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Calcium phosphate-bearing matrices induce osteogenic differentiation of stem cells through adenosine signaling

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Synthetic matrices emulating the physicochemical properties of tissue-specific ECMs are being developed at a rapid pace to regulate stem cell fate. Biomaterials containing calcium phosphate (CaP) moieties have been shown to support osteogenic differentiation of stem and progenitor cells and bone tissue formation. By using a mineralized synthetic matrix mimicking a CaP-rich bone microenvironment, we examine a molecular mechanism through which CaP minerals induce osteogenesis of human mesenchymal stem cells with an emphasis on phosphate metabolism. Our studies show that extracellular phosphate uptake through solute carrier family 20 (phosphate transporter), member 1 (SLC20a1) supports osteogenic differentiation of human mesenchymal stem cells via adenosine, an ATP metabolite, which acts as an autocrine/paracrine signaling molecule through A2b adenosine receptor. Perturbation of SLC20a1 abrogates osteogenic differentiation by decreasing intramitochondrial phosphate and ATP synthesis. Collectively, this study offers the demonstration of a previously unknown mechanism for the beneficial role of CaP biomaterials in bone repair and the role of phosphate ions in bone physiology and regeneration. These findings also begin to shed light on the role of ATP metabolism in bone homeostasis, which may be exploited to treat bone metabolic diseases.

bone metabolism | mineralized matrix | biomimetic material | phosphate signaling

Harnessing the ability of adult stem cells to differentiate and contribute to tissue repair has enormous potential for wound healing, tissue regeneration, and restoration of organ functionality. However, controlling the fate of transplanted and/or endogenous progenitor cells to treat compromised tissues and organs remains a significant challenge (1, 2). Studies have shown that biomaterials recapitulating various physicochemical cues of the native tissue can be used to direct stem cell differentiation (3–9). Biomaterials-assisted transplantation of stem cells provides a promising approach to deliver cells to the targeted site and direct their differentiation to functional tissues. We and others have shown that biomaterials containing calcium phosphate (CaP) moieties, a major constituent of native bone tissue, can promote osteogenic differentiation of progenitor and stem cells and can facilitate in vivo bone tissue formation (10–20). However, to use CaP biomaterials efficiently for bone tissue repair, it is of paramount importance to understand the molecular mechanisms underlying the osteogenicity (osteogenic differentiation of progenitor cells in the absence of any exogenous chemical or biological osteogenic-inducing factors) and osteoinductivity (de novo bone growth in vivo even in locations where there is no vital bone) of a CaP mineral environment.

The osteogenicity and osteoinductivity of CaP minerals have been attributed to different factors, such as the ability of CaP to modulate extracellular calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions and the adsorption and release of osteoinductive growth factors like bone morphogenic proteins (BMPs) (18, 21–24). This is further supported by findings that exposure of osteoblasts and progenitor cells to Ca^{2+}- or PO_4^{3-}-rich medium promotes their osteogenic differentiation (25–27). Additionally, it has been shown that among various CaP materials, the ones that dissociate easily to Ca^{2+} and PO_4^{3-} contribute to better bone healing (13, 21). Despite the large number of studies demonstrating the potential role of CaP minerals and Ca^{2+} and PO_4^{3-} on osteogenic differentiation of osteoblasts and progenitor cells, the molecular mechanism through which these ions regulate osteogenic commitment of stem cells remains largely unknown. Recent studies have shown that influx of extracellular Ca^{2+} through L-type calcium channels promotes osteogenic differentiation of osteoprogenitor cells (28). However, very little is known about the mechanism through which PO_4^{3-} supports osteogenesis. During skeletal growth and bone remodeling, PO_4^{3-} plays an important role in apatite formation (29, 30). In addition to osteoblasts and progenitor cells, studies have shown that exposure to PO_4^{3-} alters the cell phenotype of nonskeletal tissues, such as human vascular smooth muscle cells, into osteogenic-like cells (31, 32). Central to phosphate metabolism is solute carrier family 20 (phosphate transporter), member 1 (SLC20a1, or PiT-1), a sodium-phosphate symporter that transports PO_4^{3-} ions from the extracellular milieu

Significance

A mechanistic understanding of how calcium phosphate (CaP) minerals contribute to osteogenic commitment of stem cells and bone tissue formation is a necessary requirement for developing efficient CaP-based synthetic matrices to treat bone defects. This study unravels a previously unknown mechanism, phosphate-ATP-adenosine metabolic signaling, by which the CaP-rich mineral environment in bone tissues promotes osteogenic differentiation of human mesenchymal stem cells. In addition to a mechanical perspective on how biomaterials can influence stem cell differentiation through metabolic pathways, this discovery opens up new avenues for treating critical bone defects and bone metabolic disorders.

