MicroRNA 874-3p Exerts Skeletal Anabolic Effects Epigenetically during Weaning by Suppressing Hdac1 Expression*

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Embryonic skeletogenesis and postnatal bone development require the transfer of calcium from the mother to the offspring during pregnancy and lactation. Therefore, bone resorption in the mother becomes elevated during these periods, resulting in significant maternal skeletal loss. There follows an anabolic phase around weaning during which there is a remarkable recovery of the maternal skeleton. However, the mechanism(s) of this anabolic response remain(s) largely unknown. We identified eight differentially expressed miRNAs by array profiling, of which miR-874-3p was highly expressed at weaning, a time when bone loss was noted to recover. We report that this weaning-associated miRNA is an anabolic target. Therefore, an agomir of miR-874-3p induced osteoblast differentiation and mineralization. These actions were mediated through the inhibition of Hdac1 expression and enhanced Runt2 transcriptional activation. When injected in vivo, the agomir significantly increased osteoblastogenesis and mineralization, reversed bone loss caused by ovariectomy, and increased bone strength. We speculate that elevated miR-874-3p expression during weaning enhances bone formation and that this miRNA may become a therapeutic target for conditions of bone loss.

The role of microRNAs (miRNAs)3 in regulating the skeleton is just beginning to emerge (1). Notably, the conditional deletion of the miRNA-processing endoribonuclease Dicer in cells of the osteoblast lineage demonstrates a clear requirement for miRNAs in embryonic skeletogenesis as well as in postnatal bone growth, modeling, and remodeling (2). miRNAs are small non-coding RNAs that mediate translational inhibition or degradation of a transcript by binding to complementary sites of the target mRNAs. This permits effective control of gene expression beyond genomic interactions between transcription factors and their cognate elements. Many miRNAs are evolutionary conserved with well defined developmental and cell type-specific expression patterns (2–4).

In this study, we aimed to identify miRNAs that are anabolic to the skeleton. Therefore, we examined miRNA expression during lactation and weaning, the two phases of reproduction characterized by marked fluctuations in bone resorption and formation. There is profound maternal bone loss during lactation to cope with the calcium requirement for postnatal skeletal growth and modeling. This bone loss is, however, reversed through rapid new bone synthesis that replenishes the maternal skeleton during weaning (5). If the processes become decoupled, then pregnancy- and lactation-associated osteoporosis ensue (5). There is evidence for an association between breast feeding and vertebral fractures, supporting the idea that lengthy lactation may, in fact, predispose to a greater risk of osteoporosis-related fractures in postmenopausal women (6).

Testifying to the efficiency and uniqueness of this rapid remodeling phase, rats, which typically nurse multiple offspring, can lose up to 35% of their bone mineral content over 21 days of lactation. This loss is reversed completely after weaning. For this reason, the weaning phase is considered the most anabolic phase in the reproductive cycle of a rodent and provides a vital window for the identification of new anabolic targets (7–9). For example, elevated levels of the neurohypophyseal hormone oxytocin during lactation and weaning led us to examine its action on bone (10, 11). Mice deficient in oxytocin or its receptor have been found to be osteopenic, and this led us to speculate that oxytocin plays a role in enabling maternal skeletal replenishment during weaning (12).

Here we report the identification of eight miRNAs whose expression was modulated specifically during weaning. We chose to further explore the action of miR-874-3p on the osteoblasts in vitro and in vivo. We conclude that miR-874-3p stimulates osteoblast differentiation, mineralization, and bone

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* The abbreviations used are: miRNA, microRNA; qPCR, quantitative PCR; BV, bone volume; TV, tissue volume; Tb.N, trabecular number; Alp, alkaline phosphatase; Hdac1, histone deacetylase 1; BMCs, bone marrow cells; Ct.Th, cortical thickness; CT, computed tomography.
MicroRNA 874-3p Exerts Skeletal Anabolic Effects

formation and, therefore, that it might not only mediate post-weaning skeletal recovery but also have wider implications as a potential target for conditions of bone loss.

