Connective Tissue Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617769

Collagen XXIV (Col24a1) Gene Expression is a Specific Marker of Osteoblast Differentiation and Bone Formation

Noritaka Matsuo [ab]; Shizuko Tanaka [a]; Hidekatsu Yoshioka [b]; Manuel Koch [c]; Marion K. Gordon [d]; Francesco Ramirez [e]
[a] Research Division of the Hospital for Special Surgery, Laboratory of Genetics and Organogenesis, New York, New York, USA
[b] Department of Anatomy, Biology, and Medicine, Oita University, Oita, Japan
[c] Institute of Biochemistry II, University of Cologne, Cologne, Germany
[d] Environmental and Occupational Health Institute and Department of Pharmacology and Toxicology, Ernest Mario School of Pharmacy, Rutgers University, Piscataway, New Jersey, USA
[e] Child Health Institute of New Jersey, Robert W. Johnson Medical School, New Brunswick, New Jersey, USA

Online Publication Date: 01 March 2008
To cite this Article: Matsuo, Noritaka, Tanaka, Shizuko, Yoshioka, Hidekatsu, Koch, Manuel, Gordon, Marion K. and Ramirez, Francesco (2008) 'Collagen XXIV (Col24a1) Gene Expression is a Specific Marker of Osteoblast Differentiation and Bone Formation', Connective Tissue Research, 49:2, 68 - 75
To link to this article: DOI: 10.1080/03008200801913502
URL: http://dx.doi.org/10.1080/03008200801913502

Connective Tissue Research, 49:68–75, 2008

ISSN: 0300-8207 print / 1521-0456 online
DOI: 10.1080/03008200801913502

informa
healthcare

Collagen XXIV (Col24a1) Gene Expression is a Specific Marker of Osteoblast Differentiation and Bone Formation

Noritaka Matsuo
Laboratory of Genetics and Organogenesis, Research Division of the Hospital for Special Surgery, New York, New York, USA; and Department of Anatomy, Biology, and Medicine, Oita University, Oita, Japan

Shizuko Tanaka
Laboratory of Genetics and Organogenesis, Research Division of the Hospital for Special Surgery, New York, New York, USA

Hidekatsu Yoshioka
Department of Anatomy, Biology and Medicine, Oita University, Oita, Japan

Manuel Koch
Institute of Biochemistry II, University of Cologne, Cologne, Germany

Marion K. Gordon
Environmental and Occupational Health Institute and Department of Pharmacology and Toxicology, Ernest Mario School of Pharmacy, Rutgers University, Piscataway, New Jersey, USA

Francesco Ramirez
Child Health Institute of New Jersey, Robert W. Johnson Medical School, New Brunswick, New Jersey, USA

Collagen XXIV is an ill-characterized fibrillar collagen that is predominantly expressed in the forming skeleton of the mouse embryo. Here we report that the Col24al gene is constitutively transcribed in the trabecular bone and periosteum of the newborn mouse as well. The bone specificity of Col24al was further documented using three well-characterized cell culture models of osteoblast differentiation. These *in vitro* analyses indicated that Col24al transcription is activated at about the same time as that of the osteocalcin gene, and gradually increases to eventually plateau as osteoblasts begin to deposit a mineralizing matrix. These findings lend further support to the hypothesis that collagen XXIV may be implicated in the formation of a mineralization-competent bone matrix.

Keywords Bone, Extracellular Matrix, Fibrillar Collagens, Mineralization, Osteoblasts

Received 26 September 2007; accepted 13 November 2007; revised 12 November 2007.

Address correspondence to Francesco Ramirez, Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1603, New York, NY 10029, USA. E-mail: francesco.ramirez@mssm.edu

INTRODUCTION

The collagens represent a very large family of extracellular proteins that impart structural integrity to the connective tissue of virtually every organ system. The collagen family comprises 28 different α-chain trimers or types that give rise to a large variety of suprastructures, such as fibrils, filaments, and networks [1, 2]. Fibril forming collagens are the most abundant and ubiquitously distributed members of the collagen family. They share a common primary structure, which consists of a triple helical domain flanked at both ends by cysteine-rich globular peptides, and are widely expressed in soft and hard tissues (types I, III, and V) or predominantly in cartilage (types II and XI).

Fibrillar collagens traditionally have been classified into major (types I, II, and III) and minor (types V and XI) collagen types based on their relative abundance in the connective tissues [1]. More recently, phylogenetic considerations have segregated the fibrillar collagen sub units into a type A clade (α1(I), α2(I), α1(II), and α2(V) chains); a type B clade (α1(V), α3(V), α(XI), and α2(XI) chains); and a type C clade α1(XXIV) and α1(XXVII) chains) [3, 4]. Members of the last clade display structural features of invertebrate collagens and are predicted

