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Original

Fibroblast growth factor 2 and forskolin induce mineralization-associated genes in two kinds of osteoblast-like cells

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Abstract: Fibroblast growth factor 2 (FGF2) and cyclic AMP (cAMP) play critical roles in controlling the differentiation of osteoblasts and mineralization of bone. We have previously reported that each of FGF2 and forskolin (FSK) alone increase transcription of the bone sialoprotein (BSP) gene, and that together (FGF/FSK) they upregulate BSP gene expression synergistically in rat osteoblast-like ROS 17/2.8 cells. However, other genes that are upregulated after stimulation by FGF2, FSK or FGF/FSK remain unclear. In the present study, we investigated candidate genes associated with mineralization after stimulation by FGF2, FSK and FGF/FSK in two kinds of osteoblast-like cells using microarray and real-time PCR. In ROS17/2.8 cells, FGF2 and FSK each increased the gene expression of c-FOS (7.2-fold and 10.7-fold, respectively). However, FGF/FSK did not induce c-FOS gene expression. FGF2 increased the expression of the dentin matrix protein 1 (DMP1, 129.8-fold) gene. In contrast, FGF/FSK increased the expression of the amphiregulin (AREG, 73-fold) gene maximally. In human osteoblast-like Saos2 cells, FGF2 increased the expression of the osteopontin (SPP1, 16.7-fold), interleukin-8 (IL8, 6.4-fold) and IL11 (4.8-fold) genes. FSK induced the expression of the IL6 (2.6-fold), IL11 (4.0-fold), chemokine ligand 13 (CXCL13, 2.8-fold) and bone morphogenetic protein 2 (BMP2, 2.5-fold) genes. These results suggest that FGF2 and FSK might be crucial regulators of mineralization and bone formation. (J Oral Sci 54, 251-259, 2012)

Keywords: transcription factor; osteoblast; FGF2; forskolin; microarray.

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Introduction

Osteoblasts produce and secrete several kinds of growth factors that are also found in the extracellular matrix of bone, such as fibroblast growth factor 2 (FGF2), transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF). These growth factors may regulate the initiation and development of bone formation and fracture callus. A particularly close relationship between FGF2 and bone formation has been reported (1,2). Overexpression of the FGF2 gene in transgenic mice causes premature mineralization, flattening and shortening of long bones (3). Moreover, disruption of the FGF2 gene leads to a decrease of both bone mass and bone formation (4). Therefore, FGF2 could be crucial for the regulation of osteoblast proliferation and proper bone development.

Forskolin (FSK) is a labdane diterpene produced by the Indian Coleus plant. FSK is commonly used to raise cyclic AMP (cAMP) levels in studies of cell physiology. FSK resensitizes cell receptors by activating the enzyme adenylyl cyclase and increasing the intracellular levels of cAMP. Neurotransmitters and parathyroid hormone (PTH), which stimulate cAMP production and activation of protein kinase A (PKA) (5), can promote (6) or inhibit (7) growth and proliferation in a cell-specific manner. FSK and 8-Br cAMP, which also elevate the intracellular level of cAMP, together with PTH, have been shown to increase the levels of both osteocalcin (OC) mRNA and protein (8).

OC and bone sialoprotein (BSP) are non-collagenous proteins expressed specifically in mineralized connective tissues (9). OC is expressed in bone matrix at the late stage of mineralization, whereas BSP is expressed in the early stage. The temporo-spatial deposition of BSP in the extracellular matrix of bone (10) and its ability to nucleate hydroxyapatite crystal formation (11) indicate a potential role of BSP in the initial mineralization of bone. Several studies have shown that FGF2 and FSK (cAMP) induce expression of the OC gene (12,13), and that Msx2, Dlx5 and Runx2 are important transcription factors for OC gene expression (13-17). A low concentration of FSK induces differentiation of osteoprogenitor cells, possibly through a non-cAMP-dependent process, and intermittent elevation of intracellular cAMP has an inhibitory effect on bone formation (18). Moreover, FSK inhibits DNA synthesis, blocks protein kinase C (PKC)-stimulated tyrosine phosphorylation of p44MAPK (ERK1) and p42MAPK (ERK2), and also inhibits PKC-stimulated MAPK and Raf-1 activities in MC3T3-E1 osteoblast-like cells (19). Phorbol-12-myristate-13-acetate (PMA) activates the intermediate MKK step of the Raf-1/MKK/MAPK cascade in the presence of FSK (20). Inducible cAMP early repressor (ICER) expression in osteoblasts also requires activation of the cAMP-protein kinase A (PKA) signaling pathway (20). We have previously reported that FGF2 and FSK (FGF/FSK) synergistically upregulate BSP gene expression through PKA, tyrosine kinase, and MAP-kinase-dependent pathways, which target the inverted CCAAT, CRE, FRE and Pit-1 elements in the rat BSP gene promoter (21).