Experimental Procedures

Chemicals and Reagents—The miRNA isolation kit (miR-Vana), miR-874-3p agomir and antagonim were purchased from Ambion (Life Technologies), and the Bmp2 ELISA was from R&D Systems (Minneapolis, MN). miRNA microarray profiling was carried out using the Agilent platform (Genotypic Technology Private Ltd.). Quantitative PCRs were performed using the StepOne real-time PCR system and a TaqMan 5’ nuclease probe method (Applied Biosystems) (miRNA transcripts normalized to U6) (13–15).

Calvarial Cell Culture—For experiments, 1-day-old mouse calvarial cells were used, harvested from nine to ten calvariae at room temperature. Briefly, individual calvariae were isolated surgically from the skull, sutures were segregated, and the pooled calvariae were kept for repeated digestion (15 min/digestion) with 0.05% trypsin and 0.1% collagenase P to release cells. The first digestion was discarded, and cells were collected from the next four digestions. Cells were cultured in α-minum Eagle’s medium containing 10% FCS and 1% penicillin/streptomycin (complete growth medium). Calvariae were allowed to reach 70–80% confluence for the experiments (16).

Alkaline Phosphatase (ALP) and Mineralization Assay of Bone Marrow Cells (BMCs)—At the end of the treatments, BMCs from the femora of mice were flushed out in osteoblast differentiation medium containing 10−7 m dexamethasone (bone marrow differentiation medium). BMCs were cultured for 21 days with a change of medium every 48 h. At the end of the experiment, ALP activity was measured at 405 nm, and mineralized nodules were stained and quantified at 405 nm (16).

Analysis of miRNA Target Site Prediction—In silico putative targets were screened for each of the differentially expressed miRNAs using TargetScan and miRanda on the basis of specific base-pairing rules (17, 18).

Transfection in Osteoblast Cells—The sequence of agomir miR-874-3p was as follows: sense, 5’-GGUCUCCUGGCCA-GGCCAGTT-3’; antisense, 5’-CUGCCUGGGCCAGGG-ACCGA-3’. The sequence of antagonist miR-874-3p was 5’-UCGGUCCUGGCCAGGG-CAG-3’. Transfections of the agomir and antagonist into mouse osteoblasts were carried out at 50–60% confluence with reduced-serum and antibiotic-free OptiMEM using the transfection agent Lipofectamine (Invitrogen). Alp activity, mineralization, and qPCR were performed using protocols published previously (19, 20). For the reporter-promoter assays, Runx2-Luc and Renilla luciferase vectors (from GeneCopoeia) were co-transfected into osteoblasts, and luciferase activity was determined using a dual assay luciferase kit (19). The full length of the Runx2 promoter is 1039 bp (catalog no. MP01230, GeneCopoeia). Western blotting used an enhanced chemiluminescence system (GE Healthcare) according to the instructions of the manufacturer (15).

In Vivo Studies—All animal care and experimental procedures were approved by the Institutional Animal Ethics Committee and Institutional Animal Care and Use Committee. Female BALB/c mice (18 ± 5 g) and female adult Sprague-Dawley rats (220 ± 30 g) were kept in a 12-h light-dark cycle with controlled temperature (22–24 °C) and humidity (50–60%) and ad libitum access to standard rodent food and water (19). Micro–computed tomography (μCT) measurements were carried out on a SkyScan 1076 CT scanner (Aartselaar, Antwerp, Belgium). Briefly, femora, tibiae, and vertebrae were scanned at a nominal resolution of 18 mm. One hundred projections were acquired (angular range, 180°), and image slices were reconstructed using a modified Feldkamp algorithm (according to Sky Scan Nrecon software). Parameters, including bone volume-to-tissue volume (BV/TV), trabecular number (Tb.N), and trabecular separation were calculated (15). Agomir and miR-C were administered with an anionic liposome (Invivofectamine 2.0, Life Technologies), which allows for fusion of the liposome-nucleic acid complex with the cell membrane and subsequent uptake by endocytosis. Bone formation was assessed by injecting tetracycline (20 mg/kg) into all groups of mice 30 days apart. Using fluorescence imaging and Leica-Qwin software (Leica Microsystems Inc., Buffalo Grove, IL), we calculated mineralized surface (mineral apposition) rate and bone formation rate.

Statistical Analysis—For experiments requiring multiple comparisons testing, we used one-way ANOVA followed by Newman-Keuls test of significance (GraphPad Prism v.5). Student’s t test was used for experiments with only two treatments.

