

## RESEARCH ARTICLE

# Muscle-derived exosomes encapsulate myomiRs and are involved in local skeletal muscle tissue communication

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## Abstract

Exosomes are extracellular vesicles that are released from most cell types encapsulating specific molecular cargo. Exosomes serve as mediators of cell-to-cell and tissue-to-tissue communications under normal and pathological conditions. It has been shown that exosomes carrying muscle-specific miRNAs, myomiRs, are secreted from skeletal muscle cells in vitro and are elevated in the blood of muscle disease patients. The aim of this study was to investigate the secretion of exosomes encapsulating the four myomiRs from skeletal muscle tissues and to assess their role in inter-tissue communication between neighboring skeletal muscles in vivo. We demonstrate, for the first time, that isolated, intact skeletal muscle tissues secrete exosomes encapsulating the four myomiRs, miR-1, miR-133a, miR-133b, and miR-206. Notably, we show that the sorting of the four myomiRs within exosomes varies between skeletal muscles of different muscle fiber-type composition. miR-133a and miR-133b downregulation in TA muscles caused a reduction of their levels in neighboring skeletal muscles and in serum exosomes. In conclusion, our results reveal that skeletal muscle-derived exosomes encapsulate the four myomiRs, some of which enter the blood, while a portion is used for the local communication between proximal muscle tissues. These findings provide important evidence regarding novel pathways implicated in skeletal muscle function.

## KEYWORDS

exosomal cross talk, exosomes, myomiRs, skeletal muscle

**Abbreviations:** C2C12, murine skeletal muscle cell line; C57BL/6, wild-type mouse strain; DMEM, Dulbecco's modified Eagle's medium; EBD, Evans blue dye; EDL, extensor digitorum longus; exo-FBS, exosome-depleted FBS; FBS, fetal bovine serum; GPx, glutathione peroxidase; HIF, hypoxia-inducible factor; HS, horse serum; miR, microRNA; miRNA, microRNA; MnSOD, manganese superoxide dismutase; mRNA, messenger RNA; myomiR, muscle-specific miRNA; P-S, penicillin-streptomycin; PCR, polymerase chain reaction; PFA, paraformaldehyde; ROS, reactive oxygen species; SEM, scanning electron microscopy; TA, tibialis anterior; TRPS, tunable resistive pulse sensing; WGA, wheat germ agglutinin.

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# 1 | INTRODUCTION

Exosomes are membranous nanovesicles with a diameter of 50–150 nm. They are a distinct subpopulation of secretory vesicles and are released from different eukaryotic cell types into the extracellular environment in response to both normal and pathological conditions.<sup>1–3</sup> Exosomes have been detected in human biofluids, such as blood, urine, and saliva, encapsulating a wide range of biological elements including proteins, lipids, RNA, and DNA, which are associated to the exosome-releasing cells.<sup>4–6</sup> Their cargo has, therefore, been characterized as the fingerprint of the producing cells and of their status. Specifically, myoblasts and myotubes have been reported to secrete exosomes with various cargo, exosomes derived from cancerous cells were found to be involved in the metastatic cascade and exosomes from dendritic cells were proved to enhance the cellular immune response.<sup>7–10</sup> Therefore, it is currently well noted that exosomes serve as mediators of cell-to-cell communication via the delivery of their molecular cargo, to neighboring or distant cells and tissues.<sup>5,11–13</sup>

The discovery of microRNAs (miRNAs) within exosomes and confirmation of their functional role in the recipient target cells revealed a novel contribution of these molecules in the communication of tissues.<sup>5,14–16</sup> miRNAs are short, non-coding RNA molecules that regulate the gene expression at a posttranscriptional level, by binding on the 3′ untranslated region (3′UTR) of target mRNAs causing either inhibition of translation or their degradation.<sup>17,18</sup> Although miRNAs are in general found ubiquitously, many of them are expressed in a tissue-specific manner.<sup>19,20</sup> Among many other tissues, a group of miRNAs has been linked with the process of myogenesis and muscle development in skeletal muscles.<sup>20–22</sup> Muscle-specific miRNAs, known as myomiRs, are predominantly expressed in muscle cells and include miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b, miR-486, miR-499a, and miR-499b. Some of these myomiRs were also found to be expressed in other cell types; however, their main function is confined to muscle development and function. Moreover, their expression relies on the activity of transcription factors that are involved in the process of myogenesis.<sup>23–25</sup> Four myomiRs, miR-1, miR-133a, miR-133b, and miR-206, have been extensively studied in relation to their expression and activity in skeletal muscle tissue.<sup>22,26,27</sup> In vitro and in vivo studies showed that miR-1 and miR-206 promote myoblast differentiation and regeneration, whereas miR-133a and miR-133b promote myoblast proliferation and muscle growth.<sup>25,27,28</sup>

It is now well established that many miRNAs are detected at significant levels in body fluids, including serum and plasma, showing an unexpected and remarkable nuclease resistance.<sup>29,30</sup> One of the suggested mechanisms

ascribed to the stability of the circulating miRNAs in blood is their exosomal encapsulation.<sup>4,31,32</sup> Packaging of miRNAs within exosomes constitutes an RNase-protected transportation from the exosome-releasing cells to the receiving target cells. Recently, it was reported that the four myomiRs (miR-1, miR-133a, miR-133b, and miR-206) are encapsulated within exosomes and are detected at higher levels in the blood of muscular dystrophy patients compared to healthy controls.<sup>33,34</sup> Furthermore, isolation of exosomes secreted from skeletal muscle fibers and skeletal muscles cut into small pieces were identified to carry the four myomiRs in recent studies.<sup>7,35,36</sup> However, no information has been provided to date regarding the exosomal sorting of the four myomiRs in skeletal muscle tissues and their potential contribution in communication between near and distant tissues.

In this study, we aim to investigate the direct release of exosomes encapsulating the four myomiRs from different skeletal muscles and to examine their role in the communication between different neighboring skeletal muscle tissues in vivo. Initially, we showed that healthy skeletal muscle tissues located on the hindlimbs secrete exosomes carrying the four myomiRs. In addition, we observed differences in the exosomal sorting and release of the four myomiRs that depend on the skeletal muscle function and the muscle fiber type composition. Finally, we propose a novel mechanism by which skeletal muscle tissues communicate locally through the exosomal route and the transfer of molecular information.

## 2 | MATERIALS AND METHODS

### 2.1 | Experimental healthy mice

Housing and handling of all mice were performed according to the European Directive 2010/63/EE and Cyprus Legislation for the protection and welfare of animals, Laws 1994–2013. Six-week-old male mice on a C57BL/6 background were used for the analysis of the four myomiRs. Mice of the same age were subjected to intramuscular injections in TA muscles with one of the following amounts of anti-miR-133b or scrambled control: 5 µg (600 pmoles), 3 µg (360 pmoles), 1 µg (120 pmoles), 0.2 µg (24 pmoles), and 0.1 µg (12 pmoles). Oligonucleotides were diluted in 10 µL of 0.9% saline. Ten microliters of injection volume was chosen in order to minimize possible leakage of the injected oligonucleotides to nearby tissues. Mice were sacrificed 10 days post-injection for tissue collection. Blood sampling was carried out by puncture of the retro-orbital veins under general anesthesia. All mice were sacrificed by cervical dislocation before muscle collection. Skeletal muscle tissues located on

the hindlimbs (EDL, soleus, TA, gastrocnemius, quadriceps) or forelimbs (triceps) were harvested from each mouse and their weights were recorded for all mice. Connective or fat tissue was carefully removed without injuring any main arteries or veins proximal to the skeletal muscles, to limit the blood contamination.

## 2.2 | Modified oligonucleotides

Modified single-stranded RNAs (antagomiR-133b and scrambled control) were designed and synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Inc, Coralville, IO, USA). The oligonucleotide sequences were as follows:

AntagomiR-133b:

5'-mU\*mA\*mGmCmUmGmGmUmUmGmAmAmG-mGmGmGmAmCmC\*mA\*mA\*mA\*-Chol-3'

Scrambled control:

5'-mG\*mC\*mGmAmCmUmAmUmAmCmGmCmG-mCmAmAmUmAmU\*mG\*mG\*mG\*-Chol-3'

The lower-case letter “m” represents 2'-OMe modification of the following nucleotide, the asterisk (\*) represents phosphorothioate linkage, and “Chol” symbolizes the linkage of cholesterol molecule at the 3' end.

## 2.3 | Evans blue dye experiment

Evans blue dye (EBD) was initially diluted in 1% of PBS stock solution. Prior to intramuscular injections, EBD was further diluted in 0.9% saline at a final concentration of 0.1%. Intramuscular injections of 10, 40, 70, and 100  $\mu$ L (final volume) were performed in TA muscles of 6-week-old male wild-type mice. Mice were euthanatized via cervical dislocation 30 minutes post-injection and five skeletal muscles of the hindlimbs were collected.

## 2.4 | Cell culture

Immortalized murine myoblasts C2C12 were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% of Exosome-Depleted Fetal Bovine Serum (Exo-FBS; System Biosciences, Palo Alto, CA, USA), 1% of antibiotic Penicillin-Streptomycin (P-S; Invitrogen, Carlsbad, CA, USA), and 2% of GlutaMAX (Invitrogen, Carlsbad, CA, USA) in a humidified incubator at 37°C and 5% CO<sub>2</sub>. At ~80% confluency, cells were transfected with 2  $\mu$ g of antagomiR-133b or scrambled control. Cells were harvested and culture medium was collected 48 hours post-transfection. Each experiment was repeated three times.

## 2.5 | Isolation of exosomes

Culture medium was collected and filtrated through 22 $\mu$ m filters to remove any larger particles or cell debris. C2C12-derived exosomes were precipitated using ExoQuick-TC Exosome Precipitation Solution (System Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions. Freshly explanted intact skeletal muscle tissues from mice were incubated in DMEM supplemented with 10% of exo-FBS, 1% of antibiotic P-S, and 2% of GlutaMAX for 24 hours in a humidified incubator at 37°C, and 5% CO<sub>2</sub>. Medium was then collected, passed through 22 $\mu$ m filters, and muscle-derived exosomes were precipitated using ExoQuick-TC Exosome Precipitation Solution according to the manufacturer's instructions. Exosomes were isolated from murine serum samples using ExoQuick Exosome Precipitation Solution (System Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions.

## 2.6 | Scanning electron microscopy

Serum and muscle-derived exosomes were isolated from 6-week-old male mice and subjected to fixation using 4% paraformaldehyde (PFA). Samples were mounted on aluminum specimen stubs and sputtered using gold/palladium (Au/Pd). High-resolution scanning electron microscopic analysis was performed at 20.00 kV in a FEI Quanta 200 microscope and images were processed using the MountainsMap scanning electron microscopy (SEM) Topo Version 7.3 software.

## 2.7 | Tunable resistive pulse sensing analysis

Exosomes were isolated from serum and muscle tissues of 6-week-old mice. The concentration and size distribution of the isolated exosomes were analyzed by tunable resistive pulse sensing (TRPS) using the qNano gold instrument (IZON Science, Oxford, UK). The instrument was set up and calibrated as per the manufacturer recommendations. The nanopore membrane NP200 (A44687, IZON Science, Oxford, UK) was used and was axially stretched to 47.01  $\mu$ m. The apparatus for both calibration and sample measurements was operated at a voltage of 0.38 V and a pressure equivalent to 20 mbar. The exosomes isolated from serum were 10-fold diluted, whereas exosomes derived from muscle tissues were 20-fold diluted. All measurements were calibrated with 210 nm polystyrene beads that were appropriately diluted (1:1000) (IZON Science, Oxford, UK). As particles move through the membrane, they cause a change in the ionic current. This change in the current was recorded and analyzed using the IZON Control Suite software v3.3 (IZON Science,

Oxford, UK). The concentrations of exosomes derived from the five skeletal muscles were obtained from six independent experiments.

