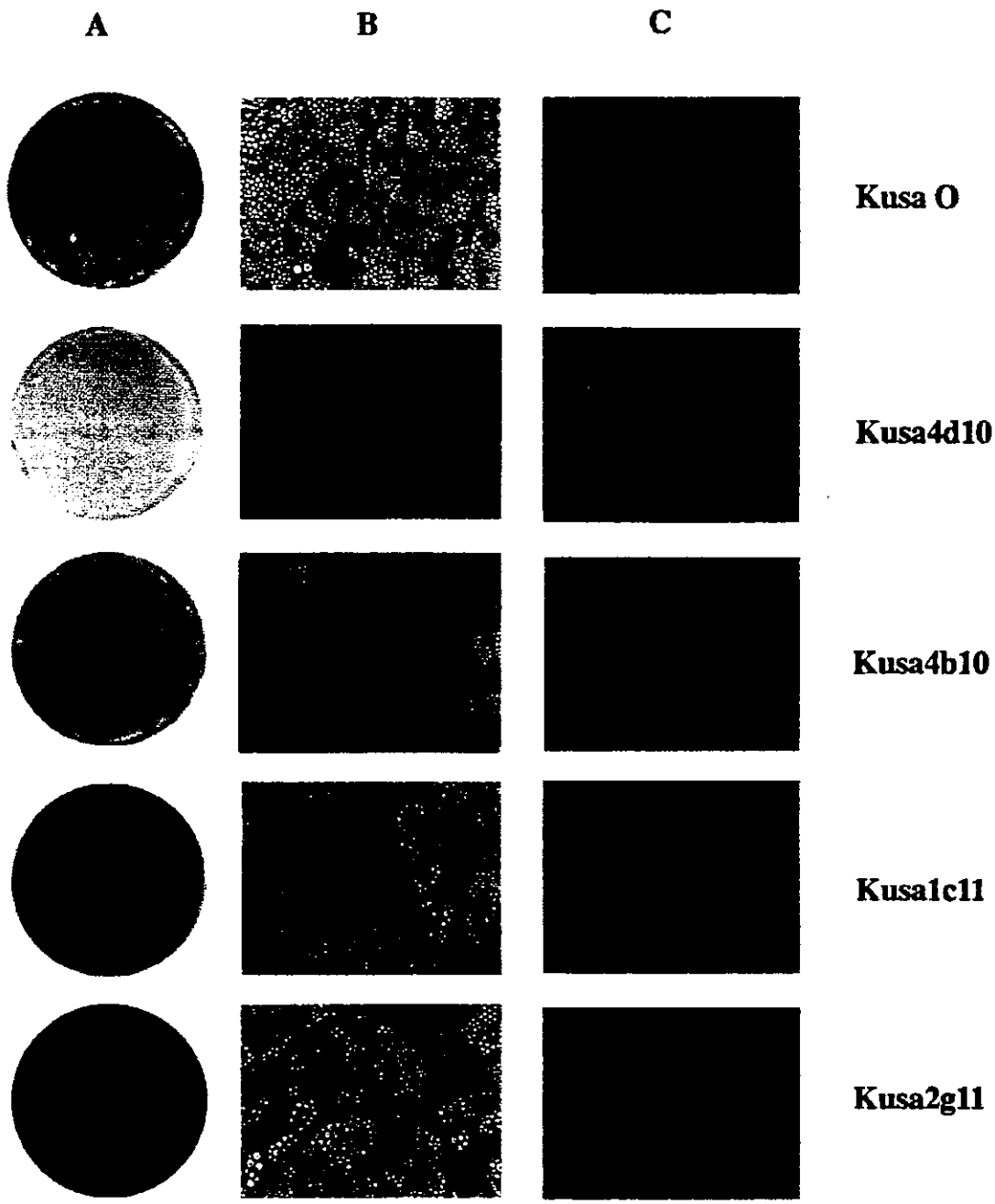

Fig. 1. Osteoblastic and adipocytic differentiation of Kusa O cells and subclones. Alizarin red was used to detect mineralization of nodules in cultures that had been incubated in osteoblast differentiation medium (see "Materials and Methods") for 21 days (A). Cells incubated in adipocyte differentiation 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].

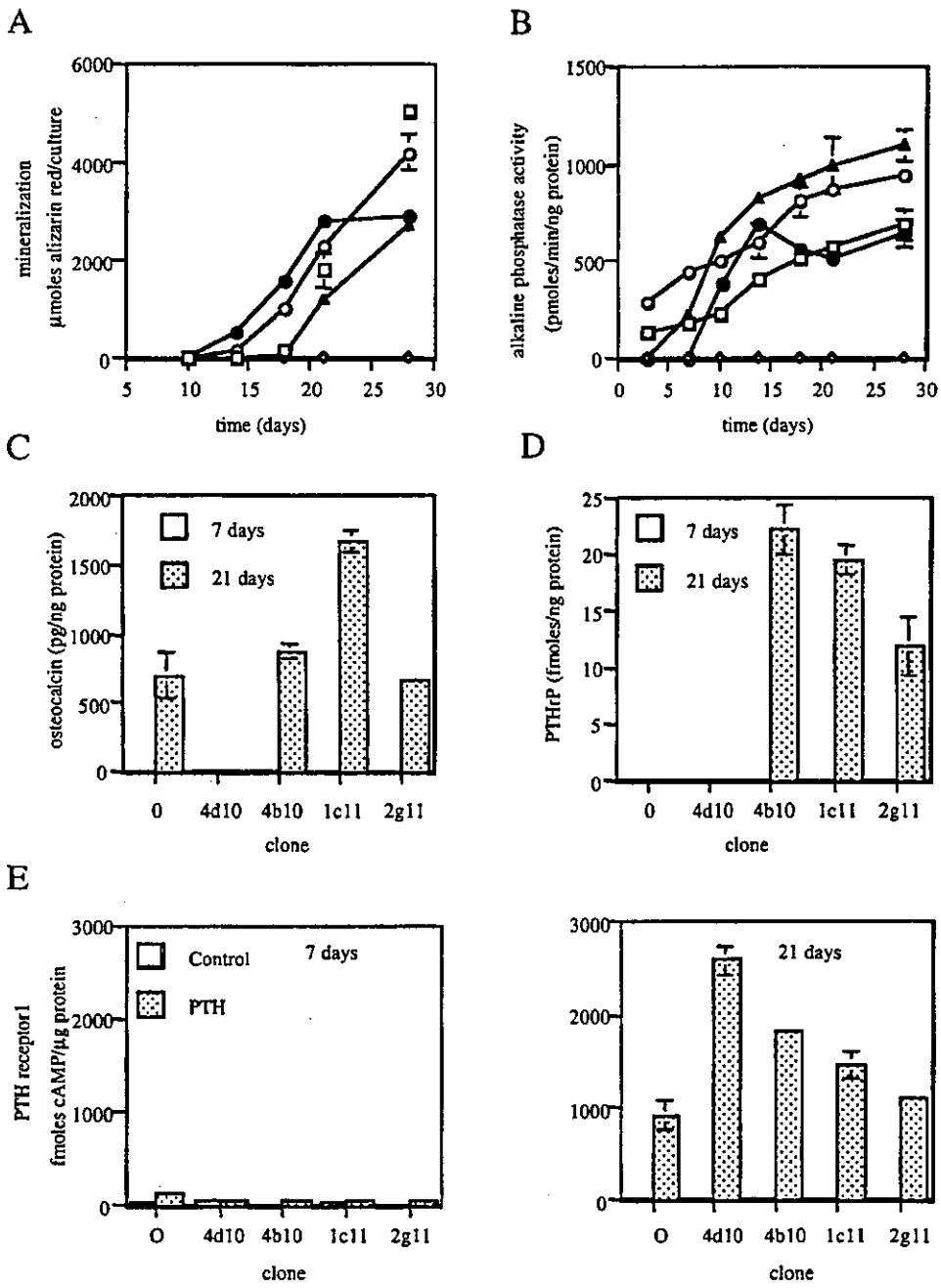

Fig. 2. Assessment of osteoblast phenotype. Cells were cultured as described in the "Materials and Methods" for the times indicated on the figure. Cell monolayers were fixed and stained for mineralized nodule formation (**A**), cell extracts were assayed for alkaline phosphatase activity (**B**) Kusa 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