Results

We first studied femur epiphyseal trabecular bone across the lactation-to-weaning transition, during which rats suffer maximum bone loss followed by complete recovery (Fig. 1A). Nuliporous rats served as controls. Micro-CT showed significant trabecular and cortical loss from day 9 onward. These changes were reversed by day 60. BV/TV, Tb.N, and cortical thickness were reduced maximally at around day 18 and normalized by day 60 (Fig. 1, B and C). Cultures of bone marrow cells isolated from rats during weaning (day 60) showed enhanced mineralized nodule formation as well as increased expression of the osteoblast genes Runx2, Bglap, and Colla1 compared with rats on day 18 (Fig. 1, D and E).

Microarray profiling of isolated bone marrow-derived osteoblasts showed that 8 of 4716 detected miRNAs were expressed differentially between peak lactation (day 18) and weaning (day 60) (Fig. 1J). Notably, miR-212, miR-327, miR-451, miR-672, and miR-874-3p were up-regulated, whereas the expression of miR-204, miR-322-3p, and miR-664 was reduced on day 60 (Fig. 1J). With the exception of miR-212, we validated the expression by qPCR of differentiating bone marrow stromal cells (Fig. 1G) and/or primary calvarial osteoblasts during mineralization (Fig. 1H). Of note was an ~5.5-fold increase in miR-874-3p during weaning, with significant expression in osteoblasts, chondrocytes, and skeletal muscle (Fig. 1F). Furthermore, miR-874-3p expression increased significantly during osteoblast differentiation, particularly in the mineralization phase (~4.0-fold) (Fig. 1F).

The highly conserved miR-874-3p is located on human chromosome 5 in the noncoding region and forms a characteristic
FIGURE 1. Bone loss and replenishment and miRNA profiling across the lactation-to-weaning transition in rats. A, representative three-dimensional images of rat femur epiphyses (trabecular) at different time points (in days) through lactation to weaning. Nulliparous rats (N) were used for comparison. Maximum loss occurs by day 18, whereas lost bone is almost completely restored by day 60. B, structural micro-CT parameters, including BV/TV and Tb.N. Data are mean ± S.E. compared with the nulliparous group (n = 5–10 rats/group). C, representative three-dimensional images of rat femur diaphyses (cortical) at different time points during lactation physiology. Bottom panel, cortical thickness (Ct.Th) of femur bone. Data are mean ± S.E. compared with the nulliparous group (n = 5–10 rats/group). D and E, ex vivo osteoblast mineralization (optical densities, n = 9) (D) and expression (qPCR) of the osteoblast genes Runx2, Bglap, and Col1a1 (E) on days 18 and 60. OD, optical density. F, heatmap showing array expression of selected miRNAs in bone marrow stromal cells on day 18 (lactation) versus day 60 (weaning). G and H, quantitative PCR validation of selected miRNAs from bone marrow stromal cells (compared with nulliparous rats) (G) or calvarial osteoblast (H) cultures (compared with day 0 osteoblast cells with mineralized osteoblast cells). I and J, expression (qPCR) of miR-874-3p in different tissues and cells (I) and in osteoblasts during proliferation, differentiation, or mineralization (J). Data are mean ± S.E. *, p < 0.05; **, p < 0.01 (qPCR in triplicate).
miRNA precursor stem-loop secondary structure (Fig. 2A). Stable transfection of primary mouse osteoblasts with the miR-874-3p agomir stimulated osteoblast differentiation. Alp activity, mineralization, and Bmp2 levels were increased significantly (Fig. 2B), consistent with increased mineralized nodule osteoblastic colony-forming units (Cfu-ob) formation (Fig. 2C). In contrast, the miR-874-3p antagomir significantly reduced Alp activity, Bmp2 levels, and Cfu-ob colonies (Fig. 2, B and C). Quantitative PCR and Western blotting showed increased Bmp2, Smad1, and Runx2 expression with the agomir in osteo-

**MicroRNA 874-3p Exerts Skeletal Anabolic Effects**

3962 JOURNAL OF BIOLOGICAL CHEMISTRY

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MicroRNA 874-3p Exerts Skeletal Anabolic Effects