## 2.8 | Western blotting

Isolated exosomes were subjected to lysis in lysis buffer (150 mM NaCl, 10 mM Tris-HCl (pH = 7.6), 10% Glycerol, 0.5% Tween, and 10 mM Mercaptoethanol) supplemented with 2X EDTA-free Protease Inhibitor cocktail. Sonication and centrifugation at 4°C of the lysate followed, for cell debris removal. A total of 40–60 µg of protein extracts were incubated overnight at 4°C with anti-CD63 (Abcam, Cambridge, UK), anti-CD81 (Abcam, Cambridge, UK), anti-TSG101 (Santa Cruz Biotechnology, Dallas, TX, USA), and anti-Calnexin (Abcam, Cambridge, UK) primary antibodies, followed by 2-hour incubation at room temperature with anti-mouse IgG or anti-rabbit IgG secondary antibodies conjugated to HRP (Santa Cruz Biotechnology, Dallas, TX, USA). Proteins were visualized using UVP BioSpectrum 810 Imaging System.

## 2.9 | Immunofluorescence

To assess tissue hypoxia, freshly explanted and incubated for 24 hours or 48 hours hindlimbs muscles of 6-week-old mice were snap-frozen in liquid nitrogen-cooled isopentane and stored at −80°C. The frozen tissues were cross-cryosectioned at 10 µm thickness and placed on positive superfrost microscope slides. The tissue sections were incubated with blocking buffer consisting of 5% bovine serum albumin (BSA; Sigma-Aldrich, St. Luis, MO, USA) in 0.1% of PBS-Triton X-100 (PBS-T) for 1 hour. Subsequently, the sections were incubated overnight at +4°C in a humid chamber with the following primary antibodies: mouse monoclonal anti-SOD2 (dilution of 1:500; Abcam, Cambridge, UK) and goat polyclonal anti-GPx (dilution of 1:100; Santa Cruz Biotechnology, Dallas, TX, USA). The sections were next washed three times with 0.1% PBS-T for 5 minutes each and then, incubated at room temperature with the following secondary antibodies: Alexa Fluor 594 anti-mouse IgG and Alexa Fluor 555 anti-goat IgG (dilution of 1:1500; Invitrogen, Carlsbad, CA, USA). Finally, membrane and nuclear counterstaining were carried out with wheat germ agglutinin (WGA), Alexa Fluor 488 conjugate (dilution of 1:100; Thermo Fisher Scientific, Waltham, MA, USA), and Hoechst 33342 (dilution of 1:2000; Invitrogen, Carlsbad, CA, USA), respectively, in PBS for 10 minutes at room temperature, followed by two PBS washes for 5 minutes each. The slides were mounted with DAKO fluorescent mounting medium (DAKO North

America, Inc, Carpinteria, CA, USA) and were examined under an Olympus IX73 fluorescent microscope.

## 2.10 | ATPase assay

ATPase functional assay was performed using the colorimetric ATPase Assay Kit (Abcam, Cambridge, UK) following the manufacturer's instructions. In brief, freshly harvested and 24-hour incubated EDL, gastrocnemius, and quadriceps were homogenized in ice-cold assay buffer. Hundred microliters of aliquots of each sample were incubated with saturated ammonium sulfate (final concentration 3.2 M) for 30 minutes on ice. The pellet was reconstituted in 200 µL of assay buffer and 4 µL of the solution was used to measure the enzymatic activity. Background controls were prepared for all samples.

## 2.11 | Labeling exosomes

TA-derived exosomes were dissolved in 100–200 µL PBS and incubated with the lipophilic dye 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine (DiD; Invitrogen, Carlsbad, CA, USA) to a final concentration 2 µM, in the dark, at room temperature for 30 minutes. The exosomes were then isolated using ExoQuick Exosome Precipitation Solution according to the manufacturer's instructions. The labeled exosomes were washed three times using PBS and finally dissolved in 100 µL of PBS.

## 2.12 | Isolation of muscle fibers and muscle satellite cells

For primary mouse muscle fiber and muscle satellite cell isolation, mouse myofibers were isolated from EDL tissues of 6-week-old mice following the adapted protocol from Rosenblatt and colleagues.<sup>37</sup> In brief, EDL muscles were digested in 0.2% of collagenase type I (Sigma-Aldrich, St. Luis, MO, USA) in DMEM, supplemented with 1% of antibiotic P-S and 2% of GlutaMAX, at 37°C for 1 hour. Single muscle fibers were next purified from fibroblasts and cell debris and transferred to a new prewarmed culture dish coated with 1 mg/mL Matrigel (Corning, NY, USA) in plating medium consisting of DMEM supplemented with 10% of horse serum (HS; Thermo Fisher Scientific, Waltham, MA, USA), 1% of P-S, and 2% of GlutaMAX for 48 hours. The isolated muscle fibers and muscle satellite cells were next incubated with the labeled TA-derived exosomes for 24 hours dissolved in medium consisting of DMEM supplemented with 10% of exo-FBS, 1% of P-S, and 2% of GlutaMAX. The muscle fibers and muscle satellite cells



were next fixed in 4% PFA and counterstained with WGA (dilution 1:200) for the membrane and Hoechst (dilution 1:2000) for the nucleus. The myofibers and the cells were mounted with DAKO mounting medium and left to dry for 30 minutes at room temperature in the dark. Exosomal uptake was assessed by fluorescent microscopy using the Olympus IX73 microscope.

## 2.13 | miRNA analysis

Total RNA, enriched in miRNAs, was extracted from homogenized muscle tissues using the mirVana miRNA Isolation Kit (Invitrogen, Carlsbad, CA, USA) and from exosomes using the Total Exosome RNA & Protein Isolation Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Ten nanograms of the extracted RNA was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real-time PCR amplification was performed using TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA, USA) specific for miR-1, miR-133a, miR-133b, miR-206, and miR-16. miRNA levels were normalized to the endogenous snoR-135 for tissue extractions or cel-miR-39 spike-in control for exosomal extractions. Data analysis was performed using the QuantStudio 7 Flex System Software (Applied Biosystems, Foster City, CA, USA).

## 2.14 | Statistical analysis

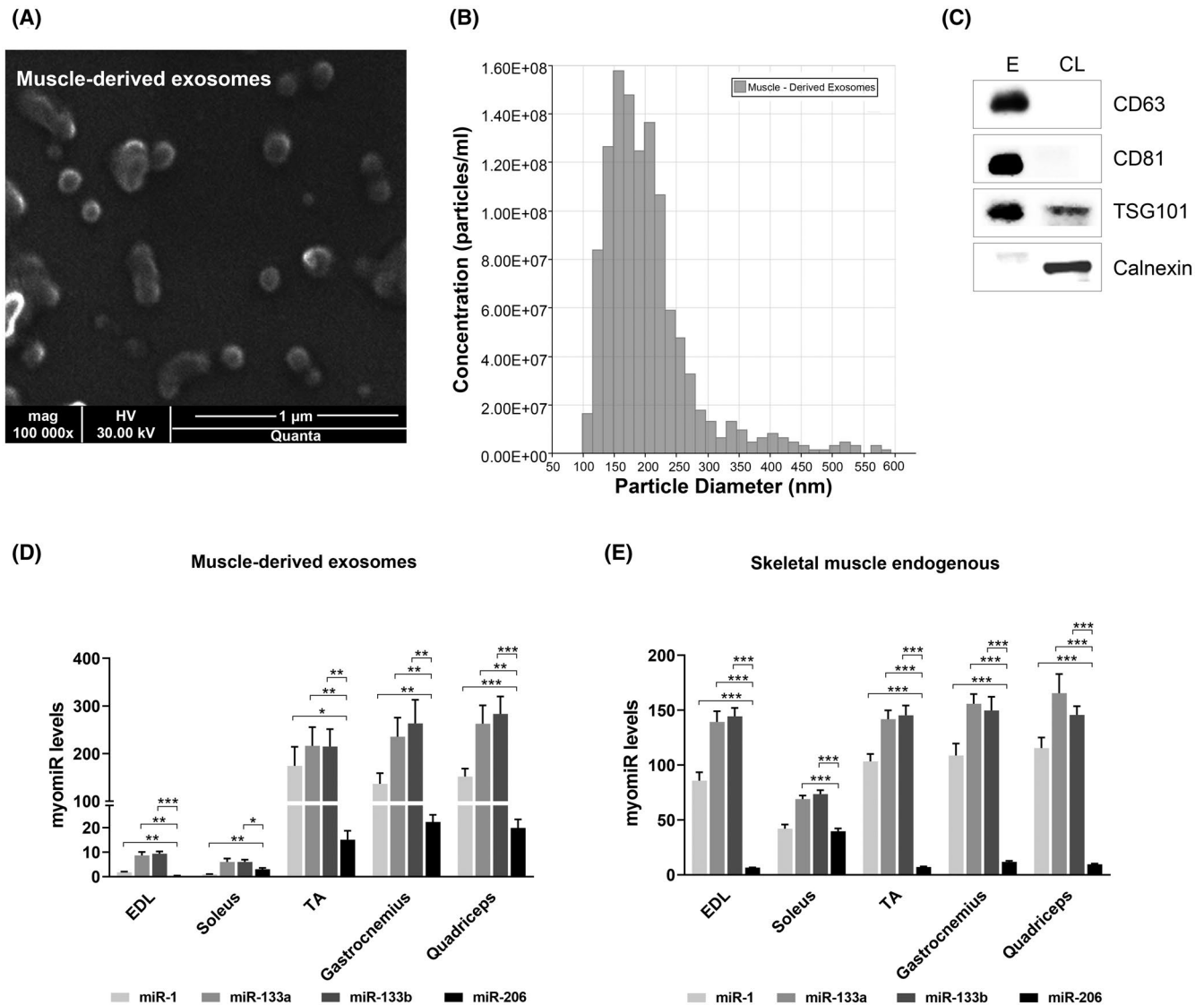
The expression and exosomal levels of the four miRNAs from six different tissues (five skeletal muscles and serum) and the ATPase activity levels from three muscles were acquired from six independent experiments ( $n = 6$ ). Any significant differences between the four miRNA levels within the skeletal muscles, muscle-derived exosomes, and serum exosomes were initially determined by one-way ANOVA analysis for each tissue separately. ANOVA was accompanied by post hoc two-sample t test with unequal variance or equal variance to further evaluate the differences in endogenous and exosomal levels between the miR-206 and the other three miRNAs. Additionally, assessment of differences in endogenous and exosomal miRNA levels between the anti-miR-133b-injected mice and the scrambled control group was carried out using the two-sample t test with unequal or equal variance, on normalized data. The equality of variance for the t test was evaluated using the F-test for all sets of data. In all cases, a value of  $P < .05$  was considered statistically significant. Correlation between endogenous levels, muscle-derived exosomal levels, and serum exosomal levels were calculated using the Pearson's correlation coefficient.

# 3 | RESULTS

## 3.1 | Skeletal muscle tissues secrete exosomes encapsulating the four myomiRs

Currently, very few studies have been conducted on muscle-derived exosomes, employing muscle cell lines, and pieces of skeletal muscles incubated in culture medium.<sup>7,35,36,38</sup> Although it has been reported that a number of miRNAs, including myomiRs, are selectively incorporated within exosomes released from myoblasts, myotubes, and cultured isolated myofibers, there is no information regarding the release of exosomal myomiRs directly from intact skeletal muscle tissues.<sup>7,38</sup> Five skeletal muscles on the hindlimbs of wild-type 6-week-old male mice were collected and incubated in culture medium to release exosomes. The tissues were excised from the tendons, where possible, and carefully separated from surrounding muscles and other tissues. Extracellular vesicles enriched in exosomes were, thereafter, isolated for the first time from the culture medium.

