RESEARCH ARTICLE

Low protein intake during reproduction compromises the recovery of lactation-induced bone loss in female mouse dams without affecting skeletal muscles

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Abstract
Lactation-induced bone loss occurs due to high calcium requirements for fetal growth but skeletal recovery is normally achieved promptly postweaning. Dietary protein is vital for fetus and mother but the effects of protein undernutrition on the maternal skeleton and skeletal muscles are largely unknown. We used mouse dams fed with normal (N, 20%) or low (L, 8%) protein diet during gestation and lactation and maintained on the same diets (NN, LL) or switched from low to normal (LN) during a 28 d skeletal restoration period post lactation. Skeletal muscle morphology and neuromuscular junction integrity was not different between any of the groups. However, dams fed the low protein diet showed extensive bone loss by the end of lactation, followed by full skeletal recovery in NN dams, partial recovery in LN and poor bone recovery in LL dams. Primary osteoblasts from low protein diet fed mice showed decreased in vitro bone formation and decreased osteogenic marker gene expression; promoter methylation analysis by pyrosequencing showed no differences in Bmpr1a, Ptch1, Sirt1, Oxx, and Igf1r osteoregulators, while miR-26a, -34a, and -125b expression was found altered in low protein fed mice. Therefore, normal protein diet is indispensable for maternal musculoskeletal health during the reproductive period.

KEYWORDS
bone loss, lactation, microRNAs, protein restriction, recovery

Abbreviations: Alp, alkaline phosphatase; Bglap, bone gamma-carboxyglutamate protein; Bmpr1a, bone morphogenetic protein receptor 1a; Colla1, collagen type I alpha 1 chain; EDL, extensor digitorum longus; Igf1r, insulin-like growth factor 1 receptor; microCT, micro-computed tomography; miR, microRNAs; NMJ, neuromuscular junction; Obs, osteoblasts; Oxx, osterix; Ptc1, patched 1; Runx-2, runt-related transcription factor-2; Sirt1, sirtuin 1; TA, tibialis anterior; TRAP, tartrate-resistant acid phosphatase.
in milk through proteolysis and this process is in fine balance with maternal dietary protein intake.\textsuperscript{21,22} Muscle metabolic events, such as fatty acid oxidation, are also linked to food intake via nervous system mediated regulation.\textsuperscript{22} To our knowledge, while muscle proteolysis has been largely studied during lactation, there is no evidence for maternal skeletal muscle fiber morphological changes or neuromuscular junctions (N MJ s), the synapse between a motor neuron and a muscle fiber, in conjunction with protein under-nutrition.

Dietary calcium (Ca) and vitamin D supplements during gestation and lactation have been extensively studied, suggesting beneficial effects on both maternal and fetal/offspring skeletal homeostasis.\textsuperscript{23} Ca supplementation in the maternal diet has been proved beneficial for both maternal and offspring bone health\textsuperscript{24}; the National Academy of Sciences recommends 1000 mg of daily Ca consumption for pregnant and breastfeeding women. Although some studies have explored the consequences of other nutrients, such as soy isoflavones\textsuperscript{25} and prebiotics\textsuperscript{26} on the maternal skeleton and other organs during lactation and recovery, very little is known about the effects of the maternal protein intake.

In recent years, there has been increasing evidence of the epigenetic regulation of bone health. Epigenetic mechanisms are potential therapeutic targets due to their reversible nature. Several studies have suggested that methylome changes play an important role in osteoblast differentiation and activity.\textsuperscript{27-30} Hypomethylation of the promoters of the \textit{runt-related transcription factor-2} (\textit{Runx-2}), \textit{bone gamma-carboxyglutamate protein} (\textit{Bglap}), the coding gene for osteocalcin), and \textit{os terix} (\textit{Osx}) genes is involved in osteogenic differentiation of adipose-derived mesenchymal stromal cells (MSCs).\textsuperscript{31} Other regulatory mechanisms involve microRNAs (miRs) that can regulate posttranscriptional gene expression. MiRs have been shown to control bone-related genes.\textsuperscript{32-34} For example, miR-204/211 levels are increased, suppressing \textit{Runx-2} gene expression and promoting adipogenic over osteogenic differentiation of mesenchymal stromal cells (MSCs),\textsuperscript{33} while miR-15b induces osteoblast differentiation by inhibiting Runx-2 degradation.\textsuperscript{35} Moreover, overexpression of miR-2861 and miR-3960 promotes BMP2-induced osteoblastogenesis, and their suppression inhibits osteoblast differentiation.\textsuperscript{36} It is also known that miRs are essential for endochondral ossification since osteoblast-specific Dicer knockout mice have deficient cortical bone formation and bone integrity.\textsuperscript{37,38}

The aim of this study was to investigate the effects of protein restriction on the maternal musculoskeletal system in mouse dams during gestation and lactation as well as the postweaning recovery period. Histological structural measurements in skeletal muscle and NMJ morphology evaluations were performed. Bone microarchitecture and turnover were assessed using micro computed tomography (microCT) and bone histomorphometry. In vitro experiments on primary bone cells and expression patterns of
selected miRs were investigated to determine whether an epigenetic event may be associated with maternal skeletal recovery delay.

