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Regulation of Notch signaling genes during BMP2-induced differentiation of osteoblast precursor cells☆

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Abstract

The bone morphogenetic protein (BMP)-induced Smad signal transduction pathway is an important positive regulator of osteoblast differentiation. BMP and other members of the transforming growth factor-β (TGF-β) family have distinct effects on osteoblast differentiation, depending on cell type and cell differentiation status. In C2C12 mesenchymal cells, BMP-induced osteoblast differentiation can be blocked by TGF-β. In a search for key regulators of osteoblast differentiation we have used microarray analysis to identify genes which are differentially regulated by BMP2 and TGF-β. Within the first 24 h following the onset of differentiation, 61 BMP2-regulated genes were identified of which the BMP2 effect was counteracted by TGF-β. The majority of these differentially expressed transcripts are related to signal transduction. Notably, our data show that three Notch signal transduction pathway genes, *Lfng*, *Hey1*, and *Hes1*, are differentially regulated by BMP2 and TGF-β. This suggests that these genes might function as the focal point for interaction of Smad and Notch signaling during osteoblast differentiation.

Keywords: Osteoblast; BMP2; Differentiation; Notch; Microarrays; Transcription factors; Signal transduction; TGF-β

Bone morphogenetic proteins (BMPs) are the most potent of all inducers of osteoblast differentiation. Although the BMPs were first identified for their ability to induce ectopic bone formation in vivo [1], these proteins appear to be multifunctional regulators of morphogenesis during embryonic development [2]. Transforming growth factor-β (TGF-β), a protein closely related to the BMPs, is also able to regulate osteoblast differentiation. However, regulation by TGF-β is more complex, since it can both inhibit and stimulate osteoblast differentiation depending on the cell type and its differentiation status [3–5].

To generate biological responses, TGF-β family members use heteromeric complexes of so-called type I and type II serine/threonine kinase receptors, resulting in the activation of specific receptor-regulated Smads (R-Smads). These include Smad-2 and -3, which are specific for TGF-β signaling, while Smad-1, -5, and -8 have been shown to be BMP responsive R-Smads. In combination with the common mediator Smad-4, the respective R-Smads are assembled into heteromeric complexes, which subsequently translocate to the nucleus, where they act in concert with other proteins as transcription factor complexes [6–8].

In addition to the Smad-mediated BMP pathway, several other major signaling pathways are known to play a role during osteoblast differentiation. These include the Wnt/β-catenin pathway [9–12], the Notch pathway [13,14], and the different MAP-kinase pathways [15–19]. However, the interplay between these various pathways in osteoblast differentiation is still poorly understood.

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In the C2C12 mouse mesenchymal progenitor cell line, osteoblastic differentiation can be induced by BMPs, while TGF-β1 is not able to do so, and even inhibits BMP2-induced differentiation [20]. BMP2 and TGF-β partially share the same Smad signal transduction route and have many target genes in common. Given the differential effect of TGF-β and BMP2 on C2C12 differentiation, genes that are differentially affected by the two cytokines might function as important regulators of osteoblast differentiation. Previously, we have shown that the commitment to the osteoblast phenotype is determined within the first 24 h following stimulation with BMP2 [21]. In this study, we have compared BMP2-induced gene expression levels with those of TGF-β and a combination of these two growth factors in a 24 h time frame. Notably, several members of the Notch signaling pathway were differentially affected by BMP2 and TGF-β, suggesting that BMP-induced modulation of Notch activity may be a key element in regulating osteoblast differentiation.

Materials and methods

Tissue culture and growth factors. The C2C12 murine mesenchymal progenitor cell line (obtained from the American Type Culture Collection) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS), penicillin (100 U/ml), and streptomycin (100 μg/ml) as described previously [21]. For the various experiments, cells were plated at a density of 2.0×10^4 cells/cm^2 and grown for 24 h in DMEM supplemented with 10% NCS. Subsequently, medium was replaced by DMEM containing 5% NCS (control medium) either in the presence or in the absence of 300 ng/ml recombinant human BMP2 with or without 5 ng/ml human recombinant TGF-β1. Recombinant human BMP2 was kindly provided by Wyeth (Andover, MA) and recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN).

