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Differentiation Potential of a Mouse Bone Marrow Stromal Cell Line

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Abstract In order to study osteoblast differentiation we subcloned a cell derived from a mouse a bone marrow stromal cell line, Kusa O, and obtained a number of clones representative of three different phenotypes. One that neither differentiated into osteoblasts nor into adipocytes, a second that differentiated into osteoblasts but not adipocytes, and a third that differentiated into both osteoblasts and adipocytes. Four subclones were selected for further characterization according to their ability to mineralize and/or differentiate into adipocytes. The non-mineralizing clone had no detectable alkaline phosphatase activity although some alkaline phosphatase mRNA was detected after 21 days in osteoblast differentiating medium. Alkaline phosphatase activity and mRNA in the three mineralizing clones were comparable with the parent clones. Osteocalcin mRNA and protein levels in the non-mineralizing clone were low and non-detectable, respectively, while both were elevated in the parent cells and mineralizing subclones after 21 days in differentiating medium. PTH receptor mRNA and activity increased in the four subclones and parent cells with differentiation. mRNA for two other osteoblast phenotypic markers, osteopontin and bone sialoprotein, were similarly expressed in the parent cells and subclones while mRNAs for the transcription factors, Runx2 and osterix, were detectable in both parent and subclone cells. Runx2 was unchanged with differentiation while osterix was increased. Interestingly, PPARγ mRNA expression did not correlate with cell line potential to differentiate into adipocytes. Indian hedgehog mRNA and its receptor (patched) mRNA levels both increased with differentiation while mRNA levels of the Wnt pathway components β-catenin and dickkopf also increased with differentiation. Although we have focussed on characterizing these clones from the osteoblast perspective it is clear that they may be useful for studying both osteoblast and adipocyte differentiation as well as their transdifferentiation. J. Cell. Biochem. 90: 158–169, 2003.

Key words: stromal cells; osteoblasts; adipocytes; differentiation; plasticity

Multipotential stromal stem cells from the bone marrow can differentiate into a number of cell types including osteoblasts, adipocytes, reticulocytes, and fibroblasts [Owen, 1985]; the progenitors of which have the ability to transdifferentiate [Beresford et al., 1992; Oreffo et al., 1997]. Osteoblast differentiation of these cultures is dependent upon the addition of ascorbate, which stimulates the synthesis of collagen followed by induction of osteoblastic genes [Franceschi et al., 1994]. Commitment to and interconversion of stromal cells among the several phenotypes is likely to involve specific genes that may be required to induce or suppress a particular phenotype.

It is now well documented that the transcription factor, Runx2, is required for commitment to the osteoblast phenotype [Ducy et al., 1997] and that in Runx2 null mice osteoblast differentiation is arrested in both the endochondrial and intramembranous skeleton [Komori et al., 1997; Otto et al., 1997]. Runx2 is known to

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modulate the transcription of several genes involved in the mineralization process. The bone sialoprotein (BSP) promoter has a number of functional Runx DNA binding sites and Runx2 mediates repression of the promoter [Javed et al., 2001]. In contrast, Runx2 enhances transcription of both type1 collagen genes [Kern et al., 2001], collagenase 3 [Jiménez et al., 1999], osteoprotegerin [Thirunavukkarasu et al., 2000], osteopontin [Sato et al., 1998], and osteocalcin [Gutierrez et al., 2002]. Furthermore, Runx2 appears to interact synergistically with C/ERPβ, enhancing transcription of osteocalcin 30–40-fold in HeLa cells co-expressing Runx2 and C/ERPβ compared with 2–4-fold for each protein alone [Gutierrez et al., 2002]. Another transcription factor essential for osteoblast differentiation is osterix. This factor appears to be required at a later stage of differentiation than Runx2 since preosteoblasts of osterix null mice express Runx2 at comparable levels to wild type osteoblasts while no expression of osterix was apparent in Runx2 mice [Nakashima et al., 2002]. Other transcription factors are involved in commitment of multipotential stromal cells to the adipocytic pathway, of these PPARγ has been reported to have divergent effects on adipocyte and osteoblast differentiation by playing a role in the function of many adipocyte specific genes, such as αP2 and PEPCK, as well as suppression of Runx2 [Lecka-Czernik et al., 1999] and synthesis of α1(1)-procollagen, osteopontin, alkaline phosphatase, and osteocalcin [Lecka-Czernik et al., 2002].

Alkaline phosphatase, osteopontin, BSP, PTH receptor-1 (PTHR1), and osteocalcin are all proteins whose significance in osteoblast differentiation has been studied extensively [Bellows et al., 1991]. Although variation occurs among species and cell types, osteopontin, BSP, PTHR1, and alkaline phosphatase are generally associated with early osteoblasts while osteocalcin is an indicator of the mature osteoblast [Aubin, 1998] and is implicated in the inhibition of the mineralization process [Ducy et al., 1996]. In the last few years, new insights into osteoblast differentiation have been provided by the discovery of several new transcription factors and signaling pathways. The present work was undertaken to identify cells in which osteoblast differentiation could be studied in vitro, with the aim of putting the new control pathways into context with what was known previously.

Kusa O cells are a subclone of Kusa cells that were cloned from mouse bone marrow stromal cells and which differentiate into osteoblasts, adipocytes, and myoblasts [Umezawa et al., 1992]. The Kusa O cells demonstrated plasticity and were found to support osteoclastogenesis [Horwood et al., 1998]. When incubated in the presence of ascorbate and β-glycerophosphate, a subpopulation developed an osteoblastic phenotype and formed mineralized nodules while another developed an adipocytic phenotype. We therefore subcloned the Kusa O cells with the view to obtaining genetically related clones with different phenotypes.

MATERIALS AND METHODS

Cell Culture

Kusa O cells were derived from a multipotential bone marrow stromal cell line [Umezawa et al., 1992] and were maintained in α-modified Eagle's minimal medium (αMEM) plus 10% FCS. Subclones were obtained by limiting dilution and frozen at passage 3. Cells were used between passages 5 and 20 and maintained in the same medium as the parent Kusa O cells. The characteristics of the clones appeared stable up to 25 passages, thereafter the mineralizing clones lost their capacity to mineralize.

Bone Nodule Assay

Cells were subcultured at a density of 3,000 cells/cm^2 in αMEM plus 10% FCS for 24 h before changing to an osteoblast differentiating medium of αMEM plus 15% heat-inactivated FCS (HIFCS) together with ascorbate and β-glycerophosphate (50 μg/ml and 10 mM, respectively). Cells were maintained in this medium for the times indicated and the medium was replaced three times a week. At the end of the incubation, the cells were washed three times in PBS, fixed in ice cold 70% ETOH for 1 h, and stained with 0.5% alizarin red pH 4.2 for 30 min. Cells were washed three times with deionized water and twice with PBS for 10 min. Alizarin red was eluted in 10% cetylpyridinium chloride in PBS and measured spectrophotometrically as described by Stanford et al. [1995].

