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Expression and Function of Periostin-Isoforms in Bone

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Abstract Periostin was originally identified in MC3T3-E1 osteoblast-like cells. We have identified an isoform of periostin referred to as periostin-like-factor (PLF). It is homologous to other proteins such as fasciclin I (fas I), MPB70, βIG-H3, and Algal-CAMs. All of these proteins are implicated in regulating cell adhesion. PLF and the other isoforms of periostin differ in their C-terminal sequences. PLF and periostin differ in two specific regions, between 673 and 699 amino acids (aa) and 785–812 aa. Periostin isoforms are expressed in vivo and in vitro during the stages of osteoblast differentiation and maturation. Their mRNAs are present in pre-osteoblast cells as detected by in situ hybridization, and the proteins are between 86 and 93 kD in size as determined by Western blot analysis. Antisense oligonucleotides and antibodies directed against the isoforms of periostin were used to block the activity of these proteins. In both cases, the levels of osteoblast-specific-differentiation markers were markedly reduced suggesting a role for these proteins in osteoblast differentiation. J. Cell. Biochem. 92: 1044–1061, 2004.

Key words: bone; periostin isoforms; MC3T3-E1; osteoblast differentiation

Understanding the role of molecules in signaling pathways that regulate the process of osteoblast differentiation is the focus of our research. We identified an isoform of periostin using the READS technique to isolate differentially expressed mRNAs during murine development. Many proteins expressed in bone including cbfa1, alkaline phosphatase, and collagen are present as multiple isoforms, each isoform contributing independently to the structure and/or function of bone. Because isoforms clearly have distinct roles in tissues and organs, we pursued our studies to determine the pattern of expression of periostin isoforms during bone development and suggest possible functions for this molecule.

Osteoblast-specific factor 2 (OSF2), also referred to as periostin, was first identified using subtractive hybridization techniques on MC3T3-E1 osteoblast-like cells and was thought at that time to be bone-specific [Takeshita et al., 1993]. Periostin/OSF2 has a signal peptide, is secreted into the matrix, and regulates MC3T3-E1 cell adhesion, an event that is essential and requisite for differentiation of osteoblasts [Horiuchi et al., 1999]. It is approximately 90 kD, does not contain a transmembrane domain and has four potential *N*-glycosylation sites that are not glycosylated in bone cells [Horiuchi et al., 1999]. Isoforms of periostin have been identified in mice and humans and are over-expressed by stromal cells in several human ovary, breast, colon, and brain tumors [Terasaka et al., 1989; Skonier et al., 1992; Huber and Sumper, 1994; LeBaron et al., 1995; Ulstrup et al., 1995; Lal et al., 1999; Sasaki et al., 2001]. In osteoblasts and tumors,

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periostin supports cellular adhesion and spreading in vitro [Horiuchi et al., 1999; Gillan et al., 2002]. It has been shown that purified recombinant periostin supports adhesion of ovarian epithelial cells that can be inhibited by monoclonal antibodies against $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin, but not by anti-β_1 integrin antibody. Furthermore, $\alpha_v\beta_3$ integrin, but not β_1 integrins have been shown to co-localize at focal adhesion plaques formed on periostin in CSOC cells (ovarian epithelial cells). Cells plated on periostin form fewer stress fibers and are more motile compared to those plated on fibronectin [Gillan et al., 2002].

A similar molecule identified in insects, fasciclin I (fas I), is homologous to periostin [Zinn et al., 1988]. It is involved in axonal guidance, providing a pathway from a neuron to its target. Two forms of fas I have been described. They are 48% identical, approximately 70 kD, are spatially and temporally regulated during embryonic development and are proposed as regulators of neural cell adhesion [Hortsch and Goodman, 1990]. fas I is associated with the cell membrane and may be involved in cell–cell interactions [Zinn et al., 1988; Hortsch and Goodman, 1990; McAllister et al., 1992; Hu et al., 1998]. The fas I homozygous *Drosophila* mutant [McAllister et al., 1992] showed that fas I not only mediated adhesion and guidance of axons, but was also involved in signal transduction pathways necessary for axonal guidance and growth cone extension. Other proteins homologous to periostin identified in many other systems are believed to be critical mediators of differential adhesivity and attachment. For example, (a) MPB70 which may be significant in the interaction of bacteria and host cells [Terasaka et al., 1989; Ulstrup et al., 1995], (b) βIG-H3 which is implicated in mediating growth and differentiation of cells when stimulated with TGF-β [Skonier et al., 1992; LeBaron et al., 1995], and (c) Algal-CAMs involved in cell adhesion in the alga Volvox [Huber and Sumper, 1994].

Periostin and fas I contain four homologous domains referred to as 'Repeat Domains' each containing 150 amino acids (aa), and an *N*-terminal signal sequence. Within each Repeat Domain there are two regions of sequence that are highly conserved and as suggested by Horiuchi et al. [1999] may be involved in protein–protein interactions. The function of many of these motifs has not been tested and, therefore, one cannot make any structure–function correlations. Because so little is known about the function of the periostin isoforms in bone, we examined whether periostin isoforms play a role in osteoblast differentiation and function.

In addition, we identified periostin-like-factor (PLF), an isoform of periostin, by the 'READS' differential display of mRNAs isolated at various stages during mouse heart development. The periostin isoforms including PLF are also expressed in bone. Based on their pattern of expression and sequence analysis we have chosen to study PLF and the related periostin isoforms in bone. PLF is related to OSF2/periostin but differs in its *C*-terminal sequence, suggesting that it is an alternatively spliced isoform. The findings presented here focus on the expression and putative function(s) of periostin isoforms in osteoblast development and function.

MATERIALS AND METHODS

Identification of PLF

Mouse embryos were isolated at 7.5 day post-conception (pc) [Downs and Davies, 1993; Auda-Boucher et al., 2000] and carefully dissected away from the closely attached placental and uterine tissues. Hearts were collected from 13.5 day pc embryos, neonatal, and adult mice. Total RNA was prepared using TRIZOL (Invitrogen, Carlsbad, CA). These RNAs were used in the 'READS' technique [Prashar and Weissman, 1999] to identify cDNAs that were differentially expressed in embryos at 7.5 day pc, 13.5 day pc, neonatal and adult heart tissue. Briefly, double-stranded cDNA was digested by restriction enzymes and ligated to an adapter sequence that allowed for PCR amplification of 3′-end fragments of the cDNAs. The amplified cDNAs were separated on native polyacrylamide gels. Differentially expressed cDNA were excised, amplified, sequenced, and analyzed for homology in GenBank.

Cloning of the Full-Length PLF cDNA

The PLF READS EST (expressed sequence tag) of 169 nucleotides was 99% identical over its entire length to the published sequence of Periostin (GenBank accession no.: NM_015784). To obtain the full-length cDNA, we extracted total RNA from 1-day-old neonatal mouse heart tissue solubilized in TRIZOL. Oligo-dT primed first strand cDNA was generated from this RNA

and used to amplify the full-length PLF cDNA. The 3′ oligonucleotide primer (5′GAGAGAAAACATTTGTATTGCAAGAAGC) was designed based on the READS EST sequence. The 5′ end oligonucleotide primer (5′GGCTGAAGATGGGGCTGAAGATGGTTCCTCTCCTGC) was homologous to the sequence at the 5′ end of the published OSF-2/Periostin. For PCR amplification, the XL-Gene Amp PCR kit (Applied Biosystems, Foster City, CA) was used with the following parameters: initial denaturation at 94°C for 1 min, followed by 25 cycles of denaturation at 94°C for 15 s, and annealing and extension at 66°C for 10 min, and finally extension at 72°C for 10 min. The full-length PLF cDNA was ligated into the pgem-3zf- vector (Promega, Madison, WI), and was sequenced in both directions to prevent errors in the nucleic acid sequence.

RT-PCR of Periostin and PLF

To determine the temporal expression of periostin and PLF during bone development and in MC3T3-E1 cells, RNA from embryonic day 13.5 calvaria and MC3T3-E1 cells on days 7, 14, and 21 were analyzed by RT-PCR. For amplification the following specific primers were used: Primer no. 1 (P1) forward: 5′-GATAAAATACATCCAAATCAAGTTTGCTCG-3′, P2 reverse: 5′-CGTGGATCACTTCTGTCACCGTTTCGC-3′, P3 forward: 5′-CTGAAAAACAGACTCGGGAAGAACG-3′, and P4 reverse: 5′-AAACTCTGTGGTCTGGCCTCTGGG-3′. These primers flank regions of difference between PLF and periostin (Fig. 1D). The amplification parameters were as follows: initial denaturation at 94°C for 3 min followed by 25 cycles of denaturation at 94°C for 30 s, and annealing and extension at 67.7°C for 45 s, and a final extension at 72°C for 10 min. Amplified PCR products were separated on 3% NuSeive 3:1 agarose gels. The DNA bands were excised sequenced and analyzed for homologues.

Northern Blot Analysis

Tissues or cells were collected and solubilized in TRIZOL for isolation of total RNA. Ten micrograms of total RNA were separated on 1% formaldehyde-denatured agarose gels, transferred to Nytran membranes, and probed with radiolabeled PLF full-length cDNA. We were interested in examining the expression of the periostin isoforms. Therefore, a PLF-specific region was not used. The probe used was expected to recognize all of the isoforms of periostin. The Nytran was exposed to X-Ray film and the image analyzed by densitometry to determine the level of mRNA. In order to adjust for equal loading of RNA in each lane the blots were stripped and re-probed with an 18S rRNA radiolabeled cDNA probe, and the amount of mRNA was represented as a ratio of mRNA/18S rRNA.

In Situ Hybridization

Mouse embryos were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned. Seven μm sections were deparaffinized and rehydrated. Sections were processed as described previously [Wilkinson, 1995; Redkar et al., 2001]. Briefly, sections were treated with 10 μg/ml proteinase K for 10 min at 37°C, re-fixed in glutaraldehyde, prehybridized, and then hybridized with the digoxygenin labeled PLF anti-sense (AS) riboprobe at 55°C (generated as recommended by the manufacturer: Boehringer Mannheim Biochemica, Indianapolis, IN). The probe used was expected to recognize all of the isoforms of periostin. Following the substrate reaction, stained sections were photographed using a Nikon microscope. Controls included tissues treated with a digoxygenin-labeled- sense probe.