RNA isolation. Total RNA was extracted according to an acid guanidium thiocyanate–phenol–chloroform method (TriPure Isolation Reagent, Roche Diagnostics, Germany) and RNA concentrations were determined by measuring the absorbance at 260 nm. Poly(A)$^+$ RNA was prepared from total RNA using the Oligotex method (Qiagen, Valencia, CA). Quantification of poly(A)$^+$ RNA was carried out according to the Ribogreen RNA quantitation assay (Molecular Probes, Eugene, OR).

Gene microarray analysis. Microarray analysis was performed as described previously [22]. For the hybridization experiments, RNA from growth factor-treated cells was hybridized versus RNA from control cells using the mouse Unigene 1 array from Incyte Genomics (Palo Alto, CA). The hybridization experiments were performed in duplicate with a fluor reversal to minimize possible bias caused by the molecular structure of the Cy3 and Cy5 dyes. Background-subtracted element signals were used to calculate Cy3/Cy5 ratios. Genes were selected for further analysis if the signal to background ratio for a particular gene was >2.5 and at least 40% of the area on chip could be used for signal computation in at least one of the two channels. To account for systematic errors caused by non-linear differences in signal intensities of the Cy3 or Cy5 channel, statistical analysis was performed as described previously [22]. Genes with significant ($p < 0.05$, >1.5-fold) differential expression in at least one of the treatments were selected for further analysis. Subsequently, the genes were subdivided into 9 categories according to their differential regulation by BMP2 (B), BMP2 plus TGF-β1 (BT), and TGF-β1 (T) as follows: category 1, B > BT > T; category 2, B < BT < T; category 3, B > BT = T; category 4, B < BT = T; category 5, B > BT < T; category 6, B < BT > T; category 7, B = BT < T; category 8, B = BT > T; and category 9, B = BT = T; with the assumption that B = BT or BT = T when the absolute difference between induction levels was less than 0.1. Protein functions of the genes were identified according to the GeneOntology Database.

Real-time RT-PCR. For real-time RT-PCR, the ABI Prism Sequence Detection System 5700 and Primer Express software (PE Biosystems) were used. For each gene, a set of primers was designed using sequences obtained from GenBank. All primers generated amplicons of 50–100 bp with melting temperatures between 58 and 60 °C. Prior to complementary DNA (cDNA) synthesis, 2 μg of total RNA was DNase (Invitrogen) treated for 15 min at 37 °C, after which RNA was reverse transcribed using random hexamer primers (Amersham–Pharmacia) in a total volume of 50 μl using the SUPERSCRIPT II reverse transcriptase kit (Invitrogen). Subsequently, aliquots of first strand cDNA (2 μl) were amplified using SYBR Green PCR Mastermix (Applied Biosystems) under the following conditions: initial denaturation for 10 min at 94 °C followed by 40 cycles consisting of 15 s at 94 °C and 1 min at 60 °C. Fold inductions and expression ratios were calculated from differences in threshold cycles at which an increase in reporter fluorescence above a baseline signal could first be detected (C_T) between two samples, and averaged for duplicate experiments.

Results

Expression profiles of genes differentially regulated by BMP2 and TGF-β

Upon BMP2 treatment, C2C12 mesenchymal progenitor cells differentiate into cuboidal-shaped osteoblastic cells, whereas they form myotubes in response to low serum conditions without BMP2 (Fig. 1). TGF-β1 is unable to induce differentiation of C2C12 cells, but it inhibits BMP2-induced osteoblast differentiation, as well as myotube formation. This is reflected by morphology (Fig. 1A) as well as by expression regulation of marker genes. Whereas BMP2 induces the expression of the osteoblast markers *alkaline phosphatase (ALP)* and *osteocalcin (OCN)*, TGF-β inhibits this BMP2-induced expression (Fig. 1B). Our previous experiments have indicated that C2C12 mesenchymal progenitor cells already become committed to osteoblast differentiation within 1 day after BMP treatment, as evidenced by the up-regulation of the osteoblast-specific transcription factor *core-binding factor* α1 *(Cbfa1)* within this time frame [21]. In this study, we therefore focused on genes which are regulated within the first 24 h of growth factor treatment to identify potential key regulators of osteoblast differentiation.