Oil Red-O Lipid Staining

Cells were cultured in an adipocyte differentiating medium (6.6×10^{-8} M insulin, 2.5×10^{-10} M 3-isobutyl-1-methylxanthine,

and 10^{-8} M dexamethasone) with medium changes three times a week. Cultures were washed and fixed as for the nodule assay before staining for lipid by the Oil Red-O method [Kuri-Haruch and Green, 1978].

Alkaline Phosphatase Assay

Cells were washed three times with PBS, scraped in ice cold 10 mM Tris HCl, pH 7.4, sonicated for 10 s on ice, and stored at −20°C until assayed. Extracts were assayed for alkaline phosphatase activity at 37°C for 30 min using *p*-nitrophenyl-phosphate as substrate [Partridge et al., 1981]. Aliquots of the cell extracts were assayed for protein using a BCA protein assay kit (Pierce, Milwaukee, WI).

PTH/PTHrP Receptor

Cells were subcultured and incubated as for the nodule assay for 7 or 21 days with the exception that no β-glycerophosphate was added to the medium. At the end of the incubation, cells were treated with 10 nM PTH (1–34) in the presence of isobutylmethylxanthine and cyclic AMP formation assayed as described by Houssami et al. [1994].

PTHrP and Osteocalcin Protein

Cells were subcultured at the same density as for the nodule assay. After 24 h, the medium was replaced with αMEM plus 15% HIFCS and 50 μg/ml ascorbate, and cells were incubated for 7 or 21 days. Three days prior to sample collection, the medium was replaced with αMEM plus 2% FCS together with ascorbate (50 μg/ml). At the end of the incubation time, the medium was aspirated, centrifugued briefly to remove any cell debris, and supernatants stored at −20°C until assayed for PTHrP by radioimmunoassay [Grill et al., 1991] or osteocalcin using an ELISA for rat osteocalcin (Osteometer BioTech A/S, Herlev, Denmark). The cells were washed, extracts prepared as for alkaline phosphatase measurements and assayed for protein using a BCA protein assay kit (as described above).

Gene Expression Analysis by Real-Time PCR

Cells were subcultured at the same density as for the nodule assay. After 24 h, the medium was aspirated and replaced with αMEM plus 15% HIFCS for the zero time cultures while ascorbate (50 μg/ml) was added to cultures which were examined after growth for 7 and 21 days. Zero time samples were lysed when cultures were just confluent while the 7 and 21 day cultures were lysed 7 and 21 days after the addition of ascorbate. Total RNA was prepared according to the method of Chomczynski and Sacchi [1987] and treated with DNase to remove any contaminating DNA. RNA was reversed transcribed using random hexamers and AMV reverse transcriptase. Authenticity of product was assessed for each primer pair according to size and by hybridizing the PCR product with an internal oligonucleotide probe. Aliquots of the RT mix were diluted so that they fell within the linear portion of the standard curve generated from dilutions of cDNA. All PCR reactions were performed in duplicate and the mean cycle threshold values were used to calculate gene expression with normalization to 18s. Results are represenative of three independent experiments. Amplification was carried out using AmpliTaq Gold (Perkin-Elmer) with SYBR Green (Molecular Probes) as probe and specific oligonucleotide primers (Table I). Cycling conditions were 95°C for 15 s, 60°C for 60 s for 40 cycles in a GeneAmp 5700 Detection System (Perkin-Elmer Applied Biosystems, Inc.).

RESULTS

Mineralization of Subclones

Of 10 clones that were obtained by subcloning the Kusa O cells, 4 that were representative of clones that had different osteoblastic and adipocytic potential were selected for the study. Figure 1 shows mineralization and lipid formation in these clones together with the parent Kusa O cells. The Kusa O cells could form mineralized nodules and adipocytes, Kusa4d10 failed to form mineralized nodules or adipocytes, Kusa4b10 formed mineralized nodules but failed to differentiate into adipocytes in osteoblast differentiating medium although small numbers were apparent in cultures incubated in adipocyte differentiating medium as shown in the Figure 1. The remaining two clones, Kusa1c11 and Kusa2g11, like the parent Kusa O cells, formed large numbers of mineralized nodules and differentiated into adipocytes in both osteoblast differentiating medium and adipocyte differentiating medium.

Comparison of the osteoblast phenotype of the four subclones with the parent Kusa O cells is illustrated in Figure 2. Mineralization first became apparent around day 15 and increased

TABLE I. Primers Used in Real Time PCR Analysis of Mouse Reverse Transcribed RNA

	Sequence	
Gene	5′f	5′r
Alkaline phosphatase	aaa ccc aga aca caa gca ttc c	tcc acc agc aag aag aag cc
BSP	cga tca gaa aaa gca gca cc	gta gcc ttc ata gcc atg cc
β-Catenin	agc tgg cct ggt ttg ata c	aaa acc att ccc acc cta c
Dickkopf	gac cac agc cat ttt cct c	tgt ctt gca caa cac agc c
Indian hedgehog	gga gac acc att gag act tga a	tga aga atc gca gcc aga g
Lrp5	gga ctt cat cta ctg gac cga c	tgc acc ctc cat ttc cat c
Osteocalcin	tct ctc tga cct cac aga tcc c	tac ctt att gcc ctc ctg ctt g
Osteopontin	cca tct cag aag cag aat ctc c	atg gtc atc atc gtc gtc c
Osterix	tat gct ccg acc tcc tca ac	aat agg att ggg aag cag aaa g
Patched	caa act ttg acc cct tgg aa	aaa aca agg ggc aca tca ag
PPARγ	gga aag aca acg gac aaa tca c	tac gga tcg aaa ctg gca c
PTHR	ttc cag gga ttt ttt gtt gc	agt cca tgc cag tgt cca g
Runx2	ctc cgc tgt gaa aaa cc	tga aac tct tgc ctc gtc c
Smoothened	cag gag ctc tcc ttc agc at	ttg ttc ttc tgg tgg cac tg
18s	cga tgc tct tag ctg agt gt	ggt cca aga att tca cct ct

rapidly over 28 days in all but the Kusa4d10 subclone (Fig. 2A). Alkaline phosphatase activity was indicative of mineralization capacity, with clones Kusa4b10, Kusa1c11, and Kusa2g11 showing that increasing alkaline phosphatase activity preceded mineralization (Fig. 2B). No alkaline phosphatase activity was detected in the non-mineralizing Kusa4d10 clone by this assay. Osteocalcin protein, a late marker of the osteoblast phenotype, was undetectable at day 7 but expressed by day 21 in all but the non-mineralizing Kusa4d10 cells (Fig. 2C). PTHrP was not detected in the medium from any of the clones at day 7 but was detected by day 21 in clones Kusa4b10, Kusa1c11, and Kusa2g11 but not in the Kusa4d10 cells or surprisingly the parent Kusa O cells (Fig. 2D). Induction of the PTH receptor was not apparent at day 7 but by day 21 the parent cells and four subclones demonstrated considerable PTH responsiveness (Fig. 2E).

