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Different Roles of *GNAS* and cAMP Signaling During Early and Late Stages of Osteogenic Differentiation

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Abstract

Progressive osseous heteroplasia (POH) and fibrous dysplasia (FD) are genetic diseases of bone formation at opposite ends of the osteogenic spectrum: imperfect osteogenesis of the skeleton occurs in FD, while heterotopic ossification in skin, subcutaneous fat, and skeletal muscle forms in POH. POH is caused by heterozygous inactivating germline mutations in *GNAS*, which encodes G-protein subunits regulating the cAMP pathway, while FD is caused by *GNAS* somatic activating mutations. We used pluripotent mouse ES cells to examine the effects of *Gnas* dysregulation on osteoblast differentiation. At the earliest stages of osteogenesis, *Gnas* transcripts *G_s α*, *XLαs* and *1A* are expressed at low levels and cAMP levels are also low. Inhibition of cAMP signaling (as in POH) by 2',5'-dideoxyadenosine enhanced osteoblast differentiation while conversely, increased cAMP signaling (as in FD), induced by forskolin, inhibited osteoblast differentiation. Notably, increased cAMP was inhibitory for osteogenesis only at early stages after osteogenic induction. Expression of osteogenic and adipogenic markers showed that increased cAMP enhanced adipogenesis and impaired osteoblast differentiation even in the presence of osteogenic factors, supporting cAMP as a critical regulator of osteoblast and adipocyte lineage commitment. Furthermore, increased cAMP signaling decreased BMP pathway signaling, indicating that G protein-cAMP pathway activation (as in FD) inhibits osteoblast differentiation, at least in part by blocking the BMP-Smad pathway, and suggesting that *GNAS* inactivation as occurs in POH enhances osteoblast differentiation, at least in part by stimulating BMP signaling. These data support that differences in cAMP levels during early stages of cell differentiation regulate cell fate decisions.

Keywords

progressive osseous; heteroplasia; POH; fibrous dysplasia; FD; BMP; SMAD

Introduction

Heterozygous inactivating mutations in the *GNAS* gene occur in patients with progressive osseous heteroplasia (POH) with the effect of redirecting progenitor cells to an aberrant osteogenic cell fate. Patients with POH initially form ectopic, extra-skeletal bone in skin and subcutaneous tissues with subsequent progression into deep connective tissues such as skeletal muscle [1–3]. By contrast, fibrous dysplasia (FD) of bone is caused by activating *GNAS* mutations in somatic cells, leading to abnormal osteogenesis and weakened areas of atypical woven bone [4, 5]. Both conditions identify the *GNAS* locus as a regulator of cell differentiation. Among the multiple transcripts synthesized from the *GNAS* locus, $G_s \alpha$ and XL α s mRNAs encode G-protein subunits that signal via 3,5-adenosine monophosphate (cAMP) to intracellular effectors to regulate cell growth and differentiation [6–11]. However, whether *GNAS* gene activity is important at early (cell commitment and initiation of differentiation) and/or late (differentiation progression and cell maturation) stages of osteoblast differentiation and whether these effects are mediated through cAMP signaling or an alternative pathway remain undetermined.

$G_s \alpha$ is ubiquitously expressed and activated by many G-protein coupled receptors (GPCR) to increase cAMP activation [12–15]. A number of studies collectively suggest that cAMP signaling activity has different effects on osteoblasts and osteogenesis during various stages of the differentiation process [16–27]. Many studies of osteoblast differentiation used cells that are committed osteoblast precursors that have a differentiation potential already directed toward the osteoblast lineage [28], thereby providing limited information about the earliest stages of cell differentiation.

