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# Milk peptide induced miRNA-369 facilitates bone functions in the rat osteoblasts

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ABSTRACT

The animal's health and productivity is chiefly influenced by its anatomy and physiology. Moreover, bone growth in animals is also associated to overall health and production. Besides, bone fractures accounts to constrained food intake, reduced immune function and general productivity losses. In poultry animals, bone deformities, renders severe costs in the meat production and egg quality. The treatment for various bone related issues is both time and capital seeking. Besides, currently available therapies, i.e. bisphosphonates, raloxifene are associated with various side effects like jaw osteonecrosis, vaginal bleeding etc. The present study reports the bone promoting effect in milk peptide induced miRNA, miR369. Findings represents an enhanced bone proliferation and differentiation under the influence of miR369 in *in vitro* rat osteoblasts culture. The bone marker enzyme alkaline phosphatase and osteoblast specific protein expression were tested using ELISA. miR369 significantly stimulated the marker protein levels. Additionally, miR369 when injected to neonatal rat pups, showed improved osteoblast marker gene(s) expression. The study demonstrates the positive role of miR369 in the bone functions and hence, advocates its functional utility for the management of various bone ailments prevailing in animals and/or humans. Besides miR369, a putative molecular gene therapeutic agent can prove revolutionary in controlling the animal health losses ascribed to deteriorating bone health.

**Keywords**: Animal, Bone, miRNAs, Osteoblast, Peptide

The anatomy and physiology of an animal evidently influences its health and productivity. Moreover, the complete wellbeing of the animal is very essential for maintenance of the productivity across the globe (Bork *et al.* 2013). Several diseases, viz. swine fever, fractures, anaemia are the prevailing obstructions to the progress of animal productivity in the society. Bone health is one such crucial matter that seeks the most attention. Bone injuries are associated with numerous subjects of concern, e.g. reduced diet intake and decreased immunity in the body (Karpouzos *et al.* 2017). Bone fractures in equines are very common and totally hamper their movement, running and weight carrying capacity. Furthermore, osteopathies in dogs especially craniomandibular osteopathy, that targets the mandible region of the dogs suffers from limitation of precise treatment due to the constraint of unidentified causes for the disease (Krastev *et al.* 2015). The recovery from bone disorders demands capital and time. Besides, technical expertise is indispensable for the treatment and post-operative care of the animals in the orthopaedic surgeries. Hence, compounds based on natural food sources having less side effects can serve as a boon in the diseased situation. Within this context, osteoporosis is regarded as bone metabolic disease, marked by reduced bone content and strength. It is established as a condition of compressed bone mineralization, perturbed bone architecture and enhanced bone instability (Hak 2018, Lupsa and Insogna 2015). During osteoporosis, balance between bone formation and disruption is lost that has adverse repercussions on bone connectivity and resulting into fractures (Eastell *et al.* 2016). It is identified as one of the significant public health issue generally in individuals beyond 50 years of age (Ishimi 2015). To this end, 2 therapeutic methods, viz. osteoanabolic and anti-resorptive agents are accessible at present. Nevertheless, these strategies suffer from convinced limitations. *De novo* bone formation and incapability in replenishing the old injured bone is one prime restriction of the antiresorptive agents, whilst osteoanabolic agents are unable to resist unwanted bone destruction (Upadhyay *et al.* 2020). This necessitates the development of novel compounds with definite action on bone formation and loss.

microRNA (miRNA) driven gene therapy may corroborate as one of the crucial remedial measures in the control of osteoporosis. miRNAs are short, 18–23 nucleotides, RNAs that are non-coding; controlling the post-transcriptional gene expression (Karagkouni *et al.* 2020). They can target multiple genes and may subsequently affect the expression of several proteins (Tran and Montano 2017). miRNA mostly binds with 3' untranslated region of the gene and influences biological responses like cellular

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differentiation, cell cycle progression and apoptosis along with crucial genes for the bone regeneration (O'Brien *et al.* 2018). Furthermore, several studies have detailed the role of miRNA in the osteoclast resorptive activity. miR-503 supresses osteoclast differentiation in the peripheral blood mononuclear cells, suggesting miRNA based gene antagonism a vital tool in controlling various osteogenic processes (Chen *et al.* 2014). Additionally, the fact that miRNA can provide multiple gene therapy targets makes them eligible to be employed as novel next-generation therapeutics in distinct disorders, including osteoporosis (Kaur *et al.* 2021b).