Messenger RNA levels of the osteoblastic markers measured in Figure 2 as well as osteopontin and BSP were detected by quantitative RT-PCR. Alkaline phosphatase mRNA (Fig. 3A) expression increased with differentiation in the Kusa O, Kusa1c11 and Kusa2g11 cells, while the level in the Kusa4b10 cells appeared to peak earlier in the process and decline at 21 days although the level was still higher than for the other clones. A low level of alkaline phosphatase mRNA was detectable at 21 days in the Kusa4d10 cells and although no activity was detected by the alkaline phosphatase assay (Fig. 2A), a small number of cells stained for alkaline phosphatase in parallel experiments (data not shown). Osteocalcin mRNA (Fig. 3B) was not detectable at 0 and 7 days but was highly expressed on day 21 in all but the Kusa4d10 cells, results that are in agreement with the osteocalcin protein (Fig. 2C). PTHR-1 mRNA (Fig. 3C) was very low in the undifferentiated cells but was highly expressed on day 21, data that were also reflective of the measurement of receptor activity (Fig. 2E). BSP mRNA (Fig. 3D) levels rose dramatically between days 7 and 21 in the Kusa O cells, the subclones and the primary osteoblasts, although the level of BSP mRNA in the Kusa4d10 cells was lower than in the other cells. Osteopontin mRNA (Fig. 3E) levels were low in the non-differentiated cells, but were elevated early in the process, consistent with published data [Aubin, 1998]. These levels were further elevated in the Kusa1c11 and Kusa2g11 clones at 21 days.

Having established a phenotypic profile for the Kusa O cells and four subclones we proceeded to measure mRNA levels of three transcription factors known to regulate expression of the osteoblastic and adipocytic genes. Runx2 mRNA expression did not vary substantially with differentiation or between clones and cultures of primary mouse calvarial osteoblast-like cells (Fig. 4A). Osterix, involved later in differentiation than Runx2, was more highly expressed in more differentiated cells. At 21 days osterix (Fig. 3B) expression in the Kusa4d10 cells was lower than for the Kusa O cells and other subclones but was comparable to the levels in the primary osteoblasts and was still higher than in the undifferentiated cells.

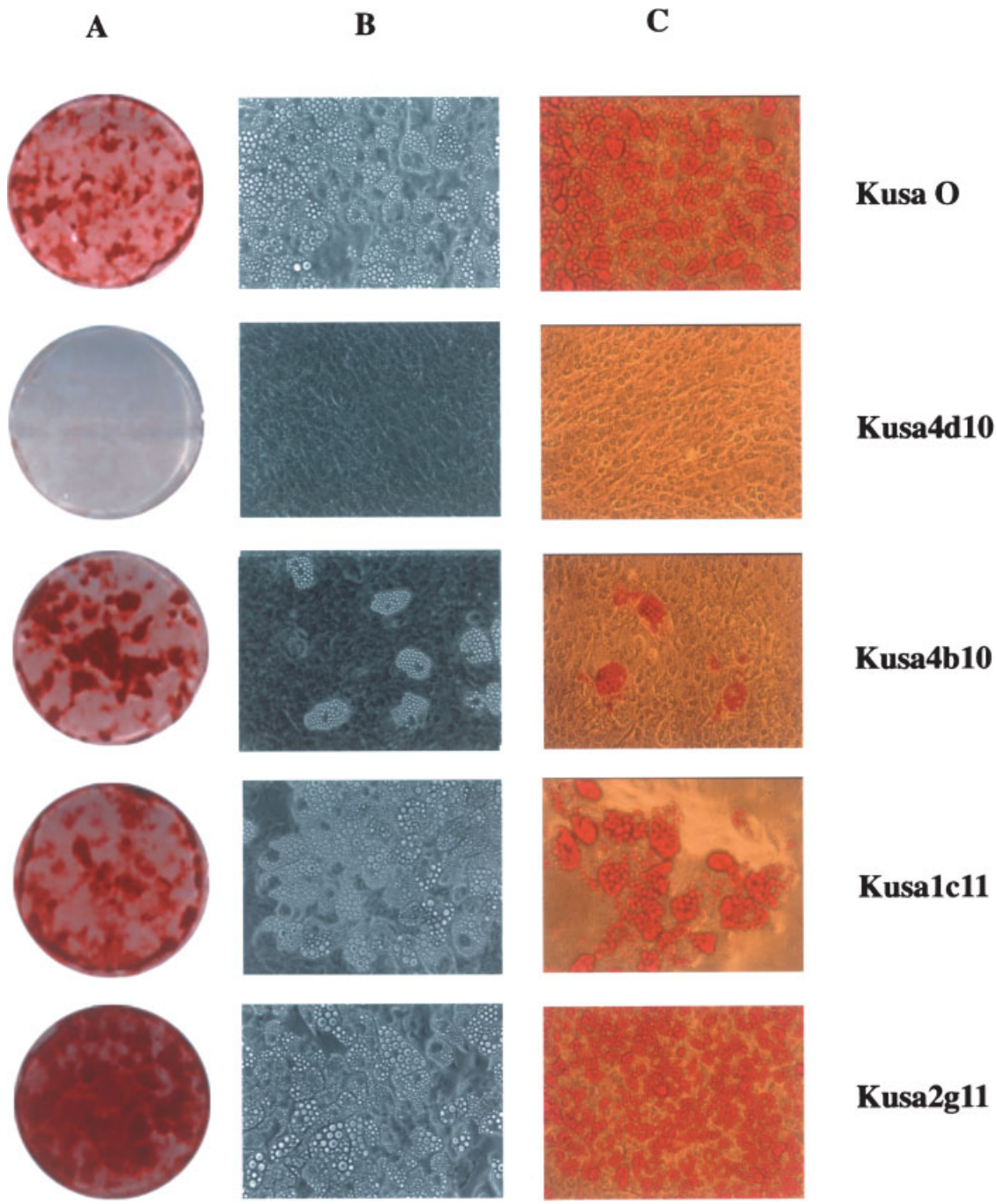

Fig. 1. Osteoblastic and adipocytic differentiation of Kusa O cells and subclones. Alizarin red was used to detect mineralization of nodules in cultures that had been incubated in osteoblast differentiation medium (see ''Materials and Methods'') for 21 days (**A**). Cells incubated in adipocyte differentiation medium (see ''Materials and Methods'') were digitally photographed (**B**) and stained for lipid with Oil red-O (**C**).

The adipocyte transcription factor PPARγ was expressed early and levels had decreased by 21 days (Fig. 3C). It is interesting to note that this gene was similarly expressed in the parent cells and four subclones despite there being no detectable adipocytes in cultures of Kusa4d10 and Kusa4b10 cells under osteoblast differentiating conditions. It is possible that this is a reflection of the "repressive" effect that PPARγ has been reported to have on several parameters of the osteoblast phenotype [Lecka-Czernik et al., 1999, 2002].