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The authors declare no conflict of interest.

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into the cytoplasm and plays a key role in mineralization of both vascular smooth muscle cells and osteoblasts (33, 34).

Here, we unravel a previously unknown mechanism, centered on phosphate metabolism, through which the CaP-rich mineral environment promotes osteogenic differentiation of human mesenchymal stem cells (hMSCs) by using an engineered matrix containing CaP moieties. Our studies show that the extracellular PO_4^{3-} plays an important role in promoting osteogenic differentiation of hMSCs by regulating intramitochondrial phosphate content and ATP synthesis. ATP is then secreted and metabolized into adenosine, which promotes osteogenic differentiation of hMSCs via A2b adenosine receptors.

Results

Biomineralized Matrix-Induced Osteogenic Differentiation of Stem Cells Uses SLC20a1. Recently, we developed a mineralized matrix containing CaP minerals by using the principles of biomineralization (35). This mineralized matrix recapitulates different static and dynamic physicochemical cues of native bone ECM, including its composite structure, CaP-rich environment, and dynamic dissolution/formation of matrix-bound CaP minerals (which establish equilibrium with the surrounding milieu) (11, 35). This mineralized matrix possesses osteogenicity, osteoconductivity, and osteoinductivity (10, 11). Analyses of the mineralized matrix with SEM showed the presence of irregularly shaped spherulites (Fig. S1*A*, *Right*). Elemental analysis revealed that these minerals are mainly CaP with a Ca/P ratio of ∼1.43; this is close to the Ca/P ratio observed in other bioactive ceramics, such as β-tricalcium phosphate (1.5) and hydroxyapatite (1.67) (36). No such minerals were observed in corresponding nonmineralized matrices (Fig. S1*A*, *Left*). The presence of CaP minerals in mineralized matrices was further confirmed by X-ray diffraction analyses, which demonstrate peaks (20° ≈ 26°, 31°) corresponding to the diffraction spacing present in hydroxyapatite [PDF-4-010-6312, based on PDF4+ (International Centre for Diffraction Data)] (Fig. S1*B*). Measurement of Ca^{2+} and PO_4^{3-} contents of the mineralized hydrogels indicated that they contain 70.1 ± 1.9 mg and 105.8 ± 3.98 mg of Ca^{2+} and PO_4^{3-} per dry weight, respectively (Fig. S2*A*). As expected the CaP components of the mineralized matrix underwent dissolution to Ca^{2+} and PO_4^{3-} when exposed to a medium devoid of these ions (Fig. S2*B*).

The hMSCs cultured on these mineralized matrices in growth medium, lacking any osteogenic-inducing soluble factors, consistently up-regulated the osteogenic markers, osteopontin (OPN) and osteocalcin (OCN) (Fig. 1 *A*–*C*). In addition to osteogenic markers, hMSCs cultured on these matrices showed up-regulation of sodium-phosphate symporter SLC20a1 (Fig. 1*D* and Fig. S3*A*). Interestingly, knockdown of SLC20a1 with siRNA (Fig. S3*B*) resulted in the down-regulation of OCN and OPN gene expression (Fig. 1 *E* and *F*) and decreased immunofluorescent staining for OCN (Fig. 1*G*).

