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Establishment and Characterization of an Immortomouse-Derived Odontoblast-Like Cell Line to Evaluate the Effect of Insulin-like Growth Factors on Odontoblast Differentiation

Javier Catón, Pablo Bringas Jr., and Margarita Zeichner-David*

Center for Craniofacial Molecular Biology, University of Southern California School of Dentistry, Los Angeles, California 90033

Abstract Insulin-like growth factors (IGF-I and IGF-II) play important roles in regulating growth and differentiation of many different organs including teeth. The presence of these factors in the developing tooth has been demonstrated. In vitro studies using tooth explants grown in the presence of IGFs suggest that they promote differentiation of ameloblast and odontoblasts cells. This is achieved by inducing or repressing gene expression associated with these cells. Since some of the genes involved in tooth differentiation are expressed by both cells, to determine the effect of IGF on odontoblast cell differentiation we first need a cell line in which a controlled environment can be created. In this study, we report the establishment and characterization of an Immortomouse-derived odontoblast-like cell line. This conditional cell line can grow indefinitely under permissive conditions in the presence of INF-γ at 33°C, differentiate into odontoblast-like cells and produce a mineralized extracellular matrix when the INF-γ is removed and cell maintained at 39°C. Addition of exogenous IGFs to the media results in an accelerated production of a mineralized matrix. This is the result of increased transcription of genes associated with bone mineralization while down regulating genes associated with dentin formation like DSPP. This data suggest that IGFs induce dental papillae mesenchyme cells to produce a bone-like mineralized extracellular matrix. J. Cell. Biochem. 100: 450–463, 2007.

Key words: IGFs; dental papilla mesenchyme; cell lines; odontoblasts; differentiation; mineralization

Growth factors can regulate proliferation, determination and differentiation of specific cell phenotypes within a number of different organ systems including teeth. Insulin-like growth factors (IGF-I and IGF-II) are considered to be pleiotropic and act as mitogenic and differentiation factors. The IGF family is a complex system comprised of two ligands (IGF-I and IGF-II), two cell surface receptors (IGF1R and IGF2R), at least six IGF binding proteins (IGFBP-1 to IGFBP-6), and multiple IGFBP proteases. The receptors, binding proteins and binding protein proteases regulate the activity of the ligands in all tissues [LeRoith, 1991; Yamaguchi et al., 1991; LeRoith et al., 1995; Rubin and Baserga, 1995; Collet and Candy, 1998; Hwa et al., 1999]. IGFs play an important role in the regulation and maintenance of bone associated proteins like Collagen Type-I, osteonectin/SPARC (ONC), osteopontin (OPN), Osteocalcin (OSC), and alkaline phosphatase (AP) [Oyamada et al., 1990; Thiebaud et al., 1990; Tanaka et al., 2002]. Since many of these proteins are also expressed during tooth formation, it is likely that some of the same regulatory mechanisms involved in bone development could also be applicable to tooth development.

The expression of IGFs and their receptors during tooth development has been reported [Bellone et al., 1990; Joseph et al., 1993, 1994,

Javier Catón's present address is Department of Craniofacial Development, King's College London, Guy's Tower Floors 27-28, GKT Dental Institute, London Bridge, London SE1 9RT, UK. javier.caton@kcl.ac.uk

*Correspondence to: Dr. Margarita Zeichner-David, Center for Craniofacial Molecular Biology, USC School of Dentistry, 2250 Alcazar Street, CSA 106, Los Angeles, CA 90033. E-mail: zeichner@usc.edu

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1996]. In vitro studies using mouse mandibular molars explants grown in the presence of IGFs resulted in an increase of tooth volume, mitotic index and cell differentiation suggesting that IGF-I promotes differentiation and development of ameloblasts and odontoblasts [Young, 1995; Yamane et al., 1997; Takahashi et al., 1998; Catón et al., 2001]. Differences in the effect of IGF-I and IGF-II were found; deposition of dentin extracellular matrix was increased with IGF-I but was not affected by IGF-II while expression of DSPP mRNA was decreased with both with IGF-II being more effective [Catón et al., 2005]. Since DSPP is expressed by both, odontoblasts and pre-secretory ameloblasts and this expression is lost after ameloblasts initiate their secretory stage [MacDougall et al., 1998; Bégue-Kirn et al., 1998a; Sreenath et al., 1999] we hypothesized that IGFs selectively down-regulate the expression of DSPP in ameloblasts to favor the induction of genes involved in enamel formation like amelogenin and enamelin, while odontoblasts might not be affected. This hypothesis can be tested using an isolated odontoblast-like cell line in which a controlled environment can be created to determine the effect of IGFs on odontoblast differentiation.