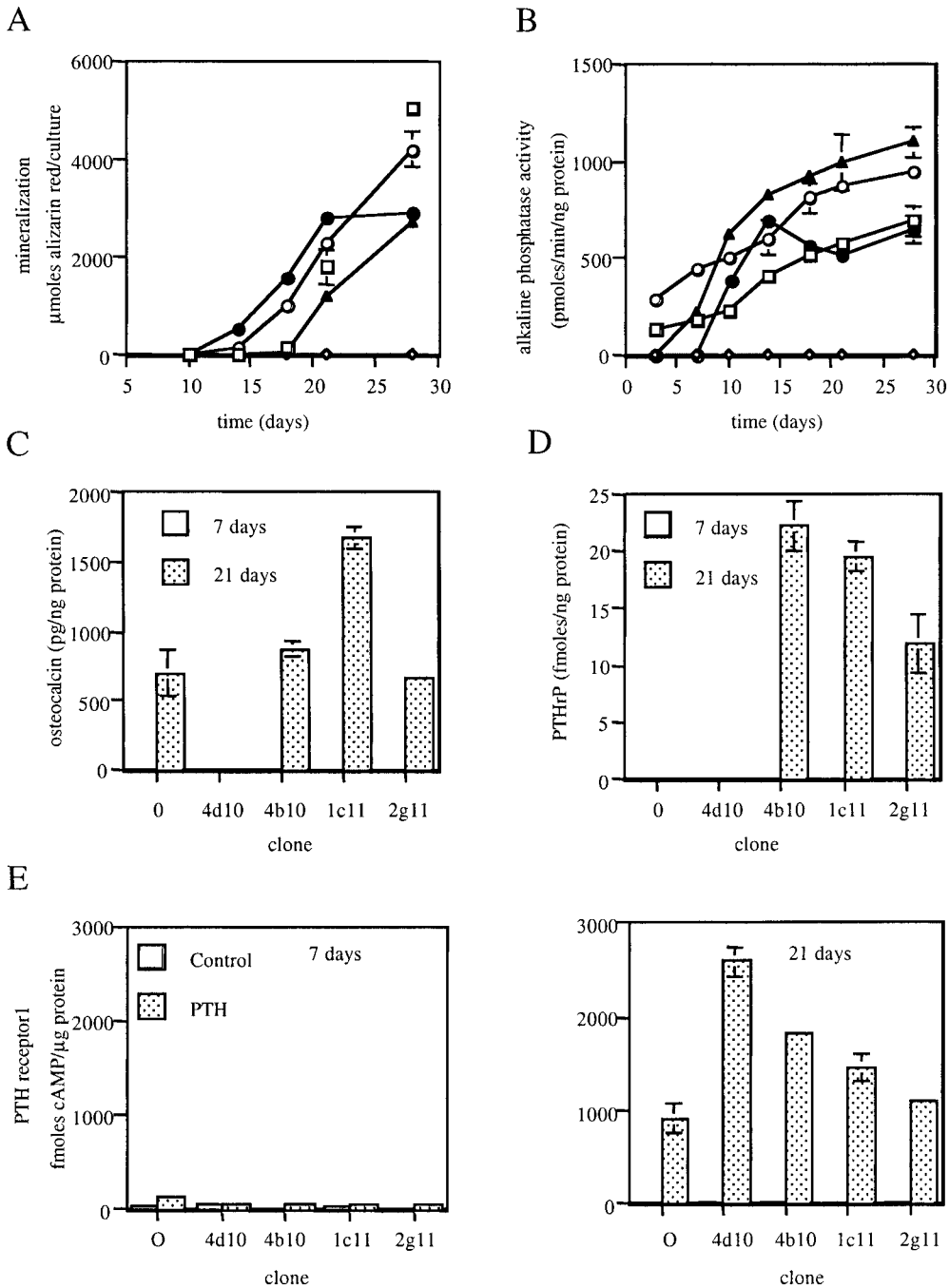

Fig. 2. Assessment of osteoblast phenotype. Cells were cultured as described in the "Materials and Methods" for the times indicated on the figure. Cell monolayers were fixed and stained for mineralized nodule formation (**A**), cell extracts were assayed for alkaline phosphatase activity (**B**) Kusa O [□], Kusa4d10 [◇], Kusa4b10 [●], Kusa1c11 [▲], Kusa2g11 [O] while the conditioned medium was assayed for osteocalcin (**C**) and PTHrP (**D**) production at 7 and 21 days. Responsiveness to PTH (**E**) was measured as described in the "Materials and Methods" in the presence and absence of PTH on day 7 and 21 of the incubation.

Other mRNAs reported to be expressed during osteoblast differentiation were measured by real time RT-PCR in Kusa O parental cells, the four subclones and primary mouse osteoblast-like cells. RT-PCR of components of the hedgehog and Wnt pathways is illustrated in Figure 5. Differentiation appeared to have no effect on smoothened mRNA (Fig. 5A) in the Kusa O cells and subclones, nor in the mouse osteoblasts-like cells while there appeared to