Moreover, lately broad scientific confirmations have accommodated the presence of bioactive peptides, extracted from proteins in the milk and milk products that shows beneficial responses on human well being (Martínez-Villaluenga and Hernández-Ledesma 2020). It has been observed that these food based peptides are physiologically active and osteoprotective in nature (Mada *et al.* 2019). A bioactive peptide (PepC/ VLPVPQK) isolated from milk protein β casein by our group, has been validated in the *in vitro* and *in vivo* systems for its osteoanabolic action (Shanmugam *et al.* 2015, Vij *et al.* 2016, Mada *et al.* 2017). Additionally the peptide has been reported bio-accessible in nature across the trans-epithelial barrier (Vij *et al.* 2016). As studies indicate that PepC possess high potency in enhancing the osteoblast proliferation, differentiation (Reddi *et al.* 2016, 2018) and considering the pre-eminence of miRNA in the bone functioning, we conjectured that one of the mechanisms by which PepC may be exercising its osteoprotective responses is by changing the expression of various miRNAs in the rat osteoblast cultures; which was previously affirmed by PepC induced miRNA profiling of the rat osteoblast cells relative to the control (Vij 2017). Understanding the mechanism of action employed by concerned miRNAs that are specific to bone signaling components not only elaborates the role of miRNA in the osteoblast differentiation but will also provide therapeutic insights for the control of several bone related ailments in the correction of mis-regulated signaling cascades (Kaur *et al.* 2021a). It is thereby essential to correctly identify and validate miRNAs in the biological systems. Therefore, present study focuses on identifying and characterizing bone specific miRNAs from the profiled data, which may establish the role of profiled miRNA in the biological bone signaling pathway and perhaps the pathogenesis of bone disorders. Through this investigation, we observed that PepC induced miR369 (rno-miR369), regulates bone differentiation positively. Additionally, the therapeutic potential of miR369 in the management of bone ailments has been suggested.

## MATERIALS AND METHODS

*Chemicals and plastic wares:* Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), antibiotics (penicillin, streptomycin and amphotericin), MTT (3 – [4,5 – dimethylthiazol – 2 – yl] – 2, 5 – diphenyl tetrazolium bromide), ascorbic acid, β -glycerophosphate, DMSO were purchased from Sigma-Aldrich Chemical Co., St. Louise, MO, USA. mirVana miRNA isolation kit, lipofectamine RNAiMAX, Opti-MEM™ media were purchased from Invitrogen. miRNA mimic, and miRNA negative control (miC) were custom prepared through Ambion (Applied Biosystems, Foster City, CA, USA). RevertAid First Strand cDNA Synthesis Kit, 2× maxima SYBR Green qPCR Master Mix (Thermo Scientific), miScript II RT kit and miScript SYBR® Green PCR Kit (Qiagen, Madrid, Spain), Osteocalcin (OCN) ELISA kit was procured from Elabscience (Elabscience Biotechnology 133 Inc., Hubei), Buffalo milk's β-casein derived novel peptide (PepC) with more than 98% purity was custom prepared from Linkbiotech Pvt. Limited, India. Plastic dishes, culture plates and flasks were purchased from Corning (Corning, NY, USA).

*Isolation and culturing of rat calvarial osteoblasts (RCO):* Neonatal rat pups were acquired with consent from the Institute Ethics Committee of Animal Experiments in the National Dairy Research Institute, Karnal, India (Approval no: 41–IAEC-18–61). The isolation of pre-osteoblast cells was executed as described by Taylor and coauthors (Taylor *et al.* 2014). The acquired cells were seeded in 25 $cm^2$ culture flasks in DMEM with 10% FBS, penicillin, streptomycin and amphotericin-B, were ultimately maintained at 37°C, 5% $CO_2$ (humidified atmosphere) in growth media and allowed to differentiate in an osteoblast specific differentiation media (OBDM), i.e. treatment with 10 mM β-glycerophosphate and 50 µg/ml ascorbic acid for 4 days.