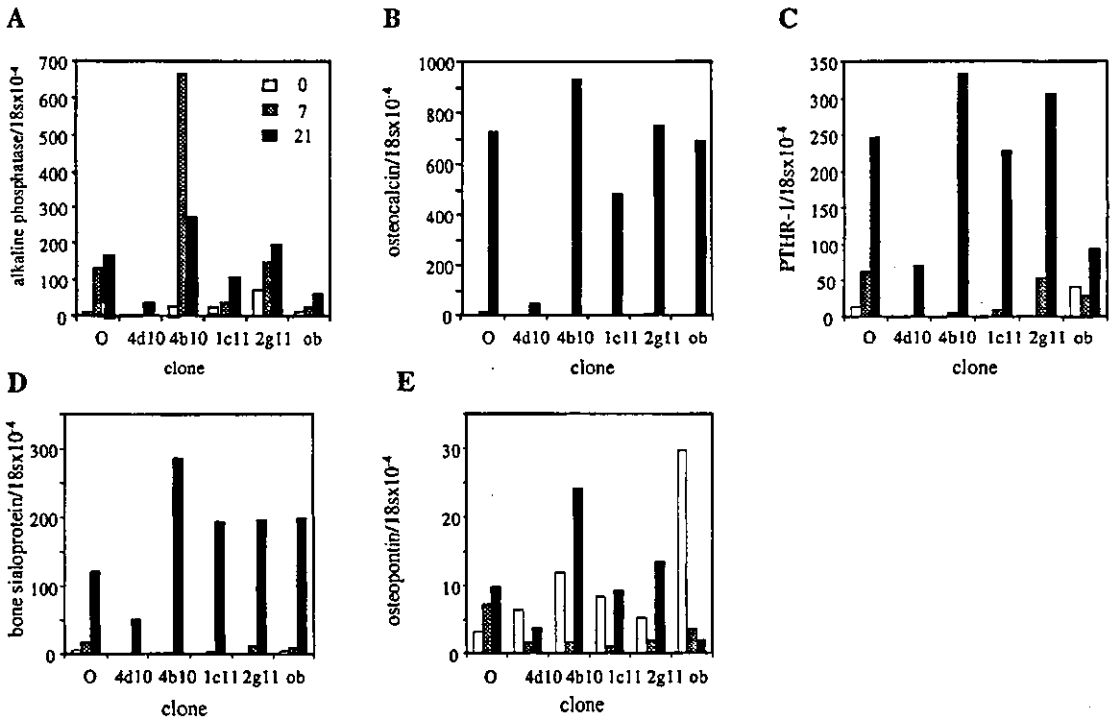

Fig. 3. Assessment of osteoblast phenotype, mRNA analysis. Messenger RNA was prepared from cells incubated as described in the "Materials and Methods," assessed by quantitative RT-PCR and expressed relative to 18s RNA at 0, 7, and 21 days.

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'

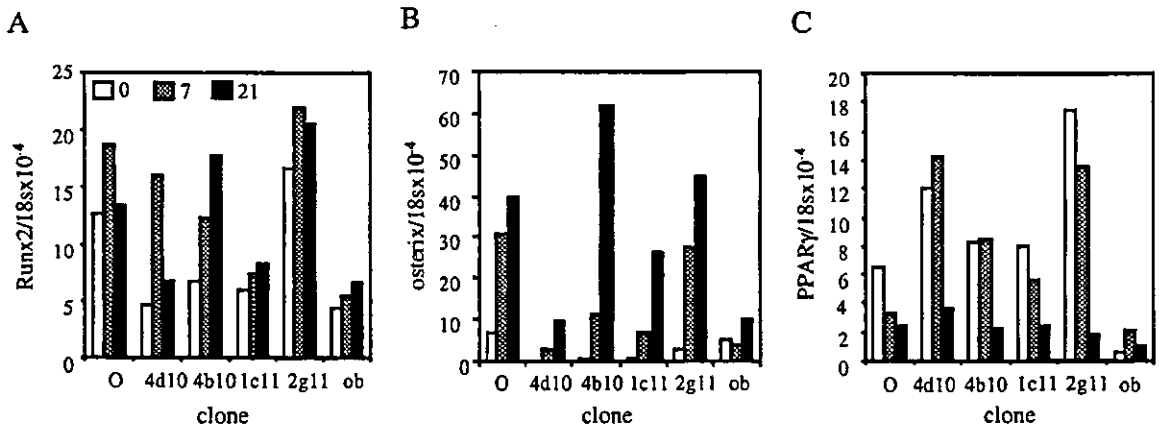

Fig. 4. Transcription factors in osteoblast differentiation, mRNA analysis. Messenger RNA was prepared as described in the "Materials and Methods," assessed by quantitative RT-PCR and expressed relative to 18s RNA at 0, 7, and 21 days.

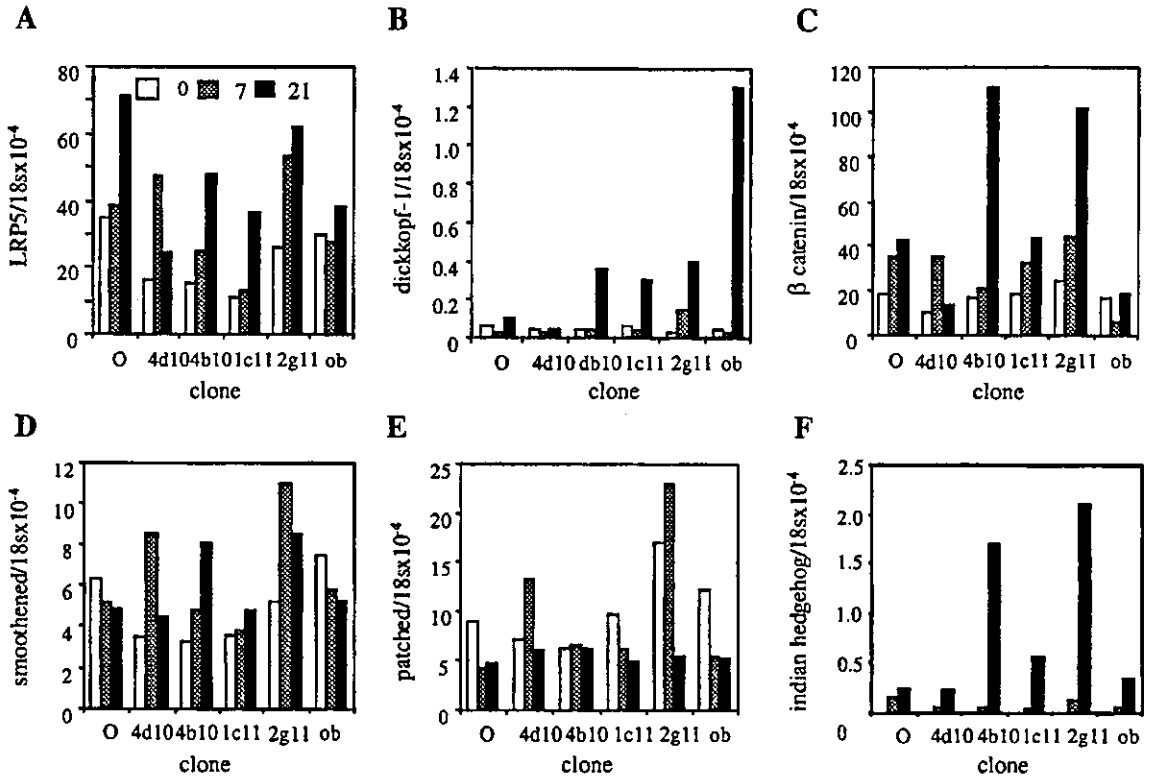

Fig. 5. Components of the Hedgehog and Wnt/β-catenin pathway in osteoblast differentiation, mRNA analysis. Messenger RNA was analyzed as described in Figure 1.