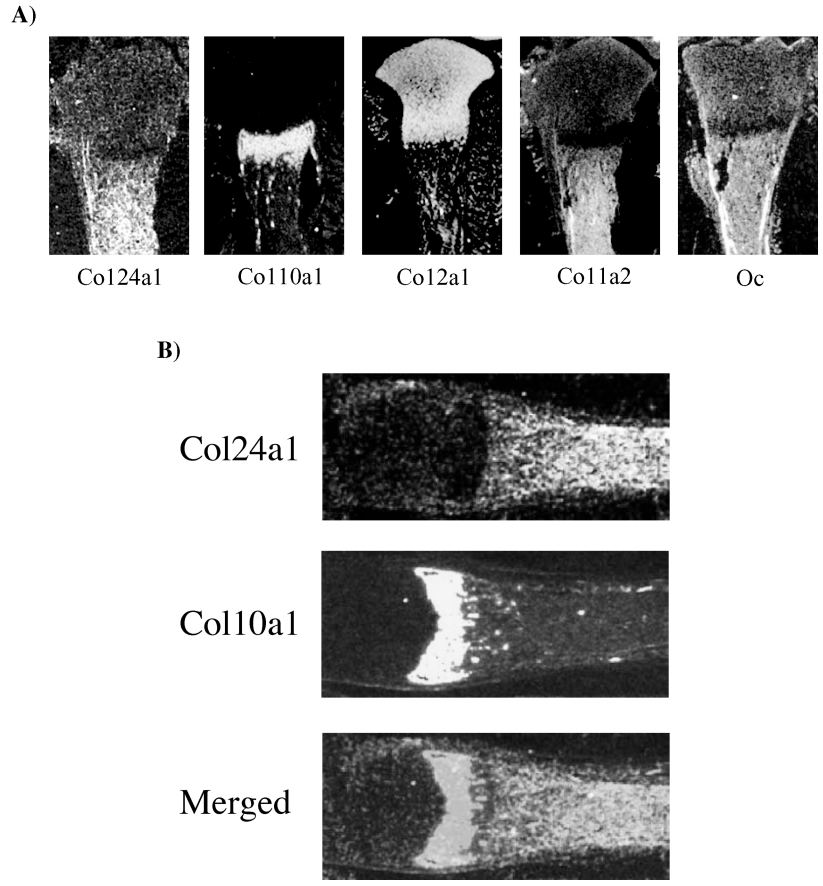

FIG. 1. Col24al gene expression in the bone of newborn mice. (A) Dark-field micrographs of longitudinal sections of P4 tibias hybridized with indicated ^{35}S-labeled riboprobes. (B) Individual and merged images of longitudinal section of P4 tibias hybridized to Col24al and Col10al probes.

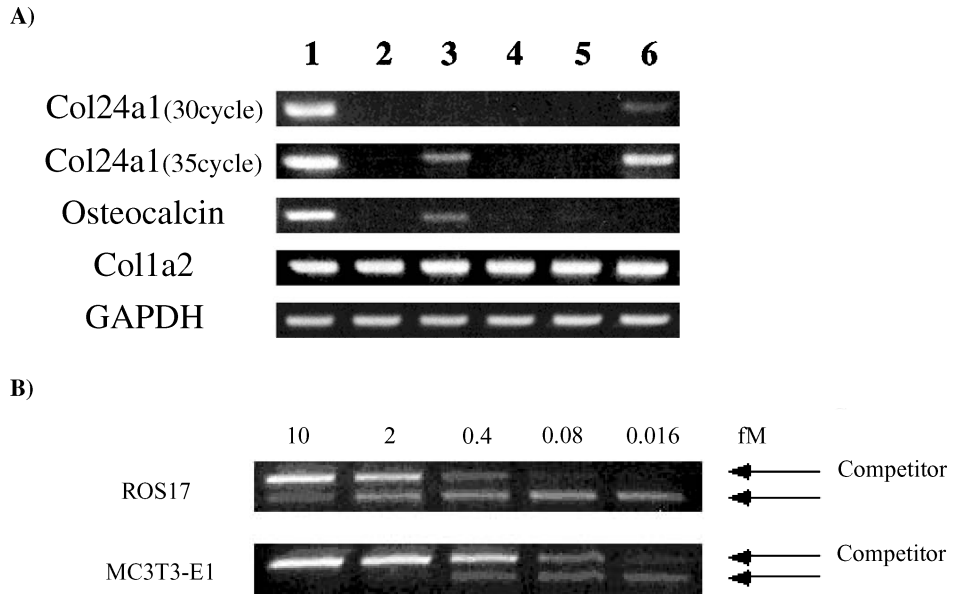

FIG. 2. Osteoblast-specific expression of Col24al. (A) RT-PCR screening of indicated transcripts in ROS17/2.8 (lane 1), ROS25 (lane 2), MC3T3-E1 (lane 3), C3H10T1/2 (lane 4) osteoblastic cell lines, NIH-3T3 fibroblasts (lane 5), and primary MCC cultures (lane 6). Col24al expression was evaluated using 30 or 35 cycles of RT-PCR amplification, whereas 25 cycles were used for the other genes. (B) Semi quantitative estimates of Col24al transcripts in the indicated osteoblastic cell lines performed using the indicated concentrations (fM) of competitor DNA.

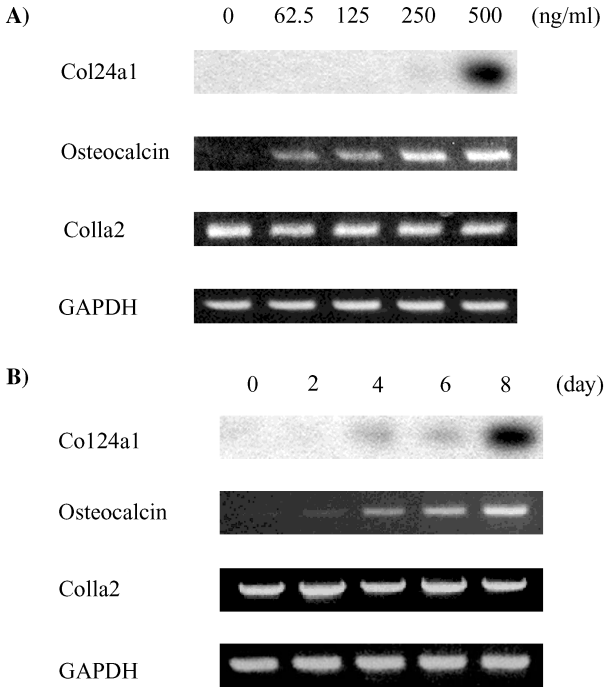

FIG. 3. BMP-2 induction of Col24al transcription in differentiating C3H10T1/2 cells. (A) RT-PCR analyses of indicated mRNAs in C3H10T1/2 cells stimulated to differentiate for 6 days with the indicated concentrations (ng/ml) of BMP-2. Transcripts were PCR-amplified for 35 cycles (Col24al) or 25 cycles (other genes) and resulting products were visualized by Southern blot hybridization or ethidium bromide staining, respectively. (B) The same experimental conditions were used to visualized PCR-amplified products from C3H10T1/2 cells that were incubated with 200 ng/ml of BMP-2 for the indicated days.

to form homotrimes (collagens XXIV and XXVII) that are predominantly expressed in the forming skeleton with mutually exclusive patterns [5–8].