2 | MATERIALS AND METHODS

2.1 | Animals

This study included 39 female mice. We used B6.Cg-Tg(Thy1-YFP)16Jrs/J mice, which express yellow fluorescent protein (YFP) only in neuronal cells (Jackson Laboratory; stock number 003709). Mice were housed in individually vented cages maintained at 21 ± 2°C on a 12-hour light/dark cycle. All experimental protocols were performed in compliance with the UK Animals (Scientific Procedures) Act 1986 regulations for the handling and use of laboratory animals and received ethical approval from the University of Liverpool Animal Welfare Ethical Review Committee (AWERB). Mice were monitored daily for any health and welfare issues.

2.2 | Experimental groups

All mice were fed ad libitum food and water. Solid food pellets of normal protein diet (N, 20% crude protein; Special Diet Services, UK; code 824226) or low protein diet (L, 8% crude protein; Special Diet Services, UK; code 824248) were of isocaloric value. Groups of 8 weeks old nulliparous female mice were fed on either L (low) or N (control) protein diet for 2 weeks prior to mating. Once adapted to the diets, the mice were mated with age-matched males on N diet and the pregnant mice were kept on the same diet throughout gestation (19-21 days) and lactation (21 days). Suckling pup number was kept the same for all animals during lactation (n = 5-6 pups) to prevent confounding effects of differences in litter size. Following lactation, mice in the normal (group Lac-N, n = 8)
and low (group Lac-L, n = 8) groups were culled (16 weeks old) and tissues were harvested (skeletal muscles and bones). The recovery (Rec) groups were comprised of dams (20 weeks old) fed on N until the end of lactation and remained on the same diet for the recovery (28 days) period (group Rec-NN, n = 5), and mice on L diet until weaning, which remained on the same diet for recovery (group Rec-LL, n = 5) or switched postweaning to N protein diet (group Rec-LN, n = 5) for the recovery period (Figure 1A). At the end of lactation or recovery mice were euthanized by a rising concentration of CO₂. Age-matched virgin mice (18 weeks old) were used as a control (group Virgins, n = 8). To reduce the number of animals according to 3Rs recommendations, the end-point age of Virgins was kept at the middle of the recovery period (2 weeks after lactation and 2 weeks before recovery end) to serve as controls for both experimental periods (Figure 1A). At least for the skeletal system, no significant change has been observed in bone mass and structure of female mice between months 3-4 of age.⁴⁹

2.3 | Muscle histology and NMJ imaging

2.3.1 | Skeletal muscles

Immediately after culling, the extensor digitorum longus (EDL) and tibialis anterior (TA) skeletal muscles were carefully dissected and weighed (n = 5). The TA muscles were embedded in Cryomatrix (Thermo Fischer, UK), immediately immersed in liquid nitrogen-frozen isopentane, and stored at −80°C for cryosectioning. TA muscles were placed at −20°C for at least 30 minutes prior to cryosectioning. Transverse sections (10 μm) were cut using a Leica cryotome and collected on Superfrost glass slides (ThermoScientific, UK). Sections were washed with PBS for 10 minutes before staining with 1:1000 dilution in 5% of NGS to identify osteoblasts. Primary antibodies were detected using a Vectastain ABC kit with a secondary goat anti-rabbit-biotinylated antibody and visualized with HRP-conjugated streptavidin using 3,3′-diaminobenzidine (DAB; Vectorlabs, UK). Histomorphometric analyses were performed according to ASBMR standards using opensource software.⁴⁰,⁴¹

2.4 | Micro-computed tomography

Hindlimbs and L5 lumbar vertebrae were scanned using a Skyscan 1272 scanner (Bruker, Belgium; 0.5 aluminum filter, 50 kV, 200 mA, voxel size 4.60 μm, 0.3° rotation angle step). Data sets were reconstructed using NRecon and 3D volumes of interest (VOI) were selected using Dataviewer and CTA software (Bruker, Belgium). Trabecular bone parameters were analyzed using CTA in the proximal tibial metaphysis and the vertebral body of L5 vertebra. Cortical bone was analyzed at the tibial midshaft. For trabecular bone analysis, VOI was selected using mineralized cartilage as a reference point. The tibial VOI analyzed was 400 slices starting 20 levels distal to the reference point, while for cortical bone measurements, a VOI (100 slices) was selected 600 slices below the reference point, as previously described.⁴²,⁴³ Trabecular bone was automatically separated from cortical bone using a macro in CTA.