*miRNA next generation sequencing (NGS) and data analysis:* NGS protocol was conducted by Illumina multiplexed sequencing platform using TruSeq Small RNA library Preparation Kit (Illumina Inc.) and analysis of the collected data was performed as stated previously in our lab (Vij 2017).

*miR369 transfection protocol:* Transfections in the present study were carried out using Lipofectamine RNAi MAX reagent in Opti-MEM® I Reduced Serum Medium (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol. RNA oligos representing mature sequences that mimic endogenous miRNAs of rno-miR-369 (miR369) and miRNA negative control (miC) (Ambion, Carlsbad, CA, USA) were supplied to the RCO at 50 nM concentration with Lipofectamine RNAi MAX for 6 h. After 6 h, transfection medium was changed to differentiation medium. After 4 days of miRNA transfection, cells were harvested for further analysis (Kaur *et al.* 2021b).

*Cell proliferation assay:* Rat osteoblast cells were treated with miC (50 nM), miR369 (50 nM) and PepC (30 ng/ml). After four days of treatment, MTT solution (0.5 mg/ml) was added to treated wells and placed on a shaker for 5 min followed by MTT assay (Mosmann 1983).

*ALP (Alkaline phosphatase) activity assay:* Approximately $3\times10^3$ rat osteoblast cells were seeded in a 96 well plate. Then the cells were transfected with miC and

Table 1. Primers used for gene expression studies

| Gene | Forward | Reverse |
|---|---|---|
| Col | TTCACCTACAGCACG CTTGTG | GATGACTGTCTTG CCCCAAGTT |
| ALP/Alp | CGTCTCCATGGTGGA TTATGC | TGGCAAAGACCG CCACAT |
| OCN | GAGCTAGCGGACCA CATTGG | CCTAAACGGTGG TGCCATAGA |
| Osx | ATTGGTTAGGTGGT GGGCAG | GGGCAAAGTCAG ACGGGTAA |
| Runx2 | CCCAGGCGTATTT CAGATG | GAACTGCCTGGG GTCTGAAA |
| miR369 | AATAATACATGGTTG ATCTTT | – |
| GAPDH | GACAACTTTGGCATC GTGGA | TGTTCTGGATGCA GGGATGA |

Col, Collagen α; ALP, Alkaline phosphatase; OCN, osteocalcin; Osx, Osterix; GAPDH, glyceraldehyde 3 phosphate.

miR369 at 50 nM concentrations in the differentiation medium for 4 days followed by ALP assay (Kaur *et al.*2021b), while PepC act as positive control.

*Expression analysis for miRNA and osteogenic marker genes:* RCO were treated with media containing PepC (positive control), miC and miR369 mimic, were maintained separately for 4 days. The cells were assessed for the expression of osteoblastogenic marker genes, viz. Alkaline phosphatase (ALP), Collagen α Type I (col), Osterix (Osx), Osteocalcin (Ocn), Runx2 and miR369 (rno-miR-369) using miScript II RT kit (Qiagen, Madrid, Spain) against the control group after reaching confluency. To prove sample equality, amplification of the housekeeping gene GAPDH was used as internal control in mRNA gene expression while RNU6 was used in miRNA expression analysis. The primers are enlisted in Table 1 and the study plan is presented in Fig. 1a.

*OCN (osteocalcin) protein expression:* Rat osteoblast cells were treated with miC and miR369 at 50 nM in the differentiation medium for 4 days. The cell culture supernatant was then used for the protein expression analysis using OCN/BGP ELISA Kit, BT Laboratory, Shanghai Korain Biotech Co Ltd as per the manufacturer's recommendations.

*Expression analysis for osteogenic marker genes in neonatal rat pups:* 1–2 days old rat pups were categorized in three groups (n=8), i.e. miC control, miR369 mimic and PepC (positive control). miC and miR369 groups were transfected with miC and miR369 respectively using transfecting reagent, *invivo*jetPEI® solution (Polyplus Transfection, Illkirch, France). Each pup was injected with 1.8 µg oligonucleotide in the calvarial region through subcutaneous route (single dose) in a span of 4 days. Solutions of oligonucleotides were prepared, following manufacturer's indications (Polyplus Transfection). While pups in the third group (PepC) were fed with 0.8 µg PepC per pup for 4 days (Mada*et al.*2017). Finally, rat pups were sacrificed and calvaria was collected for gene expression after 4 days (Kaur *et al.* 2021b) (Fig. 1b).