The goal of the present study was to use microarray and real-time PCR to investigate the candidate genes associated with mineralization after stimulation by FGF2, FSK and FGF/FSK.

Materials and Methods

Reagents

Alpha minimum essential medium (α-MEM), fetal calf serum (FCS), penicillin, streptomycin, and trypsin were obtained from Invitrogen (Carlsbad, CA, USA). A DyNAmo SYBRgreen qPCR Kit and Moloney murine leukemia virus reverse transcriptase RNase H were from Finnzymes (Espoo, Finland). FSK was from Sigma-Aldrich Japan (Tokyo, Japan). Recombinant human FGF2 was from Genzyme, Techne (Minneapolis, MN, USA). GeneChip (Human Genome U133A 2.0 Array and Rat Genome 230 2.0 Array) was purchased from Affymetrix (Santa Clara, CA, USA). All chemicals used were of analytical grade.

Cell culture

Human and rat osteoblast-like Saos2 and ROS17/2.8 cells were cultured at 37°C in 5% CO_2 air in α-MEM supplemented with 10% FCS. These cells were first grown to confluence in 60-mm tissue culture dishes in α-MEM medium containing 10% FCS, then cultured in α-MEM without serum for 12 h, and stimulated by FGF2 (10 ng/mL), FSK (1 μM) or FGF2/FSK for 6 h. Total RNA was isolated from triplicate cultures and analyzed for gene expression.

Microarray analysis

Three RNA samples were mixed together for gene expression profiling, which was performed separately for each pooled RNA sample using a GeneChip (Human Genome U133A 2.0 Array, spotted with 22,000 human genes, and Rat Genome 230 2.0 Array, spotted with 28,000 rat genes). Hybridization and scanning of the Gene Chip were done in accordance with the Affymetrix manual. Twenty micrograms of the fragmented biotinylated sample cRNA was hybridized to the GeneChip, and then washed and stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR, USA) using the EukGE-WS1 protocol by an Affymetrix GeneArray Scanner. The expressions of human and rat genes were monitored, and the data were imported into GeneSpring GX software (Agilent Technologies, Inc, Santa Clara, CA, USA)

Real-time PCR

Quantitative real-time PCR was performed using a SYBR Green qPCR Kit in a DNA Engine Opticon 2 continuous fluorescence detection system (BioRad, Hercules, CA, USA). After the RT reaction, cDNA was used as the template for real-time quantitative PCR for human WNT5A, human WNT5B, human HADC4, rat JUNB, rat MYC, rat AXIN2, rat WNT4, and rat c-FOS;

Table 1 The primers used for real-time PCR analysis in this study

Gene		Primer sequences
Human WNT5A	Forward	5'-AGAGATCGTTAGCAGCATCAGTCC-3'
	Reverse	5'-GCCTTCGTGCCTATTTGCATTA-3'
Human WNT5B	Forward	5'-GGGTGCTCATGAACCTGCAA-3'
	Reverse	5'-GCAGGCTACGTCTGCCATTTATAC-3'
Human HDAC4	Forward	5'-CATCTGTCAGCTCACTCCAGCTTC-3'
	Reverse	5'-TGGACGCTTGCTGTGGAGA-3'
Human GAPDH	Forward	5'-GCACCGTCAAGGCTGAGAAC-3'
	Reverse	5'-ATGGTGGTGAAGACGCCAGT-3'
Rat JUNB	Forward	5'-TTCGGGTCAGGGATCAGACAC-3'
	Reverse	5'-GACGATCAAGCGCTCCAGTTC-3'
Rat MYC	Forward	5'-GCTCGCCCAAATCCTGTACCT-3'
	Reverse	5'-TTTCCACAGACACCACATCAATTTC-3'
Rat AXIN2	Forward	5'-GAGCTTGACTCTGGGCCACTTC-3'
	Reverse	5'-GCAAATTCGTCACTCGCCTTC-3'
Rat WNT4	Forward	5'-AGGTGTGGCCTTTGCAGTGAC-3'
	Reverse	5'-GCTACGCCATAGGCGATGTTG-3'
Rat c-FOS	Forward	5'-GTTCCTGGCAATAGTGTGTTCCAA-3'
	Reverse	5'-TGACAATGAACATGGACGCTGA-3'
Rat GAPDH	Forward	5'-GACAACTTTGGCATCGTGGA-3'
	Reverse	5'-ATGCAGGGATGATGTTCTGG-3'