2.5 | In vitro bone cell culture and mineralization assay

For the primary bone cell cultures, immediately after microCT scans (<2 hours from sacrifice), midshafts of long bones were isolated (n = 3/group), surrounding muscles removed, and the bones centrifuged (3 minutes at 800 g) to remove the bone marrow. The bone shafts were cut into small pieces using a
scalpel and adhering cells were removed by digestion with collagenase type I (Sigma, 1 mg/mL in Hank’s balanced salt solution, HBSS) for 45 minutes in a shaking water bath at 37°C, washed in PBS, and cultured in alpha-MEM with Glutamax (Gibco, UK) and nucleosides, containing 10% of heat-inactivated FBS and penicillin (100 IU/mL)/streptomycin (100 μg/mL) (Invitrogen) in a humidified 5% of CO₂ incubator at 37°C, as previously described. Upon reaching semi-confluency, Obs grown out of the cleaned bone chips, were harvested using trypsin/EDTA (Gibco, UK) and seeded onto 6-well plates (10³ cells/well) in osteogenic medium (50 μg/mL L-ascorbic acid, 2-phosphate and 5 mM β-glycerophosphate) (Sigma, UK) for 24 days. Mineralization capacity was assessed by Alizarin Red S (ARS) (Sigma, UK) staining. Bone nodule surface area was calculated using ImageJ (NIH), as previously described.

### 2.7 | miR:target prediction and bioinformatics

To predict the targets of the differentially expressed miRs, we used the miRWalk on-line tool by applying simultaneous search from four different databases, including miRWalk, TargetScan, miRDB, and MiRTarBase using the default parameters of 7 as the minimum seed length at the 3’-UTR site and showing only the statistically significant mRNAs. A total of 174 target genes were obtained. Cytoscape v3.7.2 software was used to build the interaction networks between predicted targets and miRs as well as to determine the biological roles of the target mRNAs utilizing Gene Ontology (GO) terms of biological process and molecular functions. The enriched GO terms were presented as enrichment scores. KEGG pathway analysis was performed to determine the involvement of the predicted mRNAs targets in different biological pathways. P < .05 was considered to indicate a statistically significant result.

### 2.8 | Promoter methylation analysis

Pyrosequencing methylation assays for bone morphogenetic protein receptor 1a (Bmpr1a), patched 1 (Ptch1), osterix (Osx), insulin-like growth factor 1 receptor (Igf1r), and sirtuin 1 (Sirt1) gene promoters were designed using the Pyromark Assay Design 2.0 software (Qiagen). PCR and sequencing primers are provided in Table S1. Osteoblasts-derived genomic DNA (1 μg each) from all the experimental groups (n = 3/group) was subjected to bisulfite treatment using the EZ DNA methylation kit (Zymo Research, USA) according to the manufacturer’s protocol. Pyrosequencing templates were prepared by PCR amplification (45 cycles) of approximately 30 ng bisulfite-treated DNA using HotStarTaq Master Mix (Qiagen), 150 nM biotinylated primer, and 300 nM nonbiotinylated primer (Table S1). Optimized annealing temperatures were 52°C for Bmpr1a, 51°C for Ptch1, 48°C for Sirt1, 50°C for Osx, and 55°C for Igf1r. PCR efficiency and specificity were verified by agarose gel electrophoresis. PCR products were immobilized on streptavidin coated sepharose beads, and pyrosequenced on PyroMark Q96 MD instrument (Qiagen) according to the manufacturer's instructions. The sequence runs were analyzed using the Pyromark Q962.5.8 software Q-CpG software.

### 2.9 | Statistical analysis

All data were analyzed with GraphPad Prism 6 software and expressed as the mean ± SD. Data sets were tested for normal distribution with the D’Agostino-Pearson normality test. Comparisons between four groups were performed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons post hoc test where appropriate. For comparisons between two groups, unpaired Student’s t test or Mann-Whitney U test was applied. In all cases, P values less than .05 were considered statistically significant.

### 3 | RESULTS

#### 3.1 | Body weights and bone length

Total body weights were recorded after euthanasia. There was no difference between groups Lac-N and Lac-L (16 weeks
old) while weight was increased in both these groups compared to the Virgins group (18 weeks old), as expected (Figure 1B). For the recovery period, similar weights were observed for all groups of animals (Rec-NN, Rec-LN and Rec-LL) (Figure 1B). Additionally, there was no difference in tibial and L5 vertebral lengths, as measured by microCT (Figure 1C).