*Statistical analysis:* The data were analyzed by GraphPad Prism software (v.8), statistically, via. student T test, one-way ANOVA, and finally Newman–Keuls test. These experiments were repeated 3 times and representative values and/or average values are reported in the manuscript in form

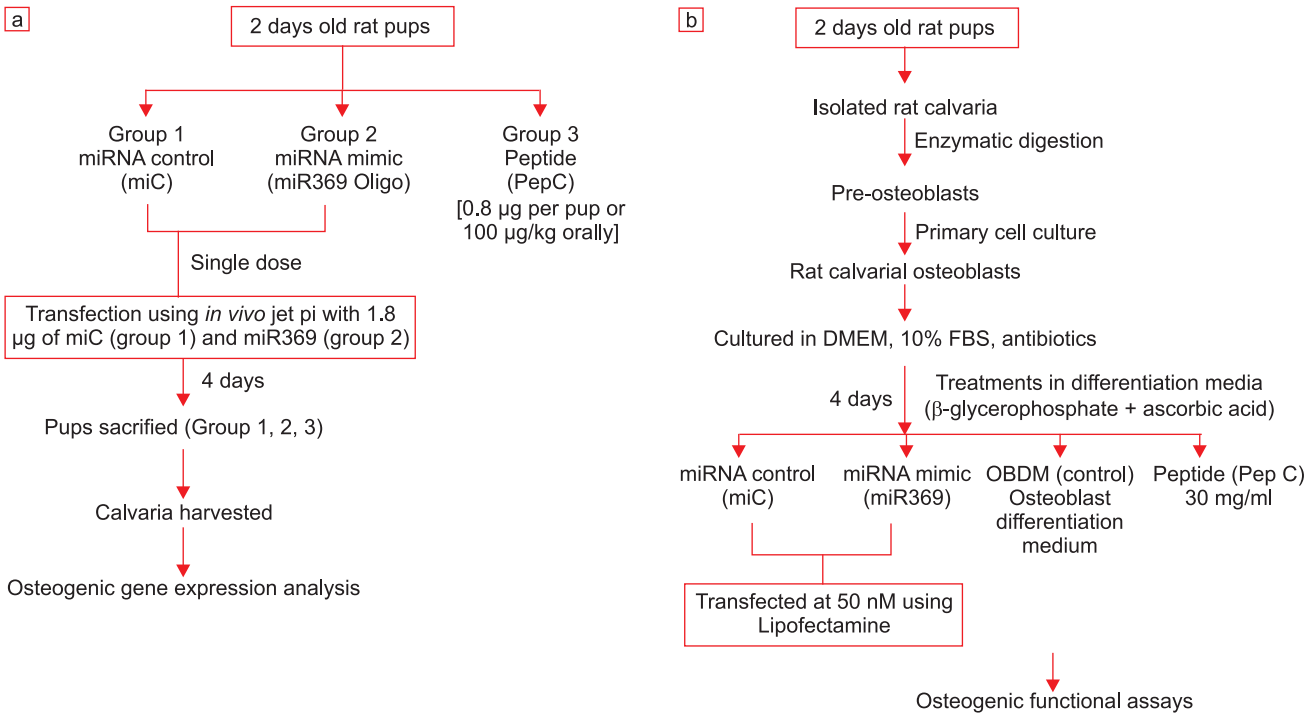


Fig. 1. Detailed study plan. (a) *In vitro* studies (rat calvarial osteoblasts); (b) *Ex vivo* studies (neonatal rat pups).

of mean±SEM, value of P<0.05 was represented as statistically significant.

## RESULTS AND DISCUSSION

*Profiled miRNAs in response to PepC and selection of promising miRNA(s):* Previous studies in our lab have offered a repertoire of differentially expressed miRNAs as induced by milk derived bioactive peptide, PepC. The osteoblast cells were treated with PepC at 30 ng/ml in the growth media supplemented with osteoblast differentiation components, β-glycerophosphate and 50 µg/ml ascorbic acid (OBDM) for 4 days and finally these treated cells were subjected to next generation sequencing run for significant regulation in miRNAs relative to the control (non-treated cells). Fig. 2a displays the list of profiled 191 miRNAs obtained in response to treatment with PepC.