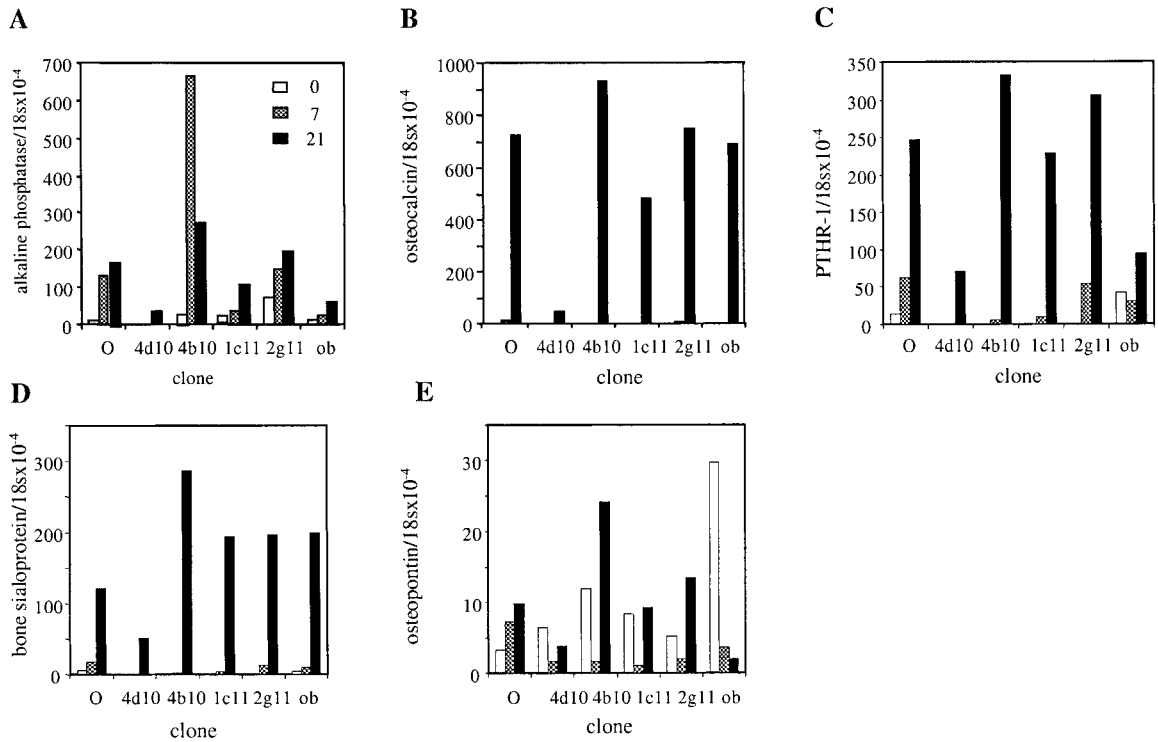

Fig. 3. Assessment of osteoblast phenotype, mRNA analysis. Messenger RNA was prepared from cells incubated as described in the "Materials and Methods," assessed by quantitative RT-PCR and expressed relative to 18s RNA at 0, 7, and 21 days.

be a trend for patched (Fig. 5B) and Indian hedgehog (Fig. 5C) to increase with differentiation although this was not apparent for patched in the Kusa4d10 cells and mouse osteoblast-like cells. It is perhaps not surprising that the signaling component of the pathway should be constitutively expressed while the ligand and its receptor are regulated. Components of the Wnt/β-catenin pathway were examined due to the recent implication of their involvement in regulating bone mass [Kato et al., 2002]. With the exception of the Kusa4d10 cells, a trend for β-catenin (Fig. 5D) and dickkopf 1 (Fig. 5E) to increase with differentiation was seen. No consistent change in Lrp5 was observed (Fig. 5F). sFRP-1 and sFRP-3 mRNAs'

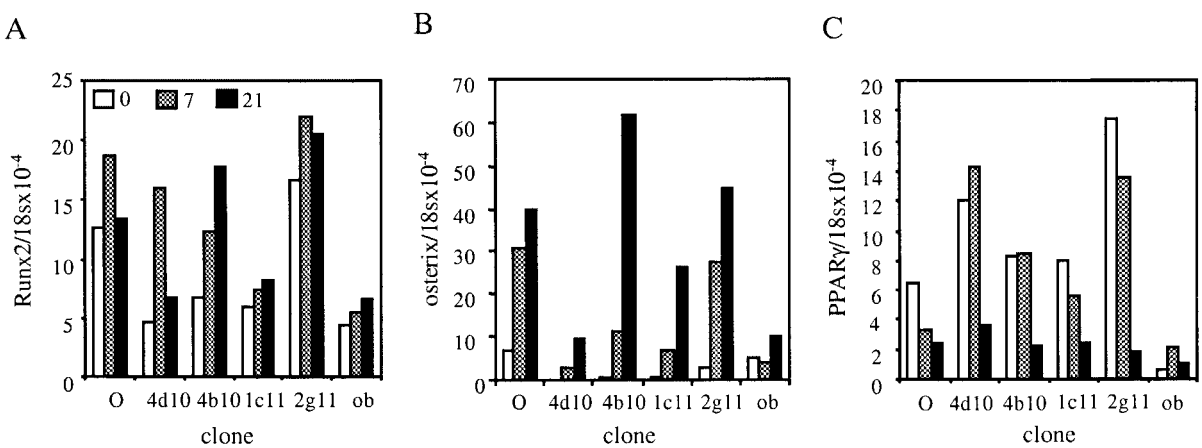

Fig. 4. Transcription factors in osteoblast differentiation, mRNA analysis. Messenger RNA was prepared as described in the "Materials and Methods," assessed by quantitative RT-PCR and expressed relative to 18s RNA at 0, 7, and 21 days.

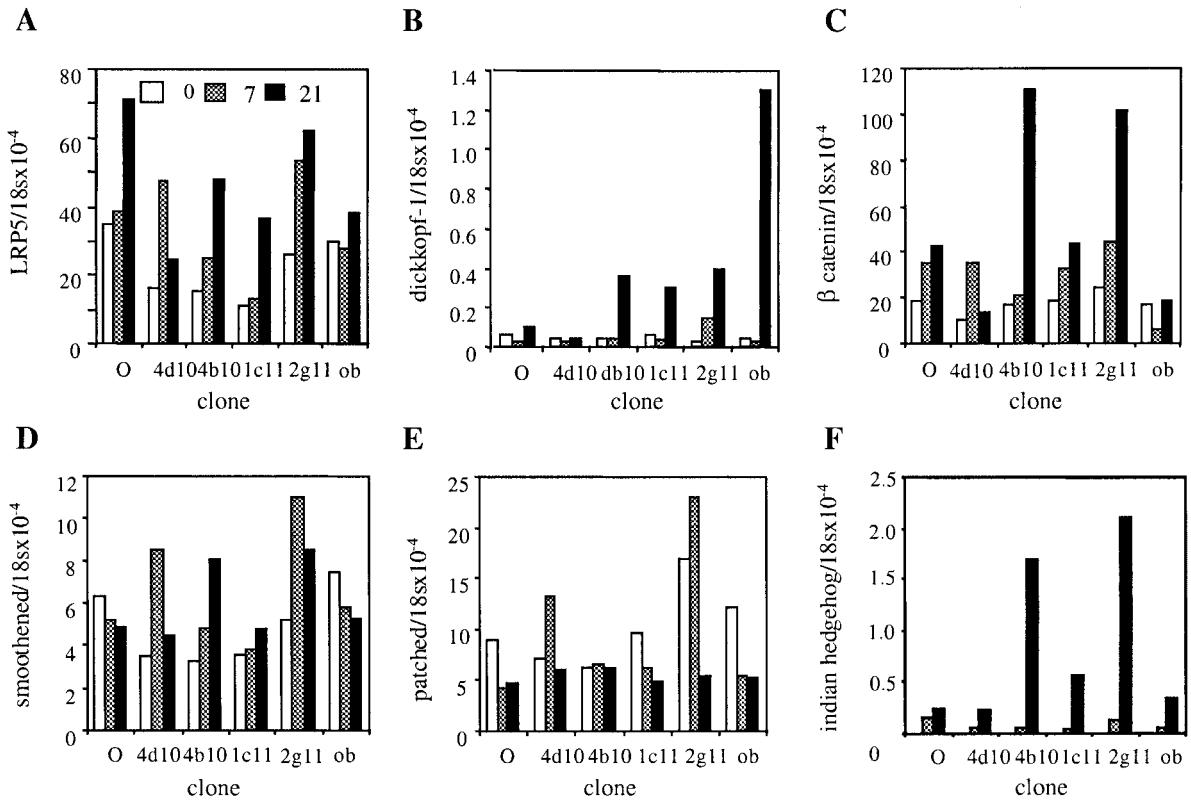

Fig. 5. Components of the Hedgehog and Wnt/β-catenin pathway in osteoblast differentiation, mRNA analysis. Messenger RNA was analyzed as described in Figure 1.

were not detectable in the KusaO and subclones but were readily detectable in the mouse osteoblast-like cells and in the case of sFRP-3 increased dramatically with differentiation (data not shown).