Mouse embryonic stem (ES) cells are pluripotent cells that can be used to investigate many aspects of cell differentiation, including early cell fate decision events such as progenitor cell commitment to specific cell lineages [29–31]. ES cells, isolated from inner cell mass of blastocysts, can be differentiated to 3-dimensional structures, called embryoid bodies (EB), that include derivatives of the 3 embryonic germ layers. In response to appropriate inductive factors, ES cells have the potential to differentiate into cells of multiple lineages [30]. The mesoderm layer is the main source of cells that undergo differentiation to an osteoblast fate during embryonic development, and EB that contain cells of the mesodermal lineage undergo osteoblast differentiation in response to osteogenic induction factors. This in vitro system supports osteoblast differentiation from the earliest stages of cell commitment and initiation of differentiation through later stages progressing to osteoblast maturation [32].

In this study, we used mouse embryonic stem (ES) cells to investigate the role of *Gnas* and cAMP signaling during early and late stages of osteogenic differentiation. Our results demonstrate that increased activity of the *Gnas*-cAMP pathway at early stages inhibits osteoblastogenesis, but acts to enhance osteoblast differentiation at later stages. Our data further suggest that osteoblast commitment and differentiation is regulated, at least in part, through crosstalk between the cAMP and the bone morphogenetic protein (BMP) signaling pathways.

Materials and Methods

Cell culture

Mouse ES cells (R1) [33] were cultured on mitomycin C treated MEF cells ($3 \times 10^6/10$ cm dish) in ES cell culture medium [DMEM with 15 % FBS, 2 mM l-glutamine, 1 $\times$ nonessential amino acids, 50 μ g/ml of gentamicin sulfate, 1000 U/ml of Leukemia Inhibitory Factor (LIF) and 0.1 mM β -mercaptoethanol] at 37 °C, 5 % CO₂. Medium was changed every day; cells were passaged every 2 days.

Embryoid body formation

Embryoid bodies were formed by suspension culture [34]. Briefly, 2×10^6 mouse ES cells (R1) were plated per 10 cm ultra low attachment dish and cultured in EB growth medium (ES cell culture medium without LIF) at 37 °C, 5 % CO₂ for 24 h, then collected by centrifugation and replated in EB growth medium; this medium was changed daily through day 5. EB at day 5 were transferred to standard cell culture plates for attachment and EB outgrowth. By EB day 5, cells decreased stem cell marker expression (Fig. 1a) and the heterogeneous cell population expresses mesodermal, endodermal, and ectodermal markers.

Osteogenic and adipogenic differentiation

Mouse ES cell osteoblast differentiation was induced as described [32] with modification of the osteogenic medium (OM). Briefly, mouse ES cells were induced to form EB; at day 5 after EB formation, EBs were cultured (100 EB/well, 24-well plate) with osteogenic medium [100 ng/ml recombinant human BMP2 (R & D Systems, USA), 5.0 µg/ml ascorbic acid (Sigma, USA), and 10 mM β-glycerophosphate (Sigma, USA) in EB growth medium] at 37 °C, 5 % CO₂. Medium was changed every 2 days. Early and late stage osteoblast marker expression was examined by qRT-PCR; mineralized nodule formation was analyzed by staining with 1 % Alizarin Red S solution (Ricca Chemical Company, USA).

Mouse ES cells were induced to adipogenesis as described [35]. Briefly, at day 2 of EB formation, EB were treated with 1×10^8 retinoic acid (RA) (Sigma, USA) for 3 days, with RA removed from EB growth medium at day 5. At day 6, EB were cultured with adipogenic medium [5 µg/ml Insulin (Sigma, USA) and 10 ng/ml triiodothyronine (T3) (Sigma, USA) in EB growth medium]. Medium was changed every 2 days. Adipogenic marker expression was examined by qRT-PCR; terminal differentiation was detected by Oil Red O staining (Sigma, USA) after being fixed in 10 % formaldehyde.

RNA isolation and quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from cells using TRIZOL (Invitrogen, USA) by the recommended protocol. Reverse transcription used SuperScript II first-strand synthesis system (Invitrogen, USA) with random primers. qRT-PCR reactions used SYBR green master mix with the 7500 fast real time PCR system (Applied Biosystems, USA). Target gene expression levels were normalized with 18 S rRNA or Gapdh.