### 3.2 Low protein diet has no effect on skeletal muscle and NMJ morphology in mouse dams

We first examined the effects of low protein diet during pregnancy and lactation on skeletal muscle and NMJ morphology. There was no difference in TA and EDL weights between the Virgins, Lan-N, and Lac-L groups (Figure 1D). Furthermore, cross-sectional area (CSA) of the TA skeletal muscle fibers, measured using transverse cryosections, was also similar between groups (Figure 1E,F). Detailed confocal image analysis of the EDL muscle also indicated that NMJ morphology was not affected by the reproduction process or the protein content of the diet, as the presynaptic YFP-labeled motor axons and the postsynaptic bungarotoxin-stained AchRs perfectly overlapped without any evidence of denervation or clustering (Figure 1G). In light of these findings, we decided not to proceed with muscle and NMJ analyses for the recovery groups.

### 3.3 Protein under-nutrition enhances lactation-induced bone loss and delays recovery

We evaluated the effect of low protein intake on tibial and L5 vertebral bone mass by measuring the trabecular and cortical parameters obtained from microCT scans. Our aim was to first examine if protein under-nutrition during gestation and lactation had an impact on skeletal mass and structure and if so, how this diet would affect bone recovery. Therefore, we compared the trabecular %BV/TV in tibiae between Lac-N and Lac-L dams, without using controls as the lactation-induced bone loss has been extensively been reported in rodents.\(^1^0\)\(^1^1\) Lac-L dams had a significant reduced bone volume in comparison with lactating Lac-N mice on a normal diet, due to decreased trabecular thickness, and decreased trabecular number as well as decreased connectivity and a more rod-like appearance of the trabecular bone (Figure 2A,G, Table S2), reflecting a compromised bone microarchitecture. Similar outcomes were obtained for the trabecular network in L5 vertebral body, showing a further bone mass reduction of 22.75% in Lac-L as compared to Lac-N mice (Figure 2E,K, Table S2). Cortical bone analyses revealed a significant decrease in the cortical thickness in the midshaft of long-bones in lactating mice with greater thickness loss in the Lac-L group, in comparison with Lac-N (Figure 2C,H).

After weaning, female mice in the Rec-NN group, which were fed a normal protein diet during gestation and lactation as well as for the recovery period of 28 d, showed full recovery of trabecular bone volume and architecture in tibiae (Figure 2B,I, Table S2) and in L5 vertebrae (Figure 2F and L, Table S2), when compared to the Virgins group. Rec-NN mice had statistically significant higher trabecular bone volume than Rec-LN and Rec-LL mice, at both skeletal sites. Cortical bone thickness showed full recovery in the Rec-NN group, partial recovery in the Rec-LN and poor recovery in the Rec-LL (Figure 2D,J).

### 3.4 Low protein intake has detrimental effects on bone remodeling

In order to evaluate bone turnover in the spines of mouse dams, we performed bone histomorphometric analyses, as previously described.\(^4^0\) Routine H&E staining (Figure S1), confirmed by Ocn IHC (Figure 3A), showed that Lac-L lactating dams had lower numbers of osteoblasts per bone surface (NOb/BS) and reduced osteoblastic surface per bone surface (ObS/BS) as compared to the Lac-N group (Figure 3B,C). In the recovery period, Rec-NN mice showed increased osteoblast numbers, in comparison with the Virgin control mice. In contrast, osteoblastic number and surface was lower in the Rec-LN mice while the Rec-LL mice showed no signs of recovery with significantly reduced osteoblast number and surface (Figure 3D-F).

TRAcP staining revealed that lactating mice on the low protein diet had increased osteoclast numbers and size compared to the Lac-N group (Figure 4A). Osteoclast number and size were also increased in the Rec-NN dams compared to the Virgin controls (Figure 4B,C), indicating a high rate of bone remodeling, most likely coupled with increased bone formation. Interestingly, Rec-LN mice showed increased osteoclastic activity compared to the Virgins but not to the Rec-NN group. On the contrary, maintenance on low protein diet during the recovery period resulted in higher numbers of bone-resorbing cells when compared to control and Rec-NN mice (Figure 4D-F).

### 3.5 Low protein diet reduces in vitro bone formation

The next sets of experiments were designed to assess the osteogenic capacity of osteoblasts in vitro. Osteoblasts were isolated from long-bones of animals of all six groups (n = 3/group) and the level of mineralization using ARS staining was evaluated after 24 days of culture in osteogenic medium. We found
that the mineralization level was correlated with the level of protein in the diet that the mice consumed by the end of lactation. Osteoblasts isolated from Lac-N lactating dams formed larger and more numerous bone nodules as compared to the Lac-L group (Figure 5A,C). Furthermore, mRNA expression levels of Alp, the master osteogenic transcription factor Runx-2, as well as Bglap, and Coll1a1, were all decreased in the Lac-L in comparison with Lac-N (Figure 5E,F,I,J), indicating decreased osteoblastic differentiation and activity.