Prior to the investigation of the exosomal release from the five skeletal muscles, we initially examined whether the incubation of the tissues within culture medium causes any level of hypoxia to the muscle tissues which consequently may interfere with the exosomes' production, cargo sorting, and release. Little information exists regarding the direct effect of tissue hypoxia on exosomes and particularly on muscle-derived exosomes. To assess the possibility that the incubated five skeletal muscles are hypoxic, immunofluorescence was carried out against two antioxidant enzymes, manganese superoxide dismutase (MnSOD) and glutathione peroxidase (GPx) on fresh (controls), 24-hour and 48-hour incubated skeletal muscle tissues. These two enzymes protect the intracellular environment and maintain normal tissue function.<sup>39,40</sup> Furthermore, they have been associated to hypoxia-inducible factors (HIF), rendering them as suitable markers for assessing the degree of hypoxia in the incubated skeletal muscles.<sup>41,42</sup> Our results revealed that the 24-hour incubated muscle sections show small deviation from the fresh sections regarding the expression of the two enzymes, suggesting that hypoxia had minor influence on the intracellular functions (Figure S1A,B). In contrast, immunofluorescent detection of the two enzymes in the 48-hour incubated muscle sections was greatly lower compared to the controls implying that these muscles are characterized by high levels of hypoxia (Figure S1A,B). To further examine whether the incubated muscle tissues are affected by hypoxia, the ATPase activity was measured. Sufficient energy metabolism is critical for normal cellular functions, including exosomal biogenesis and secretion. ATPase assay was performed for muscle tissues of different sizes, EDL (smallest), gastrocnemius, and quadriceps (largest), either fresh (controls) or incubated for 24 hours in culture medium. A small decrease of ATPase



**FIGURE 1** Muscle-derived exosomes isolated from intact skeletal muscle tissues encapsulate the four myomiRs. Five skeletal muscles, EDL, soleus, TA, gastrocnemius, and quadriceps, located on the hindlimbs of 6-week-old C57BL/6 male mice ( $n = 6$ ) were dissected and incubated in exosome-free culture medium. The successful isolation of exosomes was confirmed by SEM, TRPS, and western blot analysis. A, SEM images at 100 000 $\times$  magnification revealed spherical structures. B, TRPS analysis showed that muscle-derived exosomes have 132 nm mean diameter. C, Western blot analysis of the exosomal markers CD63, CD81, and TSG101 of the muscle-derived exosomal lysates. Calnexin was also examined as a negative control. Murine C2C12 lysates were used as negative controls. Abbreviations: E: exosomes; CL: cell lysates. D, Muscle-derived exosomes were isolated from five hindlimbs skeletal muscles and analyzed for the presence of the four myomiRs. Exosomes derived from EDL, TA, gastrocnemius, and quadriceps encapsulate the high levels of miR-1, miR-133a, and miR-133b and very low levels of miR-206. Soleus-derived exosomes carry higher levels of miR-206 similar to the levels of miR-1. E, Total RNA, including small RNA, was extracted from the five skeletal muscle tissues. EDL, TA, gastrocnemius, and quadriceps express higher levels of miR-1, miR-133a, and miR-133b compared to soleus. miR-206 levels are lower compared to the other three myomiRs in EDL, TA, gastrocnemius, and quadriceps. Only soleus expresses comparable levels of miR-206 to miR-1. The normalized data were analyzed by t test. Data illustrated on D and E are mean values  $\pm$  SEM after normalization. \* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$

activity was detected in the incubated tissues compared to controls (Figure S2). All the above results strongly imply that exosomes produced and released during the 24 hours incubation of the five skeletal muscles are characterized by limited hypoxia and, therefore, the isolation of exosomes from the incubated muscles is not caused or affected by the hypoxic conditions.

Exosomes can be distinguished from other groups of extracellular vesicles by their distinct physical properties, including their small size range, spherical morphology, and unique biochemical membrane composition with specific proteins.<sup>3,12,32</sup> The successful isolation of exosomes was verified using SEM, TRPS, and western blot analysis of exosomal markers (Figure 1A-C). SEM confirmed the

purity of exosomes and their rounded structures. Analysis of the SEM micrographs demonstrated the purity of spherical vesicles (Figure 1A). TRPS analysis showed that the isolated muscle-derived exosomes have a mean diameter of 132 nm and a mode diameter of 113 nm (Figure 1B), which are in line with the established dimensions of exosomes.<sup>43-45</sup> The isolation of exosomes from skeletal muscles was further validated by the detection of three commonly used exosomal protein markers. The exosomal markers CD63, CD81, and TSG101 were detected in the exosomes samples at higher amounts compared to whole-cell lysate extracts (Figure 1C).

Following confirmation of the successful exosomal isolation from the five skeletal muscles, we proceeded with the extraction and detection of the four myomiRs within muscle-derived exosomes. Real-Time PCR analysis revealed that miR-1, miR-133a, miR-133b, and miR-206 are encapsulated within exosomes derived from all five skeletal muscles (Figure 1D). The four myomiR levels were found significantly different within the five muscle-derived exosomes separately ( $P < .01$ , one-way ANOVA). In particular, EDL, TA, gastrocnemius, and quadriceps secreted exosomes enriched in miR-1, miR-133a, and miR-133b, whereas the detection of miR-206 was found to be much lower ( $P < .05$ ; Figure 1D). In contrast, soleus-derived exosomes displayed a different miRNA pattern encapsulating higher levels of miR-133a, miR-133b, and miR-206 compared to miR-1 (Figure 1D). In addition, gastrocnemius and quadriceps, which are the two heaviest skeletal muscles examined, released the highest levels of exosomal myomiRs, whereas EDL and soleus which are the two lightest muscles secreted the lowest levels of myomiRs (Table 1; Figure 1D).

The endogenous myomiR levels expressed in the five skeletal muscle tissues were next analyzed and compared to the corresponding exosomal levels. Real-Time PCR analysis revealed a similar endogenous myomiR pattern to that observed with muscle-derived exosomal myomiR levels. Each skeletal muscle expresses significantly different myomiR levels ( $P < .001$ , one-way ANOVA). Particularly, endogenous miR-1, miR-133a, and miR-133b levels were significantly higher compared to miR-206 in EDL, TA, gastrocnemius, and quadriceps muscles ( $P < .001$ ; Figure 1E). In contrast, the endogenous miR-206 levels in soleus tissue were found to be elevated relative to the other four skeletal muscles (Figure 1E). Considering the endogenous myomiR levels within the five skeletal muscles, EDL, TA, gastrocnemius, and quadriceps displayed similar levels, whereas soleus presented the lowest amounts (Figure 1E).

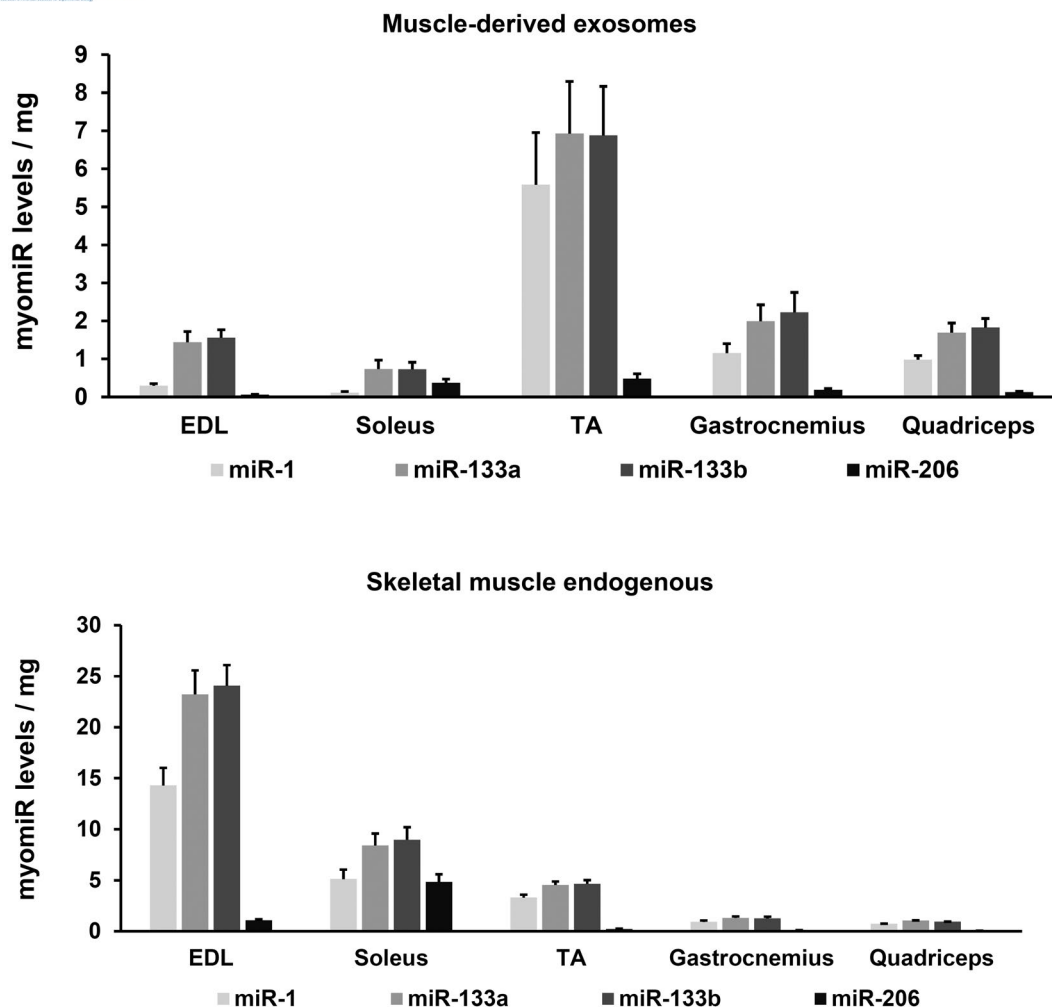
### 3.2 | Muscle-derived exosomal and endogenous myomiR levels vary between skeletal muscles

Skeletal muscle tissues vary in size and weight and they are composed of different muscle fiber types. Specifically, EDL and soleus muscles are the two lightest and smallest tissues, whereas quadriceps muscle is the heaviest one (Table 1). In addition, EDL, TA, gastrocnemius, and quadriceps muscles are mainly composed of the same glycolytic fiber type IIB, whereas soleus muscle is predominately made of oxidative type I. More importantly, the function of each skeletal muscle can be attributed to its location and connection with other tissues.<sup>46-48</sup> The association of the endogenous and the muscle-derived myomiR levels with the different properties of each skeletal muscles was, therefore, next examined. Primarily, muscle-derived exosomal myomiR levels were correlated with the weight of each muscle revealing differences between the examined skeletal muscles. TA-derived exosomes were identified to encapsulate higher levels of miR-1, miR-133a, and miR-133b per muscle weight compared to the other four muscles (Figure 2A). Exosomes derived from EDL, gastrocnemius, and quadriceps carried similar amounts of the four myomiRs, despite their weight difference and soleus-derived exosomes were found to encapsulate the lowest levels of the three myomiRs per weight (Figure 2A). In order to investigate whether these observations are due to the different number of exosomes or to the myomiR loading within the exosomes, the number of exosomes secreted by the five skeletal muscles was analyzed. TRPS analysis revealed that all five skeletal muscles secrete similar amounts of exosomes during their 24-hour incubation in culture media, suggesting that exosomes' number is independent of the size of the muscle tissue (Figure S3A). Subsequently, it is demonstrated that TA, gastrocnemius, and quadriceps encapsulate more myomiR levels within their exosomes compared to the smallest tissues EDL and soleus (Figure S3B).