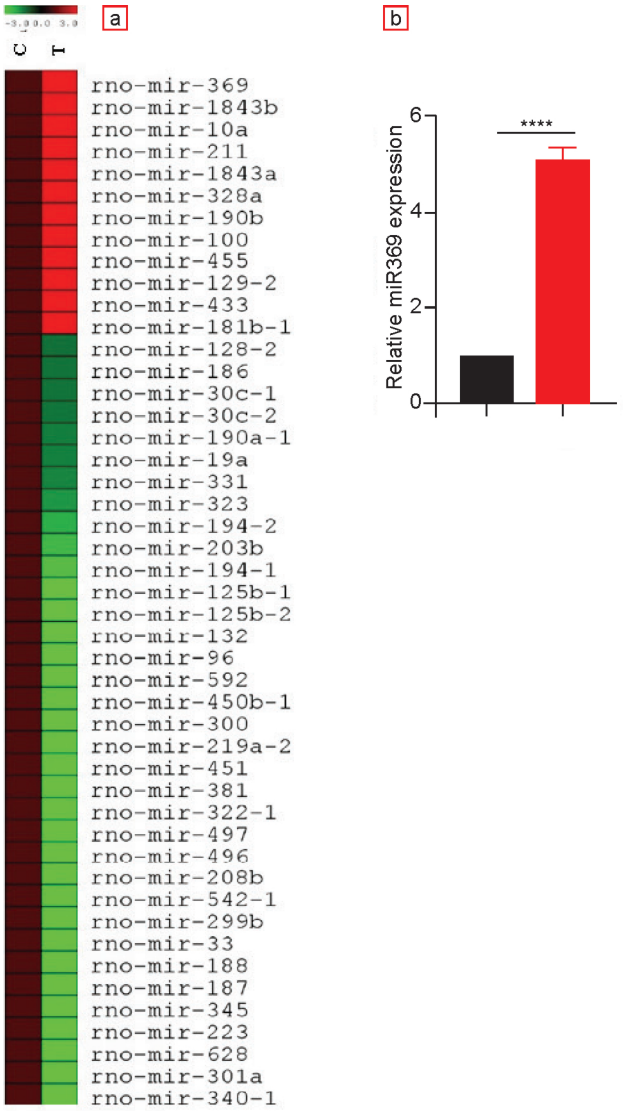


Fig. 2. PepC upregulates the expression for miR369. (a) Heat map representation for the miRNA profiled data, on treatment with PepC in the rat calvarial osteoblast cultures. Red color demarcates upregulated miRNAs, green displays downregulated miRNAs in response to PepC. (b) *In vitro* validation of miR369 expression in PepC treated cells relative to control (OBDM, Osteoblast Differentiation Medium). C, Control; P, PepC treated; PepC, VLPVPQK. All values represent means±S.E. (n=3). *P<0.05, **P<0.01, ***P<0.001 compared with the OBDM.

*Screening of promising miRNA:* Out of 191 profiled miRNA in the next generation sequencing data, rno-miR-369/miR369 was selected for further analysis as it was found significantly upregulated (11.39 folds) in the presence of PepC relative to other miRNAs in the *in silico* data. Expression of miR369 was validated *in vitro* by real time PCR, results showed increased expression of miR369 (5.09 fold) in the presence of PepC in comparison to control (OBDM) (Fig. 2b).

*miR369 upregulates osteoblast proliferation and differentiation in vitro:* Further, osteogenic functional assays were performed under the influence of miR369 in comparison to miRNA control (miC). The findings demonstrated stimulated proliferative index, ALP activity, osteoblast differentiation-marker genes and osteogenic marker protein (OCN) expressions in the miR369 treatments relative to miC (Fig. 3a-g).