human and rat GAPDH were utilized as internal controls. Details of the primers are listed in Table 1. The amplification reactions were performed in a final volume of 20 μL containing 1× SYBR Green Master Mix, 0.25 μM primer mixture and 10 ng cDNA. To reduce variability between replicates, PCR premixes, which contained all of the reagents except for cDNA, were prepared and aliquoted into 0.2-mL thin-walled strip tubes (MJ Research Inc.). The thermal cycling conditions were 30 cycles of the following protocol: 15 s of denaturation at 95°C, 50 s of annealing at 64°C, followed by 12 s of extension at 77°C. Post-PCR melting curves confirmed the specificity of single-target amplification, and the expression of target genes relative to GAPDH was determined from triplicate samples.

Statistical analysis

Triplicate samples were analyzed for each experiment, and experiments were replicated to ensure consistency of the responses to stimulation. Significant differences between controls and treatments were determined using one-way analysis of variance (ANOVA).

Results

To study the regulation of human and rat genes by FGF2, FSK or FGF2/FSK, we performed DNA microarray analyses of total RNA extracted from Saos2 and ROS17/2.8 osteoblast-like cells. In human Saos2 cells, the expression levels of 418, 68 and 345 genes were upregulated more than two-fold by FGF2 (10 ng/mL), FSK (1 μM) and FGF/FSK in Saos2 cells (Table 2 A, B, C). FGF2 increased the expression of mRNAs for synaptotagmin XII (SYT12) (60.2-fold), osteopontin (SPP1(OPN), 16.7-fold), colony stimulating factor 2 (CSF2, 15.6-fold), early growth response 1 (EGR1, 6.6-fold), interleukin 8 (IL8 6.4-fold), interleukin 11 (IL11, 4.8-fold), Wnt5B (WNT5A, 4.0-fold), histone deacetylase 4 (HDAC4, 3.5-fold), and bone morphogenetic protein 2 (BMP2, 3.3-fold) (Table 2A). FSK stimulated expression of the genes for sialophorin (LSN, 45.2-fold), synaptotagmin-like 3 (SYTL3, 6.1-fold), IL11 (4.0-fold), prostaglandin E synthase (PTGES, 2.9-fold), chemokine ligand 13 (CXCL13, 2.8-fold), IL6 (2.6-fold), and BMP2 (2.5-fold) (Table 2B). FGF/FSK induced the expression of mRNAs for SYTL3 (33.9-fold), IL11 (13.6-fold), SPP1 (12.4-fold), CSF2 (10.3-fold), HDAC4 (7.5-fold), EGR1 (6.8-fold), IL8 (5.6-fold), BMP2 (4.4-fold), and WNT5A (4.1-fold) (Table 2C).

In rat ROS17/2.8 cells, the expression levels of 591, 127 and 390 genes were upregulated more than two-fold by FGF2 (10 ng/mL), FSK (1 μM) and FGF/FSK (Table 3 A, B, C). FGF2 stimulated expression of the genes for dentin matrix protein 1 (DMP1, 129.8-fold), EGR1 (33.4-fold), c-FOS (7.2-fold), AXIN2 (4.6-fold), JUNB (4.1-fold), MYC (3.8-fold), and connective tissue growth factor (CTGF, 2.8-fold) (Table 3A). FSK increased

Table 2 Representative genes whose expression was increased by FGF2 (A), FSK (B) and FGF2 plus FSK (FGF/FSK) (C) in human osteoblast-like Saos2 cells