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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Osteogenic differentiation of the mesenchymal progenitor cells, Kusa is suppressed by Notch signaling

Kentaro Shindo,[a,*] Nobuyuki Kawashima,[a,1] Kei Sakamoto,[b,1] Akira Yamaguchi,[c] Akihiro Umezawa,[d] Minoru Takagi,[b] Ken-ichi Katsube,[b,*] and Hideaki Suda[a]

[a] *Pulp Biology and Endodontics, Graduate School of Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8549, Japan*
[b] *Molecular Pathology, Graduate School of Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8549, Japan*
[c] *Oral Pathology and Bone Metabolism, Biomedical Graduate School of Nagasaki University, 1-7-1, Sakamoto, Nagasaki 852-8588, Japan*
[d] *Department of Reproductive Biology and Pathology, National Research Institute for Child Health and Development, 3-35-31, Taishido, Setagaya-ku, Tokyo 154-8567, Japan*

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Abstract

Notch receptor plays a crucial role in proliferation and differentiation of many cell types. To elucidate the function of Notch signaling in osteogenesis, we transfected the constitutively active *Notch1* (*Notch* intracellular domain, *NICD*) into two different osteoblastic mesenchymal cell lines, KusaA and KusaO, and examined the changes of their osteogenic potentials. In *NICD* stable transformants (KusaA^{NICD} and KusaO^{NICD}), osteogenic properties including alkaline phosphatase activity, expression of osteocalcin and type I collagen, and in vitro calcification were suppressed. Transient transfection of *NICD* attenuated the promoter activities of *Cbfa1* and *Ose2* element. KusaA was capable of forming trabecular bone-like tissues when injected into mouse abdomen, but this in vivo bone forming activity was significantly suppressed in KusaA^{NICD}. Osteoclasts were induced in the KusaA-derived bone-like tissues, but lacked in the KusaA^{NICD}-derived tissues. These results suggest that Notch signaling suppresses the osteoblastic differentiation of mesenchymal progenitor cells.

Keywords: Osteogenesis; Mineralization; Mesenchymal progenitor cells; Kusa; Notch; RANKL

Introduction

The bone marrow stromal cells have been shown to have a potential to differentiate into a variety of mesenchymal cells, such as adipocytes, chondrocytes, and myocytes. The properties as multipotent progenitor cells make them an attractive target for use in therapeutic and bioengineering applications, and the regulation of their commitment to specific cell types is a field of primary interest [1,2].

Osteogenic lineage is one differentiation pathway of bone marrow progenitor cells. Many factors are involved in differentiation of osteoblasts and their importance in regulating proliferation and differentiation has been well documented, but the molecular processes controlling their lineage commitment and self-renewal are yet to be elucidated [3,4].

Notch signaling is an intercellular communication system that is conserved among the multicellular organisms, which is believed to be crucial for fate determination of stem cells and progenitor cells [5,6]. Notch is a single-pass transmembrane receptor with an extracellular domain that recognizes the DSL (Delta/Serrate/Lag2) type ligands on the surface of adjacent cells [7]. Notch signal is transduced through several different pathways. As one of them, association with the ligands induces several sequential proteolytic cleavages, which results in the release of the intracellular domain from the plasma membrane [8]. The internalized intracellular domain of Notch translocates to the nucleus, where it interacts with a DNA binding protein,

* Corresponding authors. Fax: +81-3-5803-5494 and Fax: +81-3-5803-0188.
E-mail addresses: k.shindo.endo@tmd.ac.jp (K. Shindo), katsube.mpa@tmd.ac.jp (K. Katsube).
[1] These authors contributed equally to this work.

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CBF1 to control the expression of downstream genes, including *HES* transcription factors [9,10]. In various situations, the effect of Notch signaling seems to regulate a phenomenon called "lateral specification." In lateral specification, the cells committed to the primary differentiation fate express the DSL ligand and stimulate Notch signaling pathway in juxtaposed cells, which prevents their differentiation and directs them to remain in an uncommitted state [11,12]. The concept that Notch signaling inhibits differentiation has been postulated by many studies that utilize the constitutively active form of *Notch* [13,14]. Overexpression of constitutively active *Notch* suppresses the differentiation of neurogenic or hematopoietic stem cell lines, suggesting that Notch signaling is essential for control of cell differentiation [15,16].

An accumulating body of evidence indicates that Notch signaling also mediates the generation of mesenchymal tissues, such as in myogenesis and angiogenesis [17–19]. The expression of *Notch* and its related genes is also observed in the cells that are recruited to cartilage and bone formation. *Notch2* and *Delta1* are co-expressed in chondrocytes [20]. *Notch2* is expressed by periosteal cells, osteoblasts, and osteocytes in the region of active bone formation [21]. In a human osteosarcoma cell line, SAOS-2, the expression of *Notch1, Notch2* and *Notch4* is differentially regulated upon osteogenic stimulation [22]. These observations raise the possibility that Notch signaling may also regulate the growth and differentiation of osteogenic cells, playing an important role in bone formation.

Here, we present evidence that Notch signaling has a suppressive role in osteoblastic differentiation, using mesenchymal progenitor cell lines, KusaA and KusaO [23–25].

Materials and methods

Cell culture and DNA transfection

KusaA was the original cell line established and described as Kusa [23]. Later, KusaO was subcloned as a non-osteogenic subline of Kusa during passage. KusaA and KusaO were named after this process, but the difference of their property was not well studied. KusaA is at a more advanced stage of osteoblastic differentiation compared to KusaO. Cells were cultured in α-MEM containing 10% fetal bovine serum. Constitutively active form of mouse *Notch1* (*Notch intra cellular domain, NICD*) was a gift from J. Nye. Dominant negative form of chicken *Delta* (*dnDl*) has been previously described [26]. Each DNA was recombined in a mammalian expression vector, pcDNA3 (Invitrogen). Transfection was performed using LipofectAmine 2000 (Invitrogen) according to the manufacturer's instructions. Cells stably expressing *NICD* were selected with 500 μg/ml Geneticin (Invitrogen) and expanded after single colony isolation.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted using TRIzol (Invitrogen). One microgram of total RNA was reverse-transcribed with Superscript II (Invitrogen) using oligo-dT primers. Using this cDNA as a template, polymerase chain reaction (PCR) was performed. The sequences of the PCR primers for mouse *Notch1* were: upper 5′-CTTGCAGTAGCAAGGAAGCTAAGG-3′ and lower 5′-ACTTAAATGCCTCTGGAATGTCG-3′. The PCR parameters for *Notch1* were: 25 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds. The sequences of the PCR primers for mouse *RANKL* were: upper 5′-GGTCGGGCAATTCTGAATT-3′ and lower 5′-GGGAATTACAAAGTGCACCAG-3′. The PCR parameters for *RANKL* were: 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds. The amplified products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide [27].

Western blot analysis

Cells grown in 35 mm^2 culture vessels were washed twice with phosphate buffered saline and lysed with TNTC buffer (100 mM Tris-Cl, pH 7.6; 150 mM NaCl; 1 mM $CaCl_2$; 1% Triton X-100 containing protease inhibitors (Complete; Roche)). The lysate was mixed with 2× loading buffer containing 40 mM DTT and boiled for two minutes. The proteins were electrophoresed in an 8% polyacrylamide gel containing SDS and transferred to a nitrocellulose membrane (Hybond-C Extra; Amersham Pharmacia Biotech). Protein detection was performed using anti-Notch1 cytoplasmic domain antibody (Upstate Biotechnology).