*miR369 improves bone formation in rat pups:* The investigations from *in vitro* trials were extended to *ex vivo* level. The calvariae collected from rat pups tested for the expression of osteogenic genes, miR369 improved the expression of all osteogenic genes in comparison to miRNA control, miC (Fig. 4a-e).

miRNAs, the post-transcriptional gene silencers, are regarded as critical regulators for the gene (majorly acting against 3'UTR regions) and protein expression (Kaur *et al.* 2020a). Interestingly, they have been stated to target 1/3rd total gene count in the genome of human (Paul *et al.* 2020). They majorly work in favour or against the gene transcripts partaking in the bone signaling pathways regulating osteoblast proliferation, differentiation and mineralization (Nakasa *et al.* 2015). Moreover, miRNAs are documented as critical players in the process of osteogenic differentiation and mineralization, such as induced expression of miR-143 in the MC3T3-E1 cells, encourages their differentiation capacity (Wang *et al.* 2020). Likewise, suppression of miR-19b-3p leads to diminished osteoblast proliferation and differentiation (Xiaoling *et al.* 2020). Besides, studies have reported for coupling action of miRNAs in the maintenance of osteoblast cum osteoclast mediated bone functions, miR-21 upkeeps osteogenic differentiation associated with viability of the osteoclasts (Smieszek *et al.* 2020). Apart from osteoblasts, prominent role of miRNAs has also been exhibited in other accessory bone lineages, miR-411 persuades autophagy related processes in chondrocytes facilitating an essential molecular therapy platform for osteoarthritis (Yang *et al.* 2020). Hence, the present study embarks to delineate the regulatory significance of miRNAs that are induced by a milk derived osteoanabolic peptide (VLPVPQK/PepC) in the osteoblast differentiation. The investigation is focused on demarcating the role of rno-miR-369/miR369 in the osteogenic progression by predicting its influence on the proliferation, differentiation of rat osteoblast cultures and rat neonatal pups.