DISCUSSION

We have established cell lines that exhibit three different phenotypes according to their ability to mineralize or differentiate into adipocytes. Cells of one line, Kusa4d10, were unable to mineralize or differentiate into adipocytes; another line, Kusa4b10, mineralized but was unable to differentiate into adipocytes; and two, Kusa1c11 and Kusa2g11, differentiated into both adipocytes and osteoblasts. In these subclones, the pattern of expression of genes known to be associated with osteoblast differentiation were broadly as expected. This applies particularly to alkaline phosphatase mRNA and activity, seen at high levels in the mineralizing subclones, Kusa1c11, Kusa2g11, and Kusa4b10, but virtually undetected in the non-mineralizing Kusa4d10 cells. In addition, osteocalcin mRNA and protein, produced late in differentiation was induced in the three mineralizing subclones by ascorbate, but not in the non-mineralizing Kusa4d10 cells. Messenger RNA for PTHR-1 was expressed in abundance in the four differentiating subclones, reflected also in the greatly enhanced PTH-responsive cyclic AMP response. This differs in timing from the data of Kondo et al. [1997], who reported that PTHR-1 receptor mRNA was expressed early in the osteoblast differentiation process while the fold increase in cAMP in response to PTH was higher in more mature bone marrow cultures. The reason for the lag is unclear at this point but the phenomenon raises the possibility for such a delay for other proteins.

Runx2 mRNA was present in the parent cells and subclones at similar levels at the times measured here. There was no temporal correlation of regulation of responsive genes by this transcription factor, however, post-translational modifications and/or protein–protein interactions may be important in the regulation of Runx2 activity by osteogenic factors [Xiao et al., 1998; Selvanmurugan et al., 2000;

Franceschi and Xiao, 2003; Krishnan et al., 2003; Shui et al., 2003]. In recent work, Byers et al. [2002] showed that forced over expression of Runx2 in cells of the osteoblastic lineage enhanced expression of a number of osteoblast-specific genes, as well as enhancing matrix mineralization assessed as described herein. Osterix levels in the Kusa4d10 cells are lower than in the other clones but are comparable with the levels in mouse osteoblast-like cells. In all cases, there was a small increase in mRNA with differentiation, suggesting osterix may be required throughout the process. The basic helix-loop-helix transcription factor, TWIST, could also play a role in osteoblast differentiation, since the promoter regions of several osteoblastic genes have putative binding sites for it [Yousfi et al., 2002]. TWIST has been reported to maintain cells in an undifferentiated state [Lee et al., 1999; Oshima et al., 2002], therefore, it will be of interest to study it in these clonal lines.

The Kusa4d10 and Kusa4b10 cells, which failed to differentiate into adipocytes in the presence of ascorbate, exhibit similar levels of PPARγ mRNA as the Kusa O cells and subclones Kusa1c11 and Kusa2g11 that differentiate into adipocytes under these conditions. This could indicate that these cells are early preadipocytic. In addition, the three mineralizing subclones, Kusa4b10, Kusa1c11, and Kusa2g11 appeared to be preosteoblastic since they differentiated into cells that mineralize and have a profile of osteoblastic markers typical of the osteoblast phenotype. The Kusa4d10 cells were difficult to characterize. The only differences between these and the mineralizing cells that we have observed here are in alkaline phosphase and osteocalcin gene expression. In vitro cultures of osteoblasts from alkaline phosphatase knockout mice failed to mineralize [Wennberg et al., 2000], whilst bone formation in osteocalcin-deficient mice is higher and of improved functional quality compared with wild type mice [Ducy et al., 1996]. Whether these two differences are sufficient to inhibit mineralization by this clone or whether post-translational modifications of some of the gene products measured here are important in this process remains to be determined.

PPARγ is a ligand-activated nuclear regulator of differentiation, cell growth and metabolism and is involved in the commitment of precursors to the adipocytic pathway. This important regulator of adipocyte differentiation appears able to induce transdifferentiation of myoblasts into mature adipocytes in cells ectopically expressing PPARγ and another adipocytic transcription factor, C/EBPγ, in the presence of PPARγ activators and adipocytic hormones [Hu et al., 1995]. Transdifferentiation between osteoblasts and adipocytes has been reported for bone marrow cells [Bennett et al., 1991; Nuttal et al., 1998; Park et al., 1999] while there are a number of reports showing that bone marrow cells will differentiate into adipocytes or osteoblasts when given the appropriate stimuli [Gori et al., 1999; Spinella-Jaegl et al., 2001; Dang et al., 2002]. PPARγ appears one likely determining factor in this process. It is interesting that PTHrP has been found to modulate the activity of PPARγ and inhibit differentiation of the preadipocytic MTC3T3 cells [Chan et al., 2001] while estrogen has been reported to down regulate PPARγ2 and inhibit adipogenesis in mouse KS483 cells [Dang et al., 2002]. PPARγ also directly affects osteoblast differentiation since it inhibits Runx2 transcription and hence synthesis of a number of osteoblast proteins so it would seem that PPARγ could be osteoblast repressive as well as adipocyte inductive. In the Kusa O cells and subclones, PPARγ mRNA levels were reduced with osteoblast differentiation, consistent with a role as an inhibitor of osteoblastogenesis although there was no consistent increase in Runx2 mRNA levels late in differentiation as might be expected if PPARγ inhibited Runx2 expression.

The hedgehog family of proteins play a role in pattern formation and cell proliferation during development [Ingham, 1998] while a number of reports now suggest a role for hedgehog signaling in osteoblast differentiation. Members of the family have been shown to regulate skeletal formation in vertebrates [Kim et al., 1998; St-Jacques et al., 1999], and furthermore sonic hedgehog has been reported to promote osteoblastogenesis and inhibit adipogenesis of pluripotent mesenchymal cells [Spinella-Jaegl et al., 2001; Yuasa et al., 2002]. The failure to find any notable changes in expression of either ligand or receptor in the experiments described here needs to be investigated further, with the possibility in mind that the receptor, smoothened, might be constitutively expressed, while signaling components could be regulated.

