, we performed all in vitro experiments at a MOI of 50.

As the death rate was higher for Lv-sh-1560, we decided to perform the remaining experiments using this lentiviral vector. Knockdown of the ASncmtRNA-1 (mAS1) and ASncmtRNA-2 (mAS2) at MOI 50 with Lv-sh-1560 was confirmed by RT-qPCR (Figure 1e). Cell death was consistent with apoptosis, as observed by DNA fragmentation (TUNEL assay, Figure 2a,b) and phosphatidylserine translocation to the outer layer of the plasma membrane (Annexin-V staining, Figure 2c).

The observed effects of these shRNA vectors are not exclusive to murine melanoma cells. We transduced human A375 melanoma cells with Lv-sh-912 vector, which encodes an shRNA directed to a common sequence in mouse and human ASncmtRNAs. For both this vector and the control shRNA (Lv-sh-C), a maximum transduction rate of around 95%, was reached at MOI 20 (Figure S2A,B). Under these

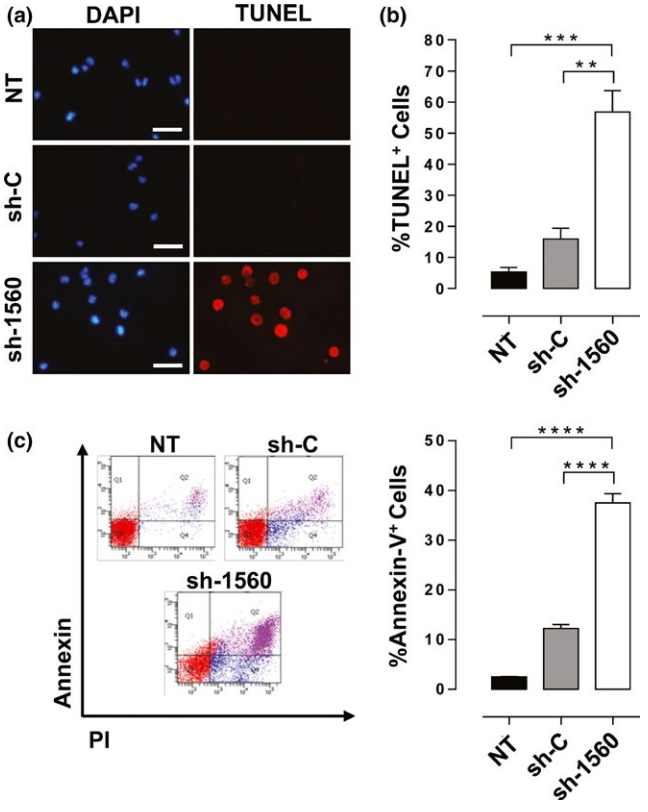

FIGURE 2 In vitro transduction of B16F10 cells with Lv-sh-1560 induces apoptotic cell death. B16F10 cells were transduced with Lv-sh-C or Lv-sh-1560 at a multiplicity of infection of 50 or left untreated for 72 hr. (a) Representative image of TUNEL assay shows significant degree of DNA fragmentation in cells treated with Lv-sh-1560, compared to controls (bars = 50 μm). (b) A triplicate analysis of the experiment in (a) revealed around 55% of TUNEL-positive cells, compared to <15% in controls (**$p < 0.01$; ***$p < 0.005$) (c) Phosphatidylserine translocation, measured by Annexin-V staining was determined by flow cytometry (see Methods). A triplicate analysis revealed almost 40% Annexin-V-positive cells for Lv-sh-1560 treatment, compared to 10% or less in controls (****$p < 0.0001$)

conditions, death rate of cells transduced with Lv-sh-912 was significantly higher at 72 hr than controls, albeit only around 35% (Figure S2C). However, the Annexin-V-positive population (Figure S2D,E) was around 90%, suggesting that the apoptotic process was delayed but under way.

To determine whether the treatment is innocuous to normal cells, we transduced human melanocytes with the same constructs. As for A375 cells (Figure S2), a MOI of 20 achieved a maximum transduction rate, of over 80% in these cells, as evidenced by EGFP expression (Figure S3A,B). In spite of the high transduction efficiency, Lv-sh-912 did not induce cell death over controls (Figure S3C), suggesting that this means of delivery is safe for RNA interference in normal human melanocytes.