In the present study, we report the establishment and characterization of an Immortomouse-derived dental papilla mesenchyme (DPM) cell line containing a temperature sensitive mutant of the SV40 large tumor antigen (T-Ag) under the control of the interferon-γ- (INF-γ)-inducible *H-2K*^{b} promoter (*H-2K*^{b}-tsA58) developed by Jat et al. [1991]. This DPM cell line behave like immortal cells under permissive conditions and terminally differentiate into odontoblast-like cells when these conditions are removed and the cells are maintained at differentiating conditions. The effects of exogenous IGFs on the proliferation, differentiation and dentin-formation of these cells were analyzed.

MATERIALS AND METHODS

Cell Source

DPM cells were prepared from 18 days old mouse embryos (E-18) mandibular first molars dissected from heterozygous Immortomouse. Homozygous *H-2K*^{b}-tsA58 males were mated overnight with DC-1 (ICR) females (Charles Rivers, Wilmington, MA). Females were inspected for vaginal plug the following morning (Day 0 of gestation) and were sacrificed after 18 days and embryos extracted by C-section. Dissected first mandibular molars were placed in 2.4 U/ml of Dispase (Boehringer Mannheim, Indianapolis, IN) and incubated for 1 h at 4°C. The mesenchymal tissue was mechanically separated from the epithelium using tungsten needles and placed in 0.25% trypsin (Gibco BRL, Gaithersburg, MD) at 37°C for 30 min. The cells were disaggregated by passing them through an 18 g needle, centrifuged, re-suspended and plated as described below. All animals used in this study were treated under the most humane conditions and all protocols were approved by the University of Southern California IACUC.

Proliferation and Differentiation Cultures

Cells were plated in 24-well plastic tissue culture plates using permissive conditions: DMEM (Gibco BRL), supplemented with 10% FBS (HyClone, Logan, UT), 1× of 5,000 U penicillin, 5 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO), and 5 U/ml of murine recombinant IFN-γ (Gibco BRL). The cells were incubated at 33°C in 95% air and 5% CO_2. Cultures were periodically monitored and when they reached confluence cells were transferred (1:5 passage rate) to new culture dishes. Stocks of these cells were stored in liquid nitrogen for future use. DPM cells were maintained under permissive conditions for 45 passages before performing differentiation studies. Cells were induced to differentiate by changing the temperature to 39°C and switching to differentiation media: DMEM supplemented with 10% FBS, 1× of 5,000 U penicillin, 5 mg/ml streptomycin, 2 mM Na β-Glycerophosphate (Sigma-Aldrich), and 50 μg/ml of Ascorbic Acid (Gibco BRL). Media was changed every other day.

Cell Proliferation Assay

To determine the rate of cell proliferation, DPM cells were plated in 6-well plates at a density of 3×10^4/plate and grown in either permissive or differentiating conditions for different time periods. The culture medium was changed every other day and at collection times, cells were washed with medium, trypsinized and the total number of cells determined using a hematocytometer. Data are expressed as mean ±SD of values obtained from one representative experiment ($n = 8$). Data were analyzed using Student *t*-test, taking the

confidence level at $P < 0.05$ using the Epistat statistic program. To determine the effect of IGFs on proliferation, different concentrations of IGFs were added and cells were collected after 24 h.

Conditions for IGF Studies

To study the effects of IGFs, DPM cells were placed under differentiating conditions but the serum in the differentiation media was replaced with 20 μg/ml Transferrin, 1% FAC + LO (1 μg/ml BSA, 1 M/ml Linoleic Acid and 1 M/ml Oleic Acid) and 1 ng/ml of fibroblast growth factor-b (FGFb, Sigma-Aldrich). In order to avoid binding of the IGFs to the plastic culture dishes 0.1 mg/ml of purified BSA was added (this media will be referred as TFF + BSA serum-free media). TFF + BSA serum-free media was supplemented with either 50 ng/ml of receptor grade human recombinant des(1–3)IGF-I or 50 ng/ml of receptor grade human recombinant des(1–6)IGF-II (GroPep, Adelaide, Australia). Untreated parallel cultures were done as controls. Cells were maintained under these conditions for up to 30 days depending of the experiment. The time course for action of the IGFs on cells was determined using the LS8 cell line transfected with a luciferase reporter gene driven by the amelogenin promoter [Zhou and Snead, 2000]. These cells displayed a significant increase on the luciferase activity after 5 h of IGF treatment compared to untreated controls, therefore RNA extraction was done 5 h after the cultures were changed to fresh media with or with out IGFs.