Extensive studies have been conducted regarding the expression and function of miR-1, miR-133a, miR-133b, and miR-206 in muscle with the majority of them focusing on muscle development and the process of myogenesis.<sup>22-27</sup> Currently, there is little information regarding their expression with the different muscle types. Following the correlation of muscle-derived exosomal myomiR levels with the different skeletal muscles, the endogenous myomiR levels were also associated to muscle weight. The results of the endogenous levels of the four myomiRs related to skeletal muscle weights revealed a different pattern from

**TABLE 1** Skeletal muscle weights  $\pm$  SEM (mg)

EDL	Soleus	TA	Gastrocnemius	Quadriceps
6.0 $\pm$ 0.3	8.2 $\pm$ 0.9	31.2 $\pm$ 0.6	118.3 $\pm$ 6.8	155.1 $\pm$ 11.1



**FIGURE 2** Endogenous and exosomal myomiR levels from skeletal muscles are associated to the tissue. The endogenous and exosomal muscle-derived levels of the four myomiRs were further normalized to the skeletal muscle weight. A, TA-derived exosomes carry higher levels of the four myomiRs per mg compared to EDL, soleus, gastrocnemius, and quadriceps. Gastrocnemius and quadriceps display less than half exosomal myomiR levels per mg compared to TA-derived myomiRs. Soleus presents the lowest levels of secreted myomiRs per mg. B, Endogenous myomiR levels per mg of tissue are reversely correlated to the weight of the skeletal muscles. The two lightest skeletal muscles EDL and soleus display the highest myomiR levels per mg, whereas the two heaviest tissues gastrocnemius and quadriceps show the lowest levels per mg

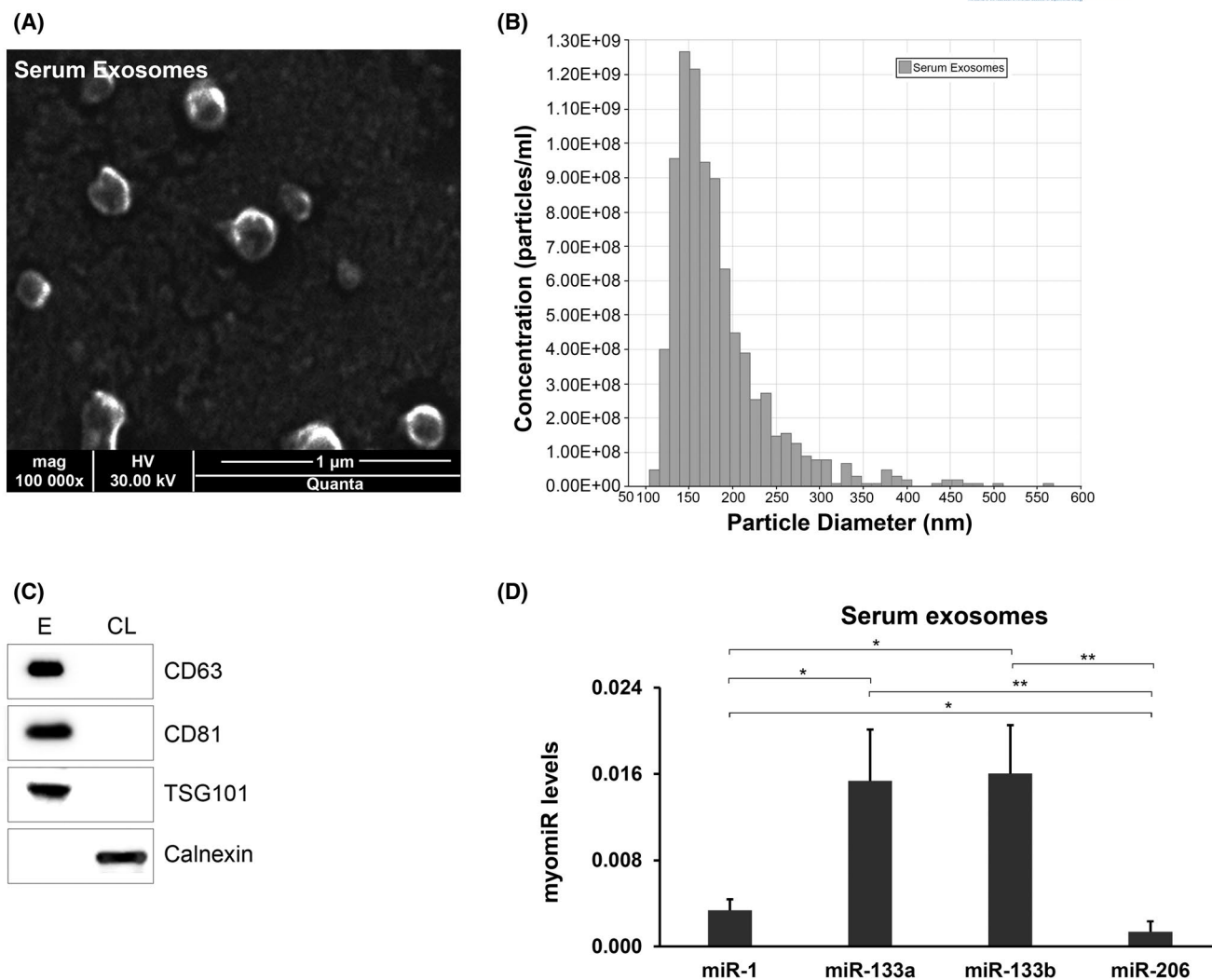
the corresponding muscle-derived analysis (Figure 2B). Notably, EDL, which is the lightest skeletal muscle, was found to express the highest levels of the four myomiRs per muscle weight (Figure 2B). Moreover, the two heaviest muscles under investigation, quadriceps and gastrocnemius, displayed the lowest myomiR levels per mg of tissue. Furthermore, even though soleus was found to secrete the lowest amounts of exosomal myomiRs per mg, it expressed endogenously higher myomiR levels compared to TA, gastrocnemius, and quadriceps (Figure 2).

### 3.3 | Exosomes encapsulating myomiRs are circulating in the serum of mice

The produced exosomes released by tissues are used as mediators of communication between neighboring tissues or

distant tissues via entering the blood circulation.<sup>11,49</sup> It has been previously shown that, in response to disease conditions, exosomal myomiR levels are elevated in the blood of patients and animal models compared to controls.<sup>33,34</sup> There is no evidence however, regarding the transport of exosomal myomiRs from skeletal muscle tissues to the blood circulation under normal conditions in mice. Such evidence will provide novel insights on the extracellular purpose of muscle-derived exosomal myomiRs. Exosomes were isolated from the serum of 6-week-old wild-type male mice and their successful isolation was confirmed using SEM, TRPS analysis, and western blotting. SEM images demonstrated spherical structures with diameter from 120-180 nm (Figure 3A). Analysis of TRPS data revealed that serum exosomes have a mean diameter of around 130 nm and mode diameter around 109 nm (Figure 3B). Western blot against the exosomal markers further verified the successful isolation of exosomes from serum



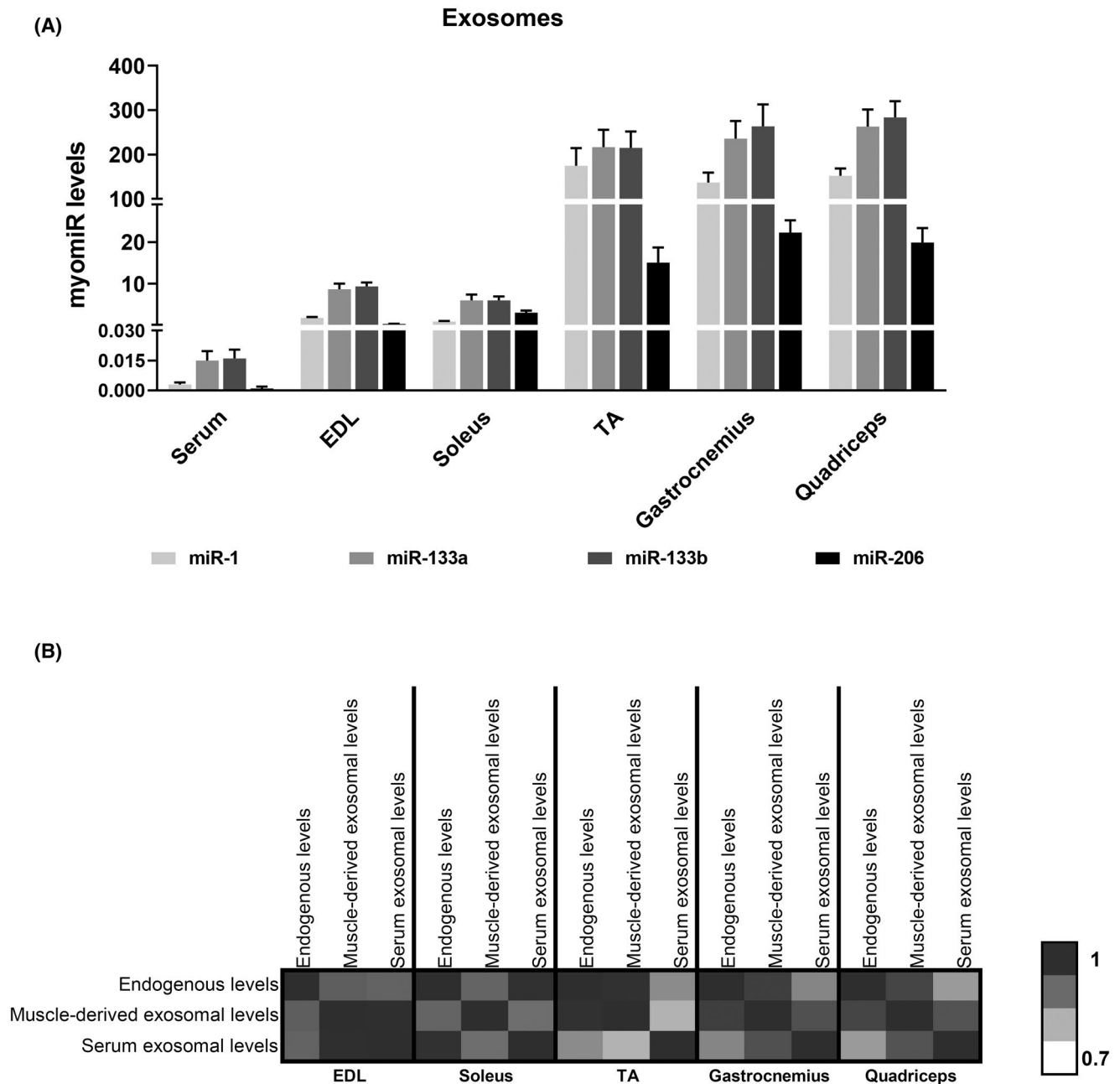


**FIGURE 3** Exosomes circulating in the bloodstream of wild-type mice encapsulate the four myomiRs. Blood was collected from 6-week-old C57BL/6 male mice ( $n = 6$ ) and exosomes were isolated from 100  $\mu$ L serum. Successful isolation of exosomes was confirmed with SEM, TRPS, and western blot analysis. A, SEM micrographs were obtained at 100,000x magnification showing vesicles with round structures. B, TRPS data analysis revealed mean diameter of around 130 nm. C, Western blot analysis of the exosomal markers CD63, CD81, and TSG101 of the exosomal lysates isolated from serum samples. Calnexin was also analyzed as negative control. Murine C2C12 lysates were used as negative controls. Abbreviations: E: exosomes; CL: cell lysates. D, Total RNA, including small RNA, was extracted from the exosomes isolated from serum. Serum exosomes encapsulate higher levels of miR-133a and miR-133b levels compared to miR-1 and miR-206. The normalized data were analyzed by unpaired t test. Data are mean values  $\pm$  SEM after normalization. \* $P < .05$ ; \*\* $P < .01$

(Figure 3C). Following the successful isolation of serum exosomes, the levels of the four encapsulated myomiRs were analyzed. All four myomiRs were found significantly different within serum exosomes ( $P < .001$ , one-way ANOVA). Notably, serum exosomal miR-133a and miR-133b levels were identified to be significantly increased ( $P < .05$ ) compared to miR-1 and miR-206, and miR-1 was detected significantly greater from miR-206 ( $P < .05$ ; Figure 3D).