Measurement of alkaline phosphatase (ALP) activity and calcium deposition

ALP activity was measured using the ALP measurement kit (ALP-K Test; Wako Chemicals). The amount of calcium deposits on culture dishes was measured using the calcium measurement kit (Ca-E Test; Wako Chemicals). Protein concentrations were measured for normalization using the DC Protein Assay Kit (Bio-Rad).

Northern blot analysis

Ten micrograms of total RNA from each cell line were electrophoresed in a 1.2% agarose formaldehyde gel. The RNA was transferred to a charged nylon membrane (Hybond-N+; Amersham Pharmacia Biotech). cDNA of *Collagen type I* (*Col I*) and *Osteocalcin* (*OC*) were labeled with [α-^{32}P]dCTP using the Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech). Hybridization was performed overnight at 42°C and the membrane was thoroughly washed with 0.1× salt sodium citrate (15 mM NaCl and 1.5 mM trisodium citrate). Radioactive signals were

detected using a digital image analyzer (Bas2500; Fuji Photo Film). Densitometrical analysis was carried out using Photoshop 5.5 (Adobe). The mouse *OC* cDNA was provided by J.M. Wozney and the α2 chain of rat type I collagen cDNA was provided by C. Genoverse.

Luciferase activity assay

Fifty percent confluent cells were transiently transfected with *NICD*. The cells were harvested 48 h after the transfection and luciferase activity was measured, using the Dual Luciferase Reporter Assay System (Promega). To see the effect of Notch signal suppression, we used the *dnDl* (dominant negative *Delta*) construct, which can inhibit Notch signal transduction to *Hes1* pathway in a cell-autonomous manner [26]. All experiments were performed in triplicate and the *firefly* luciferase activity was normalized to the co-transfected *Renilla* luciferase activity (pRL-EF, a gift from Y. Mochida). Statistical data analysis was carried out using Excel 2000 (Microsoft). The *Ose2* elements and the *Cbfa1* promoter were provided by Sumitomo Pharmaceuticals Research Center [28].

In vitro mineralization assay

To promote mineralization, 0.2 mM of L-ascorbic acid-2-phosphate (AA) and 10 mM of β-glycerophosphate (βGP) were added to the culture medium after the cells reached confluence. Cells were left confluent for several days with or without AA and βGP to allow mineralization. Calcified nodules were stained with Alizarin Red S after methanol fixation. The amount of calcification was evaluated by digitally measuring the stained areas using a computer software (Scion Image; Scion Corporation).

In vivo osteogenesis assay

Cells were grown to confluence and trypsinized to collect 1×10^8 cells/ml in 100 μl of medium. The cells were subcutaneously injected into the abdomen of C3H/He mice with an 18G syringe. Thirty days after inoculation, the animals were sacrificed and soft X-ray photos were taken to detect calcification. The tissues originating from the injected cells were dissected and processed for histological analyses. Non-decalcified 4 μm thick sections were stained with hematoxylin and eosin, von Kossa's, ALP and TRAP (tartarate resistant acid phophatase) staining methods.

Results

Constitutively active Notch suppresses the expression of osteogenic marker genes in Kusa

In order to evaluate the stem cell property of Kusa, we established the stable transformants of the constitutively active form of *Notch1* (*NICD*) driven by the cytomegalovirus promoter in both KusaA and KusaO cell lines (KusaA^{NICD} and KusaO^{NICD}). RT-PCR using the primers set within the *NICD* region revealed endogenous expression of *Notch1* in the original KusaA and KusaO, along with robust PCR amplification due to *NICD* in the KusaA^{NICD} and KusaO^{NICD} (Fig. 1A). Western blot analysis showed an identical level of NICD protein in the KusaA^{NICD} and KusaO^{NICD}, but the endogenous Notch1 protein was far below the threshold of detection (Fig. 1A). In the *NICD* transformants, *HES1* promoter was activated more than 100 fold (Fig. 1B) of its basal level. The *NICD* transformants were morphologically indiscriminate from the original cells. Each cell line proliferated at the same rate, with a doubling time of about 20 h (data not shown). To evaluate the osteogenic potential, ALP activity and the expression of *Coll* and *OC* were examined under normal culture condition and mineralization-promoting condition by addition of ascorbic acid (AA) and β-glycerophosphate (βGP) to the medium. We found that *NICD* suppressed ALP activity and the expression of *OC* in KusaA, (Figs. 2A, 2C, and 2E). The basal level of ALP activity in KusaO with or without *NICD* is far less than that in KusaA (Fig. 2B). *Coll* expression in KusaO increased after confluence, especially under mineralization-inducing condition, but this upregulation of *Col I* was suppressed by

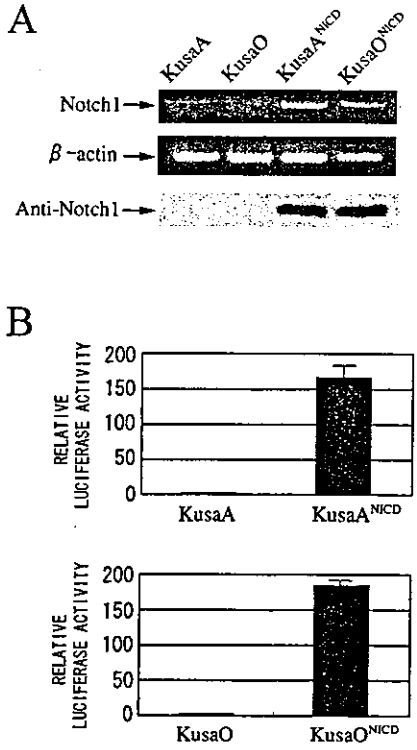


Fig. 1. Establishment of *NICD* stable transformants. (A) RT-PCR demonstrated the endogenous *Notch1* expression, and intense amplifications due to the integrated *NICD* in KusaA^{NICD} and KusaO^{NICD} (top row). Western blot analysis revealed the expression of 150 KDa NICD protein in KusaA^{NICD} and KusaO^{NICD}. The endogenous expression of Notch protein was below the threshold of detection (bottom row). (B) *HES1* promoter activity was significantly elevated in KusaA^{NICD} and KusaO^{NICD}.