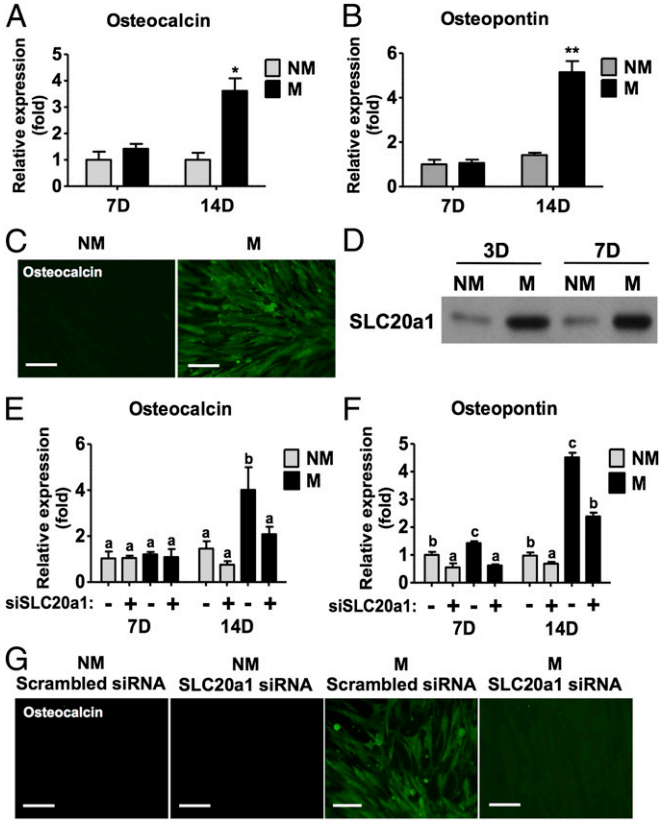

Fig. 1. Mineralized matrix containing CaP minerals promotes osteogenic differentiation of hMSCs. OCN (*A*) and OPN (*B*) gene expression after 7 d (7d) and 14 d (14d) of culture on mineralized (M) and nonmineralized (NM) matrices. (*C*) OCN immunofluorescent staining (green) after 14 d of culture on M and NM matrices. (*D*) SLC20a1 protein expression after 3 and 7 d of culture. OCN (*E*) and OPN (*F*) gene expression after 7 and 14 d of culture with and without SLC20a1 knockdown. (*G*) OCN immunofluorescent staining (green) after 14 d of culture. The plus symbol (+) denotes SLC20a1 siRNA, and the minus symbol (–) denotes corresponding scrambled siRNA. (Scale bars: 100 μm.) Data are represented as the mean ± SD (Student *t* test or one-way ANOVA followed by Bonferroni post hoc test; $*P < 0.05$; $**P < 0.01$). Groups with different letters (a–c) are significant, $P < 0.05$; $n = 3$.

Inorganic Phosphate-Regulated Osteogenic Differentiation of hMSCs Uses SLC20a1. Because SLC20a1 transports PO_4^{3-} and the biomineralized matrix contributes to the extracellular PO_4^{3-}, the role of extracellular PO_4^{3-} on osteogenic commitment of hMSCs was further validated by culturing them in medium supplemented with varying amounts of PO_4^{3-}. Similar to mineralized matrices, the hMSCs cultured in high PO_4^{3-} (5 mM) medium showed up-regulation of various osteogenic markers, such as osterix, OCN and type I collagen, compared with control cultures (Fig. 2 *A* and *B* and Fig. S3*C*). The gene expression of SLC20a1 was up-regulated in hMSCs cultured in 5 mM PO_4^{3-} medium and down-regulated upon SLC20a1 knockdown (Fig. 2*C*). Western blot analysis and image quantification demonstrated that SLC20a1 knockdown down-regulated extracellular signal-regulated kinases 1/2 (ERK1/2) activity, an important mitogen-activated protein kinase involved in osteogenic commitment during phosphate induction (Fig. 2*D* and Fig. S3*D*). Akin to mineralized matrices, the knockdown of SLC20a1 annulled the PO_4^{3-}-mediated osteogenesis of hMSCs (Fig. 2 *E* and *F*).

Increase of Intracellular ATP on Mineralized Matrices Is Dependent on SLC20a1. We observed a significant increase in intracellular PO_4^{3-} of hMSCs cultured on mineralized matrices as measured by the phosphate assay, but this increase was attenuated upon the knockdown of SLC20a1 (Fig. S4*A*). In addition to intracellular phosphate, intramitochondrial phosphate was increased on mineralized matrices but was down-regulated after partial loss of SLC20a1 (Fig. 3*A*). Because an obvious function of inorganic PO_4^{3-} is to act as a substrate for ATP synthesis in the electron transport chain of the mitochondria, we next examined ATP production. A significant increase in intracellular ATP was observed for cells cultured on mineralized matrices, as evidenced by a luminescent assay, and this increase was abrogated with the partial loss of SLC20a1 (Fig. 3*B*). This finding was corroborated by the increase of fluorescence intensity of quinacrine staining for intravesicular ATP on mineralized matrices and the decrease in intensity upon SLC20a1 knockdown (Fig. 3*C*). To examine further whether PO_4^{3-} is directly involved in ATP synthesis, we cultured hMSCs in medium containing 5 mM PO_4^{3-}. Similar to

