Overexpression of QM Induces Cell Differentiation and Mineralization in MC3T3-E1

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It has been reported that QM was highly expressed by cells isolated from epiphyseal cartilage as opposed to proliferative chondrocytes. *In vitro* investigation of the expression of QM revealed higher QM expression in nonmineralizing osteoblast and pericyte cultures as compared with mineralizing cultures. These evidences suggest that QM may play an essential role in cell differentiation before mineralization. However, our research results showed that QM overexpression in MC3T3-E1 enhanced cell differentiation and mineralization. In this study, alkaline phosphatase (ALP) activity and nodule mineralization were increased in MC3T3-E1 from QM overexpression cultures relative to normal expression QM cultures. RT-PCR revealed upregulation of the marker genes type I collagen, ALP, osteocalcin, osterix and BMP-2 and a slight decrease of a negative regulator osteopontin. These results suggest that the increasing of QM expression could stimulate osteoblast differentiation and mineralization in MC3T3-E1.

Key words QM; overexpression; osteoblast; differentiation; mineralization

QM protein, identified as a putative tumor suppressor, was first detected in a subtractive hybridization assay in Wilms' tumor cell line.[1] QM gene encodes the ribosomal protein L10 associated with 60 S ribosomes in the rough endoplasmic reticulum and is a key factor in the combination of 40 S and 60 S ribosomal subunits.[2—4] In the organs of human, QM protein level is higher in superior cervical ganglion, liver, lymphomaburkitts rail, while QM protein level is higher in the prostate and bladder of mouse. In cells of human and mouse, QM protein level is higher in $CD19^+$ B-cells and $CD4^+$ T-cells (http://expression.gnf.org). Increasing evidence indicated that QMs have several extraribosomal functions besides their roles in protein synthesis.[5,6]

QM homologues have been identified in plants, animals, and fungi. QM proteins identified are basic and hydrophilic, with molecular weights of 24 to 26 kDa. The sequence of QM proteins highly conserved suggests its fundamental and critical functions in eukaryotic organisms. Embryos and bone development studies indicate that QM proteins are highly expressed in undifferentiated cells, while differentiated and adult tissues tend not to maintain QM proteins, implying that QM may regulate development and differentiation.[7—9] A chicken QM homologue protein, Jif-1 (Jun-interacting factor 1), was able to bind c-Jun, a member of the transcription factor complex AP-1 and a target of cellular regulation, and subsequently inhibit the transcription process activated by c-Jun,[10,11] but it has been observed that QM and Jun have a rare interrelationship *in vivo*.[4] This evidence implies QM may function as a cofactor of c-Jun and is involved in transcriptional regulation. This is supported by the report that another negative regulator of Jun, presenilin 1 (PS1), also interacts with QM/Jif-1 and promotes its translocation into the nucleus.[12] QM/Jif-1 was also found to have a function in regulating proto-oncogene c-Yes and reduced c-Yes kinase activity by 70%.[13] Since c-Yes is one of the Src family kinases expressed ubiquitously in most tissues and presumably involved in many signaling pathways,[14] QM is supposed to be a regulator of various signal transduction pathways. Recently, another study showed that homozygous inactivation of the multiple endocrine neoplasia type 1 (MEN1) gene statistically correlates to higher expression of QM/Jif-1.[15]

It is interesting that QM is highly expressed by cells isolated from epiphyseal cartilage as opposed to proliferative chondrocytes. *In vitro* investigation revealed higher QM expression in nonmineralizing osteoblasts and pericyte cultures as compared with mineralizing cultures.[9] We have successfully isolated a full-length cDNA encoding a QM counterpart in pearl oyster (*Pinctada fucata*) from the mantle cDNA library using a pair of degenerated oligonucleotide primers based on the conserved regions of other organisms.[16] These evidences suggested that QM might have a role in mineralization. However, a deeply and detailed study on the role of QM in the mineralization have not been performed. Present study was aimed at determining whether overexpression of QM would lead to alterations in osteoblast differentation and mineralization, which could contribute to know the roles of QM in the cell mineralization.