Immunoblot analysis

Proteins were isolated from EB [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM glycerophosphate, 5 mM Na₄ P₂ O₇, 0.5 % NP40, and complete protease inhibitor cocktail (Roche, USA)] and electrophoresed through 4–12 % SDS-PAGE then transferred to PVDF membranes. Antibodies for phosphorylated Smad1/5/8, Smad1, or β-actin (Cell Signaling Technology, USA) in Odyssey blocking buffer (LI-COR Biosciences, USA) were used at 4 °C overnight. Antibody binding was detected with appropriate IRDye-secondary antibodies (LI-COR Biosciences, USA) in Odyssey blocking buffer for 1 h then detected by the ODYSSEY infrared imaging system.

Assay for G protein-cAMP pathway activation

One hundred EB/well were cultured with osteogenic medium, with or without 50 µM forskolin (Sigma, USA) or 0.1 mM 2',5'-dideoxyadenosine (ddA) (Sigma, USA) at various times as described for individual experiments. For ddA treatments, osteogenic medium contained 50 ng/ml BMP2 in order to evaluate osteogenic effects of ddA against a lower BMP-induction background. Following cell lysis, intracellular cAMP levels were determined by immunoassay (Amersham cAMP Biotrak Enzyme system; GE Healthcare,

USA) and detected by optical density at 450 nm. Protein concentration was quantified by Pierce BCA protein assay (Thermo Scientific, USA).

Statistical analysis

Results were obtained from 3 independent experiments. Statistically significant differences between 2 groups were determined by Student's *t*-test ($p < 0.05$).

Results

The *Gnas* transcripts *G_s α*, 1A, and XL α s are expressed at low levels during early stages of osteogenic differentiation

Inactivating mutations in the *GNAS* gene occur in the human disorder of heterotopic ossification POH in which extensive extraskelatal bone forms. To investigate the participation of *GNAS/Gnas* (human/mouse designations) during osteoblast differentiation, we used mouse ES cells since these pluripotent cells have the ability to contribute to all embryonic tissues and their developmental potential can be maintained in vitro [29, 30]. During EB formation, Oct4, a marker for ES cell pluripotency, was dramatically decreased at day 5 and maintained at low levels (Fig. 1a), indicating that EB at day 5 have exited from the pluripotent state and are committed to differentiation. Correlated with the loss of pluripotency, expression of brachyury, an early marker of the mesodermal lineage, which gives rise to mesenchymal cells such as osteoblasts [36], peaked at day 5 of EB formation (Fig. 1a). To induce differentiation, EB at day 5 were treated with osteogenic medium (OM), and this time point was considered day 0 of osteogenic induction.

During ES/EB cell osteogenic differentiation, we detected increased Alizarin Red staining (Fig. 1b), demonstrating that mouse ES cells show features of mature differentiated mineralizing osteoblasts. To further characterize osteoblast differentiation in our ES cell system, we examined the expression of early and late stage osteoblast differentiation markers by quantitative RT-PCR (Fig. 1c). Expression of the early markers *Msx2*, *osterix*, and collagen I increased significantly within 4 days ($p < 0.05$). In the following experiments we refer to this time period after osteogenic induction as early stages (including commitment and initiation of differentiation) of osteoblast differentiation. After day 4, induction is considered to be late stages (progression of differentiation and maturation). *Osterix* and collagen I remained upregulated throughout differentiation while *Msx2* expression gradually decreased. Detection of alkaline phosphatase (ALP), a intermediate stage marker, peaked at day 14. Expression of the late stage marker osteocalcin increased by day 7. These data demonstrate in vitro induction of osteoblast differentiation markers by mouse ES cells, and further support that these cells are committed to the osteoblast cell lineage within the first 4 days of induction in our ES cell differentiation system.