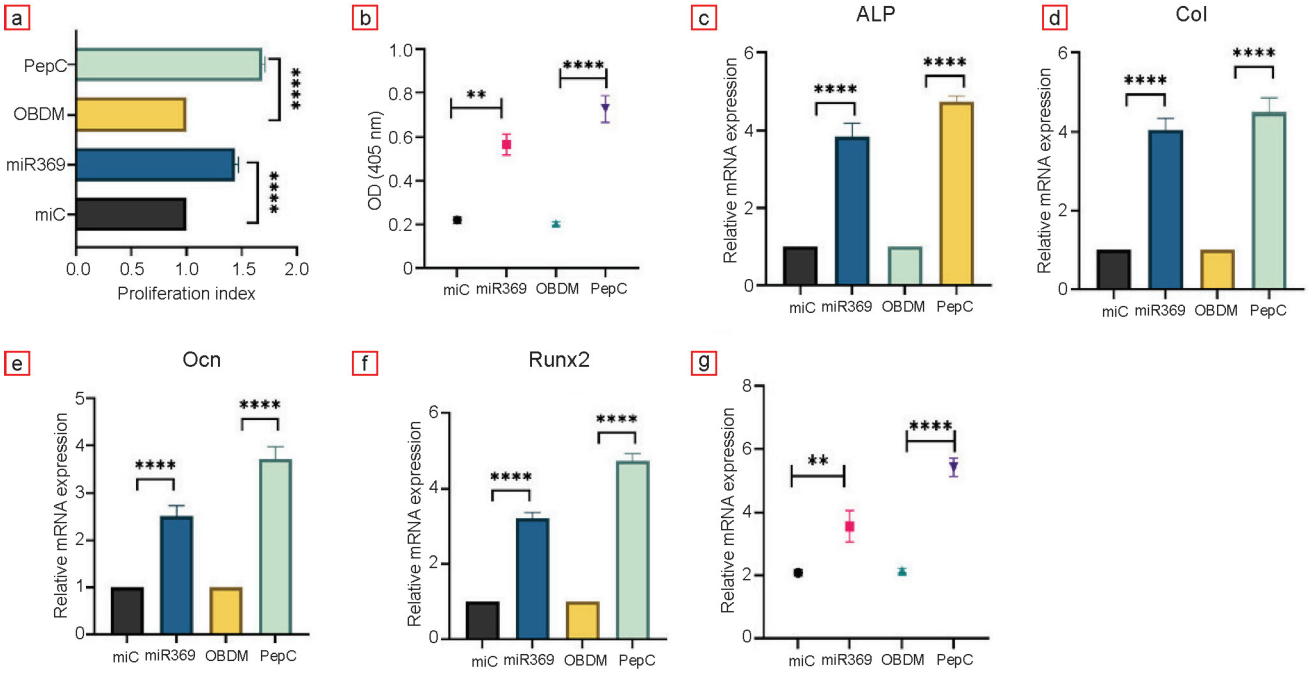


Fig. 3. miR369 stimulates osteoblast proliferation and differentiation. a. Proliferation index (PI) for the cells treated with miR369, miC (miRNA negative control), PepC (positive control), OBDM osteoblast differentiation medium (peptide control medium conditions) for 4 days in the differentiation medium. b. ALP (alkaline phosphatase) activity in terms of absorbance on treatment with miR369 relative to miC. c-f. Gene expression analysis for ALP, Col (collagen 1 alpha), Ocn (osteocalcin), Runx2. g. Osteocalcin protein expression by ELISA. All values represent means±SE (n=3). *P<0.05, **P<0.01, ***P<0.001 compared with the miC or OBDM.

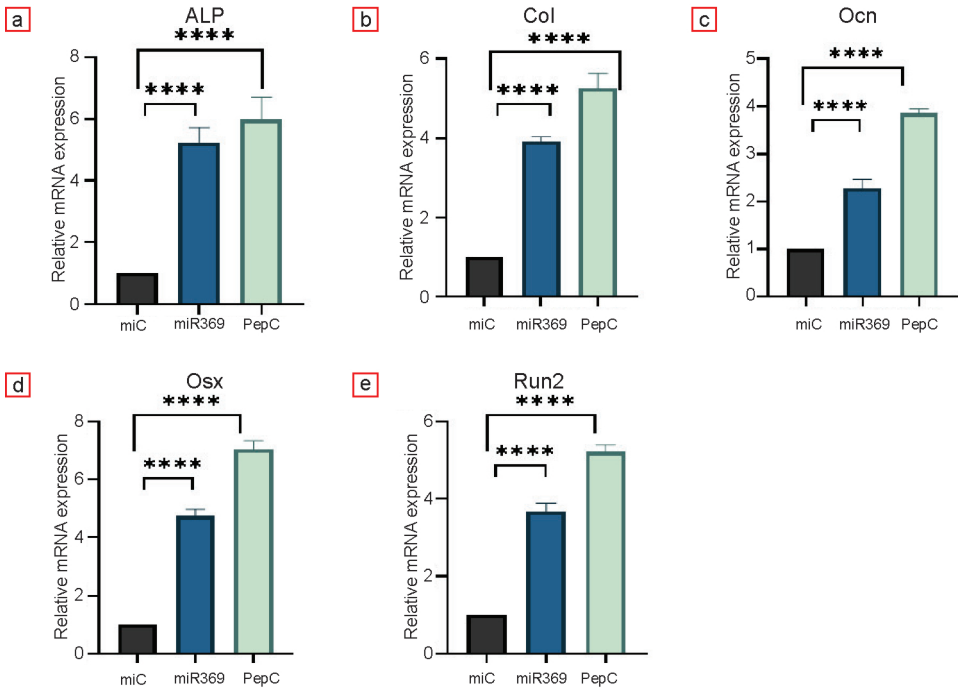


Fig. 4. miR369 stimulates osteogenic differentiation in *ex vivo* rat pup's calvaria. a-e. Gene expression analysis for Alp, Col (collagen 1 alpha), Ocn (Osteocalcin), Osx (Osterix) and Runx2 in presence of miR369 relative to miC (control). All values represent means±SE (n=8). *P<0.05, **P<0.01, ***P<0.001 compared with the miC. PepC is positive control.

Ossification is a multifarious process, controlled by biochemical signaling pathways operating in the osteoblasts and osteoclasts. Numerous growth/ transcriptions factors control this complex process of osteogenesis, viz. BMPs, Wnt, β-catenin, etc. BMPs (Bone Morphogenic Proteins), a group of metabologens are critical regulators of bone functions (Katagiri and Watabe 2016) whereas Wnt/β-catenin, a distinct signaling pathway regarded indispensable for the maintenance of bone mass via activation of numerous bone developmental events (Krishnan *et al.* 2006). Other

than this, copious evidence is available about the communication between bone-signaling pathways for the preservation of bone remodeling in the adult skeleton (Nakashima *et al.* 2005). miRNAs influence these signaling cues with subsequent up or down regulation of the pathway. Various miRNAs have been found to target wnt/β-catenin signaling in the skeletal growth that includes microRNA 141 which constrains jaw osteoporosis in the ovariectomized rats (Liu and Guo 2020). Further, miR-203 and miR-320b target BMP2 stimulated osteoblast differentiation by suppressing Dlx5 (Laxman *et al.* 2017).