MATERIALS AND METHODS

Cell Culture A robustly mineralizing subclone of the MC3T3-E1 cell line that has been previously described was used in this study.[17] Cells were seeded at 2.5×10^5 cells/cm^2 in 96-well plates (Franklin Lakes, NJ, U.S.A.) for ALP activity assays and at 1×10^5 cells/cm^2 in 6-well plates for transfection, histological assays and RT-PCR analyses. Cells were maintained in α-minimal essential medium (α-MEM) (GIBCO BRL Life Technologies, Invitrogen Corporation, CA, U.S.A.) with 10% FBS (hyclone Laboratories, Inc., UT, U.S.A.), 100 μg/ml streptomycin and 100 U/ml penicillin. Starting at 90% confluency (typically day 3; day 0=plating day), the culture medium was supplemented with 50 μg/ml ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma) to support differentiation and mineralization. The cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

RT-PCR Total RNA was isolated from treated cells using the TRIzol reagent (Sangon Corp., Shanghai) and immediately reverse-transcribed using oligo-p$(dT)_{18}$ primer and M-MuLV reverse transcriptase (Sangon) according to the

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Table 1. Primers and Conditions Used for RT-PCR[a)]

Gene	Primers	PCR conditions	bp	Ref.
QM	Fw: 5′-GGTACCCCGCCACCATTGTCATCATGGGCCGCCG-3′ Rv: 5′-GGGCCCGGAATGCAGGGCTCTCCACTTGTCCAGAG-3′	30 cycles, 94 °C 10 s, 58 °C 30 s, 72 °C 2 min 30 s	642	
Type (I) collagen	Fw: 5′-CCTGGTAAAGATGGTGCC-3′ Rv: 5′-CACCAGG TTCAC CTTTCGCACC-3′	25 cycles, 94 °C 30 s, 58 °C 30 s, 72 °C 1 min	222	21
Osteocalcin	Fw: 5′-CCTCAGTCCCCAGCCCAGATCC-3′ Rv: 5′-CAGGGCAGAGAGAGAGGACAGG-3′	25 cycles, 94 °C 30 s, 58 °C 30 s, 72 °C 1 min	219	21
Osterix	Fw: 5′-GTCAAGAGTCTTAGCCAAACTC-3′ Rv: 5′-AAATGATGTGAGGCCAGATGG-3′	25 cycles, 94 °C 30 s, 58 °C 30 s, 72 °C 1 min	123	21
ALP	Fw: 5′-GCCCTCTCCAAGACATATA-3′ Rv: 5′-CCATGATCACGTCGATATCC-3′	30 cycles, 95 °C 1 min, 55 °C 2min, 72 °C 1 min	372	22
BMP-2	Fw: 5′-AGTTCTGTCCCCAGTGACGAGTTT-3′ Rv: 5′-GTACAACATGGAGATTGCGCTGAG-3′	36 cycles, 95 °C 1 min, 63 °C 30 s, 72 °C 1 min	708	23
Runx2/Cbfa1	Fw: 5′-CCGCACGACAACCGCACCAT-3′ Rv: 5′-CGCTCCGGCCCACAAATCTC-3′	30 cycles, 94 °C 30 s, 60 °C 30 s, 72 °C 30 s	289	24
Osteopontin	Fw: 5′-TCACCATTCGGATGAGTCTG-3′ Rv: 5′-ACTTGTGGCTCTGATGTTCC-3′	30 cycles, 95 °C 1 min, 55 °C 2 min, 72 °C 1 min	437	25
β-actin	Fw: 5′-TGGACTTCGAGCAAGAGATG G-3′ Rv: 5′-ATCTCCTTCTGCATCCTGTCG -3′	23 cycles, 94 °C 1 min, 62 °C 45 s, 72 °C 45 s.	289	18

a) bp, base pairs; Fw, forward; Rv, reverse.

manufacturer's protocol.[18)] Equal cDNA input amounts were determined by β-actin cDNA amplification. The primer sequences, annealing temperature, and cycle numbers for the amplification of each gene are listed in Table 1. Then the PCR products were separated on 1.5% agarose gels, stained with EB, and photographed under UV illumination (Imagemaster® VDS Amersham Pharmacia Biotech, U.S.A.).