Microarray analysis of C2C12 cells 24 h after treatment with either BMP2, TGF-β or a combination of these factors showed 625 sequences out of the 9330 spotted on the array to exhibit significant differential expression for at least one of the conditions tested, as based on our previously described statistical analysis [22]. Subsequently, the genes were subdivided into 9

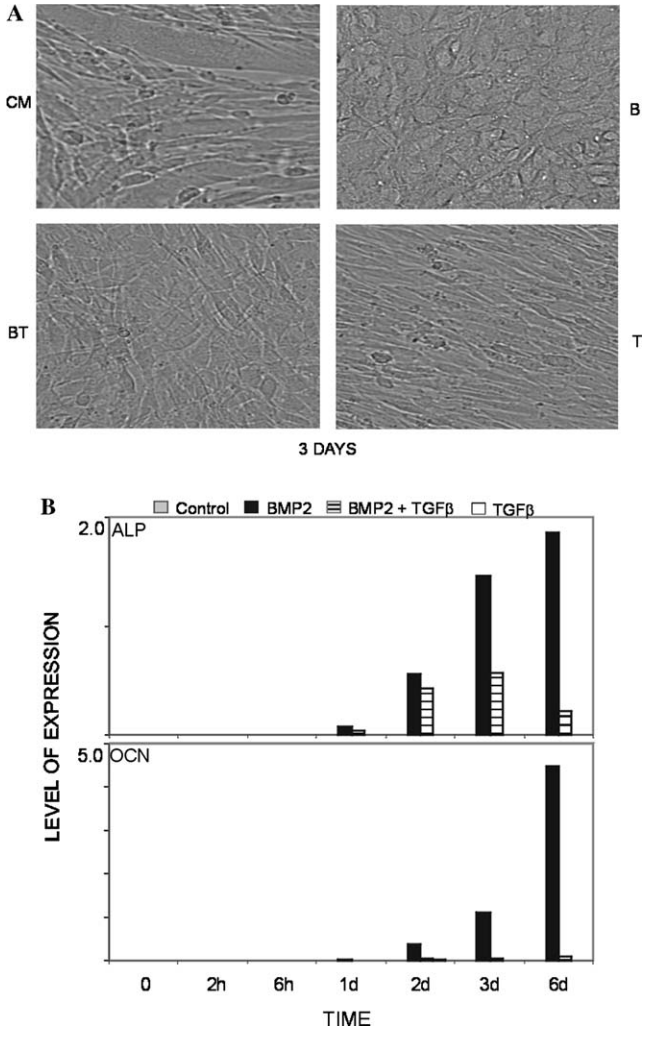

Fig. 1. Differential activities of BMP2 and TGF-β on C2C12 cells. (A) Differential morphological features of C2C12 cells treated with BMP2, TGF-β, and combinations of these growth factors. Cells were seeded and grown for 24 h upon which the medium was replaced by control medium (CM: DMEM containing 5% NCS), control medium + 300 ng/ml BMP2 (B), control medium + 5 ng/ml TGF-β (T), and control medium + 300 ng/ml BMP2 and 5 ng/ml TGF-β (BT). The figure shows micrographs taken 3 days after addition of growth factors. (B) Differential regulation of expression of the osteoblast marker genes alkaline phosphatase (ALP) and osteocalcin (OCN) by BMP2 and TGF-β. Cells were seeded and grown for 24 h upon which the medium was replaced by DMEM containing 5% NCS in the presence or absence of BMP2 (300 ng/ml) with or without TGF-β (5 ng/ml). RNA was isolated 0, 2, and 6 h, 1 day, 2, 3, and 6 days after stimulation. Expression levels were measured by real-time quantitative RT-PCR. Mean expression levels were corrected for GAPDH levels.