cells on mineralized matrices, hMSCs cultured in 5 mM PO_4^{3-} medium showed higher levels of intracellular and intramitochondrial phosphate compared with those cultured in 1 mM PO_4^{3-} medium; this exogenous PO_4^{3-}-assisted up-regulation was found to decrease upon SLC20a1 knockdown (Fig. 3*D* and Fig. S4*B*). Measurement of intracellular ATP by luminescent assay displayed a similar trend, where ATP levels increased in 5 mM PO_4^{3-} medium but were abolished with SLC20a1 knockdown (Fig. 3*E*). Additionally, quinacrine staining for intravesicular ATP demonstrated an increase in ATP fluorescent signals for hMSCs cultured in 5 mM PO_4^{3-} medium, which diminished after SLC20a1 knockdown (Fig. 3*F*).

Mineralized Matrices Promote Osteogenic Differentiation Through A2b Adenosine Receptor. The function of ATP as a signaling molecule, in addition to being an energy source, has long been established (37). ATP can mediate osteogenic signaling through purinergic receptors (38, 39). To determine the role of extracellular ATP on osteogenic differentiation, we inhibited the transport of ATP to the extracellular milieu with the vesicular transport inhibitor *N*-ethyl maleimide (NEM). The addition of NEM significantly abrogated OCN and OPN gene expression (Fig. 4 *A* and *B*) and decreased OCN immunofluorescent intensity (Fig. 4*C*) on mineralized matrices, suggesting that inhibition of ATP transport negatively affects mineralized matrix-assisted osteogenesis of hMSCs. However, pharmacological inhibition of purinergic receptors with suramin did not abrogate osteogenic differentiation of hMSCs on mineralized matrices, as shown by OCN staining (Fig. S5*A*). Additionally, we were unable to detect any significant amount of ATP in the culture medium at a measurable threshold of 100 ng/mL (Fig. S5*B*). On the contrary, HPLC measurements showed a significant amount of adenosine in cell cultures involving mineralized matrices, and this presence of extracellular adenosine was abrogated with SLC20a1 knockdown (Fig. 4*D*). To validate the role of adenosine in the mineralized matrix-mediated osteogenesis of hMSCs further, we examined the role of two likely candidates of adenosine signaling: A1 and A2b adenosine receptors. Specific pharmacological inhibition of A1 and A2b adenosine receptors by 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) and 8-[4-[4-(4-Chlorophenzyl) piperazide -1-sulfonyl)phenyl]]-1-propylxanthine (PSB603) inhibitors, respectively, demonstrated that the PSB603 down-regulated the increase in OCN and OPN gene expression on mineralized matrices, whereas DPCPX had no effect (Fig. 4 *E* and *F*). The decrease of OCN in presence of PSB603 was also demonstrated by immunofluorescent staining for OCN (Fig. 4*G*).

We also examined the effect of adenosine on osteogenic differentiation of hMSCs by culturing the cells on nonmineralized matrices in growth medium containing exogenous adenosine. We chose nonmineralized matrices because they do not support osteogenic differentiation of hMSCs in growth medium despite having similar chemical composition of the polymer network, except for the CaP moieties (Fig. 1 *A*–*C*). Supplementation of adenosine in growth medium promoted osteogenic differentiation of hMSCs on nonmineralized matrices, as evidenced by the up-regulation of OCN and OPN (Fig. 4 *E*–*G*). Furthermore, the exogenous adenosine-mediated osteogenic differentiation of hMSCs was abrogated in the presence of PSB603 but not in the presence of DPCPX, akin to mineralized matrices, thus corroborating the role of extracellular adenosine as a signaling molecule.