Generation of Anti-Periostin Isoform Polyclonal Antibody

A peptide (KKIPANKRVQGPRRRSREGRSQ) present in all known isoforms of periostin, located at the carboxy-terminus of the PLF aa sequence was used to generate polyclonal antibodies (Invitrogen). The antibody was affinity purified using the antigenic peptide.

Western Blot Analysis

Long bones (tibiae and femurs) and calvaria were harvested from embryos between 13.5 and 19.5 days pc and neonates. Bone powder was homogenized in RIPA protein extraction buffer (50 mM Tris-HCl, pH 7.5; 135 mM NaCl; 1% Triton X-100; 0.1% sodium deoxycholate; 2 mM EDTA; 50 mM NaF; 2 mM sodium orthovanadate; 10 μg/ml aprotinin; 10 μg/ml leupeptin; and 1 mM PMSF). For MC3T3-E1 cells, plates were rinsed three-times with ice-cold PBS and the cell layers were then harvested into ice-cold RIPA buffer. Homogenates were incubated for 60 min at 4°C on a rocker, centrifuged, and supernatants were collected and stored at

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                                  151                                 200
NM_015784 RefSeq mouse Periostin  IRRGLENNVN VELLNALHSH MVNKRMLTKD LKHGMVIPSM YNNLGLFINH
Mouse PLF                         IRRGLENNVN VELLNALHSH MVNKRMLTKD LKHGMVIPSM YNNLGLFINH
NM_006475 RefSeq human Periostin  IRRGLESNVN VELLNALHSH MINKRMLTKD LKNGMIIPSM YNNLGLFINH
BC007141      Truncated sequence  IRRGLENNVN VELLNALHSH MVNKRMLTKD LKHGMVIPSM YNNLGLFY..

                                  201                                 250
NM_015784 RefSeq mouse Periostin  YPNGVVTVNC ARVIHGNQIA TNGVVHVIDR VLTQIGTSIQ DFLEAEDDLS
                      Mouse PLF   YPNGVVTVNC ARVIHGNQIA TNGVVHVIDR VLTQIGTSIQ DFLEAEDDLS
NM_006475 RefSeq human Periostin  YPNGVVTVNC ARIIHGNQIA TNGVVHVIDR VLTQIGTSIQ DFIEAEDDLS
BC007141      Truncated sequence  .......... .......... .......... .......... ..........

                                  251                                 300
NM_015784 RefSeq mouse Periostin  SFRAAAITSD LLESLGRDGH FTLFAPTNEA FEKLPRGVLE RIMGDKVASE
                      Mouse PLF   SFRAAAITSD LLESLGGDGH FTLFAPTNEA FEKLPRGVLE RIMGDKVASE
NM_006475 RefSeq human Periostin  SFRAAAITSD ILEALGRDGH FTLFAPTNEA FEKLPRGVLE RFMGDKVASE
BC007141      Truncated sequence  .......... .......... .......... .......... ..........

                                  301                                 350
NM_015784 RefSeq mouse Periostin  ALMKYHILNT LQCSEAITGG AVFETMEGNT IEIGCEGDSI SINGIKMVNK
                      Mouse PLF   ALMKYHILNT LQCSEAITGG AVFETMEGNT IEIGCEGDSI SINGIKMVNK
NM_006475 RefSeq human Periostin  ALMKYHILNT LQCSESIMGG AVFETLEGNT IEIGCDGDSI TVNGIKMVNK
BC007141       Truncated sequnce  .......... .......... .......... .......... ..........

                                  351                                 400
NM_015784 RefSeq mouse Periostin  KDIVTKNGVI HLIDEVLIPD SAKQVIELAG KQQTTFTDLV AQLGLASSLK
                      Mouse PLF   KDIVTKNGVI HLIDEVLIPD SAKQVIELAG KQQTAFTDLV AQLGLASSLK
NM_006475 RefSeq human Periostin  KDIVTNNGVI HLIDQVLIPD SAKQVIELAG KQQTTFTDLV AQLGLASALR
BC007141      Truncated sequence  .......... .......... .......... .......... ..........

                                  401                                 450
NM_015784 RefSeq mouse Periostin  PDGEYTLLAP VNNAFSDDTL SMDQRLLKLI LQNHILKVKV GLSDLYNGQI
                      Mouse PLF   PDGEYTLLAP VNNAFSDDTL SMDQRLLKLI LQNHILKVKV GLSDLYNGQI
NM_006475 RefSeq human Periostin  PDGEYTLLAP VNNAFSDDTL SMVQRLLKLI LQNHILKVKV GLNELYNGQI
BC007141      Truncated sequence  .......... .......... .......... .......... ..........

                                  451                                 500
NM_015784 RefSeq mouse Periostin  LETIGGKQLR VFVYRTAICI ENSCMVRGSK QGRNGAIHIF REIIQPAEKS
                      Mouse PLF   LETIGGKQLR VFVYRTAICI ENSCMVRGSK QGRNGAIHIF REIIQPAEKS
NM_006475 RefSeq human Periostin  LETIGGKQLR VFVYRTAVCI ENSCMEKGSK QGRNGAIHIF REIIKPAEKS
BC007141      Truncated sequence  .......... .......... .......... .......... ..........

                                  501                                 550
NM_015784 RefSeq mouse Periostin  LHDKLRQDKR FSIFLSLLEA ADLKDLLTQP GDWTLFAPTN DAFKGMTSEE
                      Mouse PLF   LHDKLRQDKR FSIFLSLLEA ADLKDLLTQP GDWTLFAPTN DAFKGMTSEE
NM_006475 RefSeq human Periostin  LHEKLKQDKR FSTFLSLLEA ADLKELLTQP GDWTLFVPTN DAFKGMTSEE
BC007141      Truncated sequence  .......... .......... .......... .......... ..........

                                  551                                 600
NM_015784 RefSeq mouse Periostin  RELLIGDKNA LQNIILYHLT PGVYIGKGFE PGVTNILKTT QGSKIYLKGV
                      Mouse PLF   RELLIGDKNA LQNIILYHLT PGVYIGKGFE PGVTNILKTT QGSKIYLKGV
NM_006475 RefSeq human Periostin  KEILIRDKNA LQNIILYHLT PGVFIGKGFE PGVTNILKTT QGSKIFLKEV
BC007141      Truncated sequence  .......... .......... .......... .......... ..........

                                  601                                 650
NM_015784 RefSeq mouse Periostin  NETLLVNELK SKESDIMTTN GVIHVVDKLL YPADIPVGND QLLELLNKLI
                      Mouse PLF   NETLLVNELK SKESDIMTTN GVIHVVDKLL YPADIPVGND QLLELLNKLI
NM_006475 RefSeq human Periostin  NDTLLVNELK SKESDIMTTN GVIHVVDKLL YPADTPVGND QLLEILNKLI
BC007141      Truncated sequence  .......... .......... .......... .......... ..........

                                  651                                 700
NM_015784 RefSeq mouse Periostin  KYIQIKFVRG STFKEIPMTV YR........ .......... .........P
                      Mouse PLF   KYIQIKFARG STFKEIPMTV YTTKIITKVV EPKIKVIQGS LQPIIKTEGP
NM_006475 RefSeq human Periostin  KYIQIKFVRG STFKEIPVTV YTTKIITKVV EPKIKVIEGS LQPIIKTEGP
BC007141      Truncated sequence  .......... .......... .......... .......... ..........

                                  701                                 750
NM_015784 RefSeq mouse Periostin  AMTKIQIEGD PDFRLIKEGE TVTEVIHGEP VIKKYTKIID GVPVEITEKQ
                      Mouse PLF   AMTKIQIEGD PDFRLIKEGE TVTEVIHGEP VIKKYTKIID GVPVEITEKQ
NM_006475 RefSeq human Periostin  TLTKVKIEGE PEFRLIKEGE TITEVIHGEP IIKKYTKIID GVPVEITEKE
BC007141      Truncated sequence  .......... .......... .......... .......... ..........

                                  751                                 800
NM_015784 RefSeq mouse Periostin  TREERIITGP EIKYTRISTG GGETGETLQK FLQKEVSKVT KFIEGGDGHL
                      Mouse PLF   TREERIITGP EIKYTRISTG GGETGETLQK FLQK...... ..........
NM_006475 RefSeq human Periostin  TREERIITGP EIKYTRISTG GGETEETLKK LLQEEVTKVT KFIEGGDGHL
BC007141      Truncated sequence  .......... .......... .......... .......... ..........

                                  801                          838
NM_015784 RefSeq mouse Periostin  FEDEEIKRLL QGDTPAKKIP ANKRVQGPRR RSREGRSQ
                      Mouse PLF   .......... ..DTPAKKIP ANKRVQGPRR RSREGRSQ
NM_006475 RefSeq human Periostin  FEDEEIKRLL QGDTPVRKLQ ANKKVQGSRR RLREGRSQ
BC007141      Truncated sequence  .......... .......... .......... ........
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Fig. 1. **A**: Alignment of the predicted amino acid (aa) sequence of periostin protein isoforms in the region containing most of the differences between 151 and 838 aa. The aa's between 673 and 699 (red) are present in periostin-like-factor (PLF) and those between 785–812 (yellow) in periostin. Each isoform is designated by the accession number in GenBank. BC007141 is truncated at aa 199. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

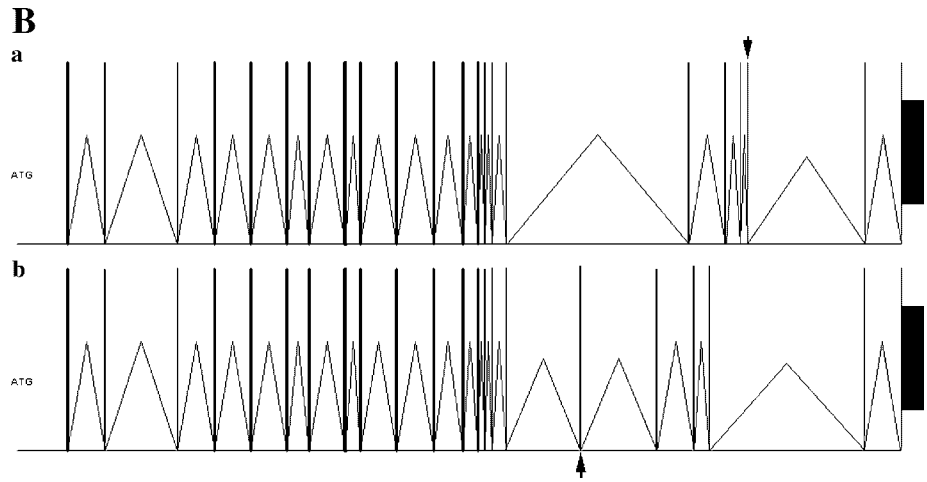

Fig. 1. **B**: The exon–intron arrangement for periostin (**a**) and PLF (**b**). The arrow in (a) corresponds to exon 21 and that in (b) corresponds to exon 17.