The *GNAS/Gnas* gene is a complex locus that uses alternate promoters and first exons to express multiple gene products, including 2 *G_s α* subunits, *G_s α* and XL α s [37]. To assess the expression of the *Gnas* gene during ES cell osteoblast differentiation, we examined the expression of 4 *Gnas* transcripts: *G_s α*, XL α s, 1A, and *Nesp* to evaluate similarities and differences in expression patterns. During the first 4 days of osteogenic induction, *G_s α*, XL α s, and 1A remained at low levels, then gradually increased after day 4 (Fig. 2b). At later time points, *G_s α*, XL α s, and 1A mRNAs were significantly upregulated in response to osteogenic medium. Consistent with low expression levels of *G_s α* and XL α s during the first 4 days of induction, intracellular cAMP, a second messenger downstream of the G-protein signaling pathway, maintained low levels of activity at early stages of osteoblast differentiation, then gradually increased (Fig. 2a). With extended time in culture, cAMP levels increased both in the presence or absence of osteogenic factors suggesting that while

cAMP may participate in the osteogenic program, it is not sufficient to promote osteogenesis.

In contrast to the $G_s \alpha$, XL α s, and 1A transcripts, Nesp expression decreased during the first 4 days of osteogenic induction (Fig. 2b) and maintained these low levels. Our data indicate that low levels of expression of the *Gnas* products encoding G-protein alpha subunits ($G_s \alpha$ and XL α s) together with low activity of G protein-cAMP signaling are associated with early stages of osteoblast differentiation, while increased $G_s \alpha$ and XL α s expression and G-protein-cAMP levels occur during later stages.

Increased cAMP inhibits osteoblast differentiation

Both $G_s \alpha$ and XL α s transduce signals through cAMP to intracellular effectors [6]. To investigate the effects of cAMP signaling on osteoblast differentiation, high levels of intracellular cAMP were induced by treatment with forskolin, a cAMP agonist. EB at day 5 were treated with forskolin in osteogenic medium for various intervals during osteogenic differentiation (Fig. 3a).

In response to osteogenic induction, high levels of mineralization were detected in cells at day 20 (Fig. 1 b, 3 c, '0 forskolin'). By comparison, cells in osteogenic medium that were treated with forskolin during the entire osteogenic induction process (days 0–20) showed decreased mineralization (Fig. 3c, 0–20) and reduced expression of later stage markers, bone sialoprotein (Bsp) and osteocalcin (Fig. 3b) as well as early osteogenic markers, osterix and collagen type I (data not shown). These results support that increased cAMP inhibits osteoblast differentiation.

To investigate the stage of differentiation at which increased cAMP inhibits osteoblast differentiation, cells in osteogenic medium were treated with forskolin only during days 0–4, days 0–8, or days 8–20, then assayed at day 20 (Fig. 3a–c). We determined that increased cAMP during the first 4 days of osteogenic induction significantly decreased Bsp and osteocalcin mRNAs and reduced mineralization (Fig. 3b,c). By contrast, increased cAMP during the later stages of osteogenic differentiation (days 8–20) had no effect on mineralization or osteocalcin and Bsp expression (Fig. 3b,c). These data support that cell differentiation is inhibited by cAMP at early, but not late, stages of osteoblastogenesis.

Decreased cAMP enhances osteoblast differentiation

To investigate the effects of decreased cAMP signaling on osteoblast differentiation, EB at day 5 in osteogenic medium were treated with the cAMP antagonist 2',5'-dideoxyadenosine (ddA) to reduce cAMP. After 20 days, ddA-treated cells showed enhanced mineralization (Fig. 3d) and high expression levels of Bsp and osteocalcin (Fig. 3e). These data demonstrate that while increased cAMP is correlated with reduced osteoblast differentiation, decreased cAMP enhances osteoblast differentiation.

To complement our results in response to increased cAMP (Fig. 3, 4a, left panel), we investigated osteogenic marker expression in response to decreased cAMP levels (Fig. 4a, right panel). Although ddA only partially suppressed cAMP levels, cells from POH patients show cAMP at 40–70 % of control levels (our unpublished data) and therefore this level of ddA inhibition may be more relevant than complete inhibition. Correlated with ddA-induced reduction of cAMP, expression levels of *Msx2* and osterix were enhanced significantly within first 2 days (Fig. 4c), supporting that decreased cAMP at early stages promotes differentiation to the osteoblast lineage.