MicroRNAs have also been implicated in regulating postnatal bone modeling and remodeling. This is testified by a high bone mass phenotype noted in mice when Dicer is deleted in mature osteoblasts (using an osteocalcin promoter) or in osteoclasts (using a CD11b or cathepsin K promoter) (26–29). However, even conditional Dicer deletion, which depletes all miRNAs in a given cell, does not inform us on how individual miRNAs regulate cell function. In fact, only a handful of miRNAs have been implicated directly in bone homeostasis. Although miR-223 and miR-155 control the osteoclast, notable regulators of osteoblastogenesis include two miRNA clusters, miR-23a–2–27a–24–2 and miR-2861–3960, that are repressed and activated, respectively, by Runx2 (28, 30–32). Specific miRNAs, miRNA-125b, miR-138, and miR-637, are also regulated by osterix during osteoblastogenesis (33–35).

To identify osteoblast-regulatory miRNAs of physiologic relevance, we profiled bone miRNAs during weaning, a period when bone formation is grossly accelerated. MicroRNA arrays during this period, day 60 in the rat, showed the differential regulation of eight of a total of 4716 detected miRNAs. Of these, miR-212, miR-327, miR-451, miR-672, and miR-874-3p were up-regulated, whereas the expression of miR-204, miR-322-3p, and miR-664 was diminished. This suggested that hitherto

FIGURE 2. miR-874-3p suppresses Hdac1 to stimulate osteoblast differentiation. A, secondary structure of pre-miR-874 predicted by miRBase (the mature miR-874-3p sequence is shown in red). B, effect of transfection of osteoblasts with the agomir or antagonim of miR-874-3p on Alp activity, mineralization, and supernatant Bmp2 (ELISA) (OD, n = 4). C, representative wells showing alizarin-positive colony (Cfu-ob) formation in osteoblast cell cultures on day 15 in osteogenic medium. D, quantitative PCR and Western blotting showing the expression of the osteoblast differentiation genes Bmp2, Smad1, and Runx2 at 24 h (tripllicate). E, effect of agomir of miR-874-3p or scrambled miR-C or Runx2 promoter activity using a luciferase (Luc) reporter (n = 6). F, in silico identification of the miR-874-3p target using the seed sequence to the 3’ UTR of Hdac1 (red, conserved seed sequence in mammals). G, expression of Hdac1 after 24 h of transfection with miR-874-3p agomir or antagonim, analyzed by Western blotting or qPCR. H, Cfu-ob formation, alkaline phosphatase activity (OD, n = 5), and mineralization (OD, n = 7) (H) and expression of Bmp2 and Runx2 mRNA (I) and protein (J) in osteoblasts transfected with siRNA against Hdac1 or scrambled siRNA. K, miR-874-3p suppresses Hdac1, which results in the up-regulation of Runx2 and osteoblast differentiation. Data are mean ± S.E., triplicate for qPCR, n for others as shown. *p < 0.05; **p < 0.01 compared with control. Other comparisons: ’p < 0.05; ”p < 0.01.

blast cultures, with modest inhibitory effects noted with the antagonist (Fig. 2D). Taken together, the results suggest that miR-874-3p directly promotes osteoblast differentiation and mineralization. To probe the mechanisms, we utilized a 1039-bp Runx2 promoter (pRunx2-Luc) (catalog no. MPRM13038, GeneCopoeia) tagged to a Dual-Luciferase reporter (GeneReporter). The agomir for miR-874-3p-stimulated Runx2 promoter activity (Fig. 2E).

We attempted to predict the target for miR-874-3p using TargetScan and miRBase. The putative targeted gene was confirmed as Hdac1, which showed an 8-nt seed sequence match with miR-874 in the 3’ UTR and was highly conserved among vertebrates (Fig. 2F) (21). In concordance with our in silico data, we found that the agonim to miR-874-3p repressed Hdac1 expression, whereas the antagonist increased its expression (Fig. 2G). These changes were corroborated at the protein level by Western blotting (Fig. 2G).

We next examined whether Hdac1 repression was required for miR-874-3p-induced Runx2 activation during osteoblast differentiation. Knocking down Hdac1 using siRNA increased Alp activity, mineralization, and nodule formation compared with scrambled siRNA-transfected cells (Fig. 2H). Furthermore, we assessed the expression of a Runx2 and its target gene, Bmp2, in Hdac1-depleted cells. The cells displayed a markedly increased expression of Runx2 and Bmp2 at the mRNA and protein levels (Fig. 2I). These data suggest that chromatin acetylation triggered by Hdac1 down-regulation was critical for miR-874-3p-induced Runx2 expression and osteoblast differentiation (Fig. 2J).