−80°C. Total protein concentration was measured using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

Protein samples were mixed with an equal volume of 2× SDS sample buffer, boiled for 10 min, and subjected to 8% SDS–PAGE and Western blot analysis as described previously [Tokuda et al., 2003]. The nitrocellulose membrane was blocked with 5% milk, Tris-buffered saline (TBS)-0.2% Tween-20 (TTBS) for 2 h, and incubated with primary antibody (0.5 μg/ml) for 2 h. The blot was then washed three-times in TTBS and incubated with 0.1 μg/ml of HRP-conjugated goat anti-rabbit IgG secondary antibody (Pierce) for 2 h at room temperature. Signal was detected by ECL (Pierce).

Cell Culture

Primary osteoblast cultures. Primary osteoblast cultures were established using neonatal rats as previously described [Selim et al., 2003]. Isolated cells were plated at 5×10^5 cells/100 mm plate in MEM (Earle's) containing 10% fetal bovine serum (FBS, Invitrogen). On the third day of culture, the initial plating medium was replaced with MEM containing 10% FBS, 10 μM β-glycerophosphate and ascorbic acid (25 μg/ml) (Sigma, St. Louis, MO).

MC3T3-E1 osteoblast cultures. The mouse osteoblast cell line MC3T3-E1 was obtained from ATCC (Manasass, VA) and routinely maintained in growth medium consisting of αMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. On the third day of culture 25 μg/ml ascorbic acid and 10 μM β-glycerophosphate were added to the medium and media was changed every 3–4 days [Owen et al., 1990].

Treatment of MC3T3-E1 cells with AS oligonucleotides. Equal numbers of MC3T3-E1 cells were transfected as previously described [Bonnelye et al., 2001]. Briefly, cells were transfected on days 0, 5, 10, and 15 with 0–5 μM AS periostin-oligonucleotide 5′-GAGAGGAACCATCTTCAGCCCTGAGCTCCG-3′ and using oligofectamine as directed by the manufacturer (Invitrogen). During the transfection period of 4 h, cells were grown in OPTI-MEM serum-free media (Invitrogen) after which, cells were grown in αMEM containing 10% FBS, ascorbic acid (50 μg/ml) and α-glycerophosphate (10 mM). Cells were harvested on days 7, 14, and 21, and extracted with TRIZOL for total RNA. DNase-treated total RNA was used for RT-PCR.

Treatment of MC3T3-E1 cells with anti-periostin isoform antibody. For antibody transfection, MC3T3-E1 cells were cultured as described above and treated as described previously [Selim et al., 2003]. Briefly, when cells reached 50–60% confluence, they were transfected with a 5 or 2.5 μg/ml of anti-periostin antibody using Chariot reagent according to the manufacturer's protocol (Activemotif, Carlsbad, CA). Control cells were either untreated or transfected with non-immune IgG and Chariot reagent. For evaluation of transfection efficiency, MC3T3-E1 cells were transfected with β-galactosidase (β-gal), fixed and stained according to the manufacturer's protocol.

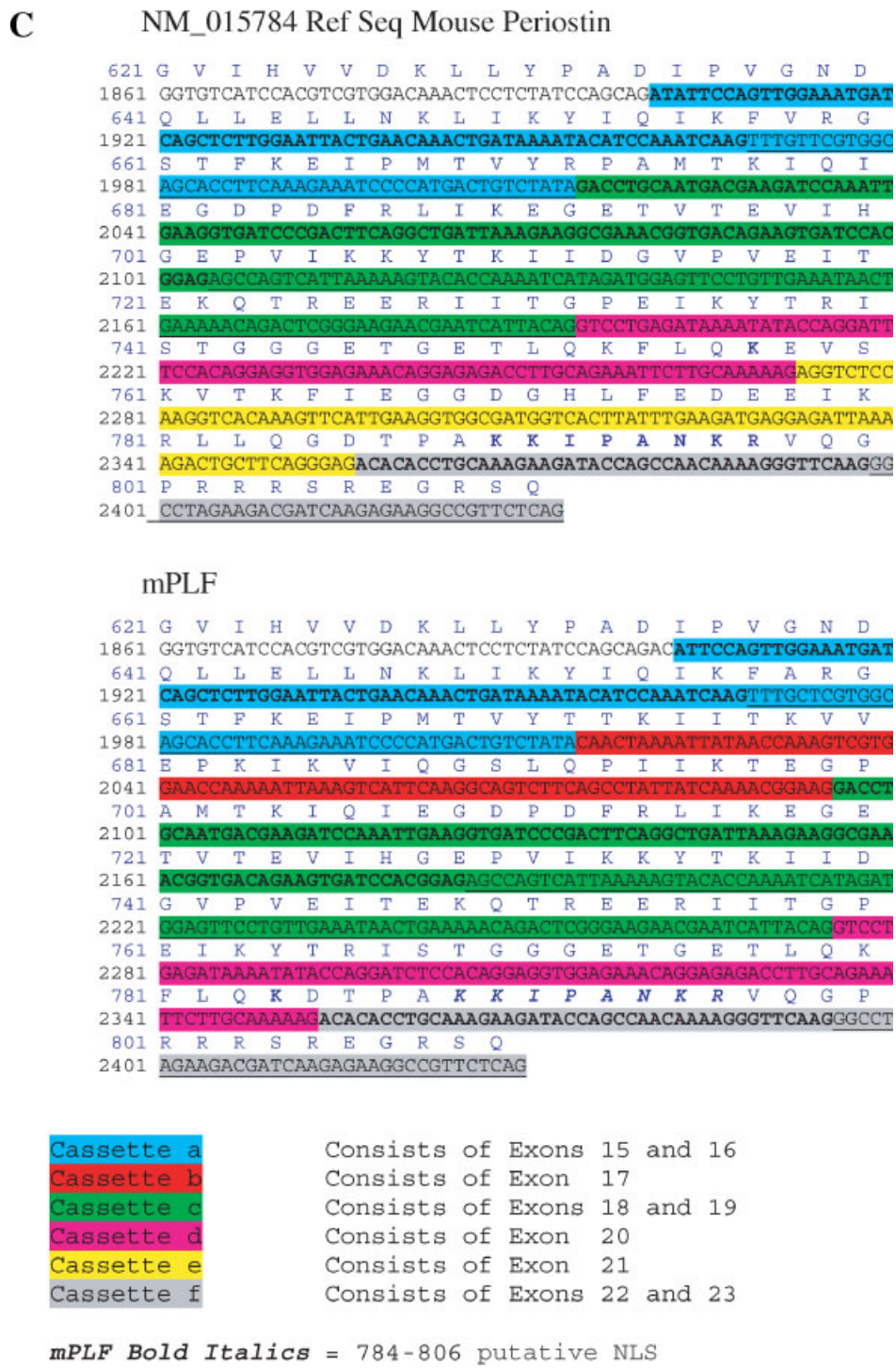

Fig. 1. **C**: The cassette arrangement of periostin and PLF. Each cassette is color coded. Cassettes are made up of either one or two exons.

Percentage of β-galactosidase transfected cells to total number of cells was calculated for transfection efficiency.

PCR analysis using gene-specific primers. Two μg total RNA isolated from MC3T3-E1 cell cultures were reverse transcribed to cDNA at 42°C for 50 min in a volume of 20 μl using Superscript II as instructed by the manufacturer (Invitrogen). One microliter aliquots of the cDNA were amplified in a 50 μl PCR

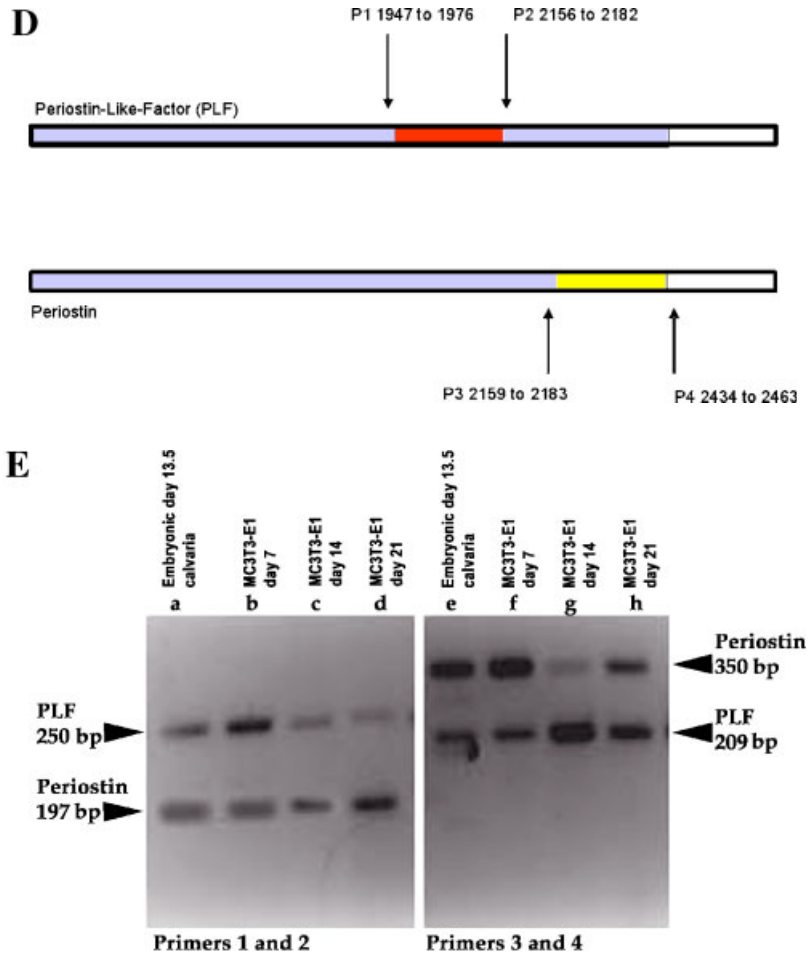

Fig. 1. **D**: Diagram representing the predicted aa sequence of PLF and periostin. The red and yellow boxes represent the 673–699 and 785–812 aa stretches that differ in these two isoforms. Primers on either side of these regions and used for RT-PCR are labeled as P1–P4. **E**: Periostin isoform expression in vivo and in vitro. Total RNA from mouse 13.5 day pc calvaria (**a** & **e**) and MC3T3-E1 cells on days 7 (**b** & **f**), 14 (**c** & **g**) and 21 (**d** & **h**) in culture were reverse transcribed and amplified by PCR using P1 and 2 (lanes a–d), P3 and 4 (lanes e–h). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