Currently, there is no information associating the skeletal muscle endogenous myomiR levels with the exosomal myomiR levels secreted by muscle tissues and the exosomal myomiR levels circulating in the bloodstream. The levels of exosomal myomiRs released from skeletal muscle tissues

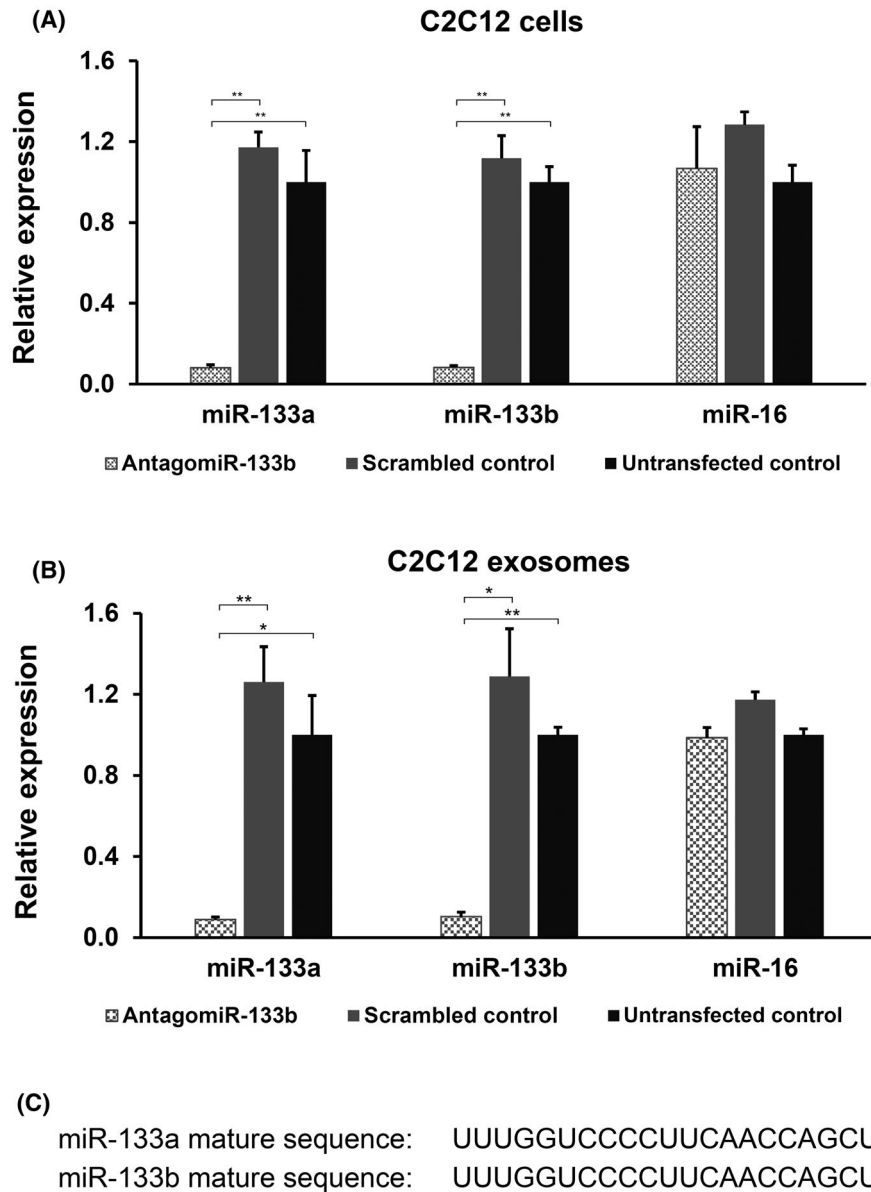
and the levels of serum exosomal myomiRs were, therefore, further analyzed. The exosomal levels of the four myomiRs in serum were found considerably lower compared to their exosomal levels derived from skeletal muscles (Figure 4A). Due to the different normalization used for the endogenous and exosomal levels of the four myomiRs, their direct comparison was not possible. However, similar biological behavior characterizing the four endogenous myomiR levels in skeletal muscles to their exosomal levels secreted from skeletal muscles and their exosomal levels circulating in blood were observed (Figures 1D,E and 3C). In order to assess the biological correlation between the endogenous and exosomal myomiR levels, Pearson's correlation coefficients were



**FIGURE 4** Muscle-derived exosomal myomiRs are strongly correlated to the circulating exosomal and the endogenous myomiR levels. A, Serum exosomal myomiR levels are much lower levels compared to the myomiR levels detected within exosomes derived from the five skeletal muscle tissues. B, Pearson's correlation coefficients were calculated for endogenous, muscle-derived exosomal, and serum exosomal myomiR levels. All four myomiRs display strong correlation for each group. In particular, endogenous and muscle-derived exosomal myomiR levels present the strongest correlation ( $r > .94$ ), whereas endogenous and serum exosomal myomiR levels the weakest correlation ( $r \sim .83$  on average), except from soleus-derived exosomes which present almost perfect correlation with serum exosomal myomiRs. The color scale indicates the degree of correlation

calculated. Strong correlation was observed in the levels of the four myomiRs expressed endogenously in skeletal muscle tissues with their levels encapsulated within muscle-derived exosomes and serum exosomes (Figure 4B). In particular, in all five skeletal muscles, the endogenous and muscle-derived exosomal myomiR levels displayed very strong

correlation ( $r > .90$ ). In addition, the endogenous levels of the myomiRs in soleus were found to be completely correlated with the serum exosomal myomiRs ( $r > .99$ ), whereas for TA, gastrocnemius, and quadriceps the corresponding correlation coefficient was lower ( $r \approx .85$  on average; Figure 4B). Furthermore, EDL-derived exosomal and serum exosomal



**FIGURE 5** In vitro transfection of antagomiR-133b reduces the exosomal levels of miR-133a and miR-133b. C2C12 cells ( $n = 3$ ) were transfected with antagomiR-133b for 48 hours. A and B, Endogenous and exosomal levels of miR-133a and miR-133b were decreased by approximately 90% in C2C12 myoblasts transfected with antagomiR-133b compared to scrambled control and untransfected cells. miR-16 was served as negative control showing no difference between antagomiR-133b transfected cells and scrambled control or untransfected cells. The normalized data were analyzed by t test. Illustrations represent relative expression of miRNA levels compared to untransfected cells. Data are means  $\pm$  SEM. \* $P < .05$ ; \*\* $P < .01$ . C, miR-133a and miR-133b have almost identical nucleotide sequences. They are different by only one nucleotide at the 3' end

myomiR levels were completely correlated ( $r > .99$ ), while TA-derived exosomal myomiRs displayed the lowest correlation coefficient with serum exosomal myomiRs ( $r \approx .80$ ; Figure 4B). High Pearson's correlations support the biological mechanism through which the endogenously expressed myomiRs are packaged within exosomes in skeletal muscles and subsequently secreted into the extracellular environment. More importantly, strong correlation with the circulating exosomal myomiRs implies that exosomes carrying myomiRs in the bloodstream originated from the skeletal muscle tissues.

### 3.4 | AntagomiR-133b downregulates the exosomal levels of miR-133b in vitro

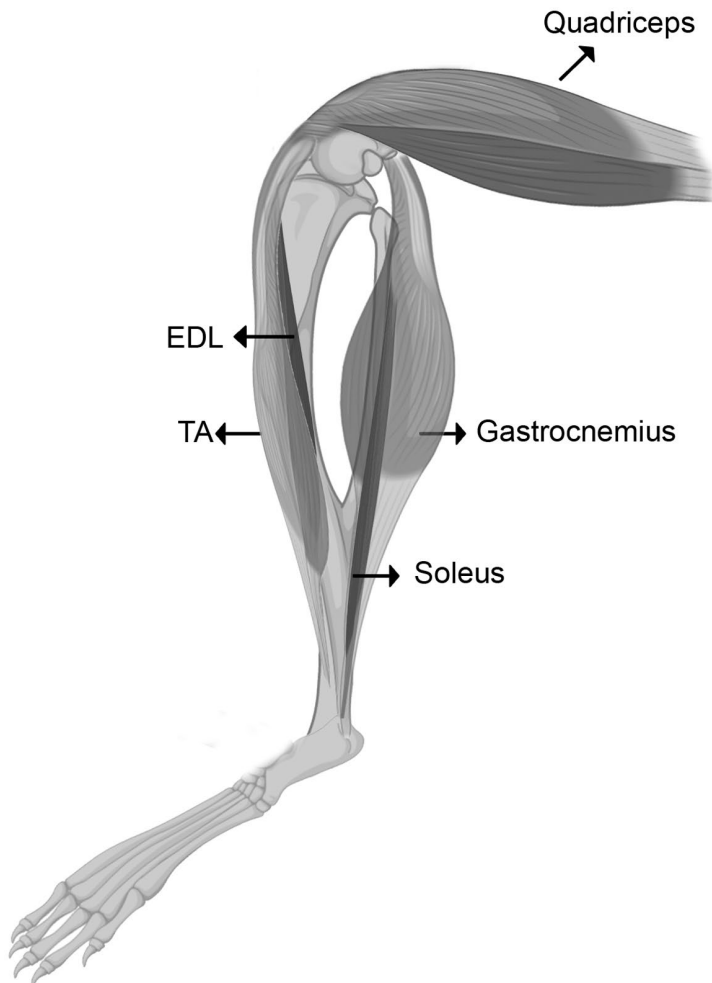
MyomiRs were found to be encapsulated within exosomes circulating in the blood of patients and mice. However, there is no evidence validating their direct release from skeletal muscle tissues. In order to investigate whether serum exosomes carrying myomiRs were directly produced and released by skeletal muscles, we proceeded to assess the effect of downregulating the endogenous myomiR levels

on their exosomal levels. AntagomiR-133b or scrambled control transfections were initially carried out on proliferating murine skeletal myoblasts cell cultures. Endogenous miR-133b levels were significantly reduced in the antagomiR-133b transfected cells compared to the control cells ( $P < .01$ ; Figure 5A). Moreover, the antagomiR-133b transfected cells were found to release significantly lower levels of miR-133b encapsulated within exosomes compared to the controls ( $P < .05$ ; Figure 5B). miR-133a sequence differs from miR-133b by only one nucleotide at the 3' end and, therefore, the possibility of a simultaneous effect occurring on the levels of miR-133a was also evaluated<sup>25</sup> (Figure 5C). As expected, the endogenous and exosomal levels of miR-133a were also significantly reduced ( $P < .01$  and  $P < .05$ , respectively), in antagomiR-133b transfected cells compared to the controls (Figure 5A,B). miR-16 was used as a control miRNA and its levels remained unchanged in all the in vitro experiments. Statistical significance of the transfection results was also confirmed with one-way ANOVA analysis between the antagomiR-133b transfected, scrambled control transfected, and untransfected cells results for each miRNA ( $P < .001$ ).

### 3.5 | AntagomiR-mediated reduction of miR-133b and miR-133a in TA muscle

In vivo experiments were next performed to investigate the exosomal transfer of miR-133b and miR-133a from skeletal muscle tissue to a neighboring muscle tissue in a wild-type mouse model. Intramuscular injections of antagomiR-133b or scrambled control were performed in the TA muscles of male wild-type mice. To evaluate the optimal injection volume with marginal leakage in surrounding tissues, intramuscular injections of different volumes of EBD in TA muscles were carried out (Figures 6 and S4). Thirty minutes post-injection, all the five hindlimb skeletal muscles were collected and visually evaluated for the range of the dye leakage. Ten microliters of administrated volume was found to be the optimal amount with minimum leakage, affecting only the EDL muscle which is fully attached to the injected TA (Figure 6).

Six-week-old wild-type male mice were next intramuscularly injected with 5  $\mu$ g of antagomiR-133b or scrambled control in both TA skeletal muscles. TA muscles were collected 10-days post-injection and analyzed for the levels of



**FIGURE 6** Neighboring skeletal muscles located on the mouse hindlimbs. Schematic diagram of the mouse hindlimbs depicting the five skeletal muscles. EDL muscle is attached to the TA muscle located on the front of the lower part of the leg. Soleus is adjacent to the gastrocnemius and are both located on the back part of the lower leg. Quadriceps is a group of muscles located on the front of the upper leg and is the most distant regarding the other four muscles