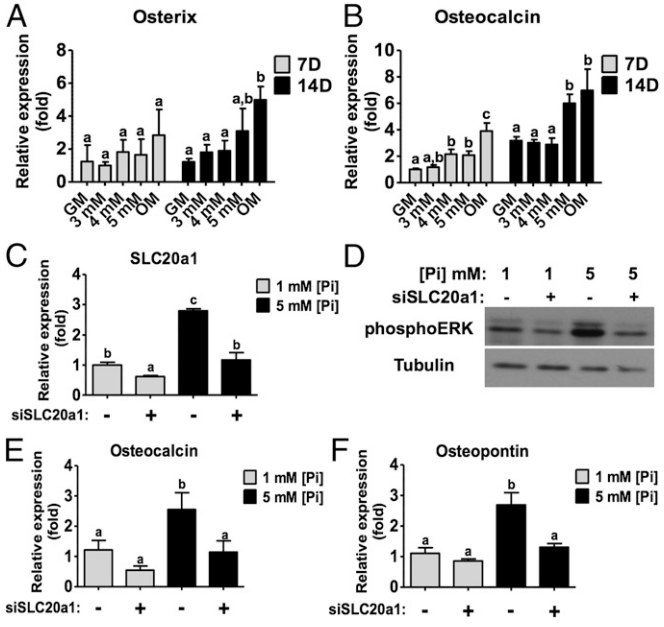

Fig. 2. Elevated levels of inorganic phosphate in culture medium promote osteogenic differentiation of hMSCs through SLC20a1. Gene expressions of osterix (*A*) and OCN (*B*) after 7 d and 14 d of culture in growth medium containing varying amounts of PO_4^{3-} ions. (*C*) SLC20a1 gene expression after 7 d of culture in normal (control; 1 mM) and high-phosphate (5 mM) medium with and without SLC20a1 knockdown. (*D*) Activation of ERK1/2 kinase after 1 d of SLC20a1 knockdown. OCN (*E*) and OPN (*F*) gene expression after 14 d of culture in low- and high-phosphate medium with and without SLC20a1 knockdown. GM, growth medium (1 mM PO_4^{3-}); OM, osteogenic medium; [Pi], concentration of PO_4^{3-}; 3 mM, 3 mM PO_4^{3-}; 4 mM, 4 mM PO_4^{3-}; 5 mM, 5 mM PO_4^{3-}. The plus (+) symbol denotes SLC20a1 siRNA and the minus (–) symbol denotes scrambled siRNA. Data are represented as the mean ± SD (one-way ANOVA, followed by Bonferroni post hoc test). Groups with different letters (a–c) are significant, $P < 0.05$; $n = 3$.

Discussion

A number of studies have shown that CaP biomaterials like bioactive glasses, ceramics, and mineralized matrices promote bone healing (13–15). In addition to the physical cues provided, studies have shown that the dissolution kinetics and the ability of the CaP minerals to undergo dissolution/formation modulating the extracellular mineral environment play a key role in osteogenic functions of CaP materials (11, 18, 21). In this study, by using a mineralized matrix, we investigated the metabolic mechanisms by which the CaP-rich microenvironment contributes to osteogenic commitment of hMSCs. We chose nondegradable matrix because it allows us to eliminate the interference of matrix degradation on osteogenic differentiation, which has previously been shown to play a role in bone tissue formation (3, 40).

The finding that osteogenic differentiation of hMSCs via mineralized matrix and high PO_4^{3-} medium can be negated through SLC20a1 knockdown suggests that PO_4^{3-} content in the extracellular milieu, and its transport through SLC20a1, is an important mediator of mineralized matrix-induced osteogenic differentiation of hMSCs. However, a fundamental question remains as to how PO_4^{3-} from the extracellular milieu promotes the osteogenic phenotype of hMSCs. Phosphate serves as the primary substrate for the F1F0-ATPase production of ATP in the mitochondria, regulates the production of mitochondrial ATP through activation of mitochondrial NADH, and improves the distribution of energy between cyto-b and cyto-c (41, 42). The increases in intracellular and intramitochondrial PO_4^{3-} provide an explanation for the observed higher ATP synthesis in hMSCs cultured on mineralized matrices or in high PO_4^{3-} medium. This is in accordance with studies showing that inhibition of SLC20a1 and ATP synthesis disturbed endochondral ossification and suppressed mineralization in conjunction with reduced PO_4^{3-} uptake in chondrocytes (43) and that ATP production affects the osteogenic commitment of hMSCs (44). The role of extracellular ATP during osteogenic differentiation, however, remains uncertain because ATP, acting through purinergic receptors, has been