2.2 | In vivo knockdown of ncmtRNAs with sh-1560 delays B16F10 tumor growth

To corroborate efficient in vivo transduction of B16F10 tumor cells with the lentiviral vector used in this study, 2 C57BL6/J mice were injected subcutaneously (sc) with 10^6 cells on the right flank. On day 7, mice were injected intratumorally with 4×10^7 transduction units (TU) of lentiviral particles assembled with pLL3.7 (Lv-EGFP) or control particles assembled with the pLKO.1 vector (Lv-empty), which does not encode EGFP. Three days after injection of lentiviral particles, mice were sacrificed and tumors were excised and sectioned. Figure 3a shows a strong fluorescent signal in tumors from mice transduced with Lv-EGFP, compared to those from mice transduced with Lv-empty, confirming entry of the vector and expression of the construct. Therefore, 17 mice were injected sc with 10^6 B16F10 cells as above and when tumors reached an average size of 100 mm^3, mice were randomized into three groups of 5, 6, and 6 mice. The first group was left untreated, while the second and third group received five intratumoral injections of 4×10^7 TU of Lv-sh-C or Lv-sh-1560, respectively, every other day. Tumor growth was monitored, and on day 20, mice were sacrificed and tumors were removed. Figure 3b depicts a significant delay in tumor growth in animals treated with Lv-sh-1560, compared to both controls. Tumors excised on day 20 post-cell injection were smaller (Figure 3c) and weighed less (Figure 3d) than