GenBank	Gene Name	
(A)		Ratio FGF2/Cont
BE881590	ETV1	63.3
AK024280	SYT12	60.2
NM_001423	EMP1	35.7
AL572488	FLJ14490	29.3
BC003143	DUSP6	19.6
NM_002135	NR4A1	19.2
M83248	SPP1 (OPN)	16.7
M11734	CSF2	15.6
AB028021	FOXA2	14.3
AW193698	TGFBR3	14.3
AK026181	PHLDA1	14.2
NM_004454	ETV5	13.1
NM_000602	SERPINE1	12.0
BC015940	NT5E (CD73)	11.0
NM_003483	HMGA2	11.0
AW151660	RGS3	9.9
NM_004369	COL6A3	8.3
AW166825	S100A6	8.1
NM_00362	TIMP3	7.3
NM_002658	PLAU	7.0
AV733950	EGR1	6.6
AF043337	IL8	6.4
M27281	VEGF	5.5
U08839	PLAUR	5.2
M57765	IL11	4.8
M92934	CTGF	4.2
NM_030775	**WNT5B**	**4.0**
NM_006037	**HDAC4**	**3.5**
NM_001200	BMP2	3.3
(B)		Ratio FSK/Cont
X52075	LSN (CD43, GPL115)	45.2
AI674404	SYTL3	6.1
AI799784	MGC45780	4.8
NM_018371	ChGn	4.7
M57765	IL11	4.0
NM_000693	ALDH1A3	3.9
D13891	ID2	3.8
AI422986	SIAT8D	3.8
AB018009	SLC7A5	3.5
AI084489	PITPNC1	3.5
BF246115	MT1F	3.3
NM_019058	DDIT4	2.9
NM_004878	PTGES	2.9
AI791187	FGFR1	2.8
NM_005985	SNAI1	2.8
NM_006419	CXCL13	2.8
NM_014746	RNF144	2.8
BF513244	DAAM2	2.7
AW118608	ATPAF2	2.7
NM_013281	FLRT3	2.7
NM_006169	NNMT	2.7
NM_005951	MT1H	2.6
NM_000600	IL6	2.6
R40917	PDE4D	2.5
NM_001200	BMP2	2.5
AI990526	AYP1	2.5
AA675892	TOB1	2.4
NM_001078	VCAM1	2.4
NM_005178	BCL3	2.3
NM_007348	ATF6	2.1
(C)		Ratio FGF2+FSK/Cont
BE881590	ETV1	66.6
AL833750	SYTL3	33.9
AI631159	SLC2A3	26.2
NM_144613	COX6B2	21.3
NM_002135	NR4A1	18.7
NM_001423	EMP1	16.8
AI638433	PDE7B	16.0
AK026181	PHLDA1	14.8
BC015940	NT5E (CD73)	13.7
M57765	IL11	13.6
M83248	SPP1 (OPN)	12.4
AI674404	SYTL3	12.2
BC003143	DUSP6	12.0
AW193698	TGFBR3	11.9
M11734	CSF2	10.3
AW003173	STC1	10.0
AA748418	MCTP2	8.6
NM_002309	LIF	7.5
NM_006037	**HDAC4**	**7.5**
AV733950	EGR1	6.8
R40917	PDE4D	6.7
NM_000584	IL8	5.6
NM_002658	PLAU	5.2
NM_000600	IL6	4.9
M27281	VEGF	4.6
NM_001200	BMP2	4.4
AY029180	PLAUR	4.4
AI968085	**WNT5A**	**4.1**
NM_002166	ID2	3.9

The indicated genes were upregulated more than two-fold compared with the control. Genes in bold print were analyzed by real-time PCR.