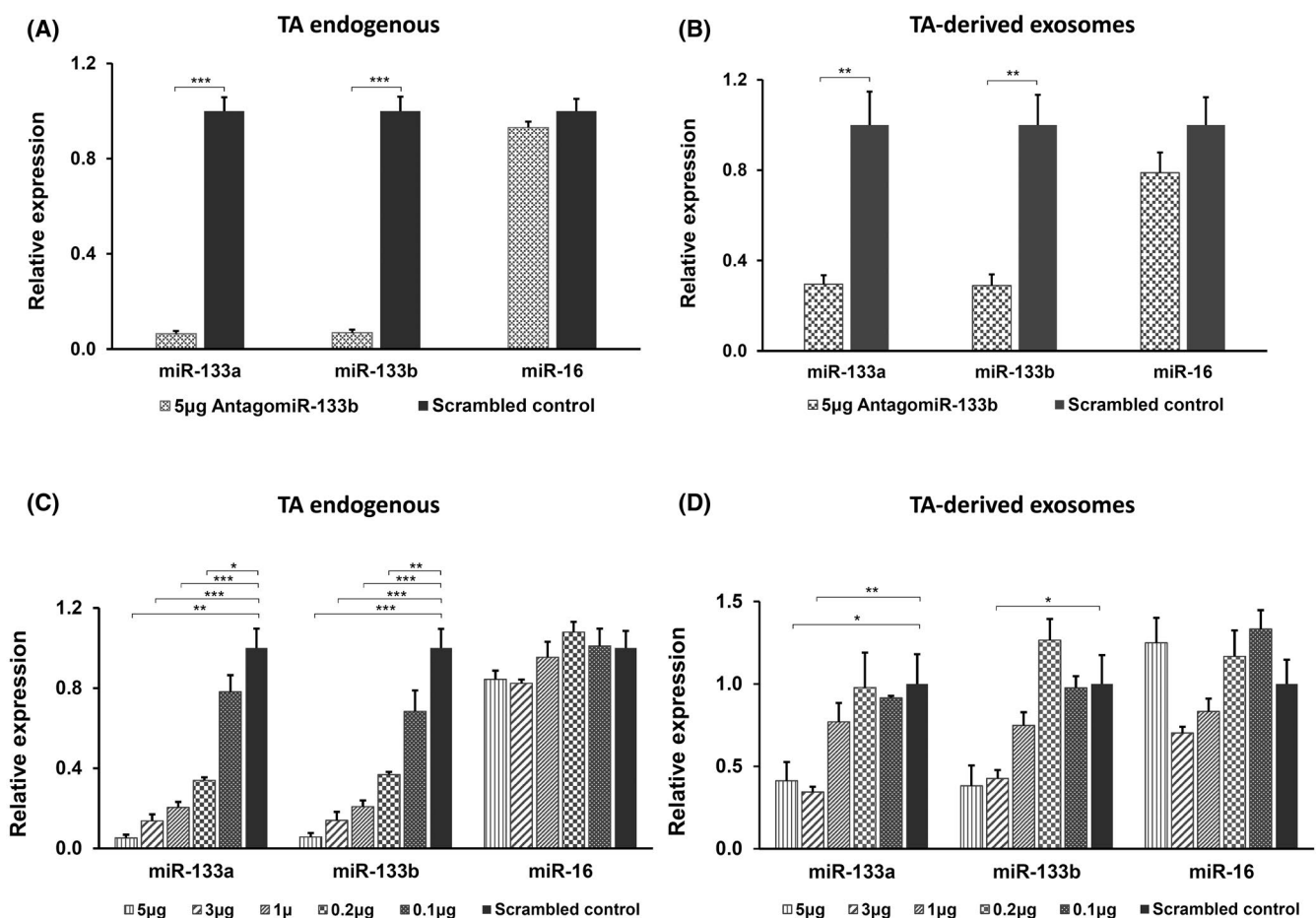


both, miR-133a and miR-133b. The endogenous levels of the two myomiRs were significantly lower (~95%) in antagomiR-133b-injected TA muscles compared to the scrambled control ( $P < .001$ ; Figure 7A). The exosomes derived from the TA-injected muscle tissues were next isolated and screened for the presence of miR-133a and miR-133b. The levels of the two myomiRs released from the antagomiR-133b-injected mice were also significantly reduced (~70%) compared to the scrambled control ( $P < .01$ ; Figure 7B). Additionally, 6-week-old wild-type male mice were intramuscularly injected with a series of different amounts of antagomiR-133b or scrambled control in both TA skeletal muscles. Administration of lower amounts of the antagomiR-133b resulted to lower reductions of the two myomiR levels compared to the control

samples, demonstrating the specificity of the designed antagomiR-133b (Figure 7C,D).

### 3.6 | Downregulation of miR-133a and miR-133b in TA muscles results in a reduction of miR-133a and miR-133b in proximal skeletal muscle tissues and in their secreted exosomes

Exosomes have been described to be involved in tissue-to-tissue communication by delivering their molecular cargo to their target tissues.<sup>11,50</sup> There is no information however, regarding skeletal muscle tissue cross talk through the exosomal route in vivo. Based on the observation that the four myomiR



**FIGURE 7** In vivo administration of antagomiR-133b in TA muscles induces downregulation of endogenous and exosomal miR-133a and miR-133b. TA muscle tissues of 6-week-old C57BL/6 male mice were intramuscularly injected with five different concentrations of antagomiR-133b and collected 10 days post-injection. A, The endogenous levels of miR-133b and miR-133a in TA muscle were downregulated (~95%) in antagomiR-133b-injected TA muscles compared to scrambled control. miR-16 was served as negative control showing no changes in antagomiR-133b-injected mice compared to the scrambled controls ( $n = 6$ ). B, The exosomal levels of miR-133a and miR-133b derived from TA muscles injected with the antagomiR-133b were significantly decreased (~70%) compared to the exosomal levels derived from TA muscle injected with scrambled oligonucleotide ( $n = 6$ ). C and D, Titration with lower concentrations of the antagomiR-133b administrated in TA muscles resulted in lower decrease of the endogenous and exosomal miR-133a and miR-133b levels indicating the specificity of the injected antagomiR-133b ( $n = 3$ ). The negative control miR-16 did not show any changes in antagomiR-133b-injected mice compared to the scrambled controls. The normalized data were analyzed by t test. Data on illustrations are relative expressions compared to scrambled-injected mice and represent means  $\pm$  SEM. \* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$

levels are higher in muscle-derived exosomes compared to serum exosomes (Figure 4A), the possibility that the muscle-derived exosomes are used for the local communication between neighboring muscle tissues without entering blood circulation was next investigated. Four skeletal muscle tissues that are near to the injected TA (EDL, soleus, gastrocnemius, and quadriceps) were collected and evaluated for the endogenous myomiR levels (Figure 6). Triceps muscles, which are located on the forelimb of mice, were also explanted, and served as distant control muscle tissues. Endogenous levels of the two myomiRs were found to be downregulated in skeletal muscle tissues located in close proximity to the TA muscles injected with the antagomiR-133b, compared to the scrambled controls (Figure 8A). EDL which is the nearest muscle to TA displayed the highest reduction in miR-133a and miR-133b levels (~85%;  $P < .001$ ), while the other three muscles presented similar percentage of downregulation (~40%;  $P < .05$ ) when compared to the scrambled control-injected mice (Figure 8A). Moreover, titration experiments revealed that the endogenous levels of the two myomiRs in the four proximal muscles depend on the amount of the antagomiR-133b injected to the TA (Figure 8B). In contrast, the two myomiR levels showed no significant alterations in triceps, which is located on the forelimbs (Figure 8C).

Considering that surrounding skeletal muscle tissues were affected endogenously by the downregulation of miR-133a and miR-133b in antagomiR-133b-injected TA muscles, the effect on exosomal myomiR levels derived from neighboring muscles was also examined. Exosomal miR-133a and miR-133b derived from three local skeletal muscles were also found to be reduced in mice injected with the highest amount of antagomiR-133b compared to the scrambled controls ( $P < .05$ ; Figure 9A). Specifically, exosomes secreted from EDL, soleus, and gastrocnemius were found to carry lower levels of the two myomiRs in the antagomiR-133b-injected mice. In contrast, quadriceps-derived exosomes did not show any significant difference in the two myomiR levels in the antagomiR-133b-injected mice, compared to the controls (Figure 9A). Intramuscular injections of lower amounts of antagomiR-133b did not lead to significant downregulation of the exosomal myomiR levels for all local tissues under investigation (Figure 9B).

The observed effect on myomiR levels in neighboring skeletal muscles after intramuscular injections of the antagomiR-133b in TA muscles, suggested that the injected muscles communicate with near muscle tissues possibly through the exosomal route. To further investigate that TA-derived exosomes are taken up by other skeletal muscles we isolated primary skeletal muscle cells and fibers from EDL muscles and incubated them with DiD-labeled TA-derived exosomes for 24 hours. Fluorescent microscopy of the muscle satellite cells and fibers revealed that the labeled exosomes are internalized by the cells, further supporting the possibility of exosomal exchange between neighboring skeletal muscles (Figure S5).

### 3.7 | Downregulation of endogenous and exosomal miR-133a and miR-133b levels in five skeletal muscle tissues reduces their exosomal levels circulating in the bloodstream

Exosomes circulating in the bloodstream are characterized by a diversity of tissue origin and, therefore, the association of the exosomal myomiRs in the blood with the myomiRs expressed endogenously was next evaluated. Serum samples were collected from the antagomiR-133b or scrambled control-injected mice 10 days post-injection followed by exosome isolation. miRNA analysis revealed that exosomal miR-133a and miR-133b levels are reduced approximately 50% in the serum of mice injected with 5 $\mu$ g of antagomiR-133b compared to the controls ( $P < .05$ ; Figure 10A). Intramuscular administrations of lower concentrations of the antagomiR-133b showed no effect on the exosomal myomiR levels in serum, in agreement with the muscle-derived exosomal myomiRs (Figure 10B).

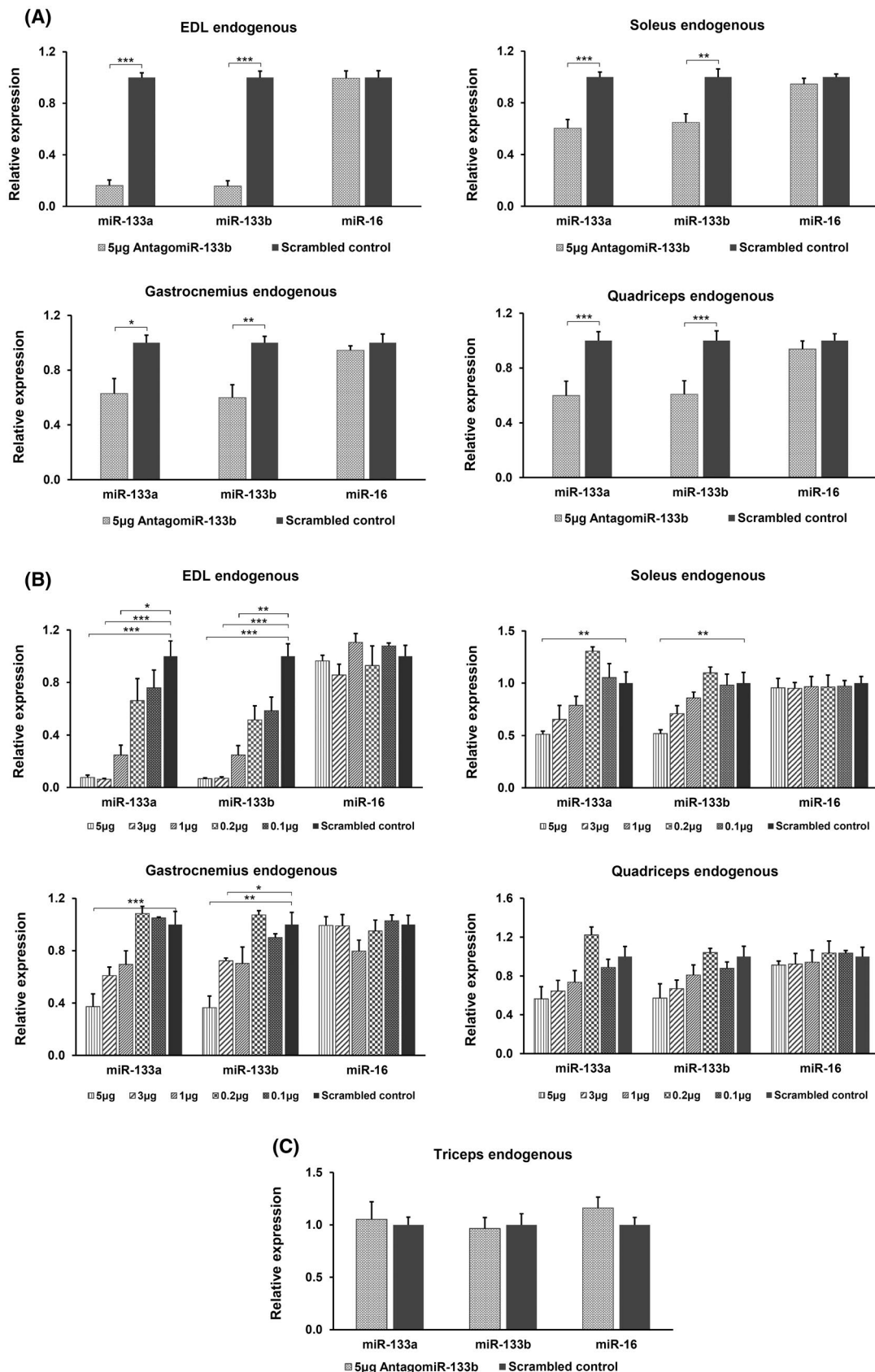
## 4 | DISCUSSION

Myogenesis is a well-orchestrated process, tightly regulated by myogenic regulatory factors (MRFs).<sup>51,52</sup> A subset of miRNAs, namely myomiRs, was found to be specifically expressed in muscle tissue and play a vital role in muscle function and development.<sup>23,53</sup> Four myomiRs, miR-1, miR-133a, miR-133b, and miR-206, have been extensively studied for their role in the process of myogenesis.<sup>25,27</sup> A lot of in vitro and in vivo experimental work has been carried out to determine the involvement of these myomiRs in muscle development, especially during embryogenesis.<sup>25,54</sup> Previous work has shown that serum levels of the four myomiRs are increased in muscle disease patients compared to healthy controls and are encapsulated within exosomes.<sup>33,34</sup> In the current years, intense emphasis has been placed on exosomes and their involvement in intercellular communication through the transportation of biological molecules.<sup>45,55</sup> Up to now, however, there is limited information regarding skeletal muscles and muscle-derived exosomal constituents in response to healthy conditions. Most of this information was obtained by experiments carried out on murine myoblasts and myotubes.<sup>7,36</sup> Few studies were conducted investigating the exosomes derived from cultured isolated muscle fibers and pieces of skeletal muscles.<sup>35,36,38</sup> During this study, the ex vivo release of exosomes and their cargo from isolated, intact wild-type skeletal muscle tissues were investigated. Moreover, the involvement of muscle-derived exosomes in local muscle communication and their correlation with serum exosomes were assessed.