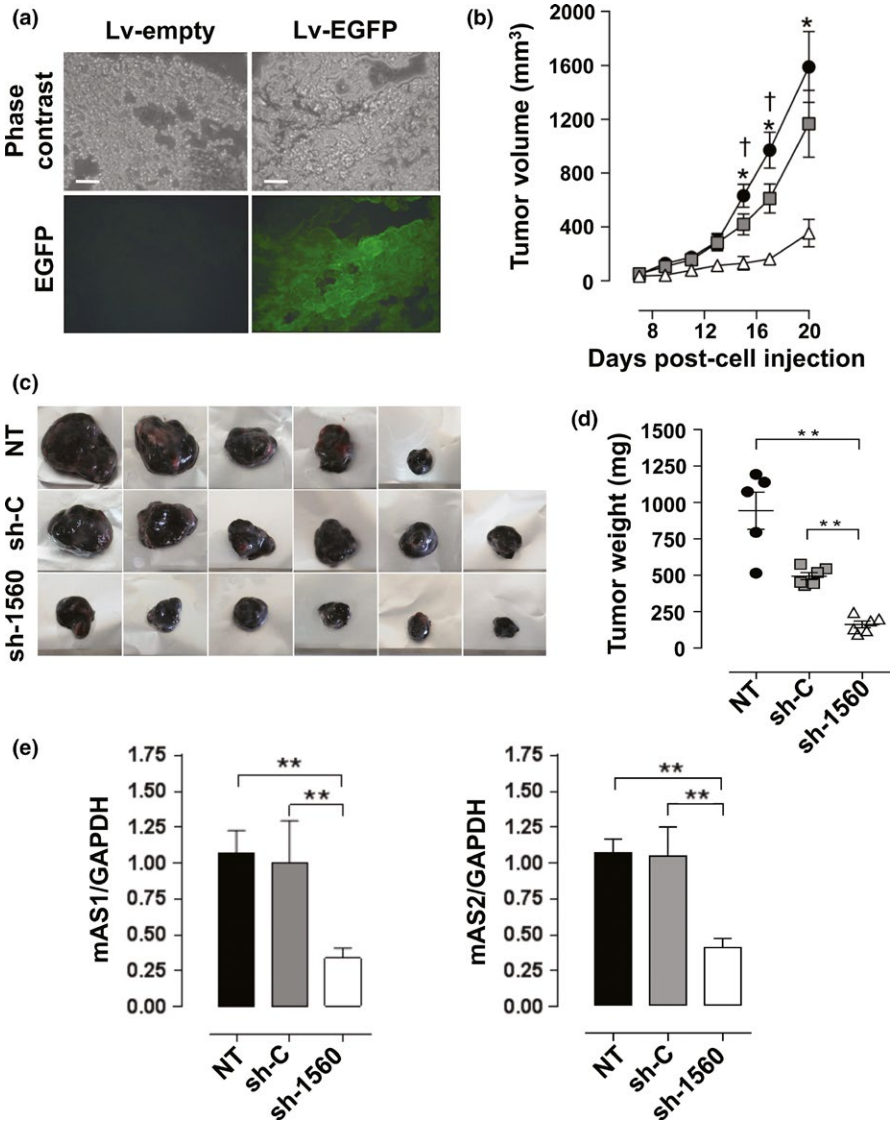

FIGURE 3 In vivo transduction of Lv-sh-1560 induces delay in tumor growth. (a) C57BL6/J mice harboring subcutaneous tumors of about 100 mm^3 were injected once intratumorally with pLL3.7 lentiviral particles encoding EGFP (Lv-EGFP) or pLKO.1 particles devoid of EFGP gene (Lv-empty). Seventy-two hours after viral injection, tumors were excised and sections were analyzed by fluorescence microscopy, showing that EGFP-expressing lentiviral particles were incorporated into tumor cells (bars = 100 μm). (b) Three groups of C57BL6/J mice were injected with Lv-sh-C or Lv-sh-1560, or left untreated, when subcutaneous tumors reached approximately 100 mm^3. Tumor growth was monitored every other day. The graph shows a significant delay in tumor growth rate in animals treated with Lv-sh-1560, compared to controls (NT, sh-C versus 1560, $^*p < 0.05$; NT versus sh-C, $^\dagger p < 0.05$). After animals were euthanized and tumors were excised, showing a significant difference in size (c) and weight ($^{**}p < 0.01$) (d). (e) RT-qPCR performed on total RNA from excised tumors corroborated the in vivo knockdown effect of Lv-sh-1560 on ASncmtRNA-1 (mAS1) and -2 (mAS2) ($^*p < 0.05$; $^{**}p < 0.01$)

those from mice that were not treated (NT) or treated with Lv-sh-C. Knockdown of ASncmtRNA-1 (mAS1) and ASncmtRNA-2 (mAS2) in tumors was confirmed by RT-qPCR (Figure 3e).

2.3 | Lv-sh-1560 treatment in vivo reduces lung colonization of B16F10 cells

To explore whether in vivo knockdown of ncmtRNAs with the lentiviral vector could exert a detrimental effect on metastasis, we performed a lung colonization assay by intravenous injection of B16F10 cells through the lateral tail vein of C57BL6/J mice. For this purpose, 24 mice were injected with 2×10^5 cells. Mice were randomized intro three groups of eight mice each, which were treated by four intravenous injections of 5×10^7 TU of Lv-sh-C or Lv-sh-1560, or saline. On day 14 post-cell injection, mice were sacrificed and lungs were removed and fixed. Figure 4a shows a representative lung from each group, in which a drastic reduction of melanoma nodules is evident in lungs from mice treated with Lv-sh-1560, compared to controls. In consequence, the weight of the lungs from treated mice was significantly lower than controls. Microscopic observation of lung tissue sections evidenced significantly fewer and smaller metastatic foci in animals treated with Lv-sh-1560, compared to controls (Figure 4c,d). A parallel experiment performed as above showed enhanced overall survival rate in mice treated with Lv-sh-1560 (Figure 4e).