categories according to their expression pattern by BMP2 (B), BMP2 plus TGF-β (BT), and TGF-β (T) as described in Materials and methods. Because TGF-β inhibits BMP2-induced differentiation, genes with an expression pattern reflecting categories 1–4 ($B > BT > T$, $B < BT < T$, $B > BT = T$, and $B < BT = T$) most likely include regulators of osteoblast differentiation. Table 1 lists the 61 genes and ESTs that are differentially regulated by BMP2 and TGF-β to at least the level (40% difference) measured for *ALP* (Fig. 1B). When classified into functional protein groups, the majority of the genes in our dataset fell in the groups "signal transduction" or "expressed sequence tags (ESTs)" (26% and 23%, respectively). Furthermore, nearly 20% of the genes encoded "structural proteins" with functions in cell adhesion, cytoskeleton or extracellular matrix and another 20% of the genes encoded "enzymes." The remaining 11% of the genes could not be classified in one of the above-mentioned groups (other).

Expression of Notch pathway genes during osteoblast differentiation

We subsequently focused on signal transduction genes that had similar expression profiles as *ALP*, i.e., the signal transduction genes present in category 1 ($B > BT > T$). As can be seen from Table 1, these genes include dihydropyrimidinase-like 3 (Dpysl-3), paired related homeobox 2 (Prrx2), MAD homolog 6 (Madh6), hairy/enhancer of split with YRPW motif 1 (Hey1), lunatic fringe (Lfng), and calcium/calmodulin dependent kinase II delta (Camk2d). To validate the expression profiles measured in this microarray experiment, the expression of these 6 genes was tested by quantitative real time RT-PCR both in original RNA used for the microarray and in RNA obtained from an identical but independent experiment. Fig. 2 shows that the expression profiles for 5 out of 6 genes were confirmed. Only for *Camk2d* the expression regulation for the various growth factor combinations was moderate and statistically not significant.

Of the 61 genes and ESTs identified in our study, two belong to the Notch signal transduction pathway: the transcription factor *Hey1* and the signal modulator *Lfng*. Previously, we have identified another transcription factor of this signal transduction pathway, *hairy/enhancer of split 1 (Hes1)*, to be differentially regulated between BMP2 and TGF-β [22]. Because this gene was not present on our current microarray, we validated the differential effect of BMP2 and TGF-β on expression of *Hes1* by quantitative real time RT-PCR in both sets of RNA. As can be seen from Fig. 2, *Hes1* is down-regulated by BMP2 and up-regulated by TGF-β. In addition, TGF-β inhibited the BMP2-induced down-regulation and caused an up-regulation of gene expression similar to that induced by TGF-β alone. The combined results indicate that at least three genes of the Notch signal transduction pathway are differentially modulated by BMP2 and TGF-β in C2C12 cells, which suggests that this pathway plays an important role in regulating the early phase of osteoblast differentiation.

Table 1
Expression profiles of the genes differentially regulated by BMP2 and TGF-β