These findings were originally interpreted to indicate that collagens XXIV and XXVII might play comparable roles in cartilage and bone tissue, respectively, by regulating collagen I and II fibrillogenesis and/or matrix calcification and mineralization [6]. Indeed, recent studies have indicated that collagen XXVII forms morphologically distinct assemblies that accumulate in the pericellular matrix of the growth plate, concurrently with cartilage calcification during endochondral bone formation [9, 10].

Osteoblasts play a central role in both intramembranous and endochondral ossification by depositing a mineralization-competent extracellular matrix that includes heterotypic collagens I, III, and V fibrils [11, 12]. Mutations in the subunits of these major and minor collagen types have underscored their importance in skeletal physiology by associating the structurally impaired matrix with perturbed bone growth and integrity [1, 2]. Primary osteoblasts or osteoblastic cell lines have been used traditionally for the *in vitro* study of the molecular factors and cellular mechanisms responsible for or associated with osteoblast differentiation and bone function [13, 14]. The present study therefore was designed to advance knowledge of collagen XXIV function in bone by further detailing the expression of the corresponding mouse gene (Col24al) in the postnatal bone and in established models of *in vitro* osteoblastogenesis. The results of these analyses further support our original hypothesis that collagen XXIV might be implicated in the formation of a mineralization-competent bone matrix.

MATERIALS AND METHODS

Cell Cultures

Rat osteosarcoma ROS17/2.8 and ROS25 cells, mouse pre osteoblast MC3T3-E1 cells, mouse C3H10T1/2 mesenchymal cells, mouse calvarial cells (MCCs), and mouse NIH-T3T fibroblasts were used in this study. ROS17/2.8, ROS25, and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified 5% CO_2 and 95% air. MC3T3-E1 and C3H10T1/2 cells were cultured in Minimum Essential Medium-α (α-MEM) supplemented with 10% heat-inactivated fetal bovine serum in a humidified 5% CO_2 and 95% air. MCC were prepared from 4-day-old mice and cultured in α-MEM supplemented with 10% heat-inactivated fetal bovine serum in a humidified 8% CO_2 and 92% air [8]. Cells were seeded at the density of 1×10^6 cells/100 mm dish.

After reaching confluency, the growth medium was replaced by differentiation medium consisting of 5% FBS/α-MEM, 100 ng/ml bone morphogenetic protein (BM)-2, 10 mM βT-glycerophosphate, and 50 μg/ml ascorbic acid (C3H10T1/2 differentiation medium), or 10% FBS/α-MEM, 10 mM βT-glycerophosphate, and 50 μg/ml ascorbic acid (MC3T3-E1 and MCC differentiation medium) [15–17]. MCC cultures were stained with Alizarin red or von Kossa to visualize calcium deposits or phosphate deposits, respectively [17].

RNA Analyses

Total RNA was isolated from cultured cells using a commercial kit (Invitrogen) according to the manufacturer's recommendations. Reverse transcriptions were carried out using Omniscript reverse transcriptase (Qiagen GmbH) with oligo(dT) primer, and the resulting single-stranded cDNA molecules were PCR amplified using primers specific for Col24al (forward primer, 5′-ATGCATTTAGGAGCCTACAG-3′; reverse primer, 5′-TCACGAGAGGTGACTCAATA 3′), Colla2 (forward primer, 5′-TGGTCCTCTGGGCATCTCAGGC-3′; reverse primer, 5′-GGTGAACCTGCTGTTGCCCTCA-3′), osteocalcin (Oc) (forward primer, 5′-ATGAGGACCCTCTCTCTGCT-3′; reverse primer, 5′-GGAGCTGCTGTGACATCCAT-3′), or GAPDH (forward primer, 5′-ACCACAGTCCATGCCATCAC-3′; reverse primer, 5′-TCCACCACCCTGTTGCTGTA-3′).