3 | DISCUSSION

As reported previously by our group, knockdown of ASncmtRNAs with a specific ASO induces apoptotic death of tumor cells, while leaving normal cells unaffected, suggesting this strategy for the development of an efficient therapeutic approach for cancer (Borgna et al., 2017; Lobos-Gonzalez et al., 2016; Vidaurre et al., 2014). We also reported that, in the syngeneic in vivo B16F10 murine melanoma model, an ASO directed to the mouse ASncmtRNAs delays tumor growth and considerably reduces metastasis, resulting in enhanced survival (Lobos-Gonzalez et al., 2016). Up to date, there are two FDA-approved ASOs, for treatment of cytomegalovirus retinitis (fomivirsen) and familial hypercholesterolemia (mipomersen) (Aartsma-Rus, 2016; McGowan et al., 2012; Vitravene Study Group, 2002a, 2002b), and there are currently many ongoing clinical trials involving ASOs for treatment of different pathologies, including cancer (clinicaltrials.gov). As a therapeutic tool, ASOs are very versatile, adjustable to many molecular targets, and the low toxicity and immunogenicity they have shown in clinical trials show promising therapeutic value, besides representing an inexpensive and highly scalable option for the treatment of diverse diseases. On the other hand, ASOs are short-lived in vivo, making it necessary to administer multiple doses. Viral vectors have the advantage of integrating into the host genome, resulting in robust and long-term transgene expression and, consequentially,

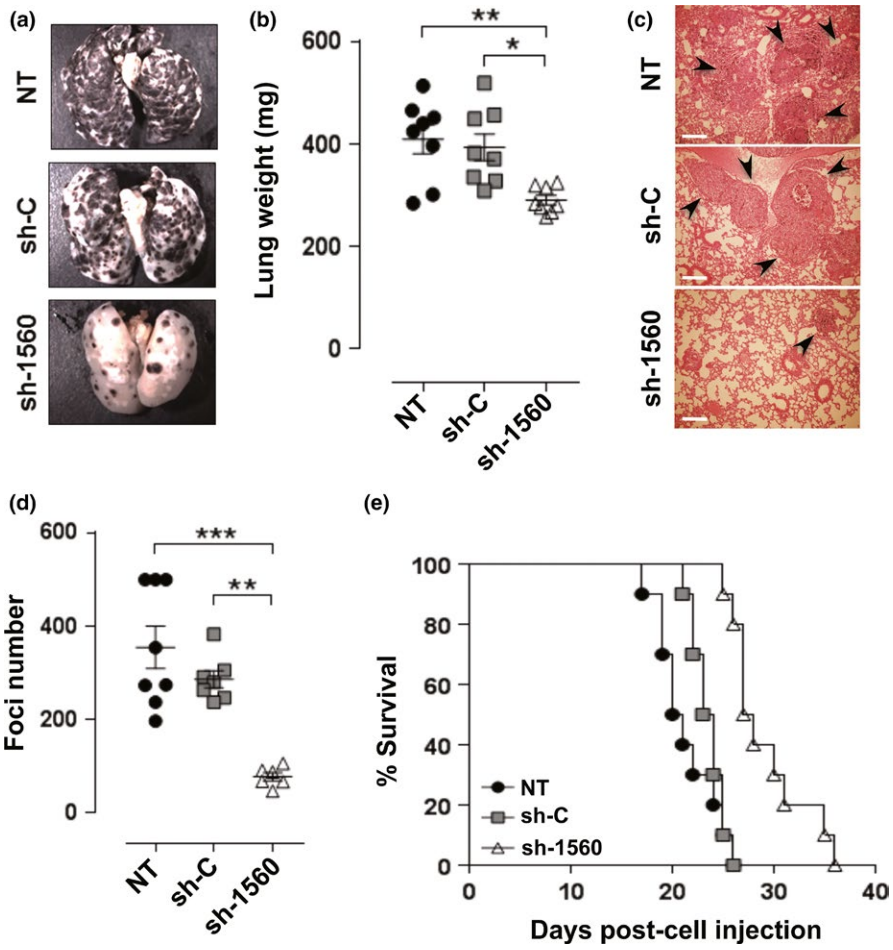

FIGURE 4 Lv-sh-1560 inhibits lung colonization of B16F10 cells. Twenty-four C57BL6/J mice were injected iv with B16F10 cells through the tail vein to induce lung colonization and randomized into three groups of eight mice each. After receiving 4 iv injections of Lv-sh-C or Lv-sh-1560 or being left untreated, mice were euthanized on day 14 post-cell injection and lungs were collected. (a) Representative images of a lung from each group show considerably less metastatic foci in animals treated with Lv-sh-1560, compared to controls, also evidenced by significantly lower weight (b). (c,d) Histological examination (c, arrowheads; bars = 100 μm) and foci counting revealed less and smaller nodules in the treatment group (**$p < 0.01$; ***$p < 0.005$). (e) A parallel tail vein metastasis assay revealed a significant difference in survival of mice treated with Lv-sh-1560, compared to controls. Median survival rates for each group were 20,5 days for NT, 23,5 days for Lv-sh-C, and 27,5 days for Lv-sh-1560 ($p = 0.031$)