To address the anabolic function of miR-874-3p in vivo, we injected ovariectomized or sham-operated mice with the agonim of miR-874-3p (7 mg kg⁻¹) (8), scrambled miRNA (miR-C, 7 mg kg⁻¹), or PBS (0.2 ml) for 6 weeks on days 1–3 of the first, third, and fifth weeks. We first measured the expression of miR-874-3p in bone extracts to demonstrate effective in vivo tissue delivery and uptake in sham-operated and ovariectomized mice (Fig. 3A). We then evaluated trabecular bone at three sites: the femoral and tibial metaphyses and the vertebral column. At all three sites, ovariectomy expectedly reduced BV/TV and Tb.N (and increased trabecular separation). This marked trabecular loss was partially reversed by the miR-874-3p agonim, whereas miR-C and vehicle were both ineffective (Fig. 3B–E). These putative anabolic actions were confirmed by a profound effect on indices of bone formation; namely, significant increases in mineral apposition rate and bone formation rate (Fig. 3F). We also found that the agonim increased bone strength and stiffness not only in ovariectomized mice but also in sham-operated controls. The latter is consistent with an increase in Tb.N noted in this group (Fig. 3C).

To explore the mechanism of the increase in bone mass by miR-874-3p, we isolated bone marrow stromal cells at 6 weeks.

Ovariectomy induced a reduction in Alp activity, mineralization, and Cfu-ob formation, consistent with low-turnover bone loss (Fig. 4, A–C). Although miR-C did not affect these ex vivo parameters, the agonim partially restored Alp, mineralization, and Cfu-ob (Fig. 4, A–C). Furthermore, consistent with in vitro transfection (Fig. 2), Bmp2 and Runx2 expression was increased and Hdac1 was repressed in bone extracts from agonim-treated mice compared with miR-C or vehicle-treated animals (Fig. 4, D and E). This suggests that the in vivo anabolic action of miR-874-3p is also mediated through Hdac1 repression and Runx2 activation.

Discussion

Hundreds of microRNAs regulate protein synthesis by blocking mRNA translation and, therefore, coordinate a wide spectrum of biological processes, such as organogenesis. There has been keen interest in understanding the potential roles of microRNAs in embryonic skeletal development, postnatal bone growth and modeling, and adult bone remodeling. For example, skeletal patterning has been found to be disrupted when miR-196 was depleted conditionally early during skeletal development (22). Likewise, growth plate abnormalities, including reduced numbers of proliferating chondrocytes and enhanced hypertrophic chondrocyte differentiation, have been noted to occur when the endoribonuclease Dicer was deleted specifically in chondrocyte lineage cells (23). Two miRNAs inhibitory to chondrogenesis have been identified as miR-199a and miR145 (24, 25).

MicroRNAs have also been implicated in regulating postnatal bone modeling and remodeling. This is testified by a high bone mass phenotype noted in mice when Dicer is deleted in mature osteoblasts (using an osteocalcin promoter) or in osteoclasts (using a CD11b or cathepsin K promoter) (26–29). However, even conditional Dicer deletion, which depletes all miRNAs in a given cell, does not inform us on how individual miRNAs regulate cell function. In fact, only a handful of miRNAs have been implicated directly in bone homeostasis. Although miR-223 and miR-155 control the osteoclast, notable regulators of osteoblastogenesis include two miRNA clusters, miR-23a–2–27a–24–2 and miR-2861–3960, that are repressed and activated, respectively, by Runx2 (28, 30–32). Specific miRNAs, miRNA-125b, miR-138, and miR-637, are also regulated by osterix during osteoblastogenesis (33–35).