Increased cAMP suppresses osteoblast commitment but upregulates the adipocyte marker PPAR- γ at early stages following osteogenic induction

To determine whether increased cAMP at early stages of differentiation alters cell commitment to the osteoblast lineage, we examined mesenchymal lineage marker expression. Osteoblasts and adipocytes can develop from common progenitors and these cell fates have been suggested to be reciprocally regulated with increased cAMP activity promoting adipogenesis [38–40]. Therefore, we compared the effects of increased cAMP on early markers of osteoblasts (Msx2 and osterix) and adipocytes (PPAR γ). In response to increased cAMP by forskolin treatment (Fig. 4a), decreased expression of Msx2, a transcriptional target of BMP signaling and a master regulator for early stage osteoblast differentiation [40–42], was detected by 24 h after osteogenic induction (Fig. 4b). Expression of osterix was also decreased (Fig. 4b). By contrast, expression of PPAR- γ , a specific transcription factor for adipocyte differentiation, was significantly increased within the first 2 days of forskolin treatment despite the presence of osteogenic induction medium (Fig. 4b). We observed these same effects using an alternate cAMP agonist, 8-Br-cAMP (data not shown). Following osteogenic induction of EB, Oct4 and brachyury mRNAs decreased, but showed no difference between cells with or without forskolin (data not shown), indicating that cAMP has no effect on loss of cell pluripotency or mesoderm commitment during osteogenic differentiation. The effects of cAMP on Msx2 and PPAR- γ , key regulators of cell lineage commitment for the osteoblast and adipocyte lineages, respectively, suggest that increased cAMP levels inhibit cell commitment to osteogenic lineage at early stages of differentiation and promote adipogenesis. These data support that cAMP signaling is critical for reciprocal cell fate decisions between osteoblast or adipocyte lineages at very early stages of the cell differentiation process.

Increased cAMP acts early during differentiation to enhance adipogenesis

To further examine the effects of increased cAMP on adipogenesis, we treated EB with forskolin in the presence of adipogenic medium. After 20 days with forskolin treatment, lipid accumulation detected by Oil Red O showed robust mature adipocyte formation from the center of the EB outgrowth (Fig. 5a). In addition, forskolin increased by 5-fold the number of adipocyte positive EB colonies (Fig. 5b) and early stage adipogenic markers (LPL and PPAR- γ) were significantly increased at day 2 of adipogenic induction (Fig. 5c). At day 20, these early stage markers, as well as the late marker aP2, are expressed at significantly higher levels in cells with forskolin treatment in the presence of adipogenic medium (Fig. 5c). Consistent with previous reports that increased IBMX-induced cAMP stimulated adipogenesis [43], our data demonstrate that increased cAMP enhances adipogenesis by acting at early stages of differentiation.

Increased cAMP blocks the BMP-Smad signaling pathway at an early stage of osteoblast differentiation

To investigate whether cAMP may regulate osteoblast differentiation through interactions with the BMP pathway, a key regulator of osteoblast differentiation [44], we assayed BMP signaling activity in response to increased cAMP. EB were treated with forskolin and BMP2 (Fig. 6a) for 0.5 to 2.5 h. Intracellular cAMP levels were rapidly and markedly increased by forskolin treatment (Fig. 6b). BMP signaling induced Smad1/5/8 phosphorylation, however this response was decreased significantly at 2.5 h in forskolin-treated cells (Fig. 6c). The expression of BMP-Smad responsive genes [45] was examined, and while no significant difference was detected in Id1 transcript levels, Msx2 and osterix expression were decreased significantly by forskolin (Fig. 6d). These results together suggest that activation of cAMP signaling interacts with and inhibits the BMP-Smad pathway at early stages of osteoblast differentiation.