Immunohistochemistry

DPM cells were grown for 20 days under differentiation conditions, fixed with 70% methanol/30% acetic acid and incubated with specific antibodies produced in our laboratory against dentin phosphoprotein (DPP) and tuftelin, or commercially obtained antibodies against vimentin, AP (Sigma-Aldrich), Collagen Type-I, Collagen Type-IV (Chemicon Int., Temecula, CA), and SV40 (Santa Cruz Biochemicals, Santa Cruz, CA), The Histomouse-SP kit (ZYMED/Invitrogen, Carlsbad, CA) was used to develop the color following the manufacturer's protocol.

RT-PCR

Total RNA was extracted from cells maintained under differentiating conditions after 0, 5, 10, 15, 20, and 25 days intervals using the Total RNA Miniprep kit (Stratagene, San Diego, CA). First strand cDNA was prepared using the cDNA Cycle kit (Invitrogen) and amplified by polymerase chain reaction (PCR) prepared from 10× PCR buffer, 10 mM dNTPs, 1.5 U of ExTaq (TaKaRa, Shiga, Japan) and 10 pM of specific primers (annealing temperatures are listed in Table I). The reactions were performed at 94°C denaturing temperature and 72°C extension temperature for 30 cycles using the PTC-100 thermocycler (MJ Research Inc, Waltham, MA). The PCR products were separated by electrophoresis on 2% agarose gels (Gibco BRL). Gels were stained with ethidium bromide and amplified bands were identified under UV light and digitally photographed.

Von Kossa Staining

Cells were grown under differentiation conditions and fixed with 70% methanol and 30% acetic acid at 5, 10, 15, 20, and 30 days intervals. Fixed cells were dehydrated in ethanol, washed and treated with a 5% solution of $AgNO_3$ for 1 h under a 100 W light source. Cells were rinsed and treated with a 5% solution of sodium thiosulfate for 3 min, washed and photographed.

Real Time iQ PCR

Specific real time PCR primers were designed for dentine sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP-1), Collagen Type-I, tuftelin, vimentin, ONC, osteopontin (OPN), osteocalcin (OCN), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Total RNA was extracted after cells were grown for 10 and 20 days in differentiation TFF + BSA serum-free media containing the IGFs. RNA was extracted and cDNA prepared as previously described. Real-time PCR was done in a two step reaction in the presence of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) using the "iCycler iQ detection system" (Bio-Rad). The annealing temperatures for each primer and their sequences are specified in Table II. Every real-time PCR reaction was done in triplicate.

Statistical Analysis

Real time PCR data was analyzed using a one-way analysis of variance ANOVA and Student's t-test, with a confidence level of $P < 0.05$.