Moreover, several evidences have dictated the existence of bioactive peptides, derived from proteins in the milk and other sources, exhibiting positive impact on human and animal health (Martínez-Villaluenga and Hernández-Ledesma 2020). It has been documented that these bioactive peptides display biologically active and osteoanabolic traits (Mada *et al.* 2019). Besides, several past investigations uncovered that, milk items and the associated bioactive peptides have appeared to increment bone mass as well as bone development (Ahn and Je 2019). These peptides have been reported to prevent bone loss related issues in the pre- and post-menopausal women (Anusha and Bindhu 2016). Tripeptide (IPP Bioactive) supports bone formation by controlling osteoclastogenesis and promoting osteoblast formation, while whey based biopeptides (YVEEL, YLLF) showed enhanced osteoblast differentiation and mineralization (Chakrabarti and Wu 2015, Pandey *et al.* 2018). Likewise, synthetic novel bioactive peptide (VLYVPQK) demonstrated augmented osteogenic behavior in the *in vitro* rat calvarial osteoblast cultures (Upadhyay *et al.* 2020).

This study majorly relies on the optimum screening and identification of promising miRNAs from a previously profiled data from our lab. This data comprehends 191 miRNAs induced by PepC in the osteoblast cultures. miR369 was selected post screening, as it was significantly upregulated in response to PepC, less explored for its osteogenic potential, its mode of action was novel to elucidate and finally its working mechanism can be traced upon understanding its novel regulatory gene targets. miR369 has been evaluated for its expression in mesenchymal stem cells previously (Bork *et al.* 2011). Moreover, its role is also evident in the regulation of inflammatory reactions (Scalavino *et al.* 2020). In the context of functional role in bone formation, *in vitro* validation of miR369 in the rat calvarial osteoblasts was examined, the investigation initiated with expression analysis for miR369 under the presence of PepC. As indicated by results presented, increased expression for miR369 in the PepC treated cases relative to non-treated, the study proceeded further to evaluate the function of miR369 on the osteoblast proliferation. miR369 and miC (miRNA control) were transfected at 50 nM concentration, so as to over-express its expression. This over-expression for miRNAs is generally performed in the *in vitro* experiments to study the effect of miRNA for the underlying biological processes (Kaur *et al.* 2021b). miR369 activated the proliferative process in the rat osteoblast cells. Likewise, earlier studies, reported the enhanced proliferation in the osteoblast cells through another miR-25 mediated induction (Li *et al.* 2018). The investigation further suspects the improved osteoblast differentiation in the presence of miR369, owing to the increased values for ALP activity, OCN protein levels and increased osteogenic marker gene expressions. This represents the ability of miR369 to persuade osteoblast development, ultimately resulting in stimulated bone formation in animals and/or humans. Similarly, several studies in the past have concluded about the importance of miRNAs in the animal developmental processes and regulation of animal biological pathways (Alvarez-Garcia and Miska 2005, Gebert and MacRae 2019). This further motivated the current findings to the *ex vivo* level, wherein it was noticed that miR369 improved the expression for osteoblast differentiation associated marker genes relative to control, show-casting the ability of miR369 in the induction of osteogenic stimulation at the *ex vivo* level as well.

In conclusion, the study reports the novel regulatory role of miR369 in the osteogenic proliferation and differentiation. It showed the mechanistic potential of PepC in the regulation of miR369. Considerably, miR369 can be employed as potential therapeutic measure for the management of diseases characterized by diminished bone function in the animals. miR369 certainly acts positive towards the bone function and application of efficient or specific miR369 delivery methodologies would emerge as a valuable therapeutic approach for the control of various bone ailments preventing the animal's health and productivity losses. Nevertheless, future detailed research work is needed in this regard for the final miRNA based specific product development using large animal models.

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