Discussion

The *GNAS/Gnas* gene is a complex locus that encodes several products, including $G_s \alpha$ and XL α s, which encode G-protein α -subunits that activate downstream cAMP activity [6]. Heterozygous inactivation of the *GNAS* gene occurs in progressive osseous heteroplasia (POH), a severely disabling human disorder of extra-skeletal osteogenesis characterized by initial bone formation events in the dermis, often in association with adipose tissue, followed by progressive ossification of cutaneous, subcutaneous, and deep connective tissues [1–3]. Cells from POH patients show decreased expression of $G_s \alpha$ and XL α s mRNAs and decreased cAMP levels [46] (our unpublished data). Conversely, somatic activation of the *GNAS* gene and increased cAMP signaling occurs in fibrous dysplasia (FD) causing imperfect osteogenesis and resultant weak, immature bone [4, 5].

The function of cAMP/G-protein signaling in osteoblast differentiation has been widely investigated in osteoblasts and/or committed pre-osteoblasts [6]. However, few studies have examined the effects of this pathway on the earliest stages of cell differentiation and lineage commitment of osteogenesis. Pluripotent embryonic stem (ES) cells provide an ideal cell model for examining early events during osteoblast differentiation [32]. In this study, we used a mouse ES cell differentiation system to demonstrate that mRNA expression of $G_s \alpha$ and XL α s, 2 G-protein α -subunits encoded by *Gnas*, are low during initial stages of osteogenic induction and then increase as osteoblast differentiation progresses. Furthermore, while cAMP is required for osteoblast differentiation at late stages of this process, cAMP inhibits osteoblast differentiation at early stages of the process. The dynamic expression of *Gnas* gene products and cAMP levels support that the roles of G-protein/cAMP signaling change at different stages of osteoblast differentiation.

Correlated with low $G_s \alpha$ and XL α s expression, the early stage cell differentiation marker *Msx2* showed high expression levels only during first 4 days of osteoblast induction. Since high expression of *Msx2* is associated with osteoprogenitor cells and prevention of osteoblast maturation [40, 42, 45], in our cell system, we consider the first 4 days of osteogenic induction to be the stages of differentiation during which early events such as commitment to cell fate decisions occur.

Several studies, using a range of committed osteoblast and preosteoblast cell types, have shown that cAMP is required for osteoblast differentiation [47]. The cells used in these studies are of varied cell types and represent osteoblasts at intermediate or late stages of differentiation [28]. These investigations are consistent with our data showing that high expression levels of $G_s \alpha$ and XL α s and high cAMP levels occur at late stages of osteoblast differentiation. However, we found that increased cAMP (induced by forskolin) during early osteoblast differentiation is inhibitory, and conversely, decreased cAMP (inhibited with ddA) enhances osteoblast differentiation (as occurs in POH). These results are consistent with *GNAS* as a suppressor of osteoblast commitment and early differentiation. *GNAS* activating mutations increase cAMP, causing impaired bone formation in FD patients. Conversely, *GNAS* inactivating mutations decrease cAMP, causing ectopic bone formation in POH patients [1, 4, 5, 48].

Osteoblasts and adipocytes can develop from common progenitors and these cell fates have been suggested to be reciprocally regulated [39–42, 49–51]. However, the question regarding how cAMP directs different cell fate decisions is complex. For example, while increased cAMP signaling is established to promote adipogenesis, inactivating mutations in *GNAS* are associated with obesity [52, 53]. Likewise, although many studies have reported a requirement for increased cAMP during osteoblast differentiation [54–60], this is an apparent contradiction to the ectopic bone formation in POH patients who carry inactivating

GNAS mutations. Cyclic AMP signaling likely has both activating and inhibitory roles that must be balanced during different stages of adipogenesis and osteoblastogenesis and therefore the timing, not only the level, of cAMP activity is important.