Another signaling pathway of importance in development and of likely importance in bone

formation and adipogenesis is the Wnt signaling pathway. Lrp5 deficient mice develop a low bone mass postnatally attributed to a decrease in osteoblast proliferation and bone matrix deposition with both defects occurring in a Runx2-independent manner [Kato et al., 2002]. TWIST has been reported to be upregulated in response to Wnt1 expression in mouse mammary cells, and expression of Wnt1 or TWIST in these cells resulted in inhibition of lactogenic differentiation [Howe et al., 2003]. Gong et al. [2001] have shown that ST2 marrow stromal cells can be induced to the osteoblastic lineage, by addition of exogenous growth factors, via the Wnt/β-catenin signaling pathway in a Smad-independent and Lrp5-dependent manner. Furthermore, Wnt1 over-expressing ST2 cells appeared to increase the rate of osteoblast differentiation over non-expressing cells and Wnt signaling has been reported to inhibit adipogenesis [Ross et al., 2000]. Therefore, by stimulation and inhibition of various components of this pathway, bone marrow cells may be driven down a particular lineage.

The mRNA and functional data described here are consistent and reflect published data for osteoblast differentiation. These subclones are closely related genetically but their phenotypes differ in ways that should provide excellent models for studying osteoblast/adipocyte differentiation and identifying genes that may be involved in their transdifferentiation.

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REFERENCES

Aubin J. 1998. Bone stem cells. J Cell Biochem Suppl 30/31:73–82.

Bellows CG, Aubin JE, Heersche JNM. 1991. Initiation and progression of mineralization of bone nodules formed in vitro: The role of alkaline phosphatase and organic phosphate. Bone Miner 14:27–40.

Bennett JH, Joyner CJ, Triffit JT, Owen ME. 1991. Adipocyte cells cultured from marrow have osteogenic potential. J Cell Sci 99:131–139.

Beresford JN, Bennett JH, Devlin C, Leboy PS, Owen ME. 1992. Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. J Cell Sci 102:341–351.

Byers BA, Pavlath GK, Murphy TJ, Karsenty G, Garcia AJ. 2002. Cell-type-dependent up-regulation of in vitro mineralization after over expression of the osteoblast-specific transcription factor Runx2/Cbfa1. J Bone Miner Res 17:1931–1945.

Chan GK, Deckelbaum RA, Bolivar I, Goltzman D, Karapalis AC. 2001. PTHrP inhibits adipocyte differentiation by down-regulating PPARγ activity via a MAPK-dependent pathway. Endocrinol 142:4900–4909.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 162:156–159.

Dang ZC, Van Bezooijen RL, Karperien M, Papapoulos SE, Löwik CWGM. 2002. Exposure of KS483 cells to estrogen enhances osteogenesis and inhibits adipogenesis. J Bone Miner Res 17:394–405.

Ducy P, Desbois C, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, Karsenty G. 1996. Increased bone formation in osteocalcin-deficient mice. Nature 382:448–452.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. 1997. Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell 89:747–754.

Franceschi RT, Xiao G. 2003. Regulation of the osteoblast-specific transcription factor, Runx2: Responsiveness to multiple signal transduction pathways. J Cell Biochem 88:446–454.

Franceschi RT, Iyer BS, Cui Y. 1994. Effects of ascorbic acid on collagen matrix formation and osteoblast differenation in murine MC3T3-E1 cells. J Bone Miner Res 9:843–845.

Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D, Zacharin M, Oexle K, Marcelino J, Suwairi W, Heeger S, Sabatakos G, Apte S, Adkins WN, Algrove J, Arslan-Kirchner M, Batch JA, Beighton P, Black GCM, Boles RG, Boon LM, Barrone C, Brunner HG, Carle GF, Dallapiccola B, De Paepe A, Floege B, Halfhide ML, Hall B, Hennekam RC, Hirose T, Jans A, Jüppner H, Kim CA, Keppler-Noreuil K, Kohlschuetter A, LaCombe D, Lambert M, Lemyre E, Lettboer T, Peltonen L, Ramesar RS, Romanengo M, Somer H, Steichen-Gersdorf E, Steinmann B, Sullivan B, Superti-Furga A, Swoboda W, van den Boogaard M-J, Hul WV, Vikkul M, Votruba M, Zabel B, Garcia T, Baron R, Olsen BR, Warman ML. 2001. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell 107: 513–523.

Gori F, Thomas T, Hicok KC, Spelberg TC, Riggs BL. 1999. Differentiation of human marrow stromal precursor cells: Bone morphogenic protein-2 increase: OSF2/CBFA1, enhances osteoblast commitment, and inhibits late adipocyte maturation. J Bone Miner Res 14:1522–1535.

Grill V, Ho P, Body JJ, Johanson SC, Lee C, Kukreja SC, Moseley JM, Martin TJ. 1991. Parathyroid hormone related protein: Elevated levels in both humoral hypercalcemia of malignancy and hypercalcaemia complicating metastatic breast cancer. J Clin Endocrinol 73:1309–1315.

Gutierrez S, Javed A, Tennant DK, van Rees M, Montecino M, Stein GS, Stein JB, Lian JB. 2002. CCAAT/enhancer-binding proteins (C.EBP) β and δ activate osteocalcin gene transcription and synergize with Runx2 at the cEBP element to regulate bone-specific expression. J Biol Chem 277:1316–1323.

Horwood NJ, Elliot J, Martin TJ, Gillespie MT. 1998. Osteotropic agents regulate the expression of osteoclast differentiation factor and osteoprotegerin in osteoclastic stromal cells. Endocrinology 39:4743–4746.

Houssami S, Findlay DM, O'Keeffe LM, Martin TJ, Sexton PM. 1994. Heterogeneity in ligand recognition of calcitonin receptors. Endocrine J 2:127–134.

Howe LR, Watanabe O, Leonard J, Brown AMC. 2003. Twist is up-regulated in response to Wnt1 and inhibits mouse mammary cell differentiation. Cancer Res 63: 1906–1913.

Hu E, Tontonoz P, Spiegelman BM. 1995. Transdifferentiation of myoblasts by the adipogenic transcription factors PPARγ and C/EBPα. Proc Natl Acad Sci USA 92:9856–9860.

Ingham PW. 1998. Transducing hedgehog: The story so far. EMBO J 17:3505–3511.

Javed A, Barnes GL, Jasanya BO, Stein JL, Gerstenfelf L, Lian JB, Stein GS. 2001. *runt* homology domain transcription factors (Runx, Cbfa, and AML) mediate repression of the bone sialoprotein promoter: Evidence for promoter context-dependent activity of Cbfa proteins. Mol Cell Biol 21:2891–2905.

Jiménez MJA, Balbín M, López JM, Alvarez J, Komori T, Lópes-Otín C. 1999. Collagenase is a target of Cbfa1, a transcriptional factor of the *runt* gene family involved in bone formation. Mol Cell Biol 19:4431–4442.

Kato M, Patel MS, Levasseur R, Lobov I, Chang BH-J, Glass DA, Hartmann C, Li L, Hwang T-H, Brayton CF, Lang RA, Karsenty G, Chan L. 2002. Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. J Cell Biol 157:303–314.

Kern B, Shen J, Starbuck M, Karsenty G. 2001. Cbfa1 contributes to the osteoblast-specific expression of *type 1 collagen* genes. J Biol Chem 276:7101–7107.