Exosomes were successfully isolated for the first time directly from five intact skeletal muscle tissues located on the

hindlimbs (EDL, soleus, TA, gastrocnemius, and quadriceps) of 6-week-old wild-type male mice. Whole tissue incubation in a humidified incubator possibly creates hypoxic conditions

under which the muscle-derived exosomes were released, in contrast to the natural environment surrounding skeletal muscles within the body. Previous studies have reported that



**FIGURE 8** Intramuscular injections of antagomiR-133b in TA muscles reduces endogenous miR-133a and miR-133b in neighboring muscles. Six-week-old C57BL/6 male mice were intramuscularly injected with five different dilutions of antagomiR-133b in TA muscles. Four neighboring skeletal muscle tissues, EDL, soleus, gastrocnemius, and quadriceps, and one distant skeletal muscle tissue, triceps, were collected 10 days post-injection. A, EDL displayed more than 80% downregulated levels of miR-133a and miR-133b in mice injected with 5  $\mu$ g antagomiR-133b compared to EDL muscles of mice injected with scrambled control. Soleus, gastrocnemius, and quadriceps showed around 40% downregulation of the miR-133a and miR-133b levels in mice injected with 5  $\mu$ g of antagomiR-133b compared to corresponding muscles of mice injected with scrambled control ( $n = 6$ ). B, Serial administrations of diluted antagomiR-133b amounts in TA muscles resulted in lower decrease of the endogenous levels of miR-133a and miR-133b in all four neighboring muscles ( $n = 3$ ). C, Triceps muscle did not show any alterations between mice injected with antagomiR-133b or scrambled control ( $n = 6$ ). miR-16 was served as control for all skeletal muscles showing no changes in antagomiR-133b-injected mice compared to scrambled control-injected mice. The normalized data were analyzed by t test. Data on illustrations are relative expressions compared to scrambled-injected mice and represent means  $\pm$  SEM. \* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$

hypoxia stimulates the release of exosomes from distinct cell lines and tumors, emphasizing that the type of the producing cells as well as the duration and severity of hypoxia regulate the exosomal response.<sup>56-58</sup> There is no evidence, however, supporting that hypoxic conditions impact the content of the muscle-derived exosomes regarding the four myomiRs under investigation, since very few studies have been conducted on the implications of hypoxia on skeletal muscles.<sup>59-61</sup> More importantly, the role of myomiRs in response to low  $O_2$  concentration is still elusive. In this study, we showed that the ex vivo incubation of the skeletal muscles in culture medium leads to limited hypoxia within the tissues. In addition, the observed strong correlation between the endogenous and muscle-derived exosomal myomiR levels for all five tissues supports that hypoxic conditions have a marginal impact on the production of the exosomes and the selection of the exosomal cargo. Moreover, the titration experiments confirmed that the release of exosomal myomiRs is specific and irrelevant to any level of hypoxia.

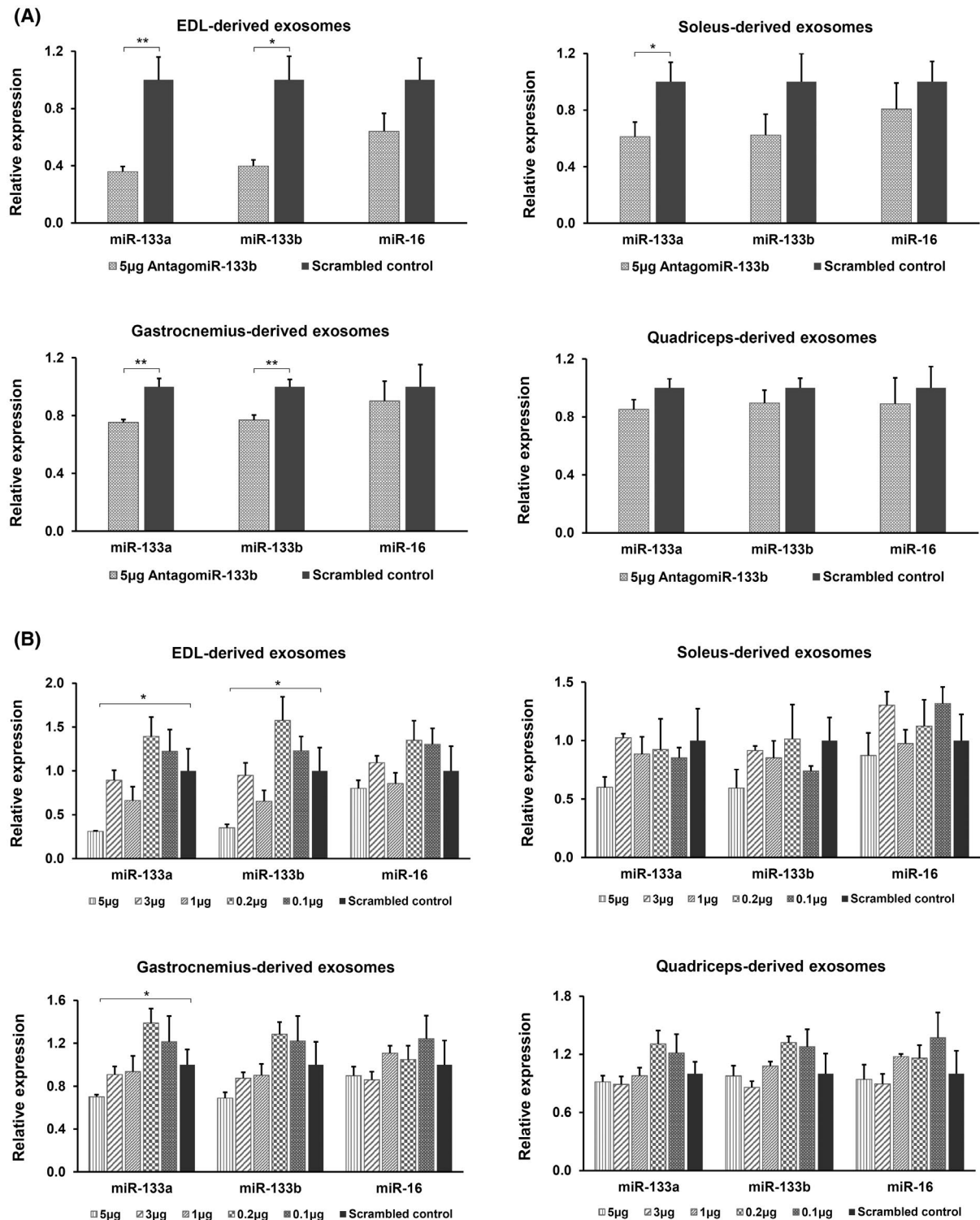
Exosomal cargo derived from all five skeletal muscles was examined for the presence of the four myomiRs. miR-1, miR-133a, and miR-133b were found to be present at higher levels compared to miR-206 within exosomes derived from all skeletal muscles except soleus. Soleus-derived exosomes were found to carry higher levels of miR-206 and lower levels of miR-1 compared to the other muscle tissues. Similarly, the endogenous levels of miR-1, miR-133a, and miR-133b were found elevated compared to miR-206 in all muscles apart from soleus. Endogenous miR-206 in soleus was detected at higher levels compared to the rest tissues and was comparable to the endogenous miR-1 levels in the same tissue. This finding implies a mechanism by which the internalization of the four myomiRs within exosomes is independent of their endogenous levels. Overall, miR-206 presented a tissue-specific pattern in both endogenous and muscle-derived exosomal levels associated with the soleus muscle. Skeletal muscles are composed of heterogeneous collection of muscle fibers.<sup>46-48</sup> This variety allows for a wide range of capabilities for skeletal muscles as well as acquiring tissue plasticity. Murine soleus mainly consists of slow and fast oxidative muscle fibers type I and type IIA, respectively, while

EDL, TA, gastrocnemius, and quadriceps consist primarily of fast glycolytic type IIB muscle fibers.<sup>40</sup> Tissues with similar muscle fiber composition, EDL, TA, gastrocnemius, and quadriceps, displayed similar myomiR behavior within the tissues and within exosomes, without considering the different myomiR levels between the tissues. This observation in combination with the abundance of miR-206 in soleus and soleus-derived exosomes, strongly indicates that the muscle fiber arrangement of the examined skeletal muscles impacts the selection of specific myomiRs for encapsulation within the multivesicular bodies.

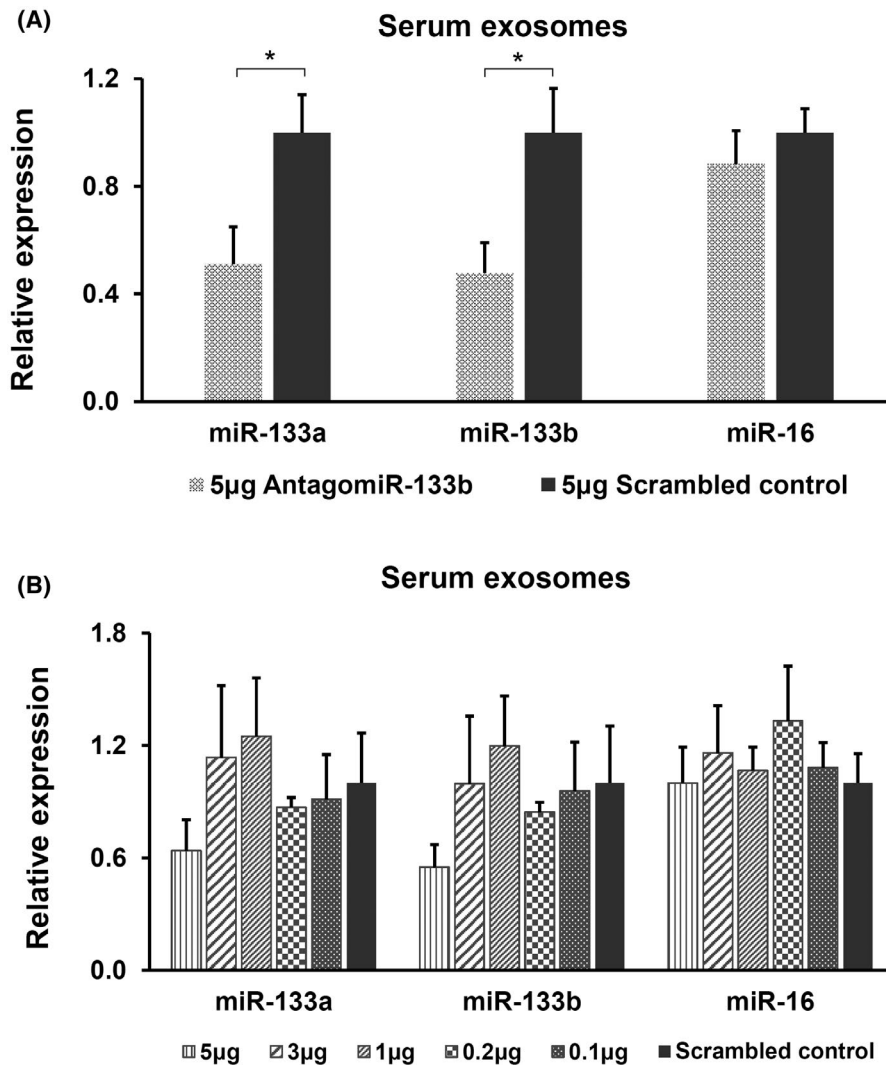
In addition to the diverse muscle tissue composition, the five skeletal muscles investigated have different weights and location on the hindlimbs; three characteristics that provide each muscle with a distinct role in the muscular system.<sup>47,62</sup> Normalization of miRNA levels over muscle weight (mg) showed that each skeletal muscle shares different exosomal and endogenous myomiR levels per muscle weight. TA muscles, which are of medium weight, were found to secrete higher levels of miR-1, miR-133a, and miR-133b within exosomes per muscle weight compared to the other hindlimb skeletal muscles. Exosomes derived from soleus presented the lowest levels of the same myomiRs, but the highest miR-206 levels when compared to the rest skeletal muscles. Interestingly, exosomes' quantification revealed that the five skeletal muscles secrete similar amounts of exosomes independently of their size. Consequently, the two smallest muscles load lower levels of myomiRs within their secreted exosomes, whereas the rest muscles package similar amounts of the four myomiRs. In addition, soleus encapsulates within its exosomes greater miR-206 levels compared to miR-1, which is in line with the fiber type specificity of this myomiR. Overall, these associations further demonstrate that myomiRs' selection and encapsulation within exosomes depends on the muscle size and the muscle fiber type.