To identify osteoblast-regulatory miRNAs of physiologic relevance, we profiled bone miRNAs during weaning, a period when bone formation is grossly accelerated. MicroRNA arrays during this period, day 60 in the rat, showed the differential regulation of eight of a total of 4716 detected miRNAs. Of these, miR-212, miR-327, miR-451, miR-672, and miR-874-3p were up-regulated, whereas the expression of miR-204, miR-322-3p, and miR-664 was diminished. This suggested that hitherto
unrecognized miRNAs were likely involved in skeletal anabolism during a physiologically critical window. We therefore tested directly the effect of modulating the expression one of these miRNAs, miR-874-3p, using specifically designed agomirs and antagomirs. Of note is that miR-874-3p has been characterized as a tumor-suppressive miRNA (21). We found that transfection of osteoblast precursors with the agomir stimulated cell differentiation into a mature, mineralizing phenotype and that this effect was associated with elevated Runx2 and Smad1 expression. Furthermore, this action was mediated epigenetically in that agomir-induced osteoblastogenesis and Runx2 expression were both dependent on Hdac1 repression (Fig. 2K). This is not unexpected because there is prior evidence for an interaction between miRNAs, Hdacs, and Runx2. For example, Runx2 can positively regulate miR-2861, which, in turn, inhibits Hdac5 to up-regulate Runx2 expression in a feed-

FIGURE 3. miR-874-3p reverses ovariectomy-induced bone loss and improves strength in mice. A, enhanced miR-874-3p expression in bone following injection of miR-874-3p (agomir) using Invivofectamine 2.0 reagent into 6-week-old female Balb/c mice. This suggests adequate tissue uptake. O VX, ovariectomy. B, representative micro-CT images of the femur epiphysis. C–E, structural micro-CT parameters, including BVTV, Tb.N, trabecular spacing (Tb.S), and/or bone strength and bone stiffness parameters (units shown) in femur epiphyses (C), tibial epiphyses (D), and vertebral bone (E). Shown are mineralizing surface (MS), mineral apposition rate (MAR), and bone formation rate (BFR). Data are mean ± S.E. *, p < 0.05; **, p < 0.01 compared with the PBS-treated group or as shown (†, p < 0.05; ††, p < 0.01; n = 6 mice/group).
forward loop (32). We likewise propose a pathway in which miR-874-3p down-regulates Hdac1 expression to enhance Runx2 expression and osteoblast differentiation.

Of note is our finding that Bmp2 expression is also regulated similarly, which is not inconsistent with miR-140-induced repression of Hdac4 and the subsequent stimulation of bone formation. It is known that Bmp2 stimulates Runx2 acetylation and prevents Smurf2-mediated degradation of Runx2 (15). In contrast, by deacetylating Runx2, Hdac1 allows the protein to undergo Smurf-mediated degradation. Therefore, we hypothesize that the inhibition of Hdac1 by miR-874-3p increases Runx2 acetylation, potentiating Bmp2-induced osteoblast differentiation.

The potential anabolic action of miR-874-3p was explored in vivo in 6-week-old mice in which the skeleton underwent active modeling. Mice were ovarioectomized and injected with agomir or scrambled miRNA intermittently over 6 weeks. Agomir treatment prevented trabecular bone loss and improved bone strength not only in ovarioectomized mice but also in the sham-operated group. Importantly, ex vivo cultures of bone marrow stromal cells from agomir-treated mice showed evidence of increased osteoblastogenesis, mineralization, and Bmp2 and Runx2 expression as well as reduced Hdac1, essentially confirming our in vitro data. Therefore, these findings document miR-874-3p as a potential anabolic target. Considering that this miRNA is elevated 4- to 6-fold during weaning, it is possible that it plays a key role in restoring skeletal mass following pregnancy and lactation.

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MicroRNA 874-3p Exerts Skeletal Anabolic Effects

FIGURE 4. miR-874-3p suppresses Hdac1 in vivo to stimulate osteoblastogenesis. A–E, Alp activity (OD, n = 6) (A), alizarin-positive colony formation (Cfu-ob, representative wells) (B), mineralization (OD, n = 7) (C), and RNA (qPCR) (D) and protein (Western blot analysis) (E) levels of Hdac1, Bmp2, and Runx2 in osteoblasts from sham-operated (Sham) or ovarioectomized (OVX) mice that were given PBS (0.2 ml), scrambled miR (miR-C, 7 mg kg⁻¹), or miR-874-3p (agomir, 7 mg kg⁻¹), as indicated under “Results.” Data are mean ± S.E. *, p < 0.05; **, p < 0.01 compared with the PBS-treated group.
MicroRNA 874-3p Exerts Skeletal Anabolic Effects

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