In the recovery period, Obs from the Rec-NN group formed a significantly increased number of mineralized bone nodules compared with Virgins and also with Rec-LN and Rec-LL, in agreement with the histological as well as the microCT observations (Figure 5B,D). Interestingly, mRNA levels of Alp, Coll1a1, Bglap, and Runx-2, reflecting osteogenic differentiation and activity, were found increased in the Rec-NN group, and suppressed predominantly in the Rec-LL group (Figure 5G,H,K,L).

### 3.6 DNA methylation analysis

To identify potential epigenetic mediators of these effects, we performed targeted pyrosequencing for DNA methylation analysis of the promoters for the Bmpr1a, Ptc1, Sirt1, Osx, and Igf1r genes, while Runx-2, Alp, Coll1a1,
and Bglap were only used as osteogenic markers. The expression patterns of these genes are known to be affected by diet.\textsuperscript{53-57} They are also considered as crucial molecular players in the major signaling pathways that control osteoblastic differentiation and activity: Bmpr1a in BMP pathway, Ptch1 in hedgehog pathway, Igf1r in IGF signaling, while Osx is a master regulator of osteoblast differentiation and Sirt1 links nutritional diet with bone formation.\textsuperscript{58} All the samples demonstrated negligible DNA methylation, consistent with the promoters being in the active state, with signal ranging within the established noise area of the technology.\textsuperscript{59} Representative pyrograms are given in Figure S2.

### 3.7 Differential expression of specific miRs may regulate bone recovery delay induced by low protein diet

Based on the in vitro results, we hypothesized that the delay of bone recovery in the Rec-LL and, partially, in the Rec-LN group might be caused by differential expression (DE) of bone-related miRs. It has been shown that, among others, some miRs were directly related with bone metabolism. For example, miR-26a regulates osteogenic differentiation of BMSCs and ADSCs by differentially activating Wnt and BMP signaling pathways.\textsuperscript{50} Furthermore, it has been shown that miR-26a attenuates osteoclastogenesis,
actin-ring formation, and bone resorption by suppressing
the expression of connective tissue growth factor/CCN
family 2 (CTGF/CCN2)\textsuperscript{61}; miR-34a is downregulated
during osteoclast differentiation\textsuperscript{62} and miR-125b controls
the osteogenic differentiation of hBMSCs by targeting
BMPR1\textsubscript{b}\textsuperscript{63} and also inhibits BMP-4-induced osteoblastic
differentiation by regulating cell proliferation in mouse
ST2 MSCs, via targeting of the receptor tyrosine kinase
Erb2.\textsuperscript{64} Therefore, we selected these three miRs, -26a, 34a,
and 125b, which according to the literature have direct ef
fects on osteoblastic differentiation,\textsuperscript{60,62,63} and performed
qPCR to determine their expression levels in Obs isolated
from the mouse groups used for the bone recovery period.
The levels of miR-26a were found slightly increased in
the Rec-NN group as compared to the Virgins and signifi
cantly suppressed in the Rec-LN and Rec-LL mice. The
endogenous expression of miR-26a is increased during the
osteogenic differentiation,\textsuperscript{65} thus, the suppressed levels in
Rec-LN and Rec-LL are consistent with decreased osteo
genesis. We also found that miR-34a was downregulated in
the Rec-LN and Rec-LL and in-keeping with previous stud
es reporting that miR-34a-overexpressing transgenic mice
exhibit lower bone resorption and higher bone mass through
transforming growth factor-\beta-induced factor 2 (Tgf2) in
hibition,\textsuperscript{62} it is revealed that miR-34a is important for bone
regulation. The levels of miR-125b followed the opposite
pattern and were elevated in the low bone-forming capac
ity Obs from the Rec-LN and Rec-LL dams in comparison

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Osteoclastic TRAcP was visualized in Lac-N and Lac-L dams during gestation/lactation (A) and quantified using the number
of osteoclasts per bone surface (N.Oc./BS) (B) and the percentage of osteoclast surface per bone surface (Oc.S/BS) (C) parameters. Similarly,
representative images (D) and data comparisons (E and F) are shown for the recovery period against Virgins. Arrows indicate TRAcP-positive
osteoclasts attached to the bone surface. All data are presented as mean ± SD. ns: not significant; \textsuperscript{\&}P < .05, \textsuperscript{\&\&}P < .01 in Lac-N versus Lac-L
comparisons. Asterisks indicate comparisons versus Virgins and crosses versus Rec-NN. \textsuperscript{*}P < .05, \textsuperscript{**}P < .01, \textsuperscript{***}P < .001, \textsuperscript{****}P < .0001}
\end{figure}
with the Rec-NN and Virgins groups (Figure 6A). This was the most important miR that we measured as it directly inhibits Runx-2 and suppresses bone formation by repressing Wnt/β-catenin negative regulators and is directly linked to our findings for decreased expression levels of Runx-2 in the Rec-LL group (Figure 5).