Expression pattern	Accession No.	Abbreviation	Description	Functional group	Expression levels		
					B	BT	T
B > BT > T	AI386062	Car3	Carbonic anhydrase 3	Enz	3.34	1.21	−1.91
	AA062324	Dpysl–3	Dihydropyrimidinase-like 3	ST	2.90	2.13	1.33
	AI157520	Omd	Osteomodulin	CAd	2.54	1.58	1.00
	W97877	Prrx2	Paired related homeobox 2	ST	2.34	1.72	−1.20
	AA028346	Krt1-19	Keratin complex 1, acidic, gene 19	Cy	2.33	1.78	−1.21
	AI427265	Madh6	MAD homolog 6	ST	2.33	1.56	1.13
	AA821963	Dnpep	Aspartyl aminopeptidase	Enz	2.14	1.50	1.00
	AI552130	Hey1	Hairy/enhancer-of-split related with YRPW motif 1	ST	2.11	1.21	−1.10
	AA197529	Lfng	Lunatic fringe	ST	2.06	1.56	1.00
	AA981032	Emilin2	Elastin microfibril interfacer 2	EM	2.05	1.51	1.18
	AA450795	Cck	Cholecystokinin	ST	2.01	1.89	−1.07
	AA596753	Lxn	Latexin	Enz	1.90	1.23	1.00
	AA545032	Ppap2b	Phosphatidic acid phosphatase 2a	Enz	1.77	1.21	−1.30
	AI323002	Nsg1	Neuron specific family member 1	Other	1.76	1.00	−1.15
	AA242413	Asgr1	Asialoglycoprotein receptor 1	ST	1.73	1.19	1.00
	AI325697	Ly6a	Lymphocyte antigen 6 complex	Other	1.69	−1.13	−1.54
	AI606539		EST		1.69	1.21	1.00
	AA415211	Musk	Muscle, skeletal, receptor tyrosine kinase	ST	1.65	1.29	−1.58
	AA606242	Ecm1	Extracellular matrix protein 1	EM	1.59	1.34	−1.05
	AA517588	ALP	Alkaline phosphatase 2, liver	Enz	1.53	1.31	1.00
	W16221	Col6a1	Procollagen, type VI, α1	EM	1.30	−1.61	−1.94
	W18632		EST		1.24	−1.52	−1.86
	AA450440	Ddef1	Development and differentiation enhancing	Enz	1.00	−1.16	−1.79
	AI552157		EST		1.00	−1.25	−1.58
	AA462389	Camk2d	Calcium/calmodulin-dependent protein kinase II delta	ST	1.00	−1.40	−1.62
	AA067331		EST		−1.02	−1.34	−1.62
B < BT < T	AA261149	Col18a1	Procollagen, type XVIII, α1	EM	−1.20	1.24	1.75
	AA437677	Col18a1	Procollagen, type XVIII, α1	EM	−1.28	1.27	1.84
	W09641	Tmsb4x	Prothymosin β 4	Cy	−1.29	1.00	1.76
	AA619789	Mthfd2	Methylenetetrahydrofolate dehydrogenase	Enz	−1.62	−1.11	1.00
	AA536716	Map4k4	Mitogen activated protein kinase kinase kinase kinase 4	ST	−1.62	−1.33	−1.15
	AW209351		EST		−1.62	−1.19	1.00
	AA815681	Pdlim1	PDZ and LIM domain 1/elfin	Cy	−1.69	−1.16	1.24
	W12942	Tnc	Tenascin C	Cy	−1.69	1.27	2.38
	AA624579		EST		−1.75	−1.35	−1.07
	AA106557	Dgkd	Diacylglycerol kinase d	ST	−1.90	−1.44	1.00
	W16059	Gsto1	Glutathione *S*-transferase like	Enz	−1.94	−1.39	1.59
	AA596866		EST		−1.98	−1.38	1.00
	AA117547	Ccnd1	Cyclin D1	Other	−1.98	−1.56	1.00
	AI390830		EST		−2.02	−1.27	1.00
	AA413490	Trfr	Transferrin receptor	ST	−2.07	−1.33	1.00
	AA066390		EST, similar to sarcalumenin	Other	−2.11	−1.60	−1.24
	W99951	Nefl	Neurofilament, light polypeptide	Cy	−3.37	−1.91	−1.34
	AI325851	Cd97	CD97 antigen	CAd	−3.37	−2.25	−1.40
B > BT = T	AA067147	Mod1	Malic enzyme, supernatant	Enz	1.72	1.35	1.27
	AA590599		EST		1.52	1.30	1.31
	AA879919	F3	Coagulation factor III	ST	1.22	−1.52	−1.58
	AA518187	Ogn	Osteoglycin	ST	1.00	−1.62	−1.61
	AA414208	Sdfr1	Stromal cell derived factor receptor 1	ST	−1.18	−1.50	−1.49
	AA755873	Mgrn1	Mahogunin, ring finger 1	Other	−1.19	−1.43	−1.52
	W17626	Snap25bp	Synaptosomal-associated protein 25 binding protein	Other	−1.24	−1.66	−1.66
	AI425448	Socs5	Suppressor of cytokine signaling 5	ST	−1.28	−1.56	−1.56