Additionally, normalization of the endogenous myomiR levels over muscle weight, revealed that they are inversely correlated. Specifically, EDL which is the lightest of the five skeletal muscles investigated, showed the highest levels of endogenous myomiRs per muscle weight. The two larger muscles, gastrocnemius and quadriceps were found to express





**FIGURE 9** Proximal to TA muscles secrete reduced miR-133a and miR-133b levels after their downregulation in TA. A, Exosomes were isolated from skeletal muscle tissues, EDL, soleus, gastrocnemius, and quadriceps, that are proximal to the injected TA muscles and examined for the presence of miR-133a and miR-133b ( $n = 6$ ). EDL secreted exosomes with more than 60% reduction of the miR-133a and miR-133b levels in mice injected with 5 µg of antagomiR-133b compared to corresponding muscles of mice injected with scrambled control. Exosomes derived from soleus and gastrocnemius of the antagomiR-133b-injected mice showed a 40% and 25% reduction, respectively, of the two myomiR levels compared to corresponding muscles of mice injected with scrambled control. Quadriceps-derived exosomal myomiRs showed no difference between the two groups of injected mice. B, Intramuscular injections of diluted antagomiR-133b in TA muscles lead to a lower decrease of the miR-133a and miR-133b levels encapsulated within EDL, soleus, and gastrocnemius-derived exosomes ( $n = 3$ ). Muscle-derived exosomal miR-16 did not show any significant alterations for all skeletal muscle tissues and for all the injected mice. The normalized data were analyzed by t test. Data on illustrations are relative expressions compared to scrambled-injected mice and represent means  $\pm$  SEM. \* $P < .05$ ; \*\* $P < .01$ .



**FIGURE 10** miR-133a and miR-133b downregulation in skeletal muscles reduces their exosomal levels circulating in the serum. A, Blood was collected from 6-week-old C57BL/6 male mice ( $n = 6$ ) intramuscularly injected with antagomiR-133b or scrambled control, 10 days post-injection. Exosomes were isolated from serum samples and screened for the levels of miR-133a and miR-133b showing ~50% reduction of the two myomiR levels in mice injected with 5 µg of antagomiR-133b compared to mice injected with scrambled control. B, Intramuscular administrations of lower amounts of antagomiR-133b did not lead to significant alterations in serum exosomes collected from the blood of the antagomiR-133b-injected mice compared to controls ( $n = 3$ ). Serum exosomal miR-16 was used as control and did not show any significant changes for all the injected mice. Data are relative expressions compared to scrambled-injected mice and represent means  $\pm$  SEM. \* $P < .05$

the lowest endogenous levels of the four myomiRs per muscle weight, although they released similar exosomal myomiR levels to the much smaller EDL. Soleus was found to secrete less exosomal myomiRs than TA even though soleus was more enriched in endogenous myomiR levels per muscle weight. Overall, these results demonstrate that different muscle tissues secrete a distinct amount of the four myomiRs, which is independent of the mass of the tissue. Alternative mechanisms could impact the cargo selection and the amount of its internalization within exosomes. For example, binding proteins that transfer miRNAs in multivesicular bodies may be muscle fiber-specific, or particular miRNAs may not be available for loading because of their high in demand cellular activities.

Exosomes circulating in serum have various tissues of origin, including skeletal muscle, heart, blood cells etc<sup>27,38,55</sup> Consequently, muscle-derived exosomes correspond to a small fraction of the total amount of exosomes isolated from serum. Exosomes circulating in the blood of wild-type mice were found to carry the four myomiRs in contrast to what is observed in human healthy serum samples in which the exosomal myomiR levels were found to be minimal.<sup>34</sup> The

differences observed in healthy human and murine serum samples may be ascribed to faster muscle turnover or faster aging and muscle wasting observed in mice. Serum exosomal levels of the four myomiRs were found much lower compared to the exosomal myomiR levels derived from skeletal muscles. A reason possibly explaining this difference is the vast diversity of the tissues that produce and secrete exosomes in circulation along with variable molecular cargo. By isolating exosomes from serum, a pool of many exosomes originated from all tissues were also collected, thus rendering the muscle-derived exosomes a minority. Although the muscle-derived exosomes circulating in blood carry high levels of myomiRs, these levels are low compared to all the miRNAs isolated from serum exosomes. Another possible reason underlying the high difference between the muscle-derived and serum exosomal myomiR levels is that a significant proportion of the muscle-derived exosomes is taken up by neighboring skeletal muscle tissues rather than entering blood circulation. Nevertheless, similar pattern between myomiRs isolated from serum exosomes, muscle-derived exosomes, and skeletal muscles was observed implying their possible biological

correlation. Overall, endogenous, muscle-derived exosomal, and serum exosomal myomiR levels were strongly correlated, with the highest correlation observed between soleus and serum exosomal myomiR levels. Serum exosomal miR-133a and miR-133b were detected at higher levels compared to miR-1 and miR-206 levels, which is in agreement to the pattern characterized the soleus-derived exosomal myomiRs. All four myomiRs presented a similar behavior between the skeletal muscle tissues, the muscle-derived exosomes, and circulating exosomes, implying that exosomal myomiRs isolated from serum are directly originated from skeletal muscles.

It is worth noting that, even though most of the skeletal muscles examined in this study secreted exosomes rich in miR-1, miR-133a, and miR-133b, miR-1 was detected at lower levels circulating in serum suggesting that muscle-derived exosomes carry different amounts of each of the four myomiRs compared to the exosomes entering the circulation. For instance, muscle-derived exosomes which carry high miR-1 levels do not enter the blood circulation, but they may, instead, be used for local tissue-to-tissue communication.

Strong correlation between the profiles of the endogenous, muscle-derived exosomal, and serum exosomal levels of myomiRs implied that there is a biological mechanism involving endogenous myomiRs with exosomal myomiRs circulating in the bloodstream and thus further experiments were carried out to confirm this correlation. Downregulation of miR-133b levels *in vitro* resulted in a significant reduction of the levels of the same myomiR encapsulated within exosomes released in cell culture medium. In addition, due to the almost identical sequences of miR-133a and miR-133b, miR-133a was also found to be significantly downregulated endogenously and within exosomes released in cell culture medium. Downregulation of miR-133a and miR-133b in TA muscles *in vivo* caused a significant reduction in miR-133a and miR-133b levels in TA-derived exosomes compared to the controls. The endogenous and exosomal reduction were eliminated after the administration of lower amounts of antagomiR-133b indicating the specificity of the injected antagomiR.

Muscle-derived exosomes could potentially either promote local communication between muscle tissues or enter the blood circulation targeting distant delivery of their cargo or their disposal. In the current study, the levels of the four myomiRs were found to be significantly higher in muscle-derived exosomes compared to exosomes circulating in serum. It can be, therefore, suggested that a significant number of exosomes secreted by skeletal muscles participates in biological processes by remaining locally in the hindlimbs. Downregulation of miR-133a and miR-133b in TA muscles caused a reduction of these two myomiR levels in all proximal skeletal muscle tissues and not in a distant muscle located on the forelimb. Furthermore, labeled TA-derived exosomes were uptaken by primary skeletal muscle cells isolated from EDL muscles. These results indicate that TA communicates

with the other neighboring skeletal muscle tissues at a local level. The observed local communication could be ascribed to different possible mechanisms. A suggested mechanism we propose is through the exosomal route by packaging the antagomiR-133b within exosomes and delivering it to the neighboring tissues. The designed sequence of antagomiR-133b, in this study, is 22 nucleotides long and resembles the complementary strand of miR-133b; two properties that could render the administrated oligonucleotide as a candidate molecule for its internalization into multivesicular bodies and subsequently secreted into the extracellular environment within exosomes. As a result, exosomes released by the TA muscle carrying the antagomiR-133b could be delivered and function in nearby skeletal muscles. Another reason for the observed local cross talk could be that the reduced levels of the two myomiRs in TA-derived exosomes lead to the reduction in the levels in the neighboring tissues. In our study, TA muscles of wild-type male mice were found to release high exosomal levels of miR-133a and miR-133b. However, due to the injections of antagomiR-133b, local skeletal muscles received significantly reduced levels of miR-133a and miR-133b from the delivery of the altered TA-derived exosomes. Consequently, it is possible that the endogenous myomiR levels of the nearby skeletal muscles were not enriched as they should have under normal conditions. Lastly, the local communication could be ascribed to an indirect effect involving signaling pathways that are activated due to the downregulation of miR-133a and miR-133b in TA muscles and, subsequently, affecting the proximal skeletal muscles.

Finally, the highest amount of antagomiR administration resulted in 95% downregulation of endogenous miR-133b and miR-133a in TA muscle and a 50% reduction of their exosomal levels in serum, suggesting that a portion of the circulating myomiRs originate from muscle tissues located on the hindlimbs. The observed impact on serum exosomes cannot be attributed only to the two altered endogenous myomiR levels in TA. Neighboring skeletal muscles, also, revealed reduced secreted miR-133a and miR-133b amounts, indicating that the lower circulating exosomal levels reflect the contribution from all the affected skeletal muscles. In addition, a greater decrease of the miR-133a and miR-133b levels encapsulated within the majority of the muscle-derived exosomes under investigation, compared to serum exosomes, was detected. This observation could be ascribed to different skeletal muscle tissues which were not affected by the antagomiR administration and release normal myomiR levels in the circulation or to the fact that some of the hindlimbs muscle-derived exosomes which carry reduced myomiR levels are used for inter-tissue communication without entering the circulation.

In conclusion, this is the first study to show that intact hindlimb skeletal muscles of wild-type mice secrete exosomes encapsulating the four myomiRs. Additionally, we



demonstrated that the endogenous and muscle-derived exosomal myomiR levels are related to muscle fiber type composition. Some of the exosomal myomiRs released by the skeletal muscle tissues enter blood circulation to be transferred to distant tissues or to be discarded. More importantly, novel evidence was provided in this study regarding the local communication of proximal skeletal muscle tissues. Nevertheless, the exact role of muscle-derived exosomes remains to be elucidated. Confirming skeletal muscle exosomal cross talk under healthy conditions will provide insights into the involvement of muscle-derived exosomes in muscle development and function.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

L.A. Phylactou conceived the study; L.A. Phylactou and A. Koutsoulidou designed the study; C. Mytidou performed most of the research; A. Anayiotos, M. Prokopi, K. Kapnisis, and A. Katsioloudi performed SEM and TRPS experiments; K. Michailidou performed the statistical analysis; L.A. Phylactou, A. Koutsoulidou and C. Mytidou analyzed and interpreted the data; C. Mytidou wrote the manuscript. L.A. Phylactou coordinated the study; and all authors read this manuscript.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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