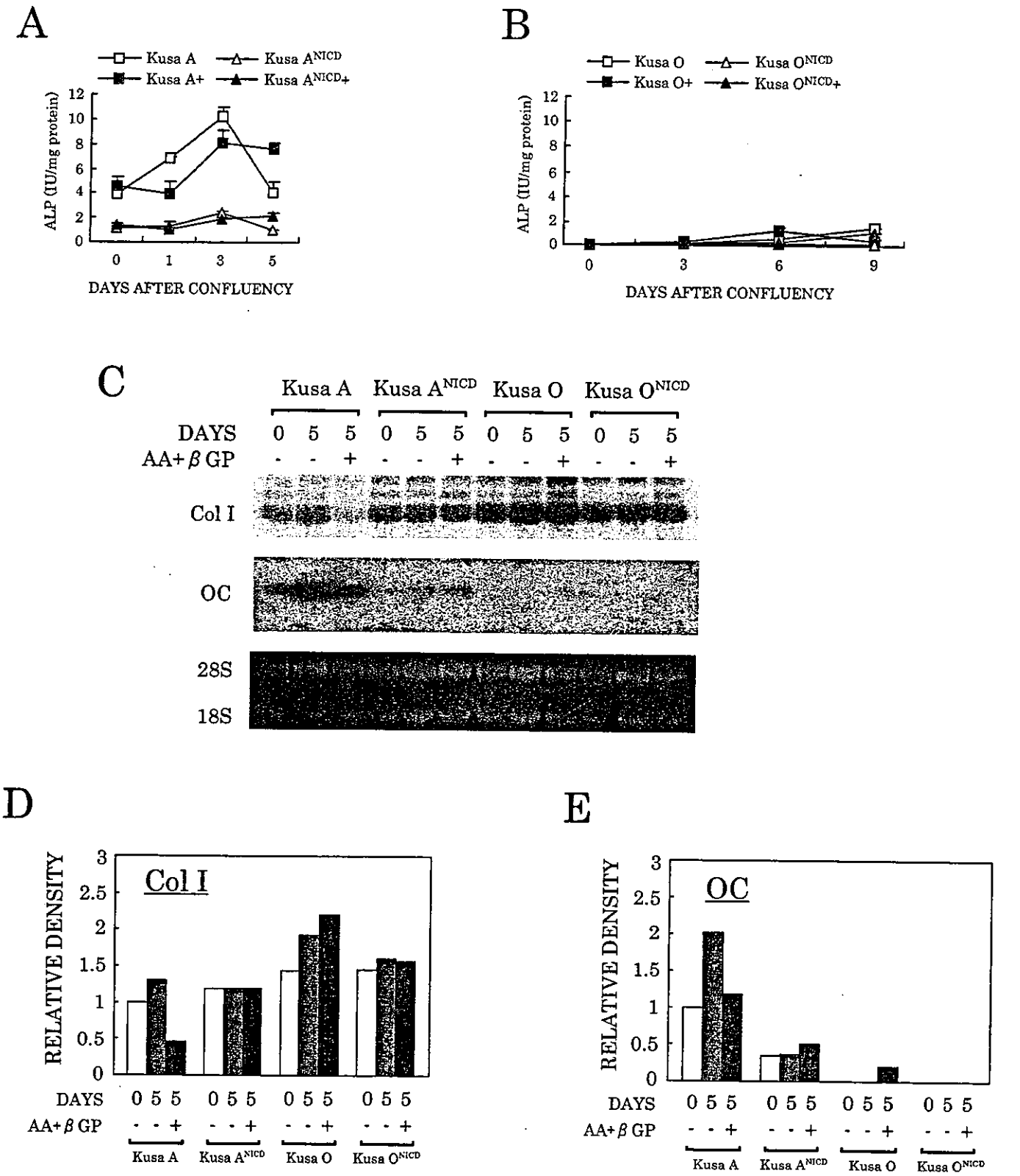


Fig. 2. Suppression of osteogenic properties in KusaA^{NICD} and KusaO^{NICD}. (A) Assay for alkaline phosphatase (ALP) activity. KusaA exhibited constantly high ALP activity, which was suppressed in KusaA^{NICD}. Cells cultured in the medium containing ascorbic acid and β-glycerophosphate are marked with a '+'. (B) ALP activity of KusaO was low with or without NICD. (C) Northern blot analysis of *Col I* and *OC*. (D, E) Densitometrical analysis of (C) shows the expression of *OC* in KusaA and *Col I* in KusaO was suppressed by NICD.

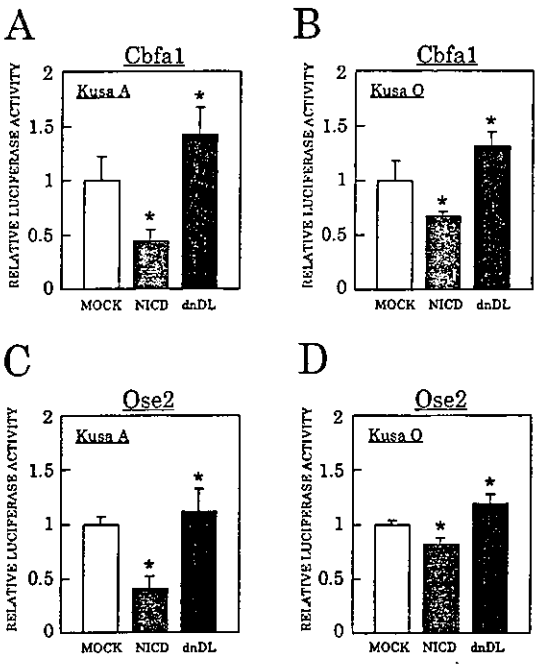


Fig. 3. Promoter activity assay for *Cbfa1* and *Ose2* elements in KusaA and KusaO with *NICD* transient transfection. (A–D) Promoter activities of *Cbfa1* and *Ose2* elements were reduced by *NICD* in both KusaA and KusaO. On the contrary, KusaA and KusaO transfected with an antagonist of Notch signaling, *dnDL* (*dominant negative Delta*), exhibited elevated promoter activities of *Cbfa1* and *Ose2*. Each bar represents the mean ± 1 SD.

NICD (Figs. 2C–D). KusaO slightly upregulated *OC* under the mineralization-inducing condition, which was not observed in KusaO^{NICD} (Figs. 2C and E).

Promoter activity of Cbfa1 and Ose2 was attenuated by NICD

Gene expression of *Col I* and *OC*, is partially under the control of *Ose2* elements in their promoters [29,30]. *Ose2* is a consensus binding site of Cbfa1 [31], which is a key transcription factor to regulate osteoblast differentiation and bone formation.

We examined the effect of Notch signal activation on the promoter activities of *Cbfa1* and *Ose2* in Kusa. The activity of *Cbfa1* promoter was attenuated by *NICD* both in KusaA and KusaO (Figs. 3A–B), indicating that the Notch signaling has an inhibitory effect on the transcription of *Cbfa1*. We also observed a modest increase of *Cbfa1* promoter activity by dnDl, implying that the expression of *Cbfa1* is being suppressed by endogenous Notch signaling in Kusa cells. Promoter activity of *Ose2* was also attenuated by *NICD* (Figs. 3C–D), which is consistent with the downregulation of *OC*, and the downregulation of *Col1* in KusaO, as shown in the northern analysis.

Calcium deposition and calcified nodule formation in vitro were reduced by NICD

In the presence of AA and βGP, calcium deposition gradually increased after confluence both in KusaA and KusaO, where KusaA exhibited a more rapid rise (Figs. 4A–B). KusaA^{NICD} and KusaO^{NICD} also showed a gradual increase in calcium deposition, but it was much slower and the amount was significantly reduced. Calcified nodules were formed only in the presence of AA and βGP. The amount of calcified nodules formed by KusaA^{NICD} was reduced to less than half of that formed by KusaA (Figs. 4C and E). KusaO also formed calcified nodules, although slower than KusaA. KusaO^{NICD} cells failed to form a calcified nodule (Figs. 4D–E).

In vivo bone formation of KusaA was suppressed by NICD

To assess the effect of Notch signaling to the osteogenic potential of Kusa in vivo, we subcutaneously injected the cultured cells into mouse abdomen and examined them after 30 days. In most cases of KusaA (9/10), the injected cells proliferated and formed a nodular mass about 7 mm in diameter that was readily identified and separated from the surrounding tissues. These masses contained calcified tissues that were observed as radiopaque foci (Figs. 5A–B). In KusaA^{NICD}, small patchy radiopaque structures were observed in some cases (4/10) (Figs. 5A and C) along with the other cases (6/10) that showed no radiopacity. In KusaO, no radiopaque image was observed with or without NICD (10/10, 10/10) (Fig. 5A).

In histology, KusaA showed a well-formed bone like structure (Fig. 6A), but KusaA^{NICD} showed small masses of calcification in fibrous tissues (Fig. 6B). In a magnified view, KusaA showed a fine structure of trabecular bone tissues (Fig. 6C), but KusaA^{NICD} showed amorphous calcified masses (Fig. 6D). The trabecular bone-like structures of KusaA were surrounded by ALP-positive spindle cells (Fig. 6E) and multinucleated TRAP-positive cells were observed adjacent to the trabecular (Fig. 6G). The other cell types such as nerve cells, capillary cells and myocytes, were not observed. No inflammatory cells were observed in the mass. KusaA^{NICD} also formed a mass that was easily separated from the surrounding tissues, but the formation of trabecular bone-like structures was not observed, except only a few small foci of amorphous calcification (Fig. 6D) that were surrounded by a few ALP-positive cells (Fig. 6F). TRAP-positive cells were not observed in the KusaA^{NICD}-derived tissues (Fig. 6H). The expression of *RANKL*, a factor of osteoclastogenesis secreted by osteoblastic cells, was detected by RT-PCR in KusaA, but was not observed in KusaA^{NICD} (Fig. 7).