Table 1 (*continued*)

Expression pattern	Accession No.	Abbreviation	Description	Functional group	Expression levels		
					B	BT	T
	AA106098	Cfh	Complement component factor H	Other	−1.30	−1.60	−1.51
	AA413899	Sat1	Spermidine/spermine N1-acetyl transferase	Enz	−1.30	−1.61	−1.56
B < BT = T	AA689893		EST		1.27	1.58	1.55
	AA473948		EST		−1.56	−1.31	−1.33
	AA638889	Rabggtb	RAB geranylgeranyl transferase, b subunit	Enz	−1.56	−1.33	−1.25
	AA794206		EST		−1.66	−1.15	−1.07
	AA684321		EST, similar to protein phosphatase	Enz	−1.69	1.00	1.00
	AA545397		EST		−1.72	−1.42	−1.35
	AI877091	Xist	Inactive X-specific transcripts	Other	−1.77	−1.26	−1.21

Genes significantly ($P < 0.05$, >1.5-fold) regulated for either BMP2 (B, 300 ng/ml), BMP2 + TGF-β (BT, 300 + 5 ng/ml, respectively) or TGF-β (T, 5 ng/ml) are shown for the expression pattern categories 1–4 (B > BT > T, B < BT < T, B > BT = T, and B < BT = T). Functional protein groups were assigned using the GeneOntology Database. CAd, cell adhesion; Cy, cytoskeletal component; EM, extracellular matrix component; Enz, enzyme; EST, expressed sequence tag; and ST, signal transduction.

Discussion

Modulation of the activity of signal transduction pathways during the initial phase of differentiation guides mesenchymal stem cells to become osteoblasts, and activating and repressing transcription factors are very important in directing the differentiation process. In our data set, we observed that "signal transduction" genes constituted over a quarter of the total set of genes regulated 24 h after growth factor treatment. Previously, we have shown that commitment of the C2C12 cells to the osteoblastic lineage occurs around 24 h after BMP treatment, when the osteoblast specific transcription factor *Cbfa1* and the novel osteoblast related genes *Tcf7* and *Hey1* become regulated [21]. Genes with expression patterns that associate with the differential effects of BMP2 and TGF-β on osteoblast differentiation in C2C12 cells might be mediators of osteoblast differentiation. Therefore, in search of novel genes implied in the commitment of mesenchymal progenitor cells to osteoblast differentiation, we have analyzed the effects of BMP2 and TGF-β on gene expression at 24 h post-stimulation.

Our study identified 5 signal transduction related genes that exhibited similar expression profiles as the osteoblast marker *ALP* after 24 h of stimulation. Of these, 4 genes have previously been reported to be involved in osteoblast differentiation or function. Prrx2 cooperates with Prrx1 in regulating the skeletogenesis of the craniofacial region, inner ear, and limbs, as was shown in knock-out experiments [23]. Madh6 is a negative regulator of the BMP signal transduction pathway capable of repressing BMP-induced osteoblast differentiation [24,25]. The function of Hey1 and Hes1 in osteoblast differentiation will be discussed below. The observation that four out of the five signal transduction genes identified here have been implicated before in osteoblast differentiation indicates that screening for genes differentially affected by BMP2 and TGF-β is a valid strategy for the identification of novel regulators of osteoblast differentiation. The dihydropyrimidinase-like protein 3 (Dpysl-3) has not previously been implicated in osteoblast differentiation. Dpysl-3 is a member of a family of dihydropyrimidinase related proteins which have been shown in humans to be intracellular components of a signaling cascade involved in the development of the nervous system [26,27]. Furthermore, studies in which we compared the expression regulation of *Dpysl-3* in osteoblastic cells and in non-osteoblastic cells showed that *Dpysl-3* is specifically regulated in osteoblastic cells ([21] and data not shown). These results indicate that Dpysl-3 might also be a regulator of osteoblast differentiation. We investigated whether additional osteoblast-related transcription factors might be identified among the 4 ESTs identified in this category. However, in silico sequence analysis did not reveal specific DNA binding domains or characteristic transcription activating domains, suggesting that these ESTs are not novel transcription factors (results not shown).