reaction mixture containing 1 nM primer set, and Stoffel Fragment of DNA polymerase (Applied Biosystems). The forward and reverse primers were as follows: collagen type 1: 5′-TCTCCACTCTTCTAGTTCCT-3′ and 5′-TTGGGTCATTTCCACATGC-3′, osteocalcin: 5′-TCTGACAAACCTTCAGTCC-3′ and 5′-AAATAGTGATACCGTAGATGCG-3′, G3PDH: 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′, cbfa1: 5′-TCTGACAAACCTCATGTCC-3′, and 5′-AAATAGTGATACCGTAGATGCG-3′, osteopontin: 5′-ACACTTTCACTCCAATCGTCC-3′ and 5′-TGCCCTTTCCGTTGTTGTCC-3′, respectively. PCR was performed using Perkin-Elmer GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: initial denaturation at 94°C for 3 min, amplification through 25 cycles of 94°C for 30 s followed by annealing and extension at the temperatures specified below for each gene for 45 s followed by a final extension step at 72°C for 10 min. PCR parameters for each gene-specific primer varied only in annealing and extension temperature which were as follows: collagen type I: 55°C, osteocalcin: 55°C, G3PDH: 65.3°C, Cbfa1: 60°C, and osteopontin: 55°C. The expected product size for each was collagen type I: 250, osteocalcin: 198, G3PDH: 452, Cbfa1: 632, and osteopontin: 239 bp, respectively. PCR products were ana-

lyzed by 1% agarose gel electrophoresis stained with ethidium bromide. A 100-bp ladder was used as a molecular weight marker (Promega). DNA gels of RT-PCR products were analyzed by densitometry. Densitometric analysis of normalized values (specific PCR product/G3PDH) were used as a semi-quantitative analysis of the PCR products.

RESULTS

Generation and Analysis of Full-Length PLF cDNA

The 169 bp sequence we identified from the READs technique was 99% identical over its entirety to the published periostin (GenBank accession no.: NM_015784). Takeshita et al. [1993] showed that periostin was bone-specific; however, our clone was isolated from the heart. We, therefore, attempted to ascertain whether our clone was the same as periostin identified in osteoblasts. Horiuchi et al. [1999] had already proposed that there were several periostin isoforms that differ in their 3′ translated sequence. It was, therefore, reasonable to assume that there might be a difference in the bone cDNA identified by Takeshita et al. [1993] and the heart cDNA. In order to get the full-length sequence, we isolated it by RT-PCR from the same cardiac RNA used in the READS technique. We used the published sequence of periostin to develop a 5′ PCR primer, because based on Horiuchi's work, we did not expect there to be any difference at the 5′ end. The 3′ primer was homologous to the 3′ end of the 169 bp READS EST.

The full-length PLF cDNA was generated and sequenced in both directions. The resulting 3,012 bp cDNA is a unique form of OSF2/periostin that will be referred to as PLF. Examination of the NCBI database revealed only high homology to BC007141. BC007141 is a cDNA sequence isolated from a mouse mammary tumor that contains a significant frame shift (caused by an extra "t" at position 592 relative to the start codon and absent in other isoforms of periostin), that produces a STOP codon, which precludes the sequence from producing a full-length protein (Fig. 1A). We also found weaker homologies to two murine[1] and three human OSF2/periostin sequences. The murine and human sequences are different from that of PLF (Fig. 1A,B). For the analysis that follows we will refer to the translated protein sequences of the isoforms in Figure 1A and will number the aa sequences based on the Reference Sequence[2] for mouse periostin (NM_015784). The predicted aa sequence of mouse PLF that we identified contains a peptide region of 27 aa (673–699 aa) that is absent from both mouse periostin (NM_015784) and the Ref Seq for human periostin (NM_006475). Further towards the COOH terminus, there is a 28 aa peptide (785–812 aa) that is absent from our translated PLF cDNA but present in the mouse and human periostins (NM_015784 and NM_006475, respectively). There are various other mouse and human periostin isoform sequences in GenBank which contain variations on these differences, none of which have the 27 aa peptide (673–699 aa) found in mouse PLF. These different proteins are most likely isoforms resulting from alternatively spliced RNAs. An analysis of the exon–intron (Fig. 1B and Table I) arrangement of periostin [Fig. 1B(a)] and PLF [Fig. 1B(b)] showed that nucleotides that code for the 673–699 aa region in mouse PLF comprise exon 17 (present in periostin intron 16–17) on chromosome 3 and the nucleotides that code for 785–812 aa comprise exon 21 of periostin (NM_015784).

Horiuchi et al. [1999] isolated multiple segments at the 3′ end of the periostin cDNA by PCR. Based on the sequence analysis of this segment they showed that there were four possible isoforms of periostin generated by a combination of six different cassettes, a–f [Horiuchi et al., 1999; Fig. 1C]. Our sequence analysis of the full-length PLF cDNA and predicted aa sequence showed that it most resembles Horuichi's isoform 3 of mouse periostin [Horiuchi et al., 1999]. Comparing the two sequences of PLF and periostin [Takeshita et al.,

[1]GenBank nomenclature: Murine OSF2 = NM015784 and BC031449, human OSF2 = NM006475, AY140646, and HUMOSF2P1.

[2]NCBI Reference Sequence (Ref Seq): "The Reference Sequence collection aims to provide a comprehensive, integrated, non-redundant set of sequences, including genomic DNA, transcript (RNA), and protein products, for major research organisms. The main features of the Ref Seq collection include non-redundancy, explicitly linked nucleotide and protein sequences, updates to reflect current knowledge of sequence data and biology, data validation and format consistency, distinct accession series, and ongoing curation by NCBI staff and collaborators, with review status indicated on each record".

TABLE I. Exon–Intron Arrangement for Periostin (NM_015784) and Periostin-Like-Factor*

	NM_015784 Periostin (bp)		Periostin-Like Factor (PLF) (bp)
Exon 1 (ATG)	125	Exon 1 (ATG)	125
Intron 1–2	1,359	Intron 1–2	1,359
Exon 2	99	Exon 2	99
Intron 2–3	3,178	Intron 2–3	3,178
Exon 3	65	Exon 3	65
Intron 3–4	1,532	Intron 3–4	1,532
Exon 4	158	Exon 4	158
Intron 4–5	1,490	Intron 4–5	1,490
Exon 5	165	Exon 5	165
Intron 5–6	884	Intron 5–6	884
Exon 6	147	Exon 6	147
Intron 6–7	486	Intron 6–7	486
Exon 7	142	Exon 7	142
Intron 7–8	1,031	Intron 7–8	1,031
Exon 8	213	Exon 8	213
Intron 8–9	553	Intron 8–9	553
Exon 9	135	Exon 9	135
Entron 9–10	800	Entron 9–10	800
Exon 10	149	Exon 10	149
Intron 10–11	1,164	Intron 10–11	1,164
Exon 11	137	Exon 11	137
Intron 11–12	882	Intron 11–12	882
Exon 12	131	Exon 12	131
Intron 12–13	522	Intron 12–13	522
Exon 13	131	Exon 13	131
Intron 13–14	90	Intron 13–14	90
Exon 14	103	Exon 14	103
Intron 14–15	151	Intron 14–15	151
Exon 15	68	Exon 15	68
Intron 15–16	328	Intron 15–16	328
Exon 16	46	Exon 16	46
Intron 16–17	5,781	Intron 16–17	2,805
		Exon 17	82
		Intron 17–18	2,894
Exon 18	90	Exon 18	90
Intron 18–19	1,006	Intron 18–19	1,006
Exon 19	90	Exon 19	90
Intron 19–20	634	Intron 19–20	634
Exon 20	78	Exon 20	78
Intron 20–21	385	Intron 20–21	4,053
Exon 21	84		
Intron 21–22	3,584		
Exon 22	42	Exon 22	42
Intron 22–23	881	Intron 21–22	881
Exon 23	773	Exon 23	773

*Exons 17 and 21 are specific to PLF and periostin, respectively.

1993; Horiuchi et al., 1999], there are some key differences. In the region at the 3′ end, analyzed by Horiuchi et al. [1999], isoform 3 and PLF contain cassettes a–d and f and lack cassette e. The mouse periostin Ref Seq contains cassettes a, c–f and lacks cassette b. Thus PLF contains a 27 aa sequence that is not found in mouse periostin and mouse periostin contains a 28 aa sequence that is not found in PLF. Horiuchi's cassettes are made up of either single or double exons in the *periostin* gene on chromosome 3 in the mouse (Fig. 1C).