Establishment of Stable MC3T3-E1 Cell Lines with Overexpressing of QM The coding sequence of *QM* was amplified by RT-PCR from MC3T3-E1 with TaKaRa advantage 2 taq DNA polymerase. Primers were designed with *Kpn*I and *Apa*I restriction sites in them, respectively. The amplified sequence was subcloned into the pUCm-T and then digested with *Kpn*I/*Apa*I (Promega Corp., U.S.A.). pcDNA.3.1/*myc*-His A vector (Invitrgen Corp., U.S.A.) was used as the expression vector. The recombinant plasmid was named pcDNA.3.1/*myc*-His A/QM (pcDNA/QM). The expression vector pcDNA/QM was then transfected into MC3T3-E1 and stable overexpression QM cell line was gained by adding 600 μg/ml G418 (Amreso Inc.) to screen. As a vector control, MC3T3-E1 cells were transfected with pcDNA.3.1/*myc*-His A under the same conditions. 24 d after G418 administration, different clones of cells were selected and collected to be assessed the expression level of QM by immunoblot analysis, as described below.

Immunoblot Analysis Cells were harvested and washed twice with ice-cold PBS. The lysates were achieved with TEN-T buffer (150 mM NaCl, 10 mM Tris–HCl pH 7.4, 5 mM EDTA pH 8.0, 1% Triton X-100, 1mM PMSF, 2 μg/ml aprotinin), and then subjected to 10000 ***g*** centrifugation at 4 °C for 10 min.[18)] Protein levels were quantified using Bicinchoninic Acid assay (Beyotime biotechnology, China). 50 μg/lane total proteins were fractioned by 12.5% SDS-polyacrylamide gels electrophoresis for QM and β-actin protein, and subsequently transferred to PVDF sheets. After blocking with TTBS (50 mM Tris–HCl pH 7.4, 0.5 M NaCl, 0.05% Tween 20) containing 5% (w/v) skim milk, the sheets were incubated with c-Myc antibody (Santa Cruz Biotechnology, Germany) or with β-actin antibody (Sigma-Aldrich) at 4 °C overnight. Blots were then detected with an ECF western blotting kit (Amersham Biosciences UK Limited, England). The densities of sample bands were determined using a fluorescence scanner, Storm 860, and analyzed with ImageQuant software (Amersham Biosciences UK Limited, England).

Measurement of Protein Concentration and Alkaline Phosphatase (ALP) Activity Cells were seeded at 2.5×10^5 cells/cm^2 in 96-well plates for ALP activity assays. Starting at 90% confluence (typically day 3; day 0=plating day), the culture medium was supplemented with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate to support differentiation. Then 4 or 6 days' cultures were washed with PBS three times, and the total cellular protein concentration was determined by incubation in BCA protein assay reagent (Beyotime biotechnology, China) containing 0.1% Triton X-100 for 30 min at 37 °C. The reaction was stopped by adding 1 M NaOH, and the absorbance was measured at 550 nm. ALP activity in the cells was assayed by washing the cells with PBS three times. Then cells were incubated in 0.1 M $NaHCO_3$–Na_2CO_3 buffer (pH 10.0) containing 0.1% Triton X-100, 2 mM $MgSO_4$, and 6 mM *p*-nitrophenol inorganic phosphate (PNPP) (Sangon) for 30 min at 37 °C. The reaction was stopped by adding 1 M NaOH, and the absorbance was measured at 405 nm.[19)] The changes of the ALP activity were calculated according to the formula: value of absorbance at 405 nm/value of absorbance at 550 nm.