Table 3 Representative genes whose expression was induced by FGF2 (A), FSK (B) and FGF2 plus FSK (FGF/FSK) (C) in ROS17/2.8 cells

GenBank	Gene Name	
(A)		Ratio FGF2/Cont
A106002	DMP1	129.8
NM_012551	EGR1	33.4
AI0059968	FLT1	25.0
AI231350	DUSP6	21.7
AI717725	MIST1	15.9
NM_017134	ARG1	13.2
NM_053714	ANK	12.8
AI602811	DUSP6	11.4
NM_032069	GRIP1	7.5
BF415939	**c-FOS**	**7.2**
NM_012620	SERPINE1	7.1
NM_012953	FOSL1	5.9
NM_012610	NGFR	4.9
BI299621	IGF2R	4.7
NM_024355	**AXIN2**	**4.6**
NM_022277	CASP8	4.2
NM_021836	**JUNB**	**4.1**
NM_012603	**MYC**	**3.8**
NM_022266	CTGF	2.8
BI275741	EMP1	2.6
NM_133578	CPG21	2.6
BM387324	GIG2	2.6
U67140	DAP4	2.6
M94288	NOPP1 40	2.6
NM_031668	MYBBP 1A	2.5
AI137869	ATP2B1	2.5
NM_130429	LEF1	2.5
(B)		Ratio FSK/Cont
BF415939	**c-FOS**	**10.7**
AW533010	BHLHB2	6.8
NM_012551	EGR1	5.6
L08595	NR4A2	4.6
AF200684	SLC7A7	4.1
BF283073	PRSS8	3.8
BF419085	HDAC4	3.6
AB020480	SNF1 LK	3.5
NM_021770	OLIG1	3.3
NM_030851	BDKRB1	3.3
BF565001	PDE4D	3.1
AW527989	GRIP1	2.9
M22586	GLS	2.8
NM_024355	**AXIN2**	**2.8**
AW535310	ADAMTS5	2.6
AI603439	ULK1	2.6
NM_024134	DDIT3	2.5
NM_017334	CREM	2.5
AA875132	TPM1	2.5
NM_057125	PEX6	2.5
BI285710	VAD	2.4
NM_053402	**WNT4**	**2.4**
AA891661	AQP1	2.4
NM_022266	CTGF	2.1
NM_012589	IL6	2.1
(C)		Ratio FGF2+FSK/Cont
NM_017123	AREG	73.0
NM_017134	ARG1	29.3
NM_012551	EGR1	22.6
AI231350	DUSP6	22.5
AI059968	FIT1	19.2
AI060002	DMP1	13.4
NM_053883	DUSP6	13.1
NM_012653	SLC9A2	13.0
NM_053714	ANK	10.9
NM_032069	GRIP1	10.6
AF007789	PLAUR	9.1
NM_133578	CPG21	7.3
NM_023981	CSF1	6.7
NM_030851	BDKRB1	6.5
AF280967	PTGES	6.4
X63434	PLAU	6.3
NM_012953	FOSL1	6.3
AW535553	CSF1	6.0
NM_031556	CAV	5.2
AA901341	SLC2A3	5.0
AA818382	DLC1	4.8
AI102530	NAB2	4.8
NM_022266	CTGF	4.5
NM_017334	CREM	4.1
NM_021836	**JUNB**	**3.8**
NM_012603	**MYC**	**3.6**
NM_012589	IL6	3.6
AF140346	SFRP4	3.6
NM_024355	**AXIN2**	**3.5**

The indicated genes were upregulated more than two-fold compared with the control. Genes in bold print were analyzed by real-time PCR.

expression of the genes for c-FOS (10.7-fold), EGR1 (5.6-fold), HDAC4 (3.6-fold), AXIN2 (2.8-fold), WNT4 (2.4-fold), CTGF (2.1-fold) and IL6 (2.1-fold) (Table 3B). Furthermore, FGF/FSK upregulated expression of the genes for amphiregulin (AREG, 73.0-fold), EGR1 (22.6-fold), DMP1 (13.4-fold), PTGES (6.4-fold), CTGF (4.5-fold), cAMP response element modulator (CREM, 4.1-fold), JUNB (3.8-fold), MYC (3.8-fold), and AXIN2 (3.5-fold) (Table 3C).