TABLE I. Primers Used for RT-PCR

Primer	Sequence	Annealing temperature	Number of cycles
Actin s	CGGTTGGCCTTAGGGTTCAGGGGG	50°C	30
Actin as	CATCGTGGGCCGCTCAGGCACCA		
SV40 s	GCTTGGCTACACTGTTTGTTGC	50°C	30
SV40 as	TGTTTCATGCCCTGAGTCTTCC		
Vimentin s	TGCGAGAGAAATTGCAGGAGG	50°C	30
Vimentin as	GCAGTAAAGGCACTTGAAAGC		
DMP-1 s	CAGGTCGGAAGAATCTAAAGG	50°C	30
DMP-1 as	TCTCAGTAACTGTCAGGTTGG		
Collagen I s	GCCTTTTCTGTTCCTTTCTCC	52°C	30
Collagen I as	CCTCCCTTGTTAAATAGCACCG		
Alkaline phosphatase s	CCTGACCAAAAACCTCAAAGGC	58°C	30
Alkaline phosphatase as	ACATTTTCCCGTTCACCGTCC		
Osteonectin s	GATCCATGAGAATGAGAAGCG	50°C	30
Osteonectin as	CTATGTGAGCACCTTATCCCC		
Osteopontin s	TTCGGATGAGTCTGATGAGACC	48°C	30
Osteopontin as	GGAAGAACAGAAGCAAAGTGC		
Osteocalcin s	TCTCTCTGACCTCACAGATCC	50°C	30
Osteocalcin as	AGAGTTTGGCTTTAGGGCAGC		
IGF-I s	GGACCAGAGACCCTTTGCGGGG	55°C	30
IGF-I as	GGCTGCTTTTGTAGGCTTCAGTGG		
IGF-II s	GGCCCCGGAGAGAGACTCTGTGC	55°C	30
IGF-II as	GCCCACGGGGTATCTGGGGAA		
IGF-IR s	ACTGACCTCATGCGCATGTGCTGG	55°C	30
IGF-IR as	CTCGTTCTTGCGGCCCCCGTTCAT		
IGF-IIR s	TGTACACTCTTCTTCTCCTGGCA	55°C	30
IGF-IIR as	AGAGATGTTGATGTAGAAGACAGG		
IGFBP-2 s	TGCCCAAAGTGTGCAGTAAACC	58°C	30
IGFBP-2 as	ATTTCTTTCCCCATTCCCAGGC		
IGFBP-3 s	TGACCGATTCCAAGTTCCATCC	57°C	30
IGFBP-3 as	AGGCAATGTACGTCGTCTTTCC		
IGFBP-6 s	CTCTATGTGCCAAACTGTGACC	55°C	30
IGFBP-6 as	TTTTTAAGCCAGAGACACCCCC		

RESULTS

Establishment and Characterization of the Conditionally Immortalized DPM Cell Line

DPM cells were grown under permissive conditions for approximately 45 passages. A typical appearance of a spindle shape characteristic of fibroblast cells can be seen in Figure 1A. After 6 months of continuous growth the cells did not show any sign of senesce and continued to divide confirming the immortal character of these cells. Growth rates were

TABLE II. Primers Used for Quantitative Real-Time PCR

Primer	Sequence sense sequence anti-sense	Annealing temperature
Vimentin	AGGACATCATGCGGCTGCGAGA TGTTCCTGAATCTGGGCCTGCA	64
Tuftelin	TCAGCACACAGGCCCGTGCCAA CCTGCGGCTACCAGGGTAGTCA	64
Collagen I	CGGAGAAGAAGGAAAACGAGGAG CACCATCAGCACCAGGGAAAC	58
Osteocalcin	CAGACTCCGGCGCTACCTTGGA TCAAGTCCCGGAGAGCAGCCAA	63
DMP-1	GAGGGACAAGACCCCAGCAGTG CAGCTTGCTCCTCGGTGGACTG	62
DSPP	GTGAGGACAAGGACGAATCTGA CACTACTGTCACTGCTGTCACT	53
Osteonectin	GAGAGCAACACCCCCATGTGTG AATCCAGGCAGGGGGCGATG	63
Osteopontin	AGAAACTCTTCCAAGCAATTCCAA AATCCTCGCTCTCTGCATGG	56

All the reactions were done for 40 cycles.