Discussion

The process of osteogenesis can be divided into several steps, consisting of cell proliferation, extracellular matrix maturation and mineralization [32,33]. Initially, osteoblasts actively proliferate and produce extracellular matrix pro-

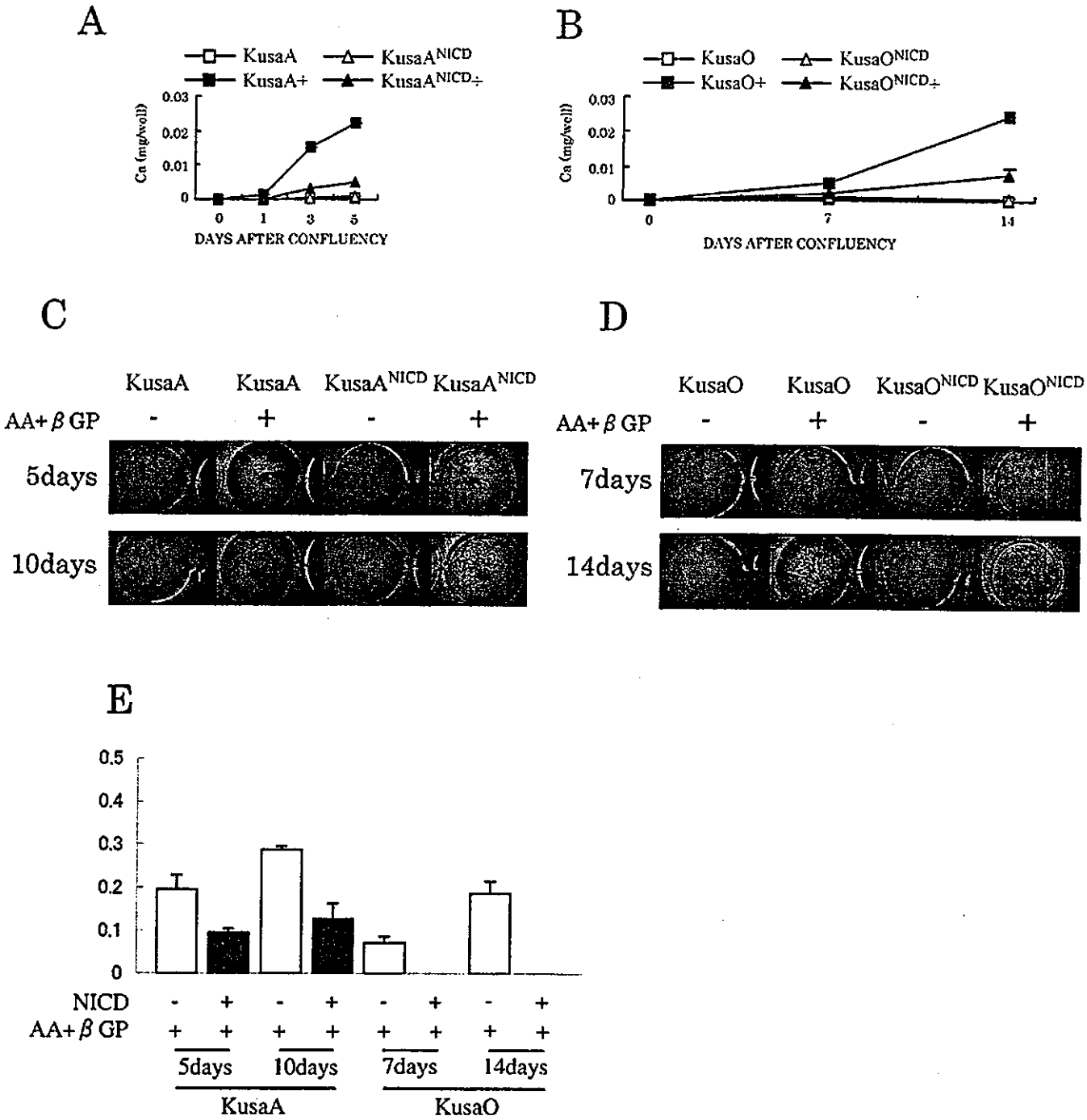


Fig. 4. In vitro calcification assay. (A, B) Quantification of calcium deposition on culture dish. '+' represents the addition of ascorbic acid and β-glycerophosphate to the culture medium (filled squares and triangles in the graph). (C, D) Calcification on culture dishes stained with Alizarin Red S. (E) Graphical representation of (C) and (D).

teins, such as fibronectin or Col I. Once the matrix has matured, osteoblasts cease to divide but become to actively engage in the synergetic synthesis of matrix proteins. This step is accompanied with enhanced expression of alkaline phosphatase (ALP) that is assumed to metabolize phosphate ions into insoluble phosphate salts, such as calcium phosphate [34,35]. Later, upregulation of *OC* and osteopontin, which are the major non-collagenous bone matrix proteins, contributes to the refinement of extracellular matrices for calcification.

We demonstrated that Notch signaling downregulated the early osteoblastic marker *Col I* in KusaO and the late osteoblastic marker *OC* in KusaA, implying that Notch signaling exerts an inhibitory effect at various stages of osteoblastic differentiation. In hematopoietic progenitor cells, *NICD* inhibits granulocyte differentiation and permits