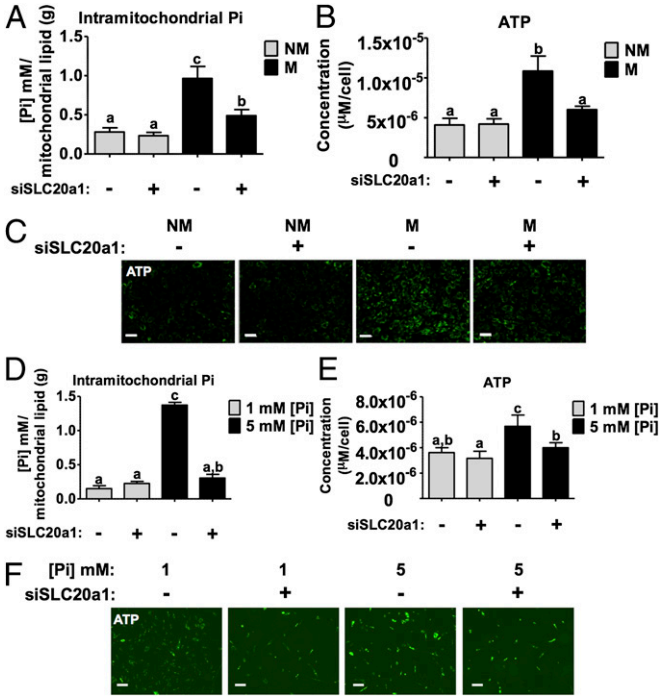

Fig. 3. Mineralized matrices regulate intracellular PO_4^{3-} and ATP content through SLC20a1. (*A–C*) hMSCs cultured on M and NM matrices with and without SLC20a1 knockdown. (*A*) Intramitochondrial PO_4^{3-} after 1 d of culture. (*B*) Intracellular ATP luminescent assay after 4 d of culture. (*C*) Quinacrine staining for ATP after 4 d of culture. (*D–F*) hMSCs cultured in normal (control; 1 mM) and high- (5 mM) $PO4^{3-}$ medium with and without SLC20a1 knockdown. (*D*) Intramitochondrial PO_4^{3-} after 1 d of culture. (*E*) Intracellular ATP luminescent assay after 4 d of culture. (*F*) Intravesicular ATP staining with quinacrine after 4 d of culture. Pi, phosphate ion. The plus (+) symbol denotes SLC20a1 siRNA and the minus (–) symbol denotes scrambled siRNA Data are represented as the mean ± SD (one-way ANOVA, followed by Bonferroni post hoc test). Groups with different letters (a–c) are significant, $P < 0.05$; $n = 3$. (Scale bars: 200 μm.)

implicated both in promoting osteogenic differentiation (38, 39) and in inhibiting formation of mineralized nodules (45, 46). The lack of detectable extracellular ATP, together with the fact that pharmacological inhibition of purinergic receptors did not have any significant effect on mineralized matrix-mediated osteogenesis of hMSCs, suggests that the increase in intracellular ATP promotes osteogenic commitment through routes other than extracellular ATP acting on its own.

The presence of a significant amount of adenosine, an ATP metabolite, in the extracellular milieu suggests that membrane-bound ectonucleotidases (CD39), such as ectonucleoside triphosphate diphosphohydrolase, ectonucleotide pyrophosphatase/phosphodiesterase, and ecto-5′nucleotidases (CD73), rapidly metabolized ATP to adenosine (47). These findings, in conjunction with the pharmacological inhibition studies, clearly identify the role of adenosine signaling through A2b receptor on mineralized environment-assisted osteogenic differentiation of hMSCs. The role of exogenous adenosine is further corroborated by the findings that hMSCs on nonmineralized matrices undergo osteogenesis in the presence of medium containing adenosine and that this phenomenon is abrogated upon pharmacological inhibition of A2b adenosine receptor. These results are consistent with emerging studies that show the pivotal role of adenosine signaling via A2b adenosine receptor in both in vivo and in vitro bone development and osteogenic differentiation of stem cells (48–50). A recent study by He et al. (51) showed the role of adenosine on bone metabolism in normal humans and patients with multiple myeloma. Furthermore, these authors have shown that osteoblast cells from A2b receptor and CD39-KO mice exhibit diminished osteogenic differentiation.