To investigate candidate genes associated with miner-

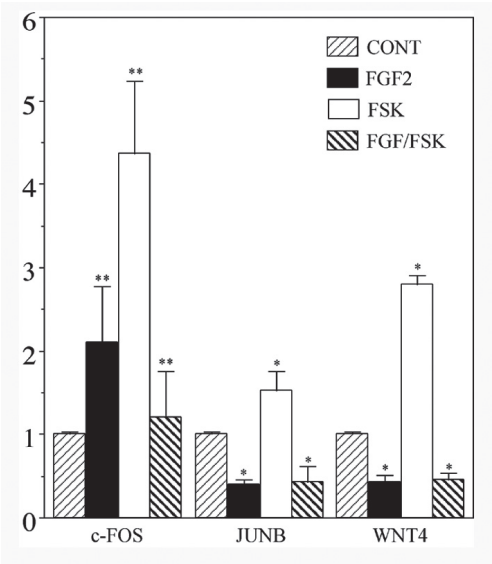

Fig. 1 Expression levels of the c-FOS, JUNB and WNT4 genes in osteoblast-like ROS17/2.8 cells after stimulation with FGF2, FSK and FGF/FSK, determined using real-time PCR. The indicated genes were selected based on the results of Gene Chip analysis. Experiments were performed in triplicate for each data point, and standard errors are shown as vertical lines. Significant differences compared to controls are shown at the following probability levels: $*P < 0.01$, $**P < 0.05$.

alization after stimulation by FGF2, FSK and FGF/FSK in human osteoblast-like Saos2 cells, we carried out real-time PCR. FGF2 increased expression of the genes for WNT5A (4.8-fold), WNT5B (5.2-fold), and HDAC4 (2.5-fold). While FSK induced expression of the genes for WNT5A (5.5-fold) and HDAC4 (2.4-fold), it suppressed expression of the mRNA for WNT5B (0.17-fold). FGF/FSK induced expression of the genes for WNT5A (11.8-fold), WNT5B (6.2-fold) and HDAC4 (6.5-fold).

Next, we performed real-time PCR to examine the mRNA levels of the candidate genes associated with mineralization after stimulation by FGF2, FSK and FGF/FSK in rat osteoblast-like ROS17/2.8 cells. FGF2 increased expression of the genes for c-FOS (2.1-fold), AXIN2 (5.1-fold), and MYC (2.4-fold). In contrast, FGF2 suppressed expression of the mRNAs for JUNB (0.4-fold) and WNT4 (0.43-fold). FSK increased expression of the genes for c-FOS (4.37-fold), AXIN2 (4.5-fold), JUNB (1.53-fold), MYC (1.6-fold) and WNT4 (2.8-fold). While FGF/FSK induced expression of the genes for c-FOS (1.2-fold), AXIN2 (10.1-fold), and MYC (3.9-fold), it reduced that of the genes for JUNB (0.43-fold) and WNT4 (0.45-fold). When we compared the mRNA levels of c-FOS, JUNB and WNT4 in rat osteoblast-like ROS17/2.8 cells after stimulation by FGF2, FSK and FGF/FSK, c-FOS mRNA levels were increased by FGF2 (2.1 ± 0.67-fold), FSK (4.37 ± 0.87-fold), and FGF/FSK (1.2 ± 0.55-fold). JUNB gene expression was induced by FGF2 (0.4 ± 0.06-fold), FSK (1.53 ± 0.22-fold), and FGF/FSK (0.43 ± 0.19-fold). Expression of WNT4 mRNA was induced by FGF2 (0.43 ± 0.08-fold), FSK (2.8 ± 0.1-fold), and FGF/FSK (0.45 ± 0.09-fold). Therefore, FSK increased the expression of these three genes to a maximal extent (Fig. 3).

Discussion

In this study, we investigated candidate genes associated with mineralization induced by FGF2, FSK and FGF/FSK in two osteoblast-like cell lines (Tables 2 and 3). These genes were distinguished from various fields depending on cell type, such as regulators of gene expression (c-FOS, JUNB, MYC, WNT5 and HDAC4), extracellular matrix proteins (OC, OPN and DMP1), and inflammatory cytokines (IL6, IL8, IL1 and PTGES). Moreover, using real-time PCR, we focused on some inducible genes associated with mineralization induced by FGF2, FSK and FGF/FSK.