Since the sequence of aa in these regions are highly conserved across species it is reasonable to assume that the isoforms are functionally significant. For instance, in PLF, between aa 784 and 806 there is a sequence recognized by 'motif analysis' as a putative nuclear localization signal (NLS). This sequence is altered in periostin by the presence of cassette 'e' so that it is no longer recognized by motif analysis as a NLS.

To investigate the complexity of periostin mRNAs in embryos and cultured MC3T3-E1 cells, total RNA was analyzed by RT-PCR using primers P1-4, flanking the regions that differed between periostin and PLF (Fig. 1D for primer position). In 13.5 day mouse embryonic calvaria and MC3T3-E1 cells on days 7, 14, and 21, two PCR products of 250 and 197 bp using P1 and P2 were identified. Using P3 and P4, bands at 300 and 209 bp were identified (Fig. 1E). Sequence analysis of these cDNA bands identified them as either PLF or periostin (Fig. 1E).

mRNA Expression is Temporally and Spatially Regulated During Mouse Embryogenesis

For an overall temporal pattern of expression of the periostin isoforms during mouse embryogenesis, we probed total mouse embryonic RNA. There was a distinct pattern of expression during embryogenesis with significantly reduced levels of expression at days 11.5 and 12.5 pc in the whole embryo (Fig. 2). In MC3T3-E1 and primary osteoblast cultures, periostin isoform mRNA expression was detected over a period of 21 days (Fig. 3). Expression markedly decreased in primary osteoblasts over the 21 days in culture, whereas in MC3T3-E1 cells mRNA levels increased and remained relatively high (Fig. 3B).

In situ hybridization was used to determine the spatial location of periostin isoforms during cartilage and bone development at day 16.5 pc. mRNAs coding for these isoforms were localized to mesenchymal tissues containing pre-osteoblasts that surround cartilage primordia of the ribs (Fig. 4E,F), vertebrae (Fig. 4B,D), and the limb (Fig. 4G,H). mRNA was also detected in cells comprising the cartilage primordia of the upper and lower jaws (Fig. 5A–D), specifically in the mesenchymal/preosteoblasts in the hard palate, in undifferentiated taste bud precursor cells (Fig. 5C,E), as well as in ameloblasts and odontoblasts in the developing tooth (Fig. 5B,E). The control sections did not show any digoxygenin reactivity.

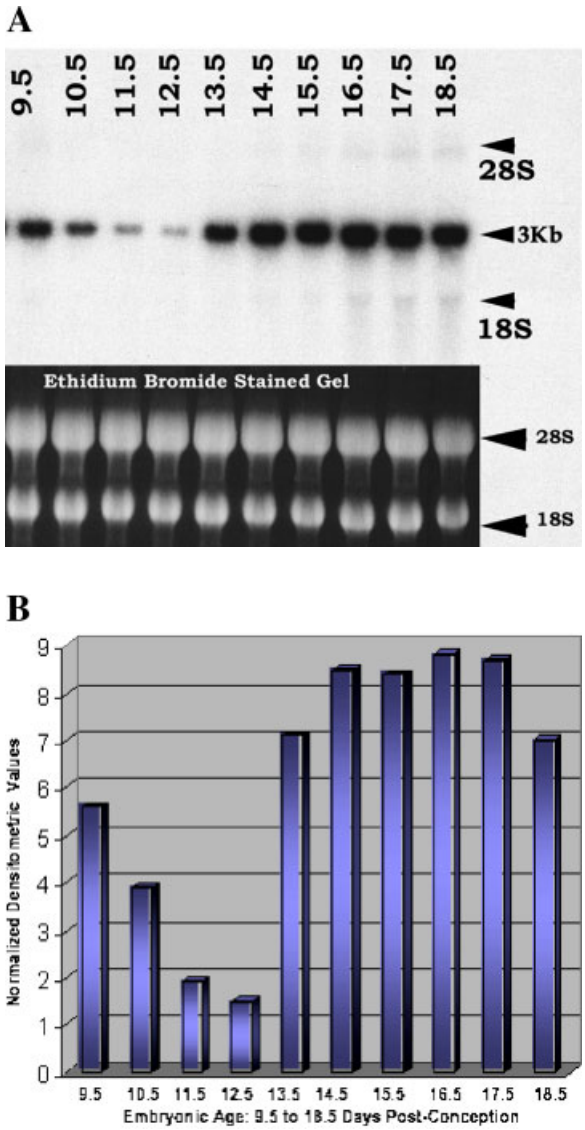

Fig. 2. Periostin isoform expression is developmentally regulated. **A**: 10 μg total RNA from whole mouse embryos from day 9.5 pc to day 18.5 pc was probed with ^{32}P labeled full-length PLF cDNA (~3 kb). Below is shown the ethidium bromide stained formaldehyde-denatured gel. Blots were stripped and re-probed with ^{32}P labeled 18S cDNA as a control for loading and transfer (data not shown). **B**: Graph of the periostin isoform mRNA/18S ratio obtained by densitometric analysis of the X-ray films. The numbers on the x-axis correspond to the age of the embryo from 9.5 to 18.5 days pc, and the values on the y-axis correspond to the mRNA ratio (periostin isoform mRNAs/18S). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Protein Expression is Temporally Regulated in Bone During Mouse Embryogenesis

We detected a difference in the pattern of periostin protein isoform expression during embryogenesis in bone and in cultured MC3T3-E1 cells. The antibody directed against periostin isoforms clearly detected multiple protein isoforms between 86 and 93 kD in both cases, but expression during embryogenesis was temporally regulated in that it was not detected by day 19.5 pc using Western blot analysis (Fig. 6A) as well as by in situ hybridization (data not shown). In MC3T3-E1 cells, expression increased over the 21 days in culture. In addition, the number of isoforms expressed increased with days in culture (Fig. 6A,B). Periostin isoforms in MC3T3-E1 cells were localized to the cytoplasm by immunostaining (Fig. 6C). The presence of a functional NLS is supported by protein detected in the nuclei of MC3T3-E1 cells immunostained with the same antibody used for Western blot analysis (Fig. 6C).

Reduction in the Amount of Periostin mRNA Resulted in a Concomitant Decrease in the Levels of Osteoblast Differentiation Markers

Since the periostin proteins were expressed in embryos and cultured osteoblast cells during the process of differentiation, we used an AS oligonucleotide and anti-periostin isoform polyclonal antibody to examine their effects on osteoblast differentiation markers. The effectiveness of the AS oligonucleotide was assessed by reduction in the amount of periostin isoform mRNA (Fig. 7A). Several bone-specific markers used here are conventionally used as differentiation markers [Stein et al., 1990]. The mRNA levels of collagen type 1, osteocalcin, osteopontin, alkaline phosphatase, and cbfa1 were appreciably reduced in AS treated cells compared to scrambled oligonucleotide controls by RT-PCR analysis (Fig. 7B). In addition, alkaline phosphatase production (Fig. 7C) and calcium levels (Fig. 7D) showed a reduction in both parameters in AS treated cells. The MTT assay (Sigma) was used to prove that the antisense oligonucleotides did not have an effect on cell viability (data not shown). These findings were corroborated when the polyclonal antibody was used to block the activity of the protein isoforms of periostin (Fig. 8B). Antibody treatment was effective in cells for up to 7 days. It is clear that by day 21 the antibody treatment was not effective possibly because of degradation over time. Efficiency of antibody transfection assessed by β-galactosidase staining was approximately 85% (Fig. 8A).