We found that increased cAMP at early stages of differentiation decreases early osteogenic markers (*Msx2*, *osterix*) but enhances expression of the adipogenic marker *PPAR-γ* even in the presence of osteogenic induction factors. Further, we found that decreased cAMP early during osteogenic induction increases *Msx2* and *osterix* expression. The reciprocal adipogenic and osteogenic responses to cAMP levels are consistent with evidence from human bone-marrow derived MSCs [61] suggesting that cAMP signaling may mediate a switch between osteogenesis and adipogenesis. Consistent with these data, expression of *G_s*, *α* and *XLas* at early stages of adipogenesis is higher than expression during early osteoblast differentiation (data not shown). These results support that low levels of cAMP signaling are required for osteoblast lineage commitment and higher levels of cAMP for adipocyte lineage commitment. Thus, cAMP modulation by *GNAS* appears critical in regulating adipogenic-osteogenic commitment.

To investigate the cellular mechanisms through which cAMP regulates cell fate decisions during osteoblast differentiation, we examined the response of the BMP signaling pathway to cAMP. BMPs, members of the *TGFβ* family, have multiple roles including cell fate decisions and cell differentiation [44, 62], and cAMP can crosstalk with the BMP pathway during cell differentiation [49, 61]. In our study, we determined that high cAMP levels at early stages of osteogenic induction decreases BMP pathway activation. Whether cross-talk between cAMP signaling and the BMP pathway is direct or mediated through other signaling pathways remains undetermined, however, our data support that interaction between these pathways is a key regulatory event.

In summary, our study demonstrates that a dynamic regulation of *Gnas* expression and cAMP levels is correlated with induction and progression of osteoblast differentiation. We have shown that the *Gnas*/cAMP pathway has important and multiple roles in cell fate decisions and differentiation during osteogenic differentiation, with low levels of cAMP required for osteoblast lineage commitment at early stages of differentiation. Our data further suggest that interactions between cAMP and BMP signaling pathways regulate these cell fate decisions. These results support the hypothesis that activating *GNAS* mutation in FD and the resulting increased cAMP signaling lead to decreased BMP pathway signals and impaired osteogenesis while inactivating *GNAS* mutations in POH patients and the resulting decreased cAMP signaling promote upregulated BMP pathway signaling in progenitor cells within soft connective tissues to induce osteogenesis and heterotopic bone formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Osteoblast differentiation of murine ES cells. **a** For differentiation assays, ES cells were induced to form embryoid bodies (EB); decreased expression of the pluripotency marker Oct4 and induction of the early mesoderm marker Brachyury were detected by qRT-PCR. **b** EBs at day 5 were treated with osteogenic medium (OM) containing BMP2 or EB culture medium (CM) and mineralized nodule formation assayed by Alizarin Red staining. **c** As in **b**, day 5 EBs were induced to osteoblast differentiation and early (Msx2, osterix, collagen I, and ALP) and late stage (osteocalcin) osteogenic markers were examined by qRT-PCR and plotted relative to cells in control medium (CM) at day 0. Data were normalized by 18S rRNA and are expressed as mean $\pm$ SEM of 3 independent experiments; # $p < 0.05$ vs. controls (spontaneous differentiation in EB culture medium) at a given time point, * $p < 0.05$ at successive time points during OM treatment vs. day 1. EB at day 5 were used in all subsequent experiments.

Fig. 2.

Expression of *Gnas* transcripts and cAMP levels during osteogenic differentiation. **a** Intercellular cAMP levels were examined by ELISA over time during osteoblast differentiation. Data were normalized by protein concentration (pmol cAMP/μg protein). **b** *Gnas* transcripts [*G_s α*, *XLα*s, *1A*, *Nesp*] were quantified by qRT-PCR during osteogenic induction. Data from 3 independent experiments were normalized by 18S rRNA and plotted relative to cells in control medium (CM) at day 0. Data are expressed as mean ± SEM, **p* < 0.05 at successive time points during OM treatment vs. day 1; #*p* < 0.05 vs. controls (spontaneous differentiation in EB culture medium). OM: osteogenic medium; CM: control (EB culture) medium.