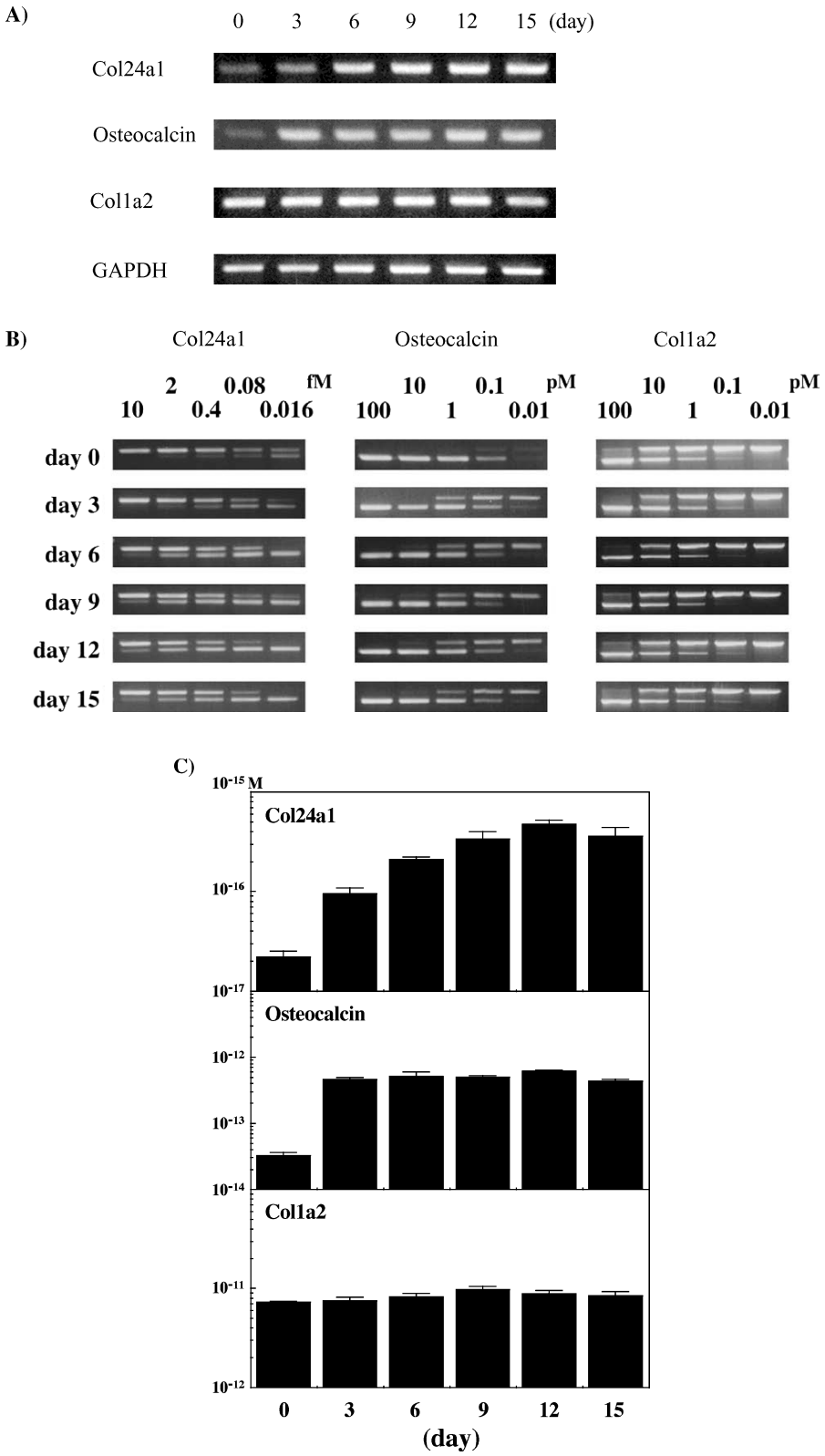

FIG. 4. Col24al accumulation in differentiating MC3T3-E1 cells. (A) RT-PCR analysis of the indicated mRNAs in cells cultured for the indicated days; amplification was 35 cycles for Col24al and 25 cycles for the other genes. (B) Semi quantitative estimates of indicated transcripts performed using the indicated concentrations (fM or pM) of competitor DNA. (C) Bar graphs represent the quantification of the amplified products in (B) whose intensity was evaluated with the NIH ImageJ software. Results represent the means ± SE ($n = 3$).

Amplification conditions included a cycle at 94°GC for 2 min followed by 25 cycles (Colla2, Oc, and GAPDH) and 35 cycles (Col24al) at 94°C for 30 sec, 56°C for 1 min, and 72°C for 1.5 min, and a final elongation cycle at 72°C for 5 min. For semiquantitative PCR analysis, competitor DNA was added to each experimental sample at the indicated concentrations and the resulting products were normalized against GAPDH levels. PCR-amplified products were separated by 2% agarose gel electrophoresis, and observed under ultraviolet illumination after staining with ethidium bromide. To estimate the degree of each transcript, relevant bands were quantified using the NIH ImageJ software.

Three independent experiments were performed in duplicate to estimate the relative amount of each gene product at a given competitor concentration. The resulting data were expressed as the mean ± SE. In the case of C3H10T1/2 cells, Col24al transcripts could only be visualized by Southern blot hybridization of the PCR-amplified products to the same ^{32}P-labeled sequence.

In Situ Hybridizations

Mouse tibias from 4-day-old mice were fixed in paraformaldehyde and embedded in to prepare frozen sections for *in situ* hybridizations to ^{35}S-labeled riboprobes [6]. Photoshop 7.0 imaging software (Adobe) was used for postprocessing of the *in situ* hybridization data.

RESULTS

We previously reported that onset of Col24al transcription in the emerging skeletal elements of the developing mouse embryo commences at about E14.5 [6]. The study did not, however, address the possibility that Col24al expression may persist post natally in bone tissue. *In situ* hybridizations therefore were performed to examine Col24al expression in the growing tibias of P4 mice. Other tissue-specific probes used in the analysis included the extracellular matrix products of osteoblasts (α2(I) collagen [Colla2], and osteocalcin [Oc]), resting and differentiating chondrocytes (α1(II) collagen [Col2al]), and hyperthrophic chondrocytes (α1(X) collagen [Col10al]). The *in situ* hybridizations detected strong Col24al signals in trabecular bone and periosteum, colocalized with Colla2 and Oc transcripts (Figure 1A). Col24al signals were instead absent in cartilage, which displayed the characteristic pattern of Col2al and Col10al positive cells in distinct regions of the growth plate (Figure 1A). In point of fact, the trabecular bone-specific expression of Col24al was more evident in a merged image showing no overlap with the Col10al-positive hyperthrophic cartilage