In this study, we identified 3 members of the Notch signaling pathway, *Lfng*, *Hey1*, and *Hes1*, to be differentially regulated by BMP2 and TGF-β. Hey1 belongs to the hairy and enhancer of split (HES) family of transcription factors and is a primary target of Notch [28]. Previously, we have shown that *Hey1* is expressed in bone during mouse embryonic development with an expression pattern that partially overlaps with that of *Cbfa1* [21]. Hey1 functions as an inhibitor of myoblast differentiation in C2C12 cells [29]. This finding is consistent with our data that show that Hey1 expression is induced when transdifferentiation of C2C12 cells to the osteoblast lineage is induced with BMP2. Hey1 is able to

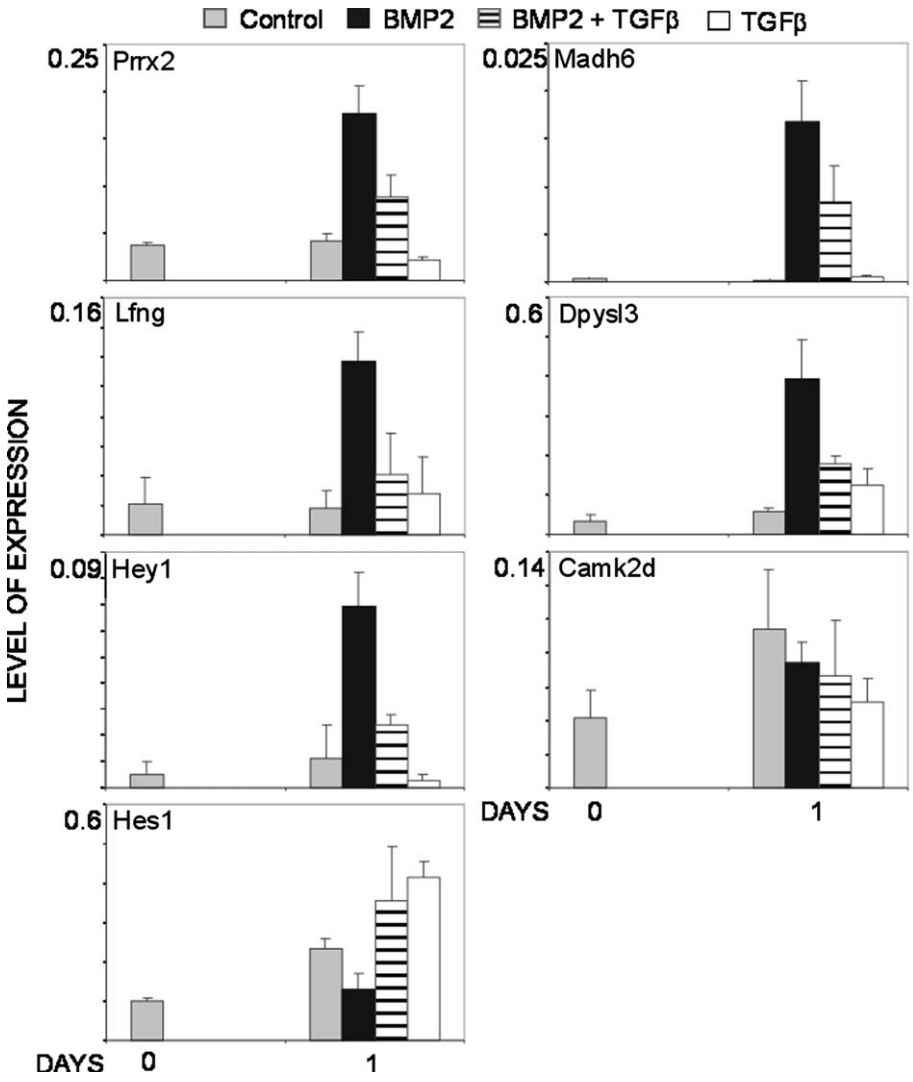

Fig. 2. BMP2 and TGF-β differentially regulate expression of signal transduction related genes. Cells were seeded and grown for 24 h upon which the medium was replaced by DMEM containing 5% NCS in the presence or absence of BMP2 (300 ng/ml) with or without TGF-β (5 ng/ml). RNA was isolated from untreated cells (day 0) and 1 day after stimulation. Expression levels were measured by real-time quantitative RT-PCR. Mean expression levels were corrected for GAPDH levels. Error bars indicate standard deviations between duplicate experiments Camk2d, calcium/calmodulin-dependent kinase II delta; Dpysl–3, dihydropyrimidinase-like 3; Hey1, hairy/enhancer of split with YRPW motif 1; Hes1, hairy/enhancer of split 1; Lfng, lunatic fringe; Madh6, MAD homolog 6; and Prrx2, paired related homeobox 2.

associate with its family member Hes1 and form stable heterodimers with Hes1 upon DNA binding [28]. The role of Hes1 in osteoblast differentiation is unclear as it has been reported to be both an inhibitor and activator of osteoblast differentiation [30,31]. Our finding that Hes1 is downregulated by the osteogenic factor BMP2, whereas it is induced by the inhibitory factor TGF-β, would be consistent with an inhibitory role of Hes1 in osteoblast differentiation.

In addition to the effect on the Notch target genes Hes1 and Hey1, we observed a differential effect of BMP2 and TGF-β on expression of *Lfng*. This protein is a modulator of Notch signaling because of its *O*-fucose-specific glycosyltransferase activity on both the Notch receptors and their ligands. However, no direct evidence has been obtained so far for a functionally relevant modification of the Notch ligands, while