Fig. 3.

Effect of cAMP on osteoblast differentiation. Mouse ES cells were treated with forskolin (**a–c**) to increase or with ddA (**d, e**) to decrease cAMP levels during osteoblast differentiation (data showing the effects of forskolin and ddA on cAMP are in Fig. 4 a). **a** Scheme of forskolin (FSK) treatments for various intervals in osteogenic medium (OM). Days of forskolin treatment period are indicated at left. **b** Bsp and osteocalcin mRNAs levels by qRT-PCR at day 20 after forskolin treatment as indicated. Data were normalized with 18S rRNA and plotted relative to samples treated with OM for 14 days. Data are from 3 independent experiments. * $p < 0.05$ vs. cells in osteogenic medium (OM) without forskolin (0 days). **c** Effect of forskolin treatment on mineralized nodule formation, assayed at day 20 by Alizarin Red. EB spontaneous differentiation in EB culture medium is shown as a control. **d** Cells were cultured in OM with ddA and mineralized nodule formation was assayed by Alizarin Red at day 14. **e** Bsp and osteocalcin mRNA levels at day 20 after ddA treatment in OM were quantified by qRT-PCR, normalized by 18S rRNA, and are shown relative to levels in cells without ddA treatment. Data represent 2 independent experiments. * $p < 0.05$ vs. cells in OM without ddA.

Fig. 4.

Effects of cAMP levels on osteogenic and adipogenic markers at early stages of osteoblast differentiation. Cells were grown in osteogenic media (OM) and treated to increase (forskolin) or decrease (ddA) cAMP. **a** cAMP levels were assayed by ELISA and normalized by protein concentration. **b, c** Msx2, osterix, and PPAR- γ mRNAs during the first 4 days in OM with forskolin **b** or ddA **c** were quantified by qRT-PCR and normalized with Gapdh. Data were plotted relative to levels at day 1 without forskolin **b** or at day 0 without ddA **c**. Data are from 3 independent experiments. * $p < 0.05$; ** $p < 0.01$ vs. cells cultured with OM.

Fig. 5.

Effects of increased cAMP on adipogenesis. EB cells were cultured in adipogenic medium (AM) or in AM with forskolin (AM + FSK) for 20 days. **a** Lipid drop formation at day 20 was assayed by Oil Red O staining. **b** Oil Red O positive colonies in each well ($n = 3$ wells) were quantified by counting. $*p < 0.05$, AM + FSK treatment vs. AM treatment. **c** Early (LPL and PPAR- γ), intermediate (EBP1), and late stage (aP2) adipogenic markers with AM or AM + FSK treatment were quantified by qRT-PCR, normalized by 18S rRNA, and plotted relative to cells in AM at day 0. Data are from 3 independent experiments. $*p < 0.05$, AM + FSK treatment vs. AM treatment.

Fig. 6.

Cross talk between cAMP and BMP signaling at early stages of osteoblast differentiation. BMP-Smad pathway activation was assayed by Smad1/5/8 phosphorylation and BMP transcriptional targets. Following initial growth in OM for 3 days to reach the early stage of osteogenic differentiation, EB cell growth was continued in EB medium with 100 ng/ml BMP2 and the presence or absence of forskolin. **a** Scheme of forskolin and BMP2 treatment. **b** Intracellular cAMP levels by ELISA were normalized by cellular protein concentration after forskolin treatment for 0.5 h or 2.5 h following 3 days of osteogenic induction in OM. **c** Smad1/5/8 phosphorylation was detected by immunoblotting after 0.5 h or 2.5 h of forskolin. **d** Following treatment of cells with forskolin for 5 h or 16 h, *Msx2*, *osterix*, and *Id1* mRNA levels were quantified by qRT-PCR, normalized to *Gapdh*, and plotted relative to samples without forskolin at the 5 h time point. Data are from 3 independent experiments. * $p < 0.05$; ** $p < 0.01$ vs. osteogenic induction without forskolin.