Using the DE of these specific miRs, bioinformatic analyses revealed a total of 174 genes predicted as potential targets, while 18 genes were common (Figure 6B, enlargement in Figure S3). Statistically significant biomolecular interactions, using constructed networks of GO and KEGG pathways, showed that, among others, some important bone regulatory mechanisms and biological processes can be affected, namely the Wnt and IL-6 signaling pathways, thyroid hormone synthesis, mesenchymal cell differentiation, and negative regulation of ERK1 and ERK2 cascades (Figure 6C, enlargement in Figure S4). Finally, it was found that the vast majority of the implicated gene targets (69.57%) were involved in Wnt signaling and pluripotency (Figure 6D).
In this study, we report that protein under-nutrition during gestation/lactation and recovery exert detrimental effects on the skeletal system of mouse dams and decreases the rate of bone accretion in the postweaning recovery period. We also examined the effects of low protein intake during gestation and lactation on skeletal muscle integrity as well as on NMJ morphology and importantly report for the first time in the literature that there were no differences when compared with normal protein consumption. Our first aim was to explore if a low protein diet during gestation and lactation can affect skeletal muscles. Lactation is linked to significant changes in maternal metabolism and subsequent adaptations, such as decreased adaptive thermogenesis, are necessary due to high-energy demands for sufficient milk production. Liver, skeletal muscle and white adipose tissue are the main metabolic tissues in mammals, and thus, their metabolic rates are modulated during lactation. Skeletal muscle protein mobilization acts as an adaptive response to meet these energy requirements and is also finely regulated in proportion to the dietary protein intake. In our cohort of mouse dams, no significant change in the TA skeletal muscle fiber CSA was observed, possibly due to activation of other metabolic pathways aiming to fulfil energy demands since both experimental diets (normal and low protein) were isocaloric. Maternal protein loss during lactation is decreased when total body protein mass exceeds certain levels to prevent exhaustion.

**FIGURE 6** Differential expressions (DE) of miR-26a, 34a and-125b (A) in Rec-NN, Rec-LN, and Rec-LL groups are shown for the recovery period and were compared to the Virgins control (n = 3/group). Using bioinformatics, the predicted target genes were mapped (B) and biomolecular interactions were designed by gene ontology (GO) and KEGG pathways analyses (C). Wnt signaling seems to be the dominant pathway affected by the selected miRs among the statistically significant regulatory mechanisms (D). All data are presented as mean ± SD. ns: not significant. Asterisks indicate comparisons versus Virgins and crosses versus Rec-NN. *P < .05, **P < .01, ***P < .001

**4 | DISCUSSION**

In this study, we report that protein under-nutrition during gestation/lactation and recovery periods reproduction exerts detrimental effects on the skeletal system of mouse dams and decreases the rate of bone accretion in the postweaning recovery period. We also examined the effects of low protein intake during gestation and lactation on skeletal muscle integrity as well as on NMJ morphology and importantly report for the first time in the literature that there were no differences when compared with normal protein consumption.
between Virgins, Lac-N, and Lac-L groups, supporting our findings that low protein intake during gestation and lactation does not dramatically alter skeletal muscles as these two systems function integrally.

Our second aim was to determine changes in the skeleton of mouse dams due to low protein diet during gestation and lactation. Pregnancy and lactation are very challenging periods for the maternal skeleton due to significant adjustments in calcium homeostasis. Our study showed that lactation resulted in a significant decrease of bone volume and deterioration of bone micro-architecture in both trabecular and cortical bone. It is known that lactation causes demineralization of the maternal skeleton, which leads to bone mass reduction. BV/TV was significantly reduced in the proximal tibia and L5 vertebral body, and this was accompanied by thinning of the trabeculae at both skeletal sites. Cortical bone thickness at the tibial mid-diaphysis was also considerably decreased by lactation. These changes were exacerbated in the Lac-L mouse dams indicating that low protein consumption leads to more profound skeletal deterioration than normal protein consumption during the reproductive period. Although some studies have reported that the extend of lactation-induced bone loss is different according to the anatomical site of the skeleton and is, particularly, higher in the spine than in the long-bones, our model did not identify any differences showing a universal effect on both sites. An important finding was that all microCT structural parameters were worsened in the Lac-L group as compared to Lac-N, and the changes in SMI and Tb.N show that low protein diet affects not only the overall bone mass, but also leads to micro-architectural changes. The increased SMI shows that the shape of trabeculae has altered from plate-like to a more rod-like structure, similar to other studies. However, these studies have significant differences from the present study, that is, mouse strain and larger litter size. Histomorphometric analyses of animal models have shown that bone turnover is increased during pregnancy and these high rates of bone formation and resorption are maintained throughout lactation. Our results indicate that mouse dams fed a normal diet during gestation and lactation, Lac-N, follow this trend but, on the contrary, the Lac-L mice show suppressed numbers of osteoblasts, and very high levels of osteoclastic bone resorption. Thus, the overall result is a significant bone loss as compared to the Lac-N group, and this is supported by the results of the microCT analysis. Our in vitro findings support our hypothesis that enhanced bone loss in the Lac-L mice was caused, at least partly, by the substantially reduced osteogenic capacity of the osteoblasts that was coupled with decreased expression of osteogenic genes in comparison with the Lac-N group. Dietary protein has beneficial effects on bone health, but very little is known regarding lactation. On the contrary, several studies have shown that a high fat diet leads to rapid bone loss in mice; however, most of the works used normal mice during other than gestational or lactational period, in which bone and energy metabolism significantly differ from the physiological state. The mechanisms that regulate lactation-related skeletal loss have been extensively studied, suggesting an interplay between increased osteocytic osteolysis, coordination by the brain-breast-bone circuit through pituitary-derived prolactin and oxytocin in response to suckling, which leads to the release of parathyroid hormone-related protein (PTHrP) and subsequent proliferation and activation of osteoclasts. However, the effects of protein intake on this molecular network are largely unknown and this needs further investigation.