sustained therapeutic action (Naldini, Blomer, Gage, et al., 1996; Sugiyama et al., 2005; Waddington et al., 2003; Zhang et al., 2002). Although the first viral vectors developed for gene therapy posed some biosafety concerns, third-generation lentiviruses have been re-engineered to render them non-replicative and self-inactivating (Dull et al., 1998). In clinical trials, these viral vectors have displayed low immunogenicity and insertional genotoxicity (Naldini, 2015). Ongoing clinical trials with this vector are mainly focused on ex vivo transduction of hematopoietical stem cells for treatment of haematological diseases (Naldini, 2015), but there is growing interest in the development of therapeutic strategies for in vivo administration of lentivirus to solid tissues such as liver (Bie et al., 2016), retina (Dalkara, Goureau, Marazova, & Sahel, 2016) and CNS (Hutson, Foster, Moon, & Yanez-Munoz, 2014), albeit at the preclinical stage. In this work, we were able to efficiently deliver shRNA-encoding lentiviral particles to subcutaneous murine melanoma tumors by intratumoral injection. The syngeneic model we used has the advantage of evidencing any immunogenic effect that could be induced by viral infection, which was not perceived in this study. As observed previously with ASO treatment (Lobos-Gonzalez et al., 2016), knockdown of ASncmtRNAs with a complementary sequence derived from the specific shRNA induced significant apoptotic cell death of B16F10 melanoma cells (Figure 1c,d), strongly delayed tumor growth (Figure 3), and inhibited metastasis (Figure 4) in the experimental animals, providing additional proof of concept for the aforementioned transcripts as potent molecular targets for melanoma therapy. Despite the robust results we obtained with ASO, which could be extrapolated to many solid tumors through systemic intravenous injection, some tissues are not readily accessible for drug administration, such as the CNS, which can only be accessed through cerebrospinal fluid, representing an inconvenience in light of multiple-dose treatments. In this respect, lentiviral-based delivery could represent a single-dose therapy which would pose a great advantage. Although in this work we administered four doses in vivo, others have reported robust transgene expression through lentiviral vectors, with single-dose treatments (Annoni et al., 2013; Stocker et al., 2009).

Taken together, our present results provide additional proof of concept that potentiates the ASncmtRNAs as effective targets for melanoma treatment and position lentiviral-based vectors as an alternative therapeutic delivery system for knockdown of these transcripts.

4 | METHODS

4.1 | Cell culture

Murine melanoma cell line B16F10 (CRL-6475), human melanoma cell line A375 (CRL-1619), and human primary epidermal neonatal melanocytes (PCS-200-012) were purchased from ATCC and cultured according to ATCC guidelines. Cells were maintained at 37°C under a 5% CO_2 atmosphere. Upon arrival, B16F10 cells were expanded and frozen in liquid nitrogen at low passage number. After resuscitation, cells were not passaged beyond 6 months. Normal melanocytes were not used beyond passage 6.