Kim HJ, Rice DP, Kettunen PJ, Thesleff I. 1998. FGF-, BMP-, and Shh-mediated signalling pathways in the regulation of cranial suture morphogenesis and calvaria bone development. Devel 125:1241–1251.

Komori T, Yagi H, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao Y-H, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. 1997. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764.

Kondo H, Ohyama T, Ohya K, Kasugai S. 1997. Temporal changes of mRNA expression of matrix proteins and parathyroid hormone and parathyroid hormone-related protein (PTH/PTHrP) receptor in bone development. J Bone Miner Res 12:2089–2097.

Krishnan V, Moore TL, Ma YL, Helvering LM, Frolik CA, Valasek KM, Ducy P, Geiser AG. 2003. Parathyroid hormone bone anabolic action requires cbfa1/runx2-dependent signaling. Mol Endocrinol 17:423–435.

Kuri-Haruch W, Green H. 1978. Adipose conversion of 3T3 cells depends on a serum factor. Proc Natl Acad Sci USA 75:6107–6109.

Lecka-Czernik B, Gubrij I, Moerman EJ, Kajkenova O, Lipschit DA. 1999. Inhibition of Osf2/Cbfa1 expression and terminal osteoblast differentiation by PPARγ2. J Cell Biochem 74:357–371.

Lecka-Czernik B, Moerman EJ, Grant DF, Lehmann JM, Manolagas SC, Jilka RL. 2002. Divergent effects of selective peroxisome proliferator-activated receptor-γ2 ligands on adipocyte versus osteoblast differentiation. Endocinol 143:2376–2384.

Lee M-S, Lowe GN, Strong DD, Wergedeal JE, Glackin CA. 1999. Twist, a basic helix-loop-helix transcription factor, can regulate the human osteogenic lineage. J Cell Biochem 75:566–577.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B. 2002. The Novel Zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108:17–29.

Nuttal ME, Patton AJ, Olivera DL, Nadeau DP, Gowen M. 1998. Human traebecular bone cells are able to express both osteoblastic and adipocytic phenotype: Implications for osteogenic disorders. J Bone Miner Res 13: 371–382.

Oreffo ROC, Virdi AS, Triffit JT. 1997. Modulation of osteogenesis and adipogenesis by human serum in human bone marrow cultures. Eur J Cell Biol 74:251–261.

Oshima A, Tanabe H, Yan T, Lowe GN, Glackin CA, Kudo A. 2002. A novel mechanism for the regulation of osteoblast differentiation: Transcription of periostin, a member of the fasciclin 1 family, is regulated by the bHLH transcription factor Twist. J Cell Biochem 86:792–804.

Otto F, Thornell AP, Crompton Denzel A, Gilmour KC, Rosewell IR, Stamp GWH, Beddongton RSP, Mumdlos S, Olsen BR, Selby PB, Owen MJ. 1997. *Cbfa1*, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89:765–771.

Owen ME. 1985. Lineage of osteogenic cells and their relationship to the stromal system. In: Peck WA, editor. Bone and mineral research, Vol 3. Amsterdam, New York, Oxford: Elsevier. pp 1–25.

Park SR, Oreffo ROC, Triffit JT. 1999. Interconversion potential of cloned human marrow adipocytes in vitro. Bone 24:549–554.

Partridge NC, Alcorn D, Michelangeli VP, Kemp BE, Ryan GB, Martin TJ. 1981. Functional properties of hormonally responsive cultured normal and malignant rat osteoblastic cells. Endocrinol 108:213–219.

Ross SE, Hemati N, Longo KA, Bennet CN, Lucas PC, Erickson RL, MacDougald OA. 2000. Inhibition of adipogenesis by Wnt signalling. Science 289:950–953.

Sato M, Morii E, Komori T, Kawahata H, Sugimoto M, Terai K, Shimizu H, Yasui T, Ogihara H, Yasui N, Ochi T, Kitamura Y, Ito Y, Nomura S. 1998. Transcriptional regulation of osteopontin gene in vivo by PEBP2-alpha CBFA1 and ETS1 in the skeletal tissues. Oncogene 17:1517–1525.

Selvanmurugan N, Pulumati MR, Tyson DR, Partridge NC. 2000. Parathyroid hormone regulation of the rat collagenase-3 promoter by protein kinase A-dependent transactivation of core binding factor α1. J Biol Chem 275:5037–5042.

Shui C, Spelberg TC, Riggs BL, Khosla S. 2003. Changes in Runx2/Cbfa1 expression and activity during osteoblastic differentiation of human bone marrow stromal cells. J Bone Miner Res 18:213–221.

Spinella-Jaegl S, Rawadi G, Kawai S, Gallea S, Faucheu C, Mollat P, Coutrois B, Bergaud B, Ramez V, Blanchet AM, Adelmant G, Baron R, Roman-Roman S. 2001. Sonic

hedgehog increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation. J Cell Sci 114:2085–2094.

St-Jacques B, Hammerschmidt M, McMahon AP. 1999. Indian hedgehog signalling regulates proliferation of chondrocytes and is essential for bone formation. Genes Dev 13:2072–2086.

Stanford CM, Jacobson PA, Eanes ED, Lembke LA, Midura RJ. 1995. Rapidly forming apatitc mineral in an osteoblastic cell line (UMR106-01BSP). J Biol Chem 270:9420–9428.

Thirunavukkarasu K, Halladay DL, Miles RR, Yang XH, Galvin RJS, Chandrasekhar S, Martin TJ, Onyia JE. 2000. The osteoblast-specific transcription factor Cbfa1 contributes to the expression of osteoprotegerin, a potent inhibitor of osteoclast differentiation and function. J Biol Chem 275:25163–25172.

Umezawa A, Maruyama T, Segawa K, Shadduck R, Waheed A, Hata J-I. 1992. Multipotent marrow stromal cell line is able to induce hematopoiesis in vivo. J Cell Physiol 151:197–205.

Wennberg C, Hessle L, Lunberg P, Mauro S, Narisawa C, Lerner UF, Millan JL. 2000. Functional characterization of osteoblasts and osteoclasts from alkaline phosphatase knockout mice. J Bone Miner Res 15:1879–1888.

Xiao G, Wang D, Douglas M, Karsenty G, Franceshi RT. 1998. Role of the α2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor. J Biol Chem 273:32988–32994.

Yousfi M, Lasmoles F, Kern B, Marie PJ. 2002. Twist inactivation reduces CBFA1/RUNX2 expression and DNA binding to the osteocalin promoter in osteoblasts. Biochem Biophys Res Commun 297:641–644.

Yuasa T, Kataoka H, Kinto N, Iwamoto M, Enomoto-Iwamoto M, Iemura S-I, Ueno N, Shibata Y, Kurosawa H, Yamaguchi A. 2002. Sonic hedgehog is involved in osteoblast differentiation by cooperating with BMP-2. J Cell Physiol 193:225–232.