Our next goal was to study the effects of low protein diet on the bone recovery period after weaning. Regardless of the accelerated bone loss that occurs during lactation, bone mass recovers promptly after weaning and termination of milk production. The recovery process is characterized by a sudden cessation of bone resorption and a remarkably elevated rate of bone formation with rapid re-mineralization of new bone matrix. Normally, the recovery period lasts approximately 6 months in humans and 4 weeks in mice and mineral content reaches the baseline values before pregnancy by gaining 2% to 3% mineral apposition per month in humans and 10% to 20% per week in mice. Some studies suggest that pharmaceutical approaches such as zoledronate or osteoprotegerin administration can prevent maternal lactation-induced bone loss and improve bone recovery, but concerns may arise for neonatal fetal growth and health. We found that after 28 days from the end of lactation only the dams that were on the normal protein diet throughout the entire experimental period (Rec-NN) achieved full recovery with microCT bone parameters that did not differ from the nulliparous control at any of the skeletal anatomical sites. Rec-LN mice had lower bone mass compared to the Rec-NN group, which shows that switching from low to normal protein diet after weaning leads to only partial bone recovery. The difference between Rec-LN and Rec-LL was of great interest, showing that maintaining mice on a low protein diet leads to an extensive delay in skeletal recovery. Interestingly, the Rec-LL group did not differ from the Lac-L during lactation, showing signs of persistent failure to recuperate lactation-induced bone reduction.

Our results indicate that a low protein diet has deleterious effects on osteoblasts and enhances osteoclastic bone resorption. Elefteriou et al have shown that activating transcription factor 4 (ATF4), a transcription factor that enhances amino-acid uptake and collagen synthesis in osteoblasts, is a crucial player highlighting the significance of protein intake in bone formation. ATF4−/− mice showed a deformed skeletal phenotype that was rescued by high protein diet intake through an increase of collagen type I synthesis and osteocalcin expression by differentiated osteoblasts due to higher amino-acid uptake. It has also been
reported that protein malnutrition stimulates bone marrow mesenchymal stem cells differentiation to adipocytes rather than osteoblasts and attenuates the bone anabolic response to PTH in female rats. Therefore, we examined the behavior of isolated Obs from the animals used for the recovery period experiments. The effect of low protein diet on osteoblasts was verified by our in vitro bone formation assay and gene expression of osteogenic markers. These results were unexpected since all cell cultures were managed under the same experimental conditions, that is, equal serum concentration and all in osteogenic medium. We assumed that when isolated Obs return to normal protein levels in vitro, they would have similar osteogenic behavior. In contrast with our expectations, osteoblastic cells retained the in vivo bone-forming capacity. These striking results led us to the novel conclusion that the nutritional protein level is able to leave a molecular signature in these cells.