Histological Demonstration of Calcium Deposition Assays Cells were plated in 6-well multiplates and grown until confluent. Media was then replaced with mineralizing media (DME supplemented with 10% FCS, 10 mM β-glycerophosphate and 50 mg/ml ascorbic acid) and was changed every 3 d. At the end of incubation on day 14, mineralization was detected with Alizarin red (Chemical Reagents Corp., Beijing) staining of calcium.[20)] Culture wells were washed twice with PBS and fixed for 1 h at 4 °C in 70% ethyl alcohol. Calcium deposits were stained for 10 min at room temperature with Alizarin red solution (40 mM, pH 4.2) that was filtered through Whatman paper prior to application. Nonspecific staining was removed by several washes in water. Quantitative analysis was performed by scanning densitometry on computer-assisted acquired images as described below.

Densitometric Analysis Scanning densitometry of the areas of interest was performed using the Molecular Analyst software quality one (Bio-Rad Laboratories) to obtain the arbitrary density units in the RT-PCR experiment. RT-PCR was using the reference genes β-actin as internal controls. The "gene of interest/reference gene" density ratio was then computed and represented in graphs.

Statistics Data are expressed as mean±S.E. of at least three independent experiments. Statistical significance was computed by the unpaired Student's t test. A $p<0.05$ was conventionally considered statistically significant.

RESULTS

Stable Overexpression of QM in MC3T3-E1 Cells To investigate the impact of QM overexpression on osteoblastic function, MC3T3-E1 cells transduced with pcDNA/QM were cultured and compared to cells transduced with pcDNA vector. After 48 h from transfection, cells were collected and analyzed if QM protein could be overexpressed. As Fig. 1A showed, pcDNA/QM transfected cells expressed QM protein with a myc tag at 28 kD which could be examined by c-myc antibody (recognized c-Myc amino acids 408—439), while vector alone transfected cells expressed no protein at 28 kD. Then stable transfected monoclonal cells were selected in complete medium containing G418. Different clones of cells were selected and collected. Total cell lysates isolated from vector-transfected or transfected pcDNA/QM clones were analyzed for QM protein by immunoblot analysis on 24 d after G418 administration. As shown in Fig. 1B, in the isolated G418-resistant cell clones, cells of pool 6 exhibited high increase in QM protein levels, compared with other clones of cells (pool 2-pool 5) and vector alone transfected cells (pool 1). Then cells of pool 6 were chosen to do following analysis.

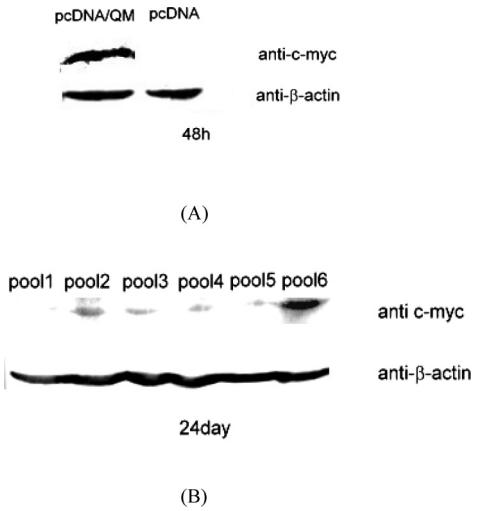

Fig. 1. Stable Overexpression of Mouse QM in MC3T3-E1 Cells

Immunoblot analysis of QM. Total cell lysates isolated from vector-transfected or stably transfected pcDNA/QM clones were analyzed for QM protein by immunoblot analysis using an anti-c-Myc antibody (1 : 500) recognizing human c-Myc (amino acids 408—439) or an anti-β-actin monoclonal antibody (1 : 500000). (A) Western blot analysis of cells on 48 h after transfection. (B) Western blot analysis of several different clones of cells on 24 d after G418 administration. pcDNA: vector alone transfected cells; pcDNA/QM: pcDNA/QM expression vector transfected cells. Pool 1: pcDNA vector alone transfected cells; pool 2—pool 6: different selected clones.