fucosylation of the Notch receptors occurs in a cell autonomous fashion [32–35]. O-fucosylation is an essential step in Notch signaling, since loss of the protein that transfers fucose to the EGF-like repeats of Notch (O-FucT-1), causes embryonic lethality of mice at midgestation with severe defects in somitogenesis, vasculogenesis, cardiogenesis, and neurogenesis. Inactivation of *Lfng* in mice causes fusion of somites, skeletal defects, and perinatal death [36,37]. It has been suggested that Lfng changes the sugar structures on Notch and thereby causes alterations in binding of Jagged and Delta. However, the reports on the effect of Lfng on Notch signaling in osteoblast differentiation as well as the effect of the

different ligands on Notch signaling in osteoblast differentiation are contradictory [13,14,33,38,39]. Strikingly, in many cases during embryonic development, it is the distribution of fringe proteins, rather than of the Notch receptors or their ligands, that is the key determinant of where Notch signaling will be active and in which form [40]. In our study, we have shown that *Lfng* and *Hey1* are regulated in a very similar manner by BMP2 and TGF-β, and that expression regulation of *Hes1* is negatively correlated with those of *Lfng* and *Hey1*. Expression of *Lfng* was shown to be initiated already 2 h after BMP2 treatment, while *Hey1* expression was induced 24 h after BMP treatment [21]. These results suggest that expression of *Lfng* causes a switch in the Notch signaling pathway such that *Hey1* becomes up-regulated and *Hes1* down-regulated.

In conclusion, we have shown that several members of the Notch signal transduction pathway are differentially regulated by BMP2 and TGF-β in C2C12 mouse mesenchymal progenitor cells. We hypothesize that Lfng might function as one of the the focal points for interaction of the Smad and Notch signal transduction pathways during osteoblast differentiation: *Lfng* expression is induced by BMP2 and subsequently the altered distribution of this gene might induce changes in the activity and form of Notch signaling, which in turn could modulate BMP-induced osteoblast differentiation. Further elucidation of the function of these members of the Notch signaling pathway in osteoblast differentiation may contribute to the identification of new therapeutics for the treatment of bone diseases such as osteoporosis.

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