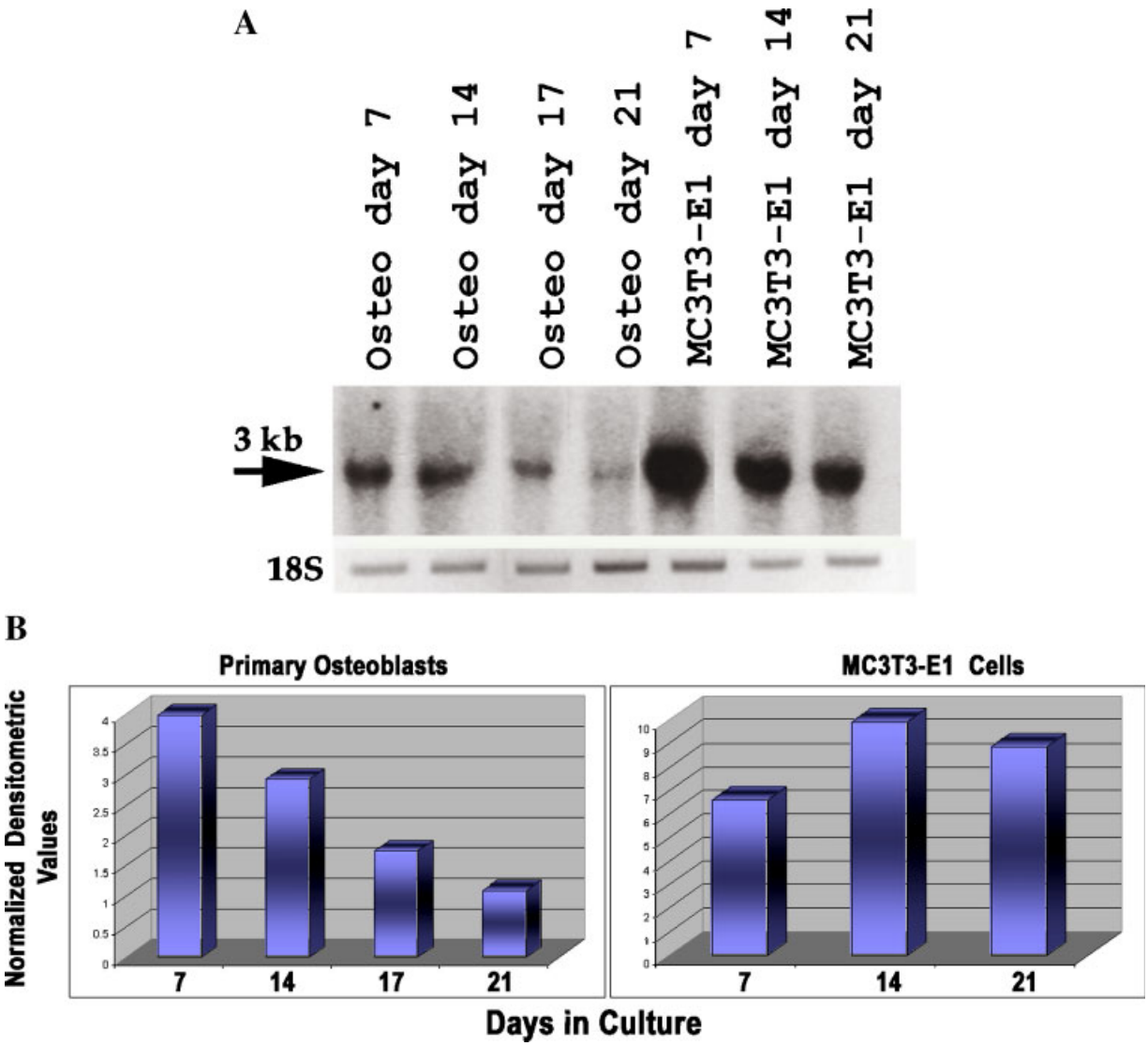

Fig. 3. Expression of periostin mRNAs decreased over time in osteoblast cultures. **A**: Primary osteoblasts isolated from neonatal rat calvaria and MC3T3-E1 cells were grown in culture for 7–21 days. Total RNA was analyzed for the expression of periostin isoforms by Northern blot analysis. Blots were stripped and re-probed with ^{32}P labeled 18S cDNA as a control for loading and transfer. **B**: Bar graph of the periostin isoform mRNA/18S ratio obtained by densitometric analysis of the X-ray films. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

We have recently isolated an additional isoform of periostin that we refer to as PLF. This isoform is expressed in bone, specifically in osteoblast cells, determined by RT-PCR and sequence analyses. We have pursued the study of this family of proteins because the specific function(s) of each isoform of periostin has not been examined and little is known about periostin itself. This study is the first step towards an in-depth analysis of periostin isoforms expressed during bone development and osteoblast differentiation.

Periostin was initially identified by Takeshita et al. [1993] using subtractive hybridization and was shown to be expressed exclusively in bone. Horiuchi et al. [1999] proposed that periostin regulated recruitment of osteogenic precursors and showed that it regulated adhesion of MC3T3-E1 cells. Structurally it shared homology with fas I [Zinn et al., 1988] which in insects regulates axonal guidance, a process involving both differential adhesion and migration.

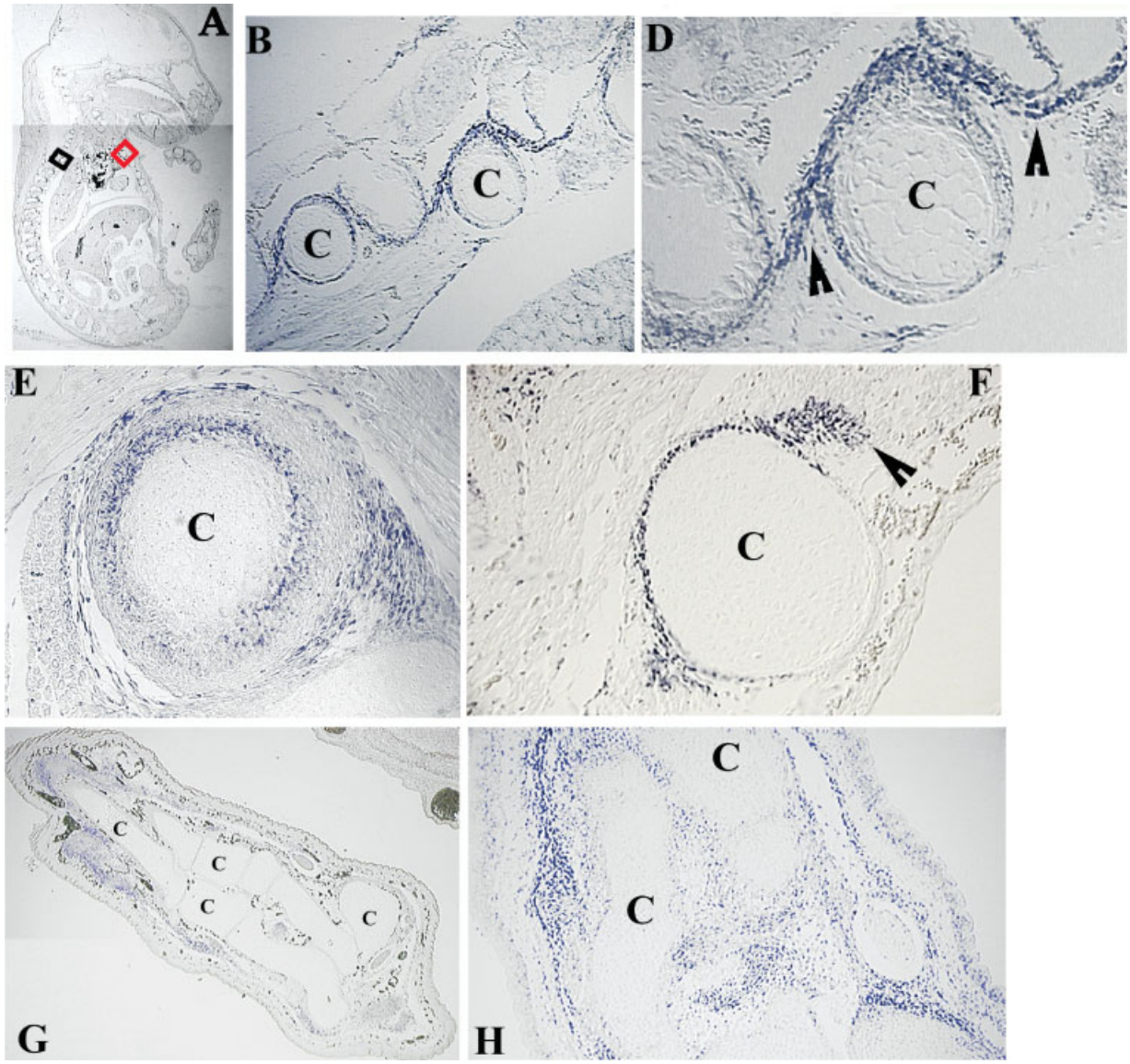

Fig. 4. Localization of mRNAs of periostin isoforms in developing bone. Mouse embryonic 16.5 day pc sections were probed with a digoxygenin labeled antisense full-length PLF riboprobe. **A**: Low magnification; montage. **B**: The region in the black box in (A) at 100× shows developing vertebrae, C = cartilage. **D**: A single developing vertebra at 200× indicates that developing pre-osteoblasts but not chondrocytes express periostin mRNAs. Pre-osteoblasts migrating away from the body of the vertebra, to form the vertebral processes, are indicated by the arrowheads. **E** & **F**: A view of presumptive ribs at 400×. Pre-osteoblasts express periostin mRNAs whereas chondrocytes do not. The red box in (A) is shown at higher magnification in (F). Cells indicated by the arrow in (F) are pre-osteoblasts migrating from the cartilaginous rib into the sternum. **G**: Developing limb at 20× magnification (**H**) image in (G) shown at 200×, mesenchymal cells expressing periostin isoforms are observed.

Recently, Kruzynska-Frejtag et al. [2001] showed that periostin was expressed in the embryonic and fetal endocardial cushions that later divide the heart into four chambers. βIG-H3, a secreted protein containing the four repeats present in periostin and fas I have a similar pattern of expression [Ferguson et al., 2003]. The function(s) of this family of proteins, all containing the conserved fas I repeat motifs is unclear. They appear to be expressed in multiple tissues during embryonic development and in adults [Zinn et al., 1988; Terasaka et al., 1989; Skonier et al., 1992; Takeshita et al., 1993; Huber and Sumper, 1994; LeBaron et al., 1995; Ulstrup et al., 1995; Horiuchi et al., 1999; Lal et al., 1999; Kruzynska-Frejtag et al., 2001; Sasaki et al., 2001; Gillan et al., 2002; Ferguson et al., 2003]. Experiments described in this study