The Activity of ALP Was Increased in Cells Transfected pcDNA/QM Expression Vector ALP activity was examined to analyze the effect on osteoblast differentiation by overexpression of QM. ALP activity was measured on 4 or 6 d. As Fig. 2 showed that ALP activity measured on 4 or 6 d was higher in QM overexpression cells than in normal cells and the difference is significant. ALP activity was higher on day 4 than on day 6.

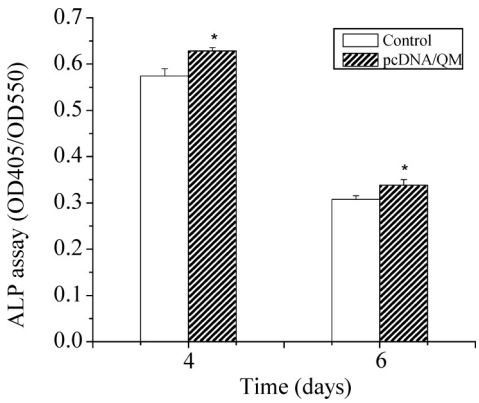

Fig. 2. Effect of Overexpression of QM on ALP Activity in MC3T3-E1

90% confluent osteoblasts were cultured with the complete medium supplemented with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate to support differentiation on 3 d. Then ALP activity was measured on 4 or 6 d. Total cell protein was determined by incubation in BCA protein assay reagent as mentioned in the Materials and Methods. Data were given as the ratio of a pool of cells' ALP activity and this pool of cells' total protein. Each value is the mean±S.E. of at least three independent experiments. Statistical significance was computed by the unpaired Student's t test. Key: (*) $p<0.05$ compared with the cells transfected pcDNA.3.1/*myc*-His A vector. Control: normal expression QM cells; pcDNA/QM: overexpression of QM cells.

Nodule Mineralization Was Increased in MC3T3-E1 From Overexpression of QM Relative to Normal Expression Nodule mineralization was detected with Alizarin red staining of calcium. MC3T3-E1 cells were cultured for 14 d in the presence of ascorbic acid and β-glycerophosphate as described in the Materials and Methods. Cultures were then fixed and subjected to Alizarin red staining for the detection of nodule mineralization (red areas). Figure 3 showed that the number of nodule mineralization of one dish was sharply increased by overexpression of QM.

Type (I) Collagen, ALP, Osteocalcin, Osterix and BMP-2 Were Upregulated in QM Overexperssion Osteoblasts It was examined whether overexpression of QM affected the expression of marker genes of the osteoblast phenotype. For this purpose we performed RT-PCR using primers for the housekeeping gene β-actin along with primers for the gene being analyzed and measured the relative ratios by densitometry. Figure 4 shows that the mRNA of ALP was increased by overexpression of QM, which is consistent with the increasing ALP activity in QM overexpression cultures mentioned above. In addition, the osteoblast markers osteocalcin (OCN), type (I) collagen (COLI), osterix (Osx) and BMP-2 were remarkably increased by overexpression of QM. However, the Osf2/Cbfa1 transcription factor was similarly transcriptionally by the overexpression of QM cultures *versus* controls. In contrast, the expression pattern of osteopontin (OPN), a bone matrix sialoprotein that is not osteoblast specific and is a negative regulator of proliferation and differentiation in MC3T3-E1 was slightly decreased by QM overexpression cultures relative to control cells.

DISCUSSION

QM, known as a key protein involved in binding of 40 and 60 S ribosomal subunits, is highly homologous throughout evolution, implying QM's critical cellular functions, which have not been well characterized yet. There were few detailed reports involving QM's roles in bone formation. In our study, analysis of osteoblast characteristics showed that QM overexpression led to a marked increase in osteoblast differentiation and mineralization in MC3T3-E1. These results suggested QM might have a potential function in bone formation.