4.2 | Construction and production of lentiviral particles encoding shRNAs

Inverted and self-complementary hairpin DNA oligonucleotides encoding short hairpin RNAs (shRNA) were chemically synthesized (Invitrogen) and ligated into HpaI and XhoI sites in the cloning site (downstream of the U6 promoter) of the self-inactivating lentiviral vector pLL3. 7 (Rubinson et al., 2003). For pLL3.7-sh-1560, targeting the ncmtRNAs, DNA oligonucleotides used were 5′ TGCACCCTCTAACCTAGAGAAGTTCAAGAG ACTTCTCTAGGTTAGAGGGTGCTTTTTTC 3′ (for) and 5′ TCGAGAAAAAAGCACCCTCTAACCTAGAGAAGTCTC TTGAACTTCTCTAGGTTAGAGGGTGCA 3′ (rev). For pLL3.7-sh-912, also directed to ncmtRNAs, oligonucleotides used were 5′ TGCCGTGCAAAGGTAGCATAATCTTCAAGAGAGATTATGC TACCTTTGCACGGCTTTTTTC 3′ (for) and 5′ TCGAGAAAAAAGCCGT GCAAAGGTAGCATAATCTCTCTTGAAGATTATGCTACCTTTGCACG GCA 3′ (rev). A control shRNA directed to Luciferase (pLL3.7-sh-C) was generated using primers 5′ TGTTCT CCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCG GAGAACTTTTTTC 3′ (for) and 5′ TCGAGAAAAAAGTTCTCCGAAC GTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAACA (rev). Constructs were analyzed by sequencing to corroborate correct insertion of shRNAs. Lentiviral production was carried out using the calcium phosphate method (Sena-Esteves, Tebbets, Steffens, Crombleholme, & Flake, 2004). Briefly, we cotransfected the lentiviral plasmids pLL3.7-sh-1560 or pLL3.7-sh-C with the packaging vector pΔ8.91 and the envelope vector pVSVg into HEK293T cells. After 60 hours, the resulting supernatant was harvested, centrifuged at 500 × *g* for 5 min at 4°C, and filtered through 0.45-μm cellulose acetate filters to eliminate cell debris. The lentiviral particles were concentrated by ultracentrifugation for 1 hr 15 min at 134,000 × *g* in a Beckman SW41 rotor and resuspended in 100 μl PBS. Viral titer was determined by infecting HEK293FT cells with serial dilutions of concentrated lentivirus, followed by flow cytometric determination (BD FacsCanto II) of EGFP expression 72 hr post-infection. For in vivo experiments, lentiviral suspensions were concentrated by ultrafiltration in 100-kDa Amicon tubes, followed by ultracentrifugation as described above.

4.3 | Cell transduction

To determine the optimal MOI in vitro, 5×10^4 B16F10 cells/well were seeded into 12-well plates (Nunc) and at 8 hr after seeding, cells were transduced with an empty lentiviral particle encoding EGFP, constructed with the pLL3.7 vector (Lv-EGFP), at different MOI (1, 5, 10, 20, 50, 100, and 150). At 72 hr post-transduction, EGFP expression was analyzed by fluorescence microscopy (Olympus BX 51) and flow cytometry (BD FacsCantoII). For shRNA treatment, B16F10 cells were transduced with Lv-sh-1560, Lv-sh-912 or Lv-sh-C lentiviral particles with the MOI selected above. For RNA extraction, cells were harvested by trypsinization at 48 hr post-transduction. Total RNA was extracted using the RNeasy Plus Mini kit (Qiagen),

according to manufacturers' directions. For other analyses, transduction was extended to 72 hr. For human cells, melanocytes or A375 cells were seeded at 5×10^4 cells/well into 12-well plates. At 8 hr after seeding, cells were transduced with Lv-sh-C or Lv-sh-912. At 72 hr post-transduction, cells were harvested and analyzed by flow cytometry to determine the optimal MOI, which was set at 20.

4.4 | Quantitative RT-PCR

DNase treatment of total RNA, cDNA synthesis, and quantitative PCR (qPCR) was carried out as described (Vidaurre et al., 2014). The primers used for qPCR were as follows: for mS, forward primer 5′ AGTCTTTCATCTTTCCCTTGCGGC and reverse primer 5′ AGAAATTCGTACATCTAGGAGC, for mAS1, forward primer 5′ CAATCCAGGTCGGTTTCTATCT and reverse primer 5′ TATATACGTACAACCTTCTCTAGGTTAGAG and for mAS2, forward primer 5′ AATAAGACGAGAAGACCCTATGGAG and reverse primer 5′ TAAACCTAATAACCTTCTCTAGGTTAGAGG. Results were normalized against the murine mRNA of GAPDH, using forward primer 5′ GATGCCCCCATGTTTGTGAT and reverse primer 5′ ATTGTGGAAGGGCTCATGACC.