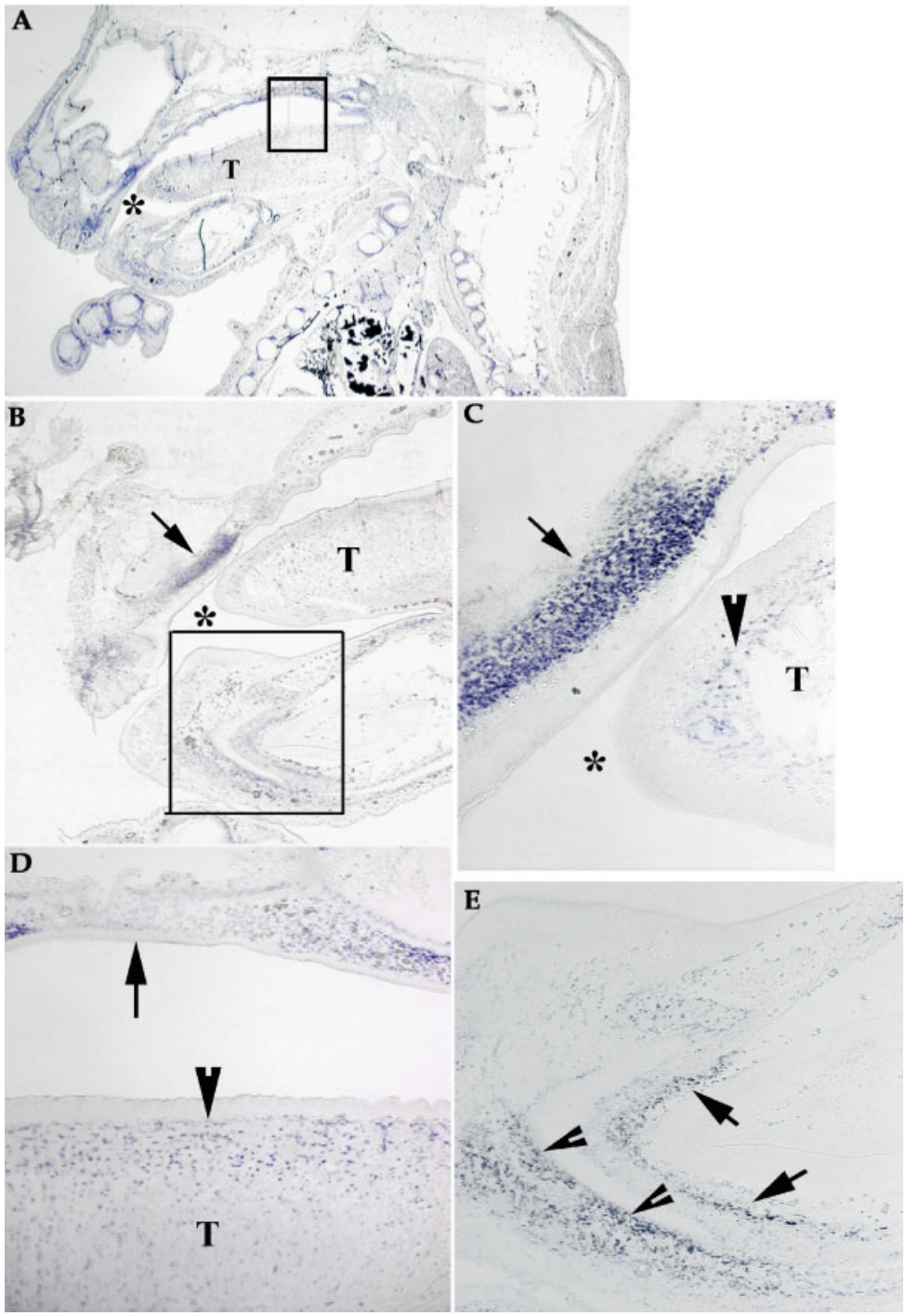

Fig. 5. mRNAs of periostin isoforms are expressed in the mesenchymal primordia of specific bony structures in the head region in the 16.5 day mouse embryo. **A**: Low magnification view (20×) of the head region. The asterisk is located at the tip of the tongue (T) in the oral cavity in (A, **B**, and **C**). The region in the box in (A) is shown at higher magnification in (**D**). B: View of the upper and lower jaws and the tongue (200×). The arrow indicates a region of the hard palate shown at higher magnification in (C). The box indicates a region of the lower jaw shown at higher magnification in (**E**). C: The arrow point to cells in the secondary palate, and the arrow head to cells in the tongue, expressing mRNAs of periostin isoforms (400×). D: The arrow points to a region that lies between the soft and hard palates that is unstained. The arrow head points to stained cells in the tongue (200×). E: The ameloblasts (arrow head) and odontoblasts (arrow) of the developing tooth express the mRNAs of periostin isoforms (400×).

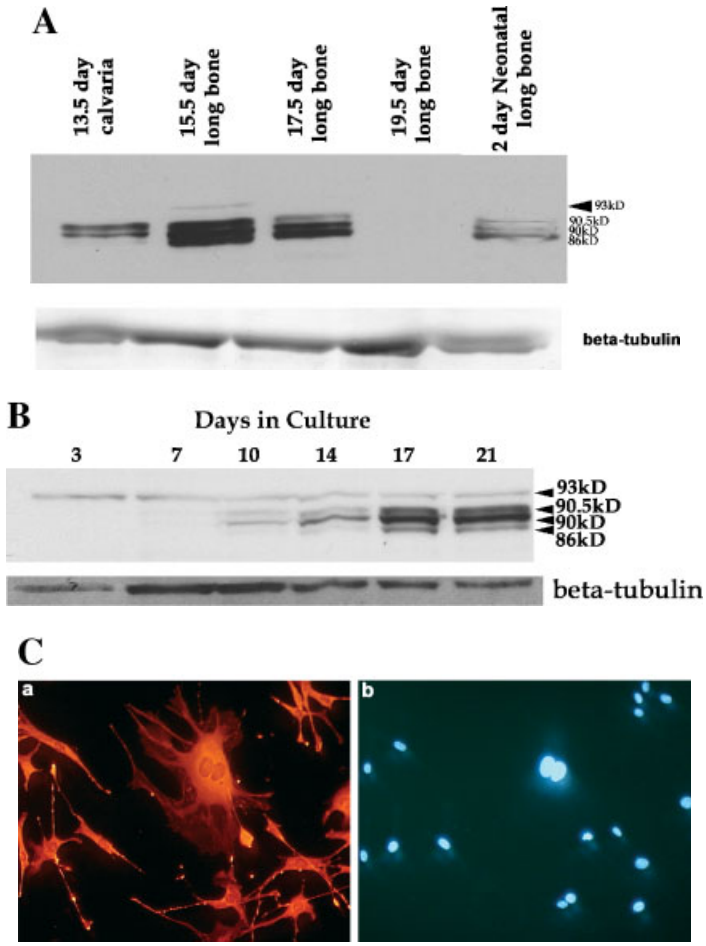

Fig. 6. Expression of protein isoforms of periostin are developmentally regulated. **A**: Protein extracts from 13.5 day embryonic calvaria, 15.5, 17.5, and 19.5 day embryonic and 2-day-old neonatal long bones were separated by SDS–PAGE. The nitrocellulose was reacted with anti-periostin antibody. **B**: Protein extracts from MC3T3-E1 cells at 3, 7, 10, 14, 17, and 21 days in culture analyzed by Western blot analysis using anti-periostin isoform polyclonal antibody. **C**: MC3T3-E1 cells stained with anti-periostin isoform antibody (**a**: red) and DAPI specific for DNA in nuclei (**b**: blue). Staining detected in both the cytoplasm and nucleus.

suggest that they are developmentally regulated and involved in osteoblast differentiation.

Horiuchi et al. [1999] isolated multiple segments at the 3′ end of the periostin cDNA by PCR. Based on the sequence analysis of this segment they showed that there were four possible isoforms of periostin generated by a combination of six different cassettes, a–f [Horiuchi et al., 1999]. Our sequence analysis of the full-length PLF cDNA and predicted aa sequence showed that it most resembles Horuichi's isoform 3 of mouse periostin [Horiuchi et al., 1999]. Noteworthy are a few key differences between the PLF and periostin sequences [Takeshita et al., 1993; Horiuchi et al., 1999]. In the region at the 3′ end, analyzed by Horiuchi et al. [1999], isoform 3 and PLF contain cassettes a–d and f and lack cassette e. Takeshita's mouse periostin sequence contains cassettes a, c–f, and lacks cassette b. Thus PLF contains a 27 aa sequence (cassette b) that is not found in mouse periostin and mouse periostin contains a 28 aa sequence (cassette e) that is not found in PLF. These cassettes correlate with exons in the *periostin* gene (chromosome 3 in the mouse). Some of the cassettes contain more than one exon. The differences amongst the periostin isoforms may have significant functional consequences. For instance, in PLF, between aa 789 and 806 there is a sequence recognized by motif analysis as a putative NLS. This sequence is altered in mouse periostin by the presence of cassette 'e,' so that it is no longer recognized by sequence analysis as a NLS. In addition,

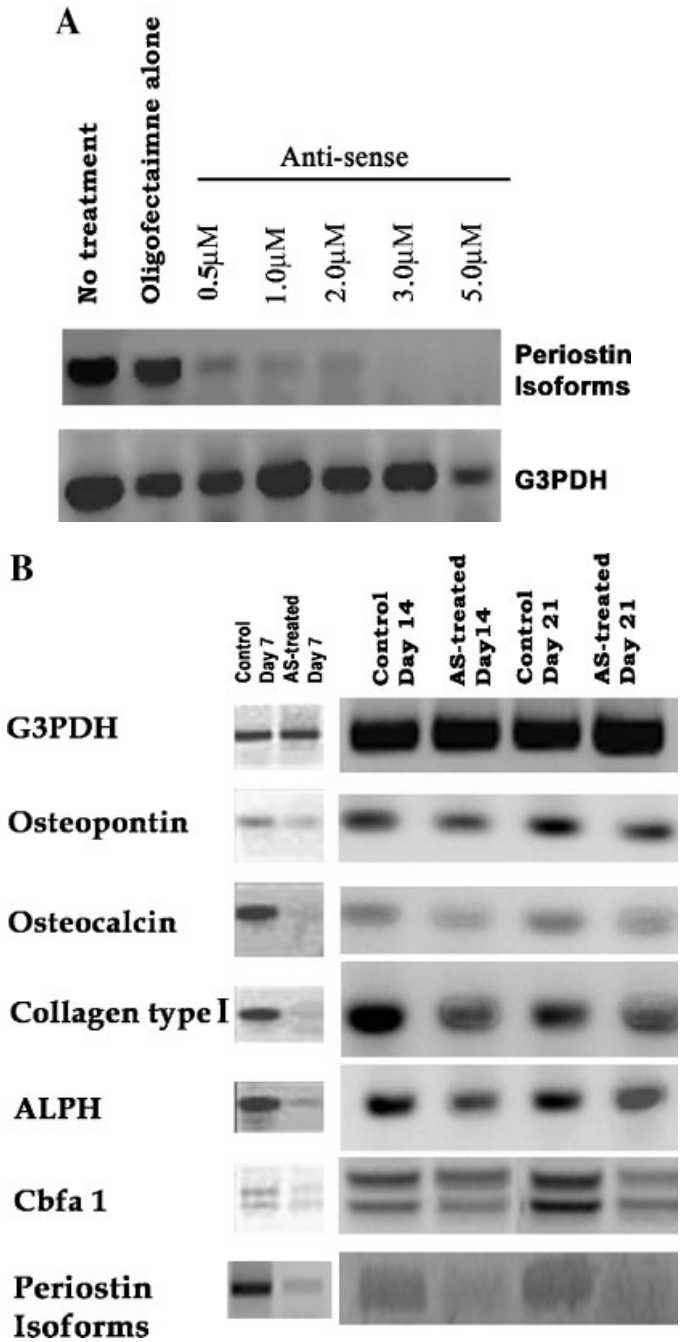

Fig. 7. The effect of periostin antisense oligonucleotide on osteoblast differentiation. **A**: MC3T3-E1 cells were transfected with varying amounts of AS oligonucleotide and the amplified products of G3PDH and periostin are shown after 3 days post-transfection. AS oligonucleotide effectively reduced mRNA levels of periostin isoforms. **B**: PCR analysis on day 7, 14, and 21 post-transfection. **C**: MC3T3-E1 cells treated with antisense-periostin-oligonucleotide and stained for alkaline phosphatase after 14 days in culture. AS treated cells (**b**), showed significantly reduced levels of ALPH production compared to scramble oligonucleotide treated control (**a**). Magnification 200×. **D**: Calcium deposition (measured as described in Safadi et al., 2003) in MC3T3-E1 cells treated with antisense-periostin-oligonucleotide after 21 days in culture. AS treated cells showed significantly reduced levels of calcium deposition compared to controls. (data presented are the mean and SEM, xxx = significant reduction in calcium deposition, $P < 0.001$ using a Student's *t*-test).