Several SH3 domains of Src family members showed interactions with QM protein that could reduce Src family kinase activity.[13] In addition, another report said that deletion/reduction of Src expression enhanced osteoblast differentiation and bone formation, contributing to the increase in bone mass.[26] On the basis of these evidences, we attempted to investigate whether changing QM expression level could affect osteoblast differentiation and mineralization or not. QM protein was overexpressed in MC3T3-E1 as shown in Fig. 1. Then ALP activity was then examined to see the QM's effect on osteoblasts differentiation. Osteoblasts always begin to differentiate on day 4 and ALP activity's heightening is a marker of the early period of cell differentiation. ALP can play a principal role in the mineralization of collagenous matrices in the animal body.[27] Weiss and coworkers[28] have demonstrated that a mutation in the L/B/K ALP gene that abolishes enzymatic activity causes profound skeletal hypomineralization. Figure 2 showed that ALP activity was higher in overexpression of QM cultures. We have done more than three experiments and at least five parallel pools in one independent experiment. The statistical analysis showed that the difference was significant. Therefore the significantly increased ALP activity might play an important role in osteoblastic differentiation and mineralization of MC3T3-E1 cells. We next examined the effect of QM overexpression on mineralization. Figure 3 showed that the number of nodules was higher in overexpression of QM cells than in normal QM expression cells. The results of ALP activity and nodule mineralization experiments clearly showed us that overexpression of QM could induce cell differentiation and mineralization. However, more experiments would be

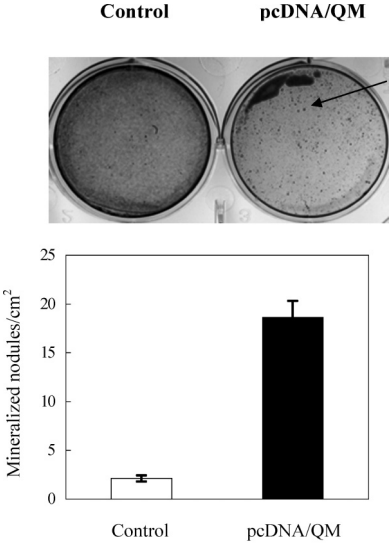

Fig. 3. Alizarin Red Staining of Day 14 Cultures: Photograph of Representative Stained Plates

MC3T3-E1 cells were cultured for 14 d, fixed, and stained histochemically to detect nodule mineralization using Alizarin red as the Materials and Methods said. Two plates represent normal expression QM cultures (Control) and QM overexpression cultures (pcDNA/QM), respectively. Positive results were seemed as red area indicated by Blank arrow. Same experiments have been done for three times. Graph represents the number of mineralized nodules/cm^2 present in each culture well 14 d after plating.