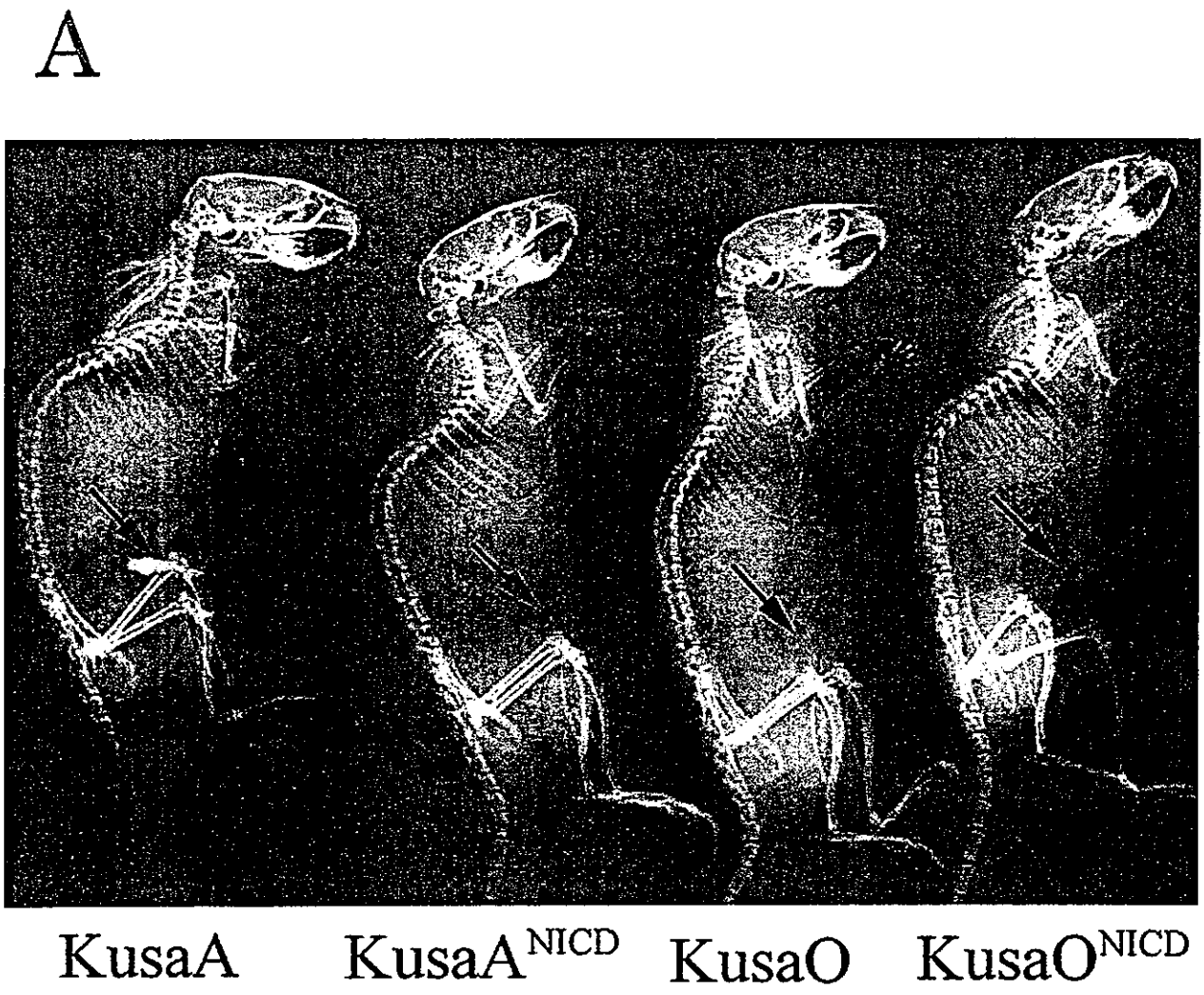
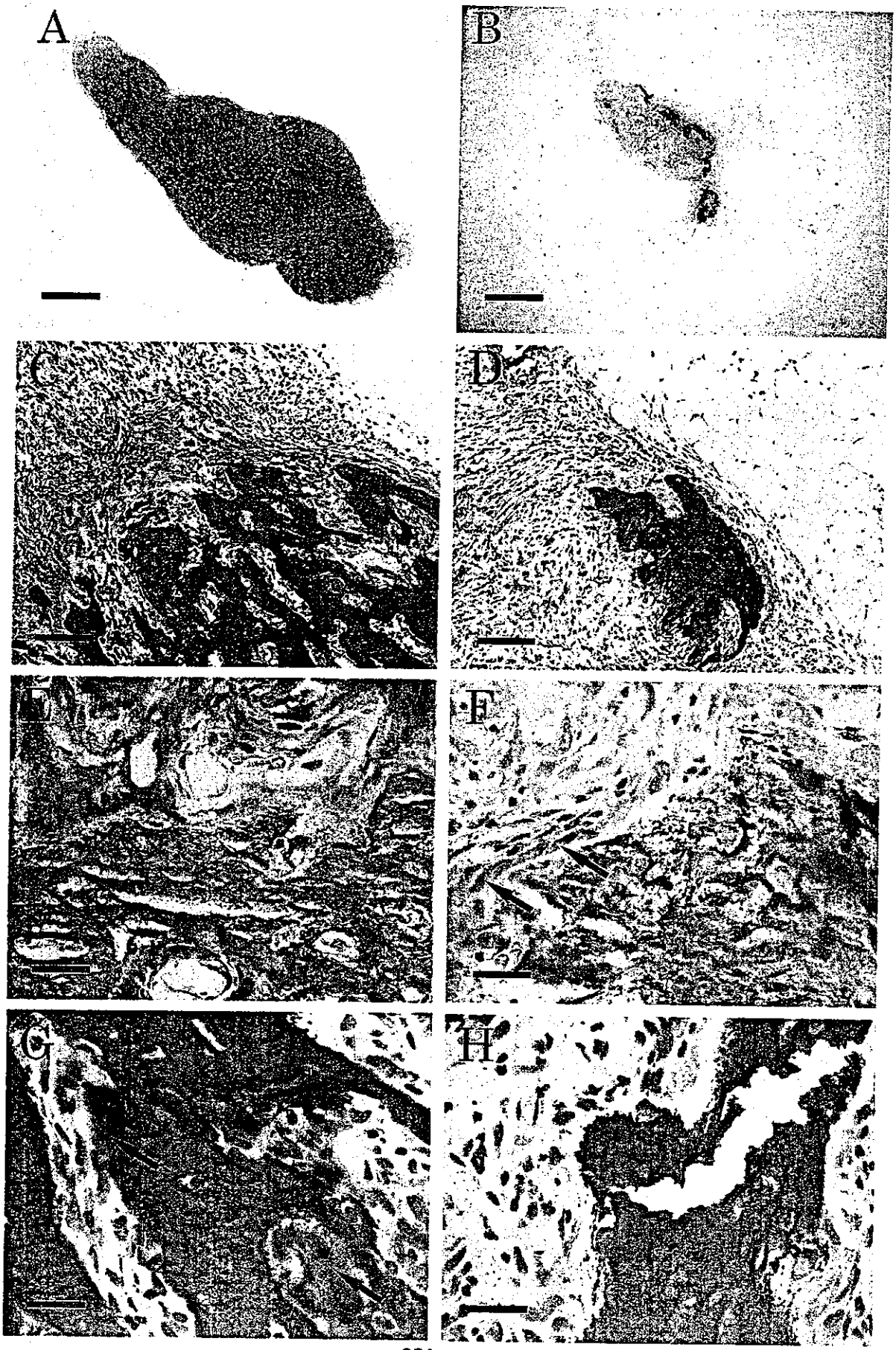


Fig. 5. Radiography of in vivo osteogenesis of Kusa cells. (A, B, C) Soft X ray photographs revealed that KusaA formed a distinct radiopaque mass in vivo, while KusaA^{NICD} formed a mass of the smaller size that is mainly radiolucent. KusaO and KusaO^{NICD} did not form a radio opaque mass.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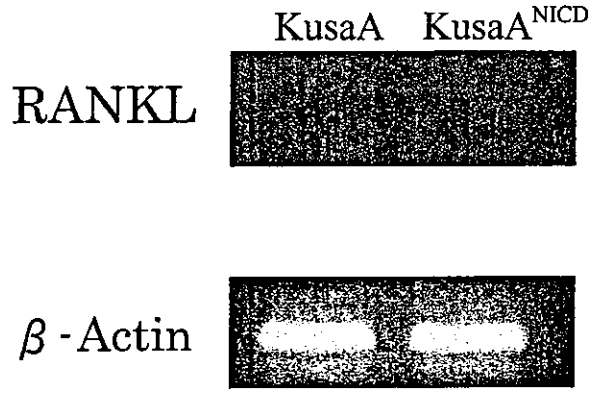

Fig. 7. *RANKL* expression in KusaA and KusaA^{NICD}. RT-PCR revealed expression of *RANKL* in KusaA, but not in KusaA^{NICD}.

the expansion of undifferentiated cells [14]. In neurogenesis, both neuronal and glial differentiation in vitro are enhanced by the attenuation of Notch signaling and suppressed by *NICD* [36,37]. These studies indicate that the activation of Notch signaling usually promotes the self-renewal of progenitor cells and inhibits their differentiation. In the present study, Notch signaling had little effect on Kusa cell proliferation, and the regulation of cell differentiation appeared to be its major role. The pattern of in vitro nodule formation was altered by *NICD*. The nodules of KusaA and KusaO had discrete margins, but those of KusaA^{NICD} and KusaO^{NICD} had ambiguous margins. This difference may reflect an activation of NICD, which inhibits "Salt and Pepper" pattern (an expression of lateral inhibition or lateral specification) formation.