It is important to note that although the focus of the current study is phosphate metabolism associated with a mineralized environment, it does not refute the beneficial effect of Ca moieties of the CaP minerals. As stated earlier, the CaP moieties of the mineralized matrix undergo dissolution/precipitation responding to the concentration of Ca^{2+} or PO_4^{3-} ions in the surrounding environment, thus creating a dynamic environment. This is very similar to exogenous supplementation of Ca^{2+} or PO_4^{3-}, which leads to CaP precipitation as the ion concentration in the medium increases to establish equilibrium. The importance of such a dynamic mineral environment and the interdependency between the extracellular Ca^{2+}, PO_4^{3-}, and CaP was demonstrated in

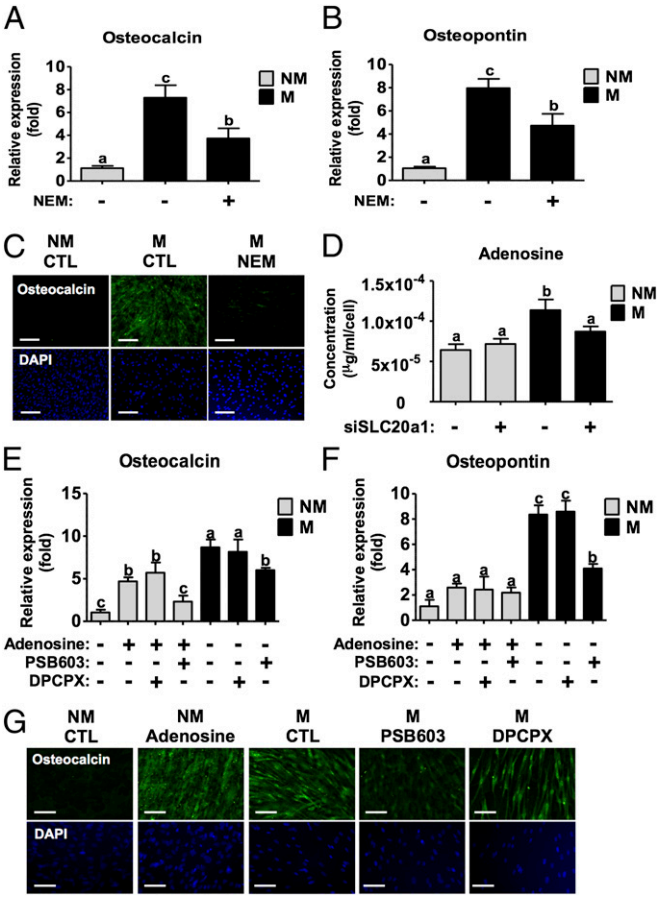

Fig. 4. Mineralized matrix-mediated osteogenic differentiation through A2b adenosine receptor. OCN (*A*) and OPN (*B*) gene expression of hMSCs after 3 wk of culture on NM matrices, M matrices, and M matrices with vesicle transport inhibitor NEM. The plus (+) and minus (–) symbols denote the presence and absence of NEM, respectively. (*C*) Immunofluorescent staining for OCN after 3 wk of culture on NM, M, and M in the presence of NEM. OCN (green) and nuclei (blue). CTL, control. (Scale bars: 200 μm.) (*D*) HPLC measurement of adenosine in culture medium after 7 d. The plus (+) symbol denotes SLC20a1 siRNA, and the minus (–) symbol denotes scrambled siRNA. OCN (*E*) and OPN (*F*) gene expressions of hMSCs cultured on M and NM for 3 wk with (+) and without (–) the presence of adenosine, A2b receptor antagonist (PSB603), or A1 receptor antagonist (DPCPX). (*G*) Immunofluorescent staining of OCN after 3 wk of culture on NM and M for 3 wk in the presence and absence of adenosine, PSB603, or DPCPX. OCN (green) and nuclei (blue). (Scale bars: 100 μm.) Data are represented as the mean ± SD (one-way ANOVA, followed by Bonferroni post hoc test). Groups with different letters (a–c) are significant, $P < 0.05$; $n = 3$.

a study by Khoshniat et al. (52), where these authors showed that inhibiting the formation of CaP minerals abrogates the extracellular PO_4^{3-}-promoted osteogenesis of osteoblasts even though CaP minerals were not endocytosed. As mentioned earlier, CaP minerals also function as a reservoir for growth factors (22, 23). It is likely that the osteoinductive factors adsorbed onto the mineralized matrices can also contribute to phosphate metabolism. Previous studies have shown that BMP-2–mediated differentiation of MC3T3-E1, preosteoblasts, and their ECM mineralization involves intracellular phosphate uptake, wherein BMP-2 promotes PO_3^{4-} transport through up-regulation of SLC20a1 (53). Similar findings were also observed in calcification of human vascular smooth muscle cells (54). In addition to BMPs, other osteoinductive molecules (e.g., NEL-like molecule-1) have been shown to promote preosteoblast mineralization through SLC20a1 (55). Conversely, supplementation of PO_3^{4-} in growth medium has been shown to up-regulate BMP-2 expression of various cells similar to other metal ions, such as Ca^{2+} and strontium (18, 24, 25, 56).