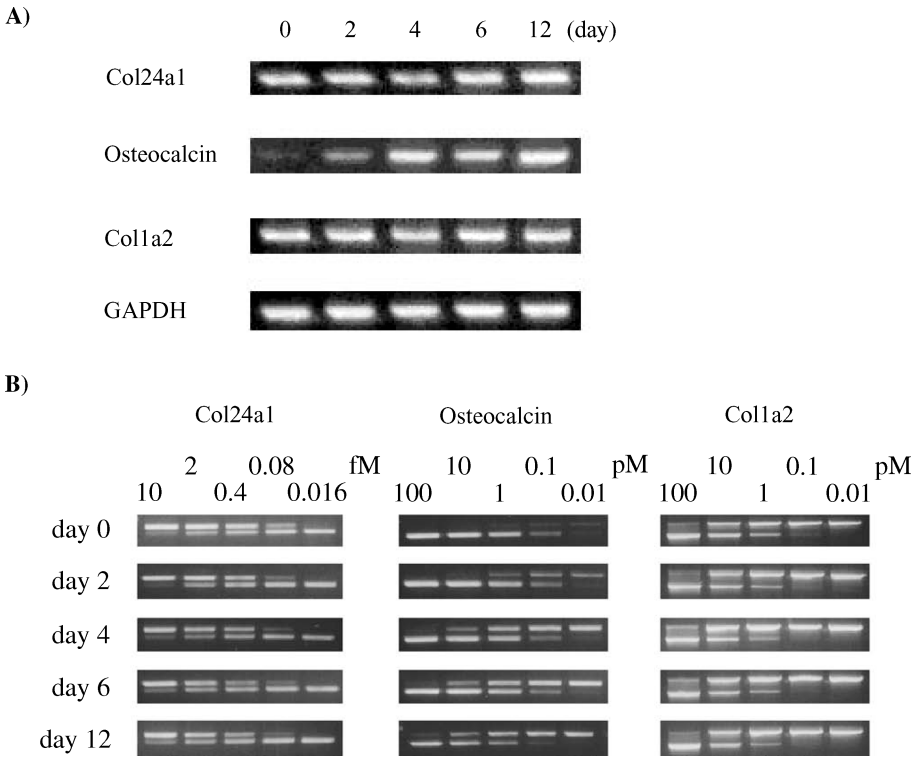

FIG. 5. Col24al expression in differentiating MCC cultures. (A) RT-PCR analysis of the indicated mRNAs in cells cultured for the indicated days; amplification was 35 cycles for Col24al and 25 cycles for the other genes. (B) Semi quantitative estimates of indicated transcripts performed using the indicated concentrations (fM or pM) of competitor DNA. (C) Bar graphs represent the quantification of the amplified products in (B) whose intensity was evaluated with the NIH ImageJ software. Results represent the means ± SE (n = 3). (D) Parallel determination of MCC terminal differentiation by assessing formation of mineral deposits using Alizarin red or von Kossa staining; untreated MCC cultures are shown in day 12 (-) samples. *(Continued)*