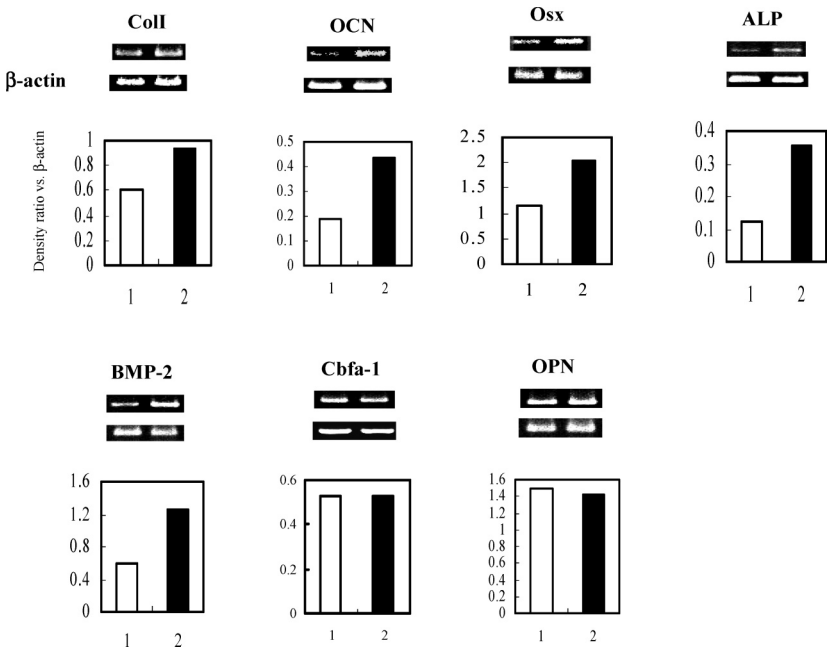

Fig. 4. Effects on the mRNA Expression of Osteoblast Marker Genes by Overexpression of QM Cells

90% confluent osteoblasts were incubated for 6 d and RNA was extracted. 1 μg total RNA was reverse-transcribed and the equivalent of 0.1 μg was subjected to PCR using primer pairs and conditions as described in the legend to Table 1. Densitometric analysis was performed as described in Materials and Methods, and then the density ratio between the gene being analyzed and the constitutive gene β-actin was computed as shown in the graphs. Similar results were observed in three independent experiments. 1: cells transfected pcDNA vector; 2: overexpression of QM cells.

needed to prove if QM could promote osteoblast differentiation and mineralization through Src famly kinase.

Increased ALP activity and mineral deposition were not the only features indicating a positive role of QM in osteoblast differentiation. Osteogenic cells are known to express various specific markers that distinguish them from other matrix-forming cells of mesenchyme origin. We have selected a few genes that characterize the osteoblast phenotype and have investigated their QM overexpression transcriptional regulation. It is observed in QM overexpression osteoblasts, as well as in normal cells, that a significant upregulation of *OCN*, *COLI*, *Osx* and *BMP-2* gene transcripts relative to normal QM-expressing cells. These markers are considered highly predictive of the differentiated osteoblast phenotype. But the transcriptional regulation of these genes is not well characterized. BMPs were discovered for their ability to induce cartilage and bone formation from non-skeletal mesodermal cells and are now of considerable interest as therapeutic agents for healing fractures and periodontal bone defects, and for inducing bone growth around implants and prostheses.[29] BMP-2 is accumulated in extracellular matrix (ECM) and is able to stimulate ectopic bone formation *in vivo* and osteoblastic differentiation *in vitro*. It stimulates the expression of three osteogenic master transcription factors: Runx2, Dlx5, and Osx.[30] Runx2/Cbfa1, an osteoblast-specific transcription factor, is a product of master gene of bone formation and its transactivation (nuclear import) is essential for osteoblastic and chondrocytic bone formation.[31] Osx was commonly stimulated in osteogenic and non-osteogenic cells in response to BMP-signaling. Our results showed that overexpression of QM remarkably increased mRNA of BMP-2 and Osx. This suggested that the effects of QM overexpreesion on osteoblast differentiation and mineralization might be through BMP-signaling and the overexpressed QM might be an upstream factor of BMP-2.

OPN is an extracellular matrix protein and a cytokine that has been reported to act in a number of physiological and pathological events including bone remodeling.[32—34] However, the role of OPN in the process of bone formation is not clearly understood. It is reported that overexpression of OPN inhibited BMP-2 responsiveness while overexpression of antisense RNA enhanced the effect of BMP-2 on alkaline phosphatase activity.[35] Increased OPN expression also caused decreases in expression of osteocalcin and bone sialoproteins while a reduction of OPN level caused the opposite. These results indicated that OPN is a negative regulator of proliferation and differentiation in MC3T3-E1 cells. But in our experiments, the OPN mRNA expression was only a very slight decrease in overexpression of QM cells (Fig. 4). This suggested that overexpression of QM might not have any effects on this pathway.

In conclusion, our study suggested that QM overexpression osteoblasts exhibit a cell autonomous alteration that lead to increased mineralization *in vitro*, as well as accelerated differentiation and increased matrix productions. These alterations were caused by genes transcription changing related to bone formation.

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