Together, the results propose a molecular mechanism, depicted in Fig. 5, in which the dynamic dissolution/precipitation of CaP minerals from the mineralized matrices dictates the concentrations of Ca^{2+} and PO_4^{3-} in the extracellular milieu. Extracellular PO_4^{3-} enters the cells through SLC20a1 and subsequently into the mitochondria, which serves as a substrate for ATP synthesis. ATP is then secreted and metabolized into adenosine, which subsequently promotes osteogenic differentiation of hMSCs through the A2b adenosine receptor via autocrine and/or paracrine signaling. The active function of PO_4^{3-} in this study reveals the underappreciated role of phosphate ions of the CaP minerals in the vicinity of osteoprogenitors during bone remodeling. The roles of PO_4^{3-} and ATP as precursors of osteogenic inducers in bone formation imply that their aberrant regulation could result in osteoporosis, a principal disease of imbalanced bone remodeling. Recent studies have found that mice lacking P2Y (13), a receptor of ADP, results in reduced bone turnover (57) and that polymorphisms in the P2X7 receptor gene are associated with reduced lumbar spine bone mineral density and accelerated bone loss in postmenopausal women (58). Validation of the PO_4^{3-}-ATP-adenosine signaling cascade in osteoporotic animal models could unravel new therapeutic targets.

In sum, by using an osteogenic, osteoinductive biomimetic matrix, we have unraveled a mechanism by which bone minerals contribute to bone tissue formation from bone marrow-derived stem cells. Furthermore, this study demonstrates the role of phosphate metabolism on osteogenic commitment of stem cells and the role of adenosine signaling in this process. These findings pave the way to new targets and approaches in treating critical bone defects and bone metabolic disorders.

Materials and Methods

Cell Culture. The hMSCs (p7071L; Institute for Regenerative Medicine, Texas A&M University) were cultured on mineralized matrices, nonmineralized matrices, or tissue culture plates. More details about mineralized matrices, cell culture, and medium are provided in *SI Text*.

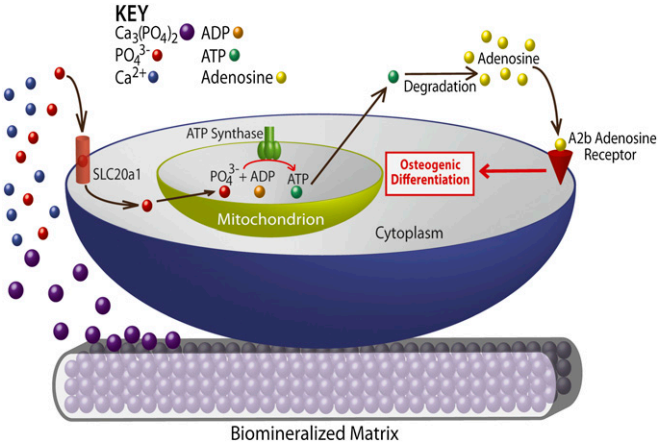

Fig. 5. Schematic model of mineralized matrix-induced osteogenic differentiation.

siRNA Knockdown. For knockdown of SLC20a1, hMSCs were transfected with siRNA oligonucleotides (Invitrogen) according to the manufacturer's instructions. Briefly, 30 nM siRNA targeting SLC20a1 (sense: GGGUGUCAAGUGGUCUGAACUGAUA, antisense: UAUCAGUUCAGACCACUUGACACCC) and scrambled control siRNA (medium GC content) were transfected with RNAimax transfection reagent (Invitrogen) under serum-free conditions for 5.5 h before cells were washed with PBS and changed to growth medium.

Characterization of Cell Phenotype. The changes in cell phenotype responding to various culture conditions were analyzed by PCR, Western blot, and immunofluorescent staining as described in *SI Text*.

HPLC Experiments. HPLC measurements were carried out to measure extracellular ATP and adenosine. Commercially available ATP and adenosine were used as controls. Fig. S5*B* shows the measurable threshold of ATP. Details are provided in *SI Text*.

Statistical Analysis. Beyond the biological replicates, experiments were repeated independently at least twice. Statistical analyses were performed with one-way ANOVA, followed by a Bonferroni post hoc test or a two-tailed Student *t* test. Different letters and asterisks represent significance at $P < 0.05$.

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