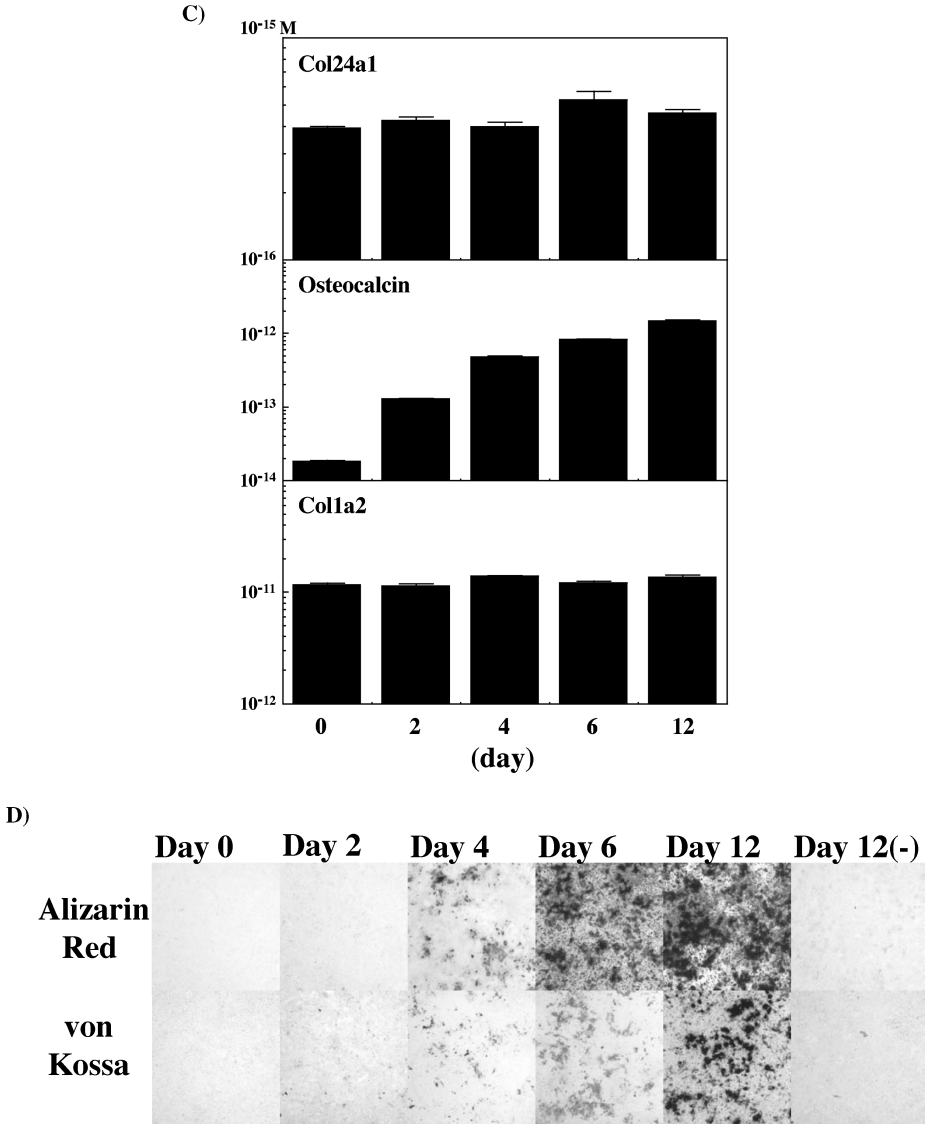

FIG. 5. (Continued).

(Figure 1B). Similar data were obtained with adult bones (data not shown), thus demonstrating that Col24al is continuously expressed by osteoblasts during postnatal bone growth and remodeling.

The *in vivo* findings were re-examined *in vitro* by comparing the dynamics of Col24al gene expression in several different models of osteoblastogenesis. First, Col24al transcript levels were evaluated by RT-PCR amplification of RNA purified from undifferentiated cell lines representing different stages of osteoblast differentiation (ROS17/2.8, ROS25, MC3T3-E1, and C3H10T1/2 cells), in undifferentiated primary mouse calvarial cells (MCC), and in NIH-3T3 fibroblasts as well. Amplification of Colla2 and Oc transcripts also was included in each experimental sample as markers of early and middle/late differentiation, respectively, while GAPDH was used as the normalizing control.

The RT-PCR screen detected Col24al transcripts only in ROS17/2.8, which correspond to mature osteoblasts, in MC3T3-E1, which are preosteoblastic cells, and in MCC, which represent an heterogeneous population of pre osteoblast cells [13, 16, 17] (Figure 2A). Semi quantitative RT-PCR analyses determined that the concentration of Col24al transcripts is about 0.5 fM in ROS17/2.8 cells, and 0.01 fM in MC3T3-E1 cells (Figure 2B). MC3T3-E1 and MCC therefore were selected to monitor Col24al mRNA accumulation during ostoblastogenesis; pluripotent C3H10T1/2 cells induced to differentiate along the osteoblastic lineage were included in this analysis as well [18, 19].

C3H10T1/2 cells were induced to differentiate into pre-osteoblasts by administration of increasing amounts of BMP-2 ranging from 62.5 to 500 ng/ml. In contrast to untreated cells, BMP-2 slightly stimulated Col24al transcription at a concentration of 250 ng/ml, and markedly increased it at a concentration of 500 ng/ml (Figure 3A). By contrast, Oc induction was first noted at a lower BMP-2 concentration (62.5 ng/ml), a finding that probably reflects the higher levels of Oc transcripts relative to those of Col24al (Figure 3A). Indeed Col24al expression in this model of osteoblast differentiation was so low that it could only be detected by Southern blot hybridization of the PCR-amplified products, thus impeding a reliable quantification of the relative amounts of the two transcripts. This last point notwithstanding, a time-course analysis of BMP-2-induced differentiation of C3H10T1/2 cells (200 ng/ml) strongly suggested that transcription of Oc and Col24al is stimulated at about the same time (day 2), and that the respective transcripts accumulate thereafter at comparable rates (Figure 3B). These results therefore established that Col24al is a BMP-2 inducible gene in osteoblastogenic C3H10T1/2 cells.

Consistent with the above data, Col24al transcripts were found to increase gradually in differentiating MC3T3-E1 cells and concomitantly with Oc mRNA accumulation Figure 4A). Semi-quantitative analyses revealed that Col24al and Oc display distinct patterns of gene expression in differentiating MC3T3-E1 cells, in that transcripts from the latter gene plateau earlier than those of the former gene (day 3 versus 9, respectively; Figures 4B and 4C). They also confirmed that the absolute amount of Col24al transcripts between days 9 and 15 is very low, notably about 1.5×10^4 and 10^3-fold less than Col2al and Oc transcripts, respectively (Figures 4B and 4C). This last finding reconciles the difficulty of Col24al detection in differentiating C3H10T1/2, as these cells are more heterogeneous and mostly correspond to the earlier stage of progenitor osteoblast commitment (Figure 3). The MC3T3-E1 data therefore indicated that Col24al is a low expressed middle/late marker of osteoblast differentiation.

The last set of *in vitro* experiments employed MCC to validate the above conclusions in a primary cell culture system that replicates terminal differentiation of osteoblasts, as evidenced by the formation of mineral nodules [17]. Accordingly, Col24al, Col1a2, and Oc mRNA levels in MCC cultures were evaluated in parallel with the emergence of calcium and phosphate deposits (by Alizarin red and von Kossa staining, respectively) (Figure 5). The analyses revealed that, similar to Colla2 and Oc, Col24al is continuously expressed through the terminal differentiation process of primary MCC (Figures 5A–C). They also showed some differences between differentiating MC3T3-E1 and MCC cultures with respect to the accumulation patterns of Col24al and Oc transcripts (Figures 4C and 5C). This apparent discrepancy is likely to reflect intrinsic differences in the differentiation properties of these two cellular models of osteoblastogenesis [16, 17]. However, the same-fold differences were noted in the absolute amounts of Col24al, Oc, and Col2al transcripts between late differentiating MC3T3-E1 and MCC cultures (Figure 4C). We therefore concluded that together the *in vivo* and *in vitro* data indicated that Col24al is constitutively expressed by osteoblasts during bone formation.