4.5 | Cell viability and apoptosis

Cell number and viability were determined by Trypan blue (Tb) exclusion, as described before (Vidaurre et al., 2014). DNA fragmentation was evaluated by the in situ cell death detection TMR red kit (Roche), following manufacturer's directions, as described (Vidaurre et al., 2014). Phosphatidylserine exposure was determined by Annexin-V binding using the Annexin-V Pacific Blue conjugate kit (Invitrogen), according to manufacturer's directions, followed by flow cytometry (BD FacsCantoII), as described before (Vidaurre et al., 2014).

4.6 | Animal studies

4.6.1 | Ethics statement

Animal experiments were conducted in accordance with the guidelines of Comisión Nacional de Investigación Científica y Tecnológica (Conicyt), Chile, and approved by the Ethical Committee of Fundación Ciencia & Vida. Male C57BL6/J mice (The Jackson Laboratory) were maintained under specific pathogen-free conditions and used at 6–8 weeks of age (20–30 g).

4.6.2 | Subcutaneous model

For generation of subcutaneous (sc) melanoma tumors, 10^6 B16F10 cells in 100 μl physiological saline were injected into the right flank. Tumor growth was monitored every other day with a caliper, and tumor volumes were calculated on the basis of the formula: tumor volume = L × W2 × 0.5236, where L is mid-axis length and W is mid-axis width. As a first approach, to evaluate whether the lentiviral particles were able to transduce B16F10 cells in solid tumors, 2 C57BL6/J mice were injected sc with B16F10 cells as described above. When tumors reached a volume of about 100 mm^3, around day 7 post-cell injection, mice received 1 intratumoral (it) injection of 4×10^7 TU of lentiviral particles assembled with vector encoding EGFP (pLL3.7) or without EGFP (pLKO.1). At 72 hr post-lentiviral injection, tumors were removed and sectioned and EGFP expression was visualized by fluorescence microscopy (Olympus BX-51). For evaluation of the therapeutic potential of shRNA against the ncmtRNAs, 17 mice were injected sc with B16F10 cells as described above. When tumors reached a volume of about 100 mm^3, around day 7 post-cell injection, mice were randomized into three groups of 6, 6, and 5 mice, which received 5 intratumoral (it) injections of 4×10^7 TU of Lv-sh-C or Lv-sh-1560 on days 7, 9, 11, 13, and 15, or left untreated (NT), respectively. On day 20 post-cell injection, mice were sacrificed and tumors were removed, flash-frozen in liquid N_2, and processed to obtain total RNA.

4.6.3 | Lung colonization model

Twenty-four C57BL6/J mice were injected intravenously (iv) through the lateral tail vein, with 2×10^5 B16F10 cells in 200 μl physiological saline. Mice were then randomized into three groups, which received iv injections of 5×10^7 TU of Lv-sh-C, Lv-sh-1560 or saline, on days 2, 5, 8, and 11. Fourteen days post-cell injection, mice were sacrificed and lungs were removed, weighed, fixed in Fekete's solution (Overwijk & Restifo, 2001), embedded in paraffin and cut into 10-μm sections, which were stained with H&E and analyzed for metastatic foci. In a parallel experiment, metastasis was induced as above and survival rate was determined by natural death or euthanasia when animals showed visible signs of distress.

4.7 | Statistical analysis

All data were expressed as mean ± *SEM*. Statistical analysis was performed using *t* test or ANOVA followed by a multicomparison test when appropriate. Statistical significance was set at $p < 0.05$. Data were analyzed using the GraphPad Prism 6.0 software.

ACKNOWLEDGEMENTS

This work was funded by Universidad Andrés Bello (DI11/11 to MV-G) and Conicyt, Chile (Fondecyt 1110845 and 1140345 to VAB, 1160889 to JT, 11150624 to MV-G, and PFB16 to Fundación Ciencia & Vida).

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How to cite this article: Varas-Godoy M, Lladser A, Farfan N, et al. In vivo knockdown of antisense non-coding mitochondrial RNAs by a lentiviral-encoded shRNA inhibits melanoma tumor growth and lung colonization. *Pigment Cell Melanoma Res*. 2018;31:64–72. https://doi.org/10.1111/pcmr.12615