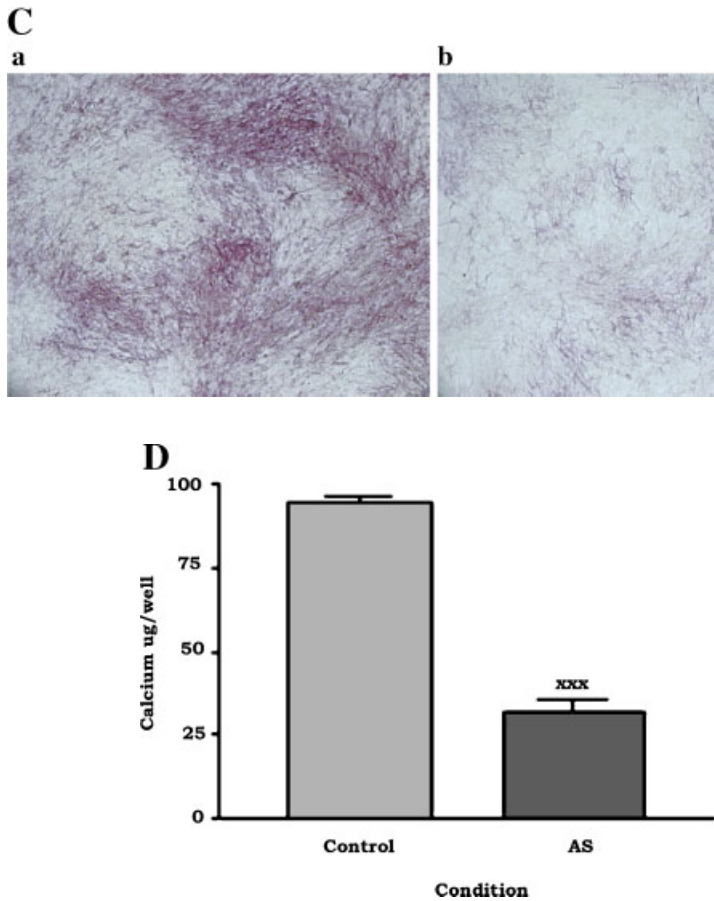

Fig. 7. (*Continued*)

Yoshioka et al. [2002] have shown by mutational analysis that the carboxy terminus of periostin (encompassing the region where changes discussed in this article are present) is sufficient to suppress anchorage-independent-growth in cancer cell lines and thereby may serve as a tumor suppressor in human cancer [Yoshioka et al., 2002].

Further analysis in this segment of the cDNA by RT-PCR analysis, showed that during embryogenesis and in cultured MC3T3-E1 cells, periostin and PLF were present. Sequence analysis of the RT-PCR DNA bands isolated from the agarose gels confirmed that the 250 and 209 bp bands identified using P1 & 2 and P3 & 4, respectively, showed 100% sequence identity at the nucleotide level to PLF. The 197 and 300 bp bands identified using P1 & 2 and P3 & 4, respectively, showed 100% sequence identity at the nucleotide level to periostin. Differences were observed in the number of isoforms detected by RT-PCR and Western blot (Figs. 1E and 6, respectively). This may be attributed to (a) the different levels of sensitivity between the two techniques, (b) isoforms may differ in regions not covered by the primers used in the RT-PCR and [Sasaki et al., 2001] isoforms not detected because they lack the epitope recognized by the antibody. We have also found additional isoforms expressed differentially during development that have not been yet characterized (unpublished findings).

We observed a decrease in the periostin isoform mRNA by embryonic day 19.5 pc in bone by in situ hybridization (data not shown) and in primary osteoblasts in culture by Northern blot analysis (Fig. 3B). The mRNA levels correlated well with protein levels that were not detected by day 19.5 pc during embryogenesis (Fig. 6A). This interesting temporal pattern of expression (Figs. 3 and 6) during osteoblast differentiation and maturation suggest different functions for the periostin isoforms at the different stages of osteoblast development. The findings in embryos and primary osteoblast cultures differed from those in MC3T3-E1 cells

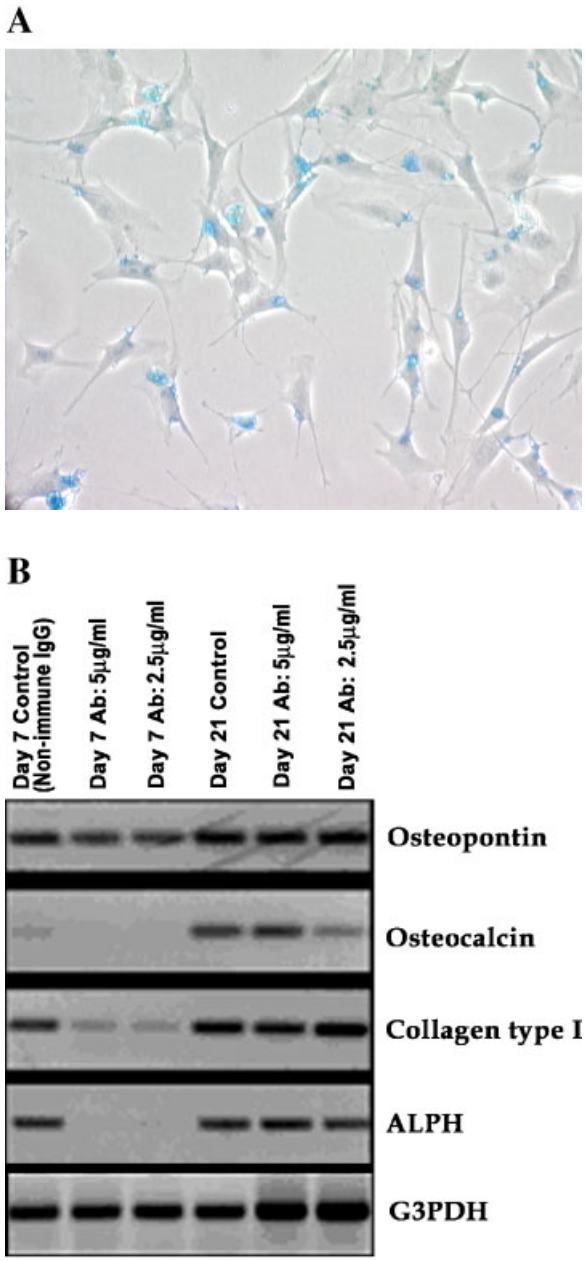

Fig. 8. The effect of periostin polyclonal antibody on osteoblast differentiation. RT-PCR analysis on RNA isolated on days 7 and 21 after treatment of MC3T3-E1 cells with anti-periostin isoform antibody and CHARIOTS. Controls were treated with mouse IgG and CHARIOTS. **A**: Cells stained for β-galatosidase 24 h after being treated with β-galatosidase and CHARIOTS, suggests efficient incorporation of protein into the cells. **B**: PCR of specific markers of osteoblast differentiation on days 7 and 21 post-treatment with either 5 or 2.5 μg/ml of anti-periostin or IgG and CHARIOTS.

where message expression was not markedly reduced over time (Fig. 3B) as in embryos and primary osteoblasts, and protein amount and the number of isoforms increased over time in culture (Fig. 6B). These differences suggest that there are inherent differences between maturation of osteoblasts during embryogenesis (in vivo), as compared to maturation of the MC3T3-E1 cells in the petri dish. Nonetheless, the presence of the periostin protein isoforms over time in MC3T3-E1 cells in culture and during embryogenesis, suggest a role for these proteins during the initial steps of differentiation. The presence of periostin isoforms early in osteoblast development suggests a role in the recruitment of these cells and possibly in the proliferation of these cells. Their presence at later stages suggests a role in differentiation. Others also suggest a role for periostin [Horiuchi et al., 1999] and βIG-H3 [Ferguson et al., 2003] in the recruitment of mesenchymal cells to osteogenic lineage and in the adhesion of MC3T3-E1 cells [Horiuchi et al., 1999].

To test the role of periostin isoforms in osteoblast differentiation, MC3T3-E1 cells were treated with AS oligonucleotides and an antibody directed against all known isoforms of periostin. The use of antibodies to inhibit protein activity, is an accepted technique, used successfully by others [Selim et al., 2003]. Markers of differentiation known to be expressed in normal osteoblasts, at increasing levels during the differentiation process, were markedly reduced by antibody and AS oligonucleotide treatment, when compared to controls. In particular, cbfa1, a transcription factor known to regulate the expression of osteocalcin, a matrix component and marker of bone differentiation was markedly reduced suggesting that the periostin isoforms may be involved in steps upstream of cbfa1. The isoforms may be able to signal and initiate intracellular events required to begin the process of differentiation possibly by binding integrin receptors [Gillan et al., 2002] and/or translocation to the nucleus.

This investigation has introduced the periostin isoform PLF, and has examined the expression and possible function of periostin isoforms. In summary, multiple isoforms of periostin are expressed in vivo during embryogenesis and in vitro in MC3T3-E1 cells and primary osteoblast cultures. The expression of these isoforms appears to be developmentally regulated and the data suggest that they are involved in regulating the process of differentiation. Future studies will address the mechanism(s) of action of periostin and PLF in osteoblast differentiation in vitro and bone formation in vivo.

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