Cbfa1 is a Runt-family transcription factor that acts as a key gene for osteoblast differentiation. Cbfa1 is a positive regulator of osteoblast-specific gene expression, where it can upregulate both *Col I* [29] and *OC* by binding to the *OSE2* elements in their promoters [31,38]. Although the relationship between Notch signaling and Cbfa1 has not been well studied, Hes1 has been reported to physically interact with Cbfa1 and cause a Cbfa1-dependent transactivation of the downstream genes [30,38]. These findings imply a possible connection between the Notch signaling pathway and Cbfa1-mediated osteogenesis. Transient expression of *NICD* in an osteoblastic cell line, MC3T3-E1 using an adenoviral vector reportedly led to upregulation of *Cbfa1* and even to increased calcified nodule formation in long-term cultures, but this effect appeared to be due to an accumulation in matrix proteins followed by delayed nodule formation [39]. Contrary to this report, we found that *NICD* modestly suppressed the promoter activity of *Cbfa1* and *Ose2*, and general suppression of osteogenesis in Kusa cells. Since the suppression of *Ose2* activity by *NICD* was also observed in COS7 cells (our unpublished data) that do not express *Cbfa1* [40], the negative effect of Notch signaling on osteogenesis may also occur in a Cbfa1-independent manner. In myogenesis, it has been postulated that the Notch signaling regulates the myoblasts differentiation by two different transduction pathways [41]. CBF1-dependent pathway appears to control the myogenic differentiation of C2C12, whereas Notch signaling in the absence of CBF1 blocks its myogenic and also osteogenic differentiation, indicating the presence of CBF1-independent pathway that leads to a pan-block of cell differentiation. The effect of CBF1 and its relationship with *Cbfa1* promoter in Kusa is now in the focus of interest.

By our preliminary experiments, slight expression of *Notch* and *Hes1* was detected, which indicates the endogenous activation of Notch signaling. Strong osteogenic properties of Kusa may be attributed to the suppression of endogenous Notch signaling by some mechanism. Interestingly, TRAP-positive osteoclasts were not observed in the bone-like tissues generated by KusaA^{NICD}. Osteoclast differentiation is in part triggered by factors from osteoblasts. RANKL, an osteoclast-differentiating factor, which belongs to the tumor necrosis factor ligand gene family, has been found in cells of osteoblastic lineage [42]. Osteoclast progenitors express RANK, a receptor of RANKL and differentiate into osteoclasts through cell-cell interactions with osteoblasts [43,44]. The *RANKL* expression was suppressed in KusaA^{NICD}, implying that this suppression resulted in the loss of osteoclast induction in vivo. Two putative Cbfa1 binding sites exist in the promoter region of *RANKL*, and upregulation of *RANKL* was observed following treatment with vitamin D3, which was accompanied with *Cbfa1* upregulation [45].

Downregulation of *RANKL* in KusaA^{NICD} may be due to the low activation of *Cbfa1*. However, a conflicting result has also been reported that *Cbfa1* overexpression had no effect on upregulation of *RANKL* [46]. The association of Notch signaling with Cbfa1 pathway remains to be elucidated.

In conclusion, we demonstrated that the constitutively active form of Notch1 suppresses the osteoblastic differentiation and osteoclastogenesis of Kusa cells, suggesting that the Notch signaling negatively regulates the bone formation and its refinement. Our findings reinforce the possibility of a new therapeutic strategy for the treatment of bone diseases

Fig. 6. In vivo osteogenesis of KusaA and KusaA^{NICD}. Histology of KusaA-derived tissues (A) and KusaA^{NICD}-derived tissues (B). (C) Higher magnification of (A) shows fibrous tissues with trabecular bone-like tissues. (D) Higher magnification of (B) shows fibrous tissues and a small mass of calcification without a trabecular structure. (E) ALP staining showed that bone-like tissues derived from KusaA were surrounded by numerous spindle shaped cells positive for ALP (colored in purple), implying the presence of osteoblasts. (F) Calcified foci derived from KusaA^{NICD} cells were surrounded by a few spindle shaped cells positive for ALP (arrows). (G) Multinuclear TRAP-positive cells (arrows) appeared adjacent to the bone-like tissues derived from KusaA, implying the presence of osteoclasts. (H) No TRAP-positive cells were observed in the KusaA^{NICD}-derived tissues Scale bar—A, B: 1 mm, C, D: 250 μm, E, F: 125 μm.

such as osteoporosis by artificially modifying the Notch signaling pathway.

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Islet cell hyperplasia in transgenic mice overexpressing EAT/mcl-1, a bcl-2 related gene

Kenichi Matsushita [a,b], Hajime Okita [a], Atsushi Suzuki [a], Kouji Shimoda [c], Mariko Fukuma [a], Taketo Yamada [a], Fumihiko Urano [a], Takahiro Honda [a], Makoto Sano [a], Shiro Iwanaga [b], Satoshi Ogawa [b], Jun-ichi Hata [a,1], Akihiro Umezawa [a,1,*]

[a] *Department of Pathology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan*
[b] *Department of Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan*
[c] *Laboratory Animal Center, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan*

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Abstract

EAT/mcl-1 (EAT), a bcl-2 related anti-apoptotic gene, is up-regulated at the early stage of differentiation of human embryonal carcinoma cells; cells which serve as a model for early embryogenesis. We generated transgenic mice for the human EAT gene driven by the EF1α promoter in order to elucidate its functional role in vivo. Histologically, these mice exhibited hyperplasia of Langerhans islet cells; pancreatic cell regions composed of both insulin- and glucagon-producing cells. Furthermore, Bax and Bag-1—possible heterodimeric partners for EAT in the anti-apoptotic process—were up-regulated in islets isolated from the EAT transgenic mice. The insulin tolerance test exhibited no significant difference between the EAT transgenic mice and non-transgenic mice, indicating that islet cell hyperplasia was not due to insulin resistance. In conclusion, EAT transgenic mice exhibit hyperplasia of pancreatic β cells. EAT may inhibit apoptosis of β cells, allowing these cells to circumvent the process of apoptosis until the adult stage.

Keywords: Apoptosis; EAT; Islet β-cell; mcl-1; Transgenic mice

1. Introduction

We isolated human EAT/mcl-1 (EAT), a bcl-2 related gene, as a gene up-regulated at an early stage of differentiation of an embryonal carcinoma (EC) cell line designated NCR-G3 (Umezawa et al., 1996). Human EC cell lines serve as model systems for early human embryogenesis based upon their multiple differentiation potential. NCR-G3, derived from a testicular EC, differentiates into multiple lineages, including trophectoderm cells, following exposure to retinoic acid (Hata et al., 1992). Human EAT (hEAT) was originally identified as mcl-1, a gene whose expression is induced during differentiation of myeloid leukemia cells (Kozopas et al., 1993); however, we further established that it is likewise up-regulated during early differentiation of EC cells (Umezawa et al., 1996). Recently, we also cloned a murine orthologue of hEAT (murine EAT, mEAT) (Okita et al., 1998).

The EAT gene is considered to be a member of the bcl-2 related gene family based upon its possession of the bcl-2 homology (BH) domains 1, 2, 3 and 4 (Bingle et al., 2000; Kroemer, 1997; Revilla et al., 1997). Bcl-2-related genes display either positive or negative regulatory effects on apoptosis in vitro and in vivo (Adams and Cory, 1998; Chao and Korsmeyer, 1998; Reed, 1998). Previous studies have established that Bcl-2, Bcl-xL, Bcl-w, Bfl-1 and A1 are anti-apoptotic molecules while Bax, Bak, Bcl-xS, Bad, Bid, Bik and Hrk are pro-apoptotic molecules (Boise et al., 1993; Chittenden et al., 1995; D'Sa-Eipper et al., 1996; Gibson et al., 1996; Inohara et al., 1997; Lin et al., 1996; Wang et al., 1996;

* Corresponding author. Tel.: +81-3-5363-3764; fax: +81-3-3353-3290.
E-mail address: umezawa@1985.jukuin.keio.ac.jp (A. Umezawa).
[1] Present address: The National Center for Child Health and Development Research Institute, Tokyo 154-8567, Japan.

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