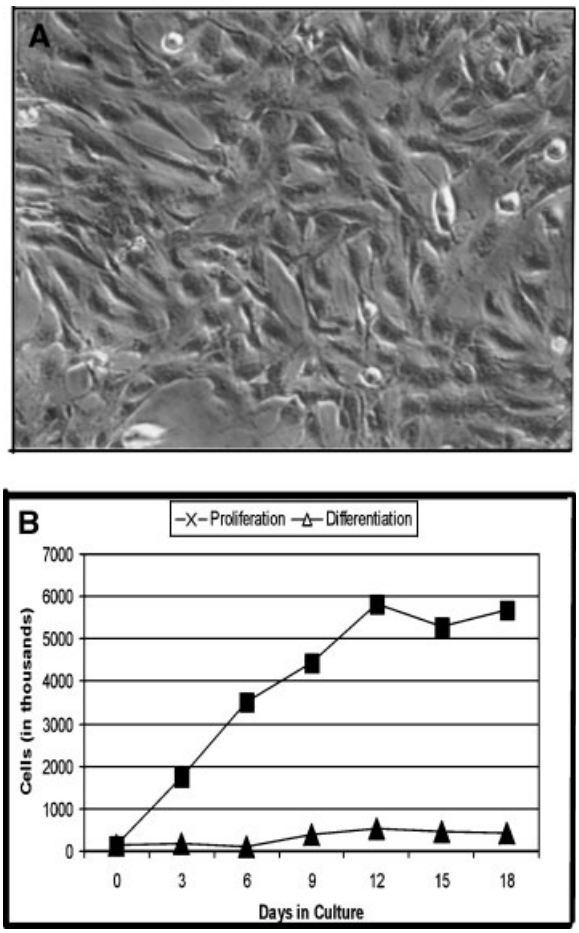

Fig. 1. DPM cells maintained under proliferation conditions shown at a magnification of 20× (**A**). Cell growth rate of the DPM cells maintained under proliferation or differentiation conditions is shown in sub part (**B**).

determined under proliferation and differentiation conditions and Figure 1B shows a linear growth under proliferation conditions until they reach confluence at Day 12. When cells are grown under differentiation conditions they stop dividing and maintain almost the same number of cells up until Day 9 when there appears to be a slight increase. These results confirm the conditional immortal character of these cells.

To determine the phenotype of the cells maintained under differentiation condition, we first tested their capacity to produce a mineralized extracellular matrix. Cells were grown for 5 days intervals up to 30 days, fixed and stained using the Von Kossa procedure to determine the presence of calcium phosphate in the extracellular matrix. As can be seen in Figure 2, DPM cells started to show formation of nodules after 20 days in culture and at 30 days in culture silver deposits are seen.

To determine the molecular phenotype of these cells, RT-PCR was performed on RNA samples obtained at 5-day intervals from cells grown under permissive conditions (0 days) and up to 25 days in cells maintained under differentiation conditions in the presence of 10% serum (Fig. 3). The integrity of the RNA was tested using β-actin and the mesenchymal nature of these cells was confirmed by determining the expression of vimentin. Transcripts for Collagen Type-I, ONC, osteopontin, tuftelin and BMP4 were expressed at all time points. DMP-1 and osteocalcin were not expressed under permissive conditions but were detected after 5 days in culture under differentiating conditions. Collagen Type-IV and AP were expressed in the initial stages of differentiation and were down-regulated after a few days in culture. Expression of DSPP was seen after 25 days in culture. No transcripts for ameloblastin or amelogenin (not shown) were detected. Expression of SV40 T-Ag transcripts appeared to continue at some level at all stages of culture.

To determine if protein expression was also present, DPM cells were grown under differentiation conditions and the presence of the proteins determined by immunocytochemistry. The data presented in Figure 4 shows the presence of SV40, vimentin, tuftelin, Collagen Type-I, Collagen Type-IV, AP and DPP protein in the DPM cell cultures.

Do DPM Cells Express Members of the IGF System?

In order to determine if IGFs, their receptors and/or binding proteins are expressed in the DPM cells maintained in vitro, RT-PCR was done using primers for the different members of the IGF system using the conditions summarized in Table I. The results presented in Figure 5 show the expression of transcripts for members of the IGF system throughout different times in culture. IGF-I and IGF-II appear to follow a similar pattern of expression. Although the data is by no means quantitative, it appears that expression of both IGF-IR and IGF-IIR is stronger in the proliferative stage (Day 0) than in the differentiation stages, Furthermore, the expression of IGF-IIR appears to be down-regulated while transcript for IGF-IR continue