DISCUSSION

Collagen XXIV is a new member of the fibrillar collagen group that exhibits structural features of invertebrate fibrillar collagens [6]. Unlike collagen XXVII, the other member of the C clade of invertebrate-like fibrillar collagens, collagen XXIV is predominantly, if not exclusively, expressed in the forming bone elements of the mouse embryo [6]. Furthermore, cell type-specific transcription of Col24al is driven by nuclear factors implicated in the regulation of osteoblastogenesis [8]. The present study provides additional evidence for a potential role of collagen XXIV in bone physiology. The evidence includes *in vitro* experiments showing that Col24al expression in pluripotent C3H10T1/2 cells is induced by BMP-2, and that Col24al transcripts accumulate progressively and concomitantly with those of Oc in differentiating MC3T3-E1 and MCC cultures. We also showed that Col24al is constitutively expressed in mineralizing primary osteoblast cultures and in the bone of adult mice.

Furthermore, the analyses enabled us to estimate that collagen XXIV is the lowest represented collagen molecule in bone. We argue from these correlative lines of evidence that deposition of collagen XXIV into the bony matrix may participate in establishing the structural requirements for tissue mineralization. Our contention is supported by preliminary data indicating that osteopenia characterizes the otherwise-normal Col24al null mice.

The extracellular matrix, and fibrillar collagen assemblies in particular, play a key role in regulating bone mineralization and bone integrity [20]. Mutations in the subunits of collagens I and V or in collagen I-modifying enzymes have been associated with significant bone abnormalities and increased risk of fractures (2, 21, 22). Moreover there is emerging evidence from the oim/oim mouse that abnormal collagen I assembly also may perturb the activity of resident cells through an ill-defined mechanism, thus exacerbating the loss of bone integrity [23]. Additional studies from genetically targeted mice have implicated nonstructural components of the extracellular matrix (biglycan, osteocalcin, and osteonectin) in the control of bone mass during physiological growth and adult remodeling through the control of local and systemic signals of osteoregulation [24–28]. In spite of its low abundance, collagen XXIV may represent yet another matrix regulator of bone mass maintenance.

There are numerous examples of severe pathologies associated with loss of function of minor collagen types [1, 2]. A case in point is collagen XIX, the least abundant of all collagen types, whose loss in mice impairs muscle differentiation and esophageal function, and leads to perinatal lethality [29]. Ongoing investigations are aimed at elucidating the precise role of collagen XXIV in bone physiology by characterizing cellular

and/or structural mechanisms responsible for reduced bone mass in Col24a1-deficient mice.

ACKNOWLEDGMENTS

We thank Bayan Sudan for excellent technical support, and Karen Johnson for organizing the manuscript. We dedicate the study to the memory of Shizuko Tanaka. This work was supported by NIH grant AR-38648.