We, therefore, hypothesized that the observed osteogenic activity during the recovery period is potentially regulated by epigenetic mechanisms or differential expression of bone-related miRs. We selected four key genes that are master regulators of osteoblastic differentiation and activation (Bmpr1a, Ptc1, Osx, and Igf1r) and Sirt1, a NAD-dependent deacetylase, which promotes osteogenic differentiation and its expression is affected by dietary protein. Specific-site DNA pyrosequencing analysis revealed no differences in methylation patterns of selected genomic loci in the promoter region of osteoblastic regulatory genes. However, other CpG rich regions may be affected and this possibility needs further study. On the contrary, we found differential expression of miRs −26a, −34a, and −125b, which have been shown to regulate bone-related genes. MiR-125b regulates the osteogenic differentiation of human MSCs by targeting BMPR1b while miR-26a reverses the bone regeneration deficit of MSCs and miR-34a inhibits osteoclastogenesis and its expression is affected by dietary protein. Specific-site DNA pyrosequencing analysis revealed no differences in methylation patterns of selected genomic loci in the promoter region of osteoblastic regulatory genes. However, other CpG rich regions may be affected and this possibility needs further study. On the contrary, we found differential expression of miRs −26a, −34a, and −125b, which have been shown to regulate bone-related genes. MiR-125b regulates the osteogenic differentiation of human MSCs by targeting BMPR1b while miR-26a reverses the bone regeneration deficit of MSCs and miR-34a inhibits osteoclastogenesis. In addition, Bglap and Osx are directly targeted by miR-125b while downregulation of its expression results in high levels of Vdr. Transfection of hBMSCs with miR-125b mimics induces decreased osteogenic differentiation with lower expression levels of Alp, Colla1, and Bglap. Thus, elevated levels of miR-125b inhibit bone formation, which we also describe in this study. The results exploring the role of miR-34a in osteogenesis are contradictory. It has been shown that miR-34a overexpression results in increased osteogenic differentiation of hASCs and reduces the inhibition of osteogenic differentiation of murine MSCs by dexamethasone. On the contrary, downregulation of miR-34a diminishes arthritis and bone loss in mice. To explain these conflicting findings, the distinct cell attributes as well as the different osteogenic regulation in health and disease has to be taken into consideration. Finally, miR-26a enhances angiogenesis-osteogenesis coupling and augments bone regeneration and repair. Furthermore, it regulates osteogenic differentiation of ADSC by targeting Smad1, which mediates BMP signaling pathway, resulting in increased levels of osteopontin. It is also of great interest that GSK3β and Smad1 has also emerged as predicted targets of miR-26a in BMSCs, which results in increased Wnt3a and, consequently, higher bone formation. During the bone recovery period, the expression of these miRs was altered favoring suppression of bone formation in combination with elevated resorption that was profound in the Rec-LL mice. This effect can be driven by dietary protein content as the Rec-LN dams showed partially recovery. Based on these observations, we performed a bioinformatic analysis aiming to examine, which genes could be potential targets of the specific miRs, and which revealed regulatory pathways involved in important bone homeostatic mechanisms. The main pathways affected appear to be the Wnt and IL-6 signaling pathways, and these have profound effects on osteoblasts and osteoclasts, respectively.

This study shows for the first time that maternal protein undernutrition during gestation/lactation leads to bone recovery retardation. We focused on the phenotypic description of our model by providing novel information on the skeletal morphology (microCT) and the major cellular events (histology, in vitro). Our data indicate that maternal protein intake and lactation-induced bone loss may be regulated by changes in miR expression. MiRs have shown long lasting effects in various pathologies and have been also established as potential biomarkers in bone disorders such as osteoporosis. However, this work is not without limitations. While we have shown that bone formation is compromised in low protein fed dams, a detailed molecular/cellular mechanism underlying remains to be unraveled. Furthermore, we anticipate verifying our bioinformatic approach for miRs both in vitro and in vivo. Next generation sequencing using Whole Genome Bisulfite Sequence, ChIP-seq, and small-RNA seq can be of great value to decipher a possible nutriepigenetic mechanism.

In conclusion, here, we report that low protein intake during the reproduction period does not affect the skeletal muscles and associated NMJs in mouse dams. However, protein under-nutrition increases lactation-induced bone loss, and maintenance on low protein diet during the recovery period delays bone restoration. We provide strong evidence to support our dual conclusion drawn by this study: a low protein diet decreases not only the total amount of cells with osteoblastic properties, but also the osteogenic potential of bone-forming cells. Importantly, isolated osteoblasts show similar in vitro osteogenic behavior to the in vivo findings, suggesting a possible epigenetic mechanism. This dietary protein-dependent effect on bone metabolism might be controlled by changes in the expression of specific miRs. Further studies are required to identify the mechanism(s) underlying these effects of low protein diet. A full understanding of the mechanisms would be expected
to lead to improved nutritional guidelines during reproduction and could identify new targets for the treatment of musculoskeletal disorders.

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CONFLICT OF INTEREST
The authors have no conflicts to declare.

AUTHOR CONTRIBUTIONS
I. Kanakis and A. Vasilaki designed the experiments with input from K. Goljanek-Whysall, R.J. van ‘t Hof, T. Liloglou, and S.E. Ozanne; I. Kanakis, M. Alameddine, and M. Scalabrin performed the experiments, acquired, and analyzed the data; I. Kanakis and A. Vasilaki wrote the manuscript, which was critically revised and approved by all coauthors.

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