The levels of WNT5A and HDAC4 mRNA were increased by each of FGF2, FSK and FGF/FSK in human osteoblast-like Saos2 cells. While expression of the WNT5B gene was induced by FGF2, it was decreased by FSK. WNT5A and WNT5B belong to the WNT family, and it is well known that the Wnt signaling pathway also plays a pivotal role in the regulation of bone mass (22). The Wnt signaling pathway is activated during postnatal bone regenerative events, such as ectopic endochondral bone formation and fracture repair. It has been reported that FGF signaling is able to control many aspects of osteoblast differentiation through induction of SOX2 and regulation of the Wnt/β-catenin pathway (23). Ambrosetti et al. showed that ERK1/2 and Akt exert distinct effects on FGF-induced osteoblast proliferation and differentiation (24). The Wnt signaling pathway, which promotes osteoblast differentiation, also induces Akt phosphorylation. Cells expressing active Akt increase the levels of stabilized β-catenin and Wnt signaling (25). Takada et al. reported that histonelysine methyltransferase activated by non-canonical Wnt signaling suppresses PPAR-gamma transactivation, induces Runx2 expression, and promotes osteoblastogenesis (26). These results suggest a close relationship between FGF2, Wnt and osteoblastogenesis.

HDAC4 is one of the histone deacetylases (HDACs) (27), and is known to regulate osteoblast differentiation. HDAC4 or HDAC5 is required for TGF-β-mediated inhibition of Runx2 function and is involved in osteoblast differentiation (28). HDAC4 deacetylates Runx2,

allowing the protein to undergo Smurf-mediated degradation. Inhibition of HDAC4 increases Runx2 acetylation and potentiates BMP-2-stimulated osteoblast differentiation, thus increasing bone formation (29). PTH regulates HDAC4 in osteoblast-like cells through a PKA-dependent pathway, leading to removal of HDAC4 from the MMP13 gene promoter and enhancing gene transcription (30). These findings suggest that HDAC4 could be related to the transcription factors induced by FGF and FSK in osteoblasts.

The level of WNT4 mRNA was increased by FSK but not by FGF2. AXIN2 gene expression was induced by each of FGF2, FSK and FGF/FSK. Also, the level of c-MYC mRNA was increased by FGF2 and FGF/FSK in ROS17/2.8 cells. AXIN2/conductin/Axil is a negative regulator of the canonical Wnt pathway that suppresses signal transduction by promoting degradation of β-catenin. AXIN2 has a role in calvarial morphogenesis and craniosynostosis (31). Studies of the function of AXIN2 and β-catenin have demonstrated that canonical signaling modulates most aspects of osteoblast physiology including proliferation, differentiation, bone matrix formation/mineralization and apoptosis, as well as coupling to osteoclastogenesis and bone resorption.

Activator protein 1 (AP1) transcription factor is formed by the c-FOS and c-JUN heterodimer. c-FOS is a key regulator of osteoblast differentiation, whereas, c-JUN, JUNB and FRA1 are essential for embryonic and/or postnatal development (32). We have previously reported that FGF2 stimulates BSP gene transcription by targeting the FGF2 response element (FRE) and AP1/glucocorticoid response element (GRE) in the rat BSP gene promoter (33). In the present study, c-FOS expression was induced by FGF2 and FSK. DNA microarray analyses demonstrated that the level of JUNB mRNA was increased by FGF2 and FGF/FSK in ROS 17/2.8 cells (Table 3), whereas real-time PCR indicated that FGF2 and FGF/FSK were unable to induce JUNB gene expression, and that FSK induced JUNB mRNA to a maximal extent (Fig. 3). Further study to clarify the reason for this discrepancy is required. Osteoblast-specific extracellular matrix proteins such as OC and BSP are known to be markedly reduced in JUNB-deficient mice (34).

Glucocorticoid increases the expression of mRNAs for alkaline phosphatase (ALP), c-MYC, c-FOS and c-JUN in human osteoblast-like cells (35). c-MYC induces differentiation and apoptosis of human ES cells (36), whereas LIF and STAT3 regulate ES cell self-renewal and pluripotency by a MYC-dependent mechanism (37). Takahashi et al. demonstrated that the four transcription factors OCT3/4, SOX2, KLF4 and c-MYC generated induced pluripotent stem cells (iPS cells) from adult human dermal fibroblasts (38). Thus, c-MYC has a close connection with cell differentiation and proliferation, and may have a role in the differentiation and proliferation of osteoblasts upon stimulation with FGF2.

In conclusion, our present findings suggest that FGF2, FSK and FGF/FSK induce many mineralization-associated genes. Therefore, the induced genes may interact with each other to regulate bone mineralization and formation.

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