REFERENCES

1. Myllyharju, J., and Kivirikko, K.I. (2001). Collagens and collagen-related diseases. *Ann. Med*., 33, 7–21.
2. Myllyharju, J., and Kivirikko, K.I. (2004). Collagens, modifying enzymes and their mutations in human, flies and worms. *Trends Genet*., 20, 33–43.
3. Boot-Handford, R.P., and Tuckwell, D.S. (2003a). Fibrillar collagen: the key to vertebrate evolution? A tale of molecular incest. *Bioessays*, 25, 142–151.
4. Wada, H., Okuyama, M., Satoh, N., and Zhang, S. (2006). Molecular evolution of fibrillar collagen in chordates, with implications for the evolution of vertebrate skeletons and chordate phylogeny. *Evol. Dev*., 8, 370–377.
5. Boot-Handford, R.P., Tuckwell, D.S., Plumb, D.A., Rock, C.F., and Poulsom, R. (2003b). A novel and highly conserved collagen (pro-α1(XXVII)) with a unique expression pattern and unusual molecular characteristics establishes a new clade within the vertebrate fibrillar collagen family. *J. Biol. Chem*., 278, 31067–31077.
6. Koch, M., Laub, F., Zhou, P., Hahn, R.A., Tanaka, S., Burgeson, R.E., Gerecke, D.R., Ramirez, F., and Gordon, M.K. (2003). Collagen XXIV, a vertebrate fibrillar collagen with structural features of invertebrate collagens: selective expression in developing cornea and bone. *J. Biol. Chem*., 278, 43236–43244.
7. Pace, J.M., Corrado, M., Missero, C., and Byers, P.H. (2003). Identification, characterization and expression analysis of a new fibrillar collagen gene, COL27A1. *Matrix Biol*., 22, 3–14.
8. Matsuo, N., Tanaka, S., Gordon, M.K., Koch, M., Yoshioka, H., and Ramirez, F. (2006). CREB-AP1 protein complexes regulate transcription of the collagen XXIV gene (Col24a1) in osteoblasts. *J Biol. Chem*., 281, 5445–5452.
9. Plumb, D.A., Dhir, V., Mironov, A., Ferrara, L., Poulsom, R., Kadler, K.E., Thornton, D.J., Briggs, M.D., and Boot-Handford, R.P. (2007). Collagen XXVII is developmentally regulated and forms thin fibrillar structures distinct from those of classical vertebrate fibrillar collagens. *J Biol. Chem*., 282, 12791–12795.
10. Hjorten, R., Hansen, U., Telfer, H.E., Fernardes, R.J., Krakow, D., Sebald, E., Wachsmann-Hogiu, S., Bruckner, P., Jacquet, R., Landis, W.J., and Pace, J.M. (2007). Type XXVII collagen at the transition of cartilage to bone during skeletogenesis. *Bone*, 41, 535–542.
11. Karsenty, G. (2001). Transcriptional control of osteoblast differentiation. *Endocrinology*, 142, 2731–2733.
12. Nakashima, K., and de Crombrugghe, B. (2003). Transcriptional mechanisms in osteoblast differentiation and bone formation. *Trends Genet*., 19, 458–466.
13. Williams, G.R., Bland, R., and Sheppard, M.C. (1994). Characterization of thyroid hormone (T3) receptors in three osteosarcoma cell lines of distinct osteoblast phenotype: Interactions among T3, vitamin D3, and retinoid signaling. *Endocrinology*, 135, 2375–2385.
14. Yamaguchi, A., Komori, T., and Suda, T. (2000). Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfal. *Endoc. Rev*., 21, 393–411.
15. Yuasa, T., Kataoka, H., Kinto, N., Iwamoto, M., Enomoto-Iwamoto, M., Iemura, S., Ueno, N., Shibata, Y., Kurosawa, H., and Yamaguchi, A. (2006). Sonic hedgehog is involved in osteoblast differentiation by cooperating with BMP-2. *J. Cell. Biochem*., 193, 225–232.
16. Sudo, H., Kodama, H.A., Amagai, Y., Yamamoto, S., and Kasai, S. (1983). In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J Cell Biol*., 96, 191–198.
17. Wada, W., Kataoka, H., Yokose, S., Ishizuya, T., Miyazono, K., Gao, Y.H., Shibasaki, Y., and Yamaguchi, A. (1998). Changes in osteoblast phenotype during differentiation of enzymatically isolated rat calvaria cells. *Bone*, 22, 479–485.
18. Taylor, S.M., and Jones, P.A. (1979). Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. *Cell*, 17, 771–779.
19. Wang, E.A., Israel, D.I., Kelly, S., and Luxenberg, D.P. (1993). Bone morphogenetic protein-2 causes commitment and differentiation in C3H10T1/2 and 3T3 cells. *Growth Factors*, 9, 57–71.
20. Tzaphlidou, M. (2005). The role of collagen in bone structure: an image processing approach. *Micron*, 36, 593–601.
21. Morello, R., Bertin, T.K., Chen, Y., Hicks, J., Tonachini, L., Monticone, M., Castagnola, P., Rauch, F., Glorieux, F.H., Vranka, J., Bachinger, H.P., Pace, J.M., Schwarze, U., Byers, P.H., Weis, M., Fernandes, R.J., Eyre, D.R., Yao, Z., Boyce, B.F., and Lee, B. (2006). CRTAP is required for prolyl 3- hydroxylation and mutations cause recessive osteogenesis imperfecta. *Cell*, 127, 291–304.
22. Cabral, W.A., Chang, W., Barnes, A.M., Weis, M., Scott, M. A., Leikin, S., Makareeva, E., Kuznetsova, N.V., Rosenbaum, K.N., Tifft, C.J., Bulas, D.I., Kozma, C., Smith, P. A., Eyre, D.R., and Marini, J.C. (2007). Prolyl 3-hydroxylase 1 deficiency causes a recessive metabolic bone disorder resembling lethal/severe osteogenesis imperfecta. *Nature Genet*., 39, 359–365.
23. Zhang, H., Doty, S.B., Hughes, C., Dempster, D., and Camacho, N. P. (2007). Increased resorptive activity and accompanying morphological alterations in osteoclasts derived from the oim/oim mouse model of osteogenesis imperfecta. *J Cell Biochem*., 102, 1011–1020.
24. Ducy, P., Desbois, C., Boyce, B., Pinero, G., Story, B., Dunstan, C., Smith, E., Bonadio, J., Goldstein, S., Gundberg, C., Bradley, A., and Karsenty, G. (1996). Increased bone formation in osteocalcin-deficient mice. *Nature*, 382, 448–452.
25. Xu, T., Bianco, P., Fisher, L.W., Longenecker, G., Smith, E., Goldstein, S., Bonadio, J., Boskey, A., Heegaard, A.-M., Sommer, B., Satomura, K., Dominguez, P., Zhao, C., Kulkarni, A.B., Robey, P.G., and Young, M.F. (1998). Targeted disruption of the biglycan gene leads to an osteoporosis-like phenotype in mice. *Nature Genet*., 10, 78–82.
26. Delany, A.M., Amling, M., Priemel, M., Howe, C., Baron, R., and Canalis, E. (2000). Osteopenia and decreased bone formation in osteonectin-deficient mice. *J. Clin. Invest*., 105, 915–923.
27. Bi, Y., Stuelten, C.H., Kilts, T., Wadhwa, S., Iozzo, R.V., Robey, P.G., Chen, X.D., and Young, M.F. (2005). Extracellular matrix proteoglycans control the fate of bone marrow stromal cells. *J. Biol. Chem*., 280, 30481–30489.
28. Lee, N.K., Sowa, H., Hinoi, E., Ferron, M., Ahn, J.D., Confavreux, C., Dacquin, R., Mee, P.J., McKee, M.D., Jung, D.Y., Zhang, Z., Kim, J.K., Mauvais-Jarvis, F., Ducy, P., and Karsenty, G. (2007). Endocrine regulation of energy metabolism by the skeleton. *Cell*, 130, 456–469.
29. Sumiyoshi, H., Mor, N., Lee, S.-Y., Henderson, S., Tanaka, S., Yoshioka, H., Rattan, S., and Ramirez, F. (2004). Esophageal muscle physiology and morphogenesis require assembly of a collagen XIX-rich basement membrane zone. *J. Cell Biol*., 166, 591–600.