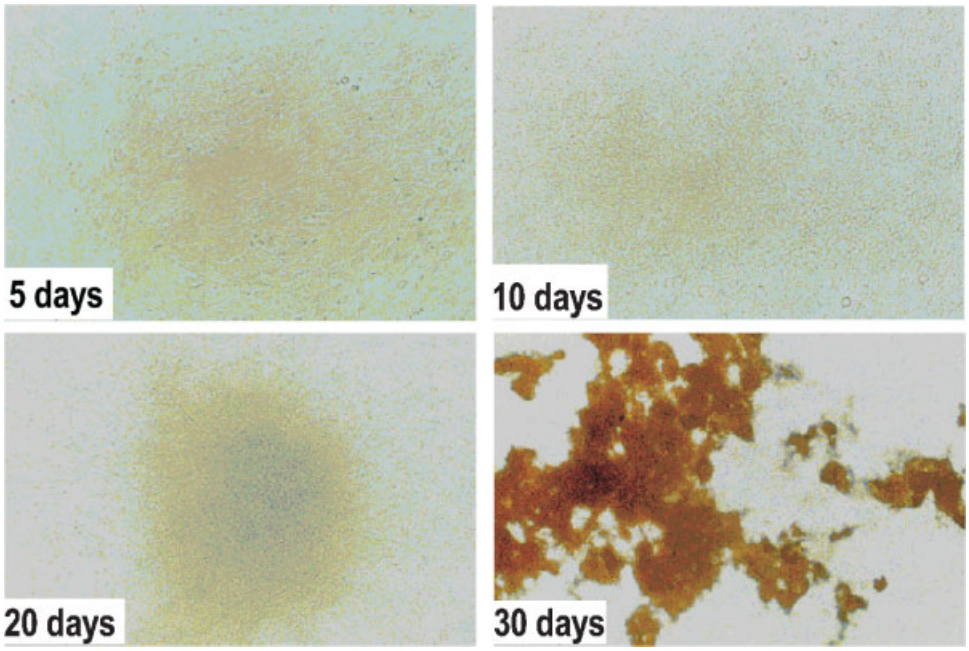

Fig. 2. Von Kossa staining on DPM cells grown under differentiation conditions for 5, 10, 20, and 30 days. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

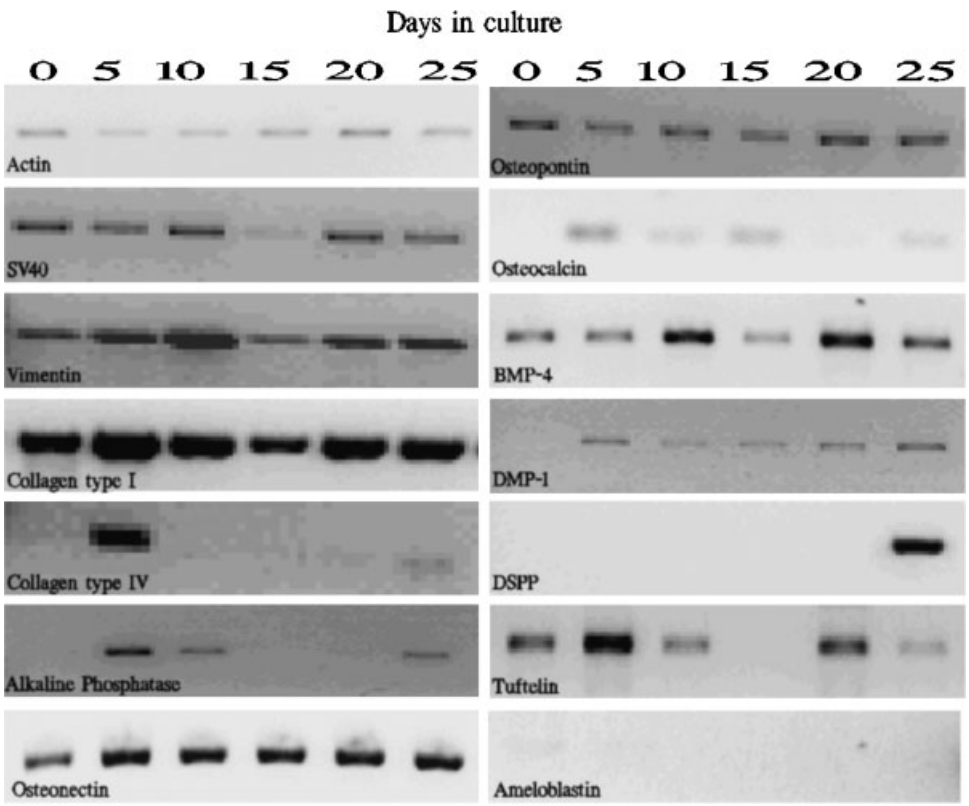

Fig. 3. The phenotype expressed by DPM cells maintained under differentiating conditions in the presence of 10% calf serum was determined using RT-PCR. DPM cells were grown for different periods of time. RNA was extracted and converted to cDNA. The primers and PCR conditions used in these experiments are documented in Table I. BMP-4, bone morphogenetic protein 4; DMP-1, dentin matrix protein 1; DSPP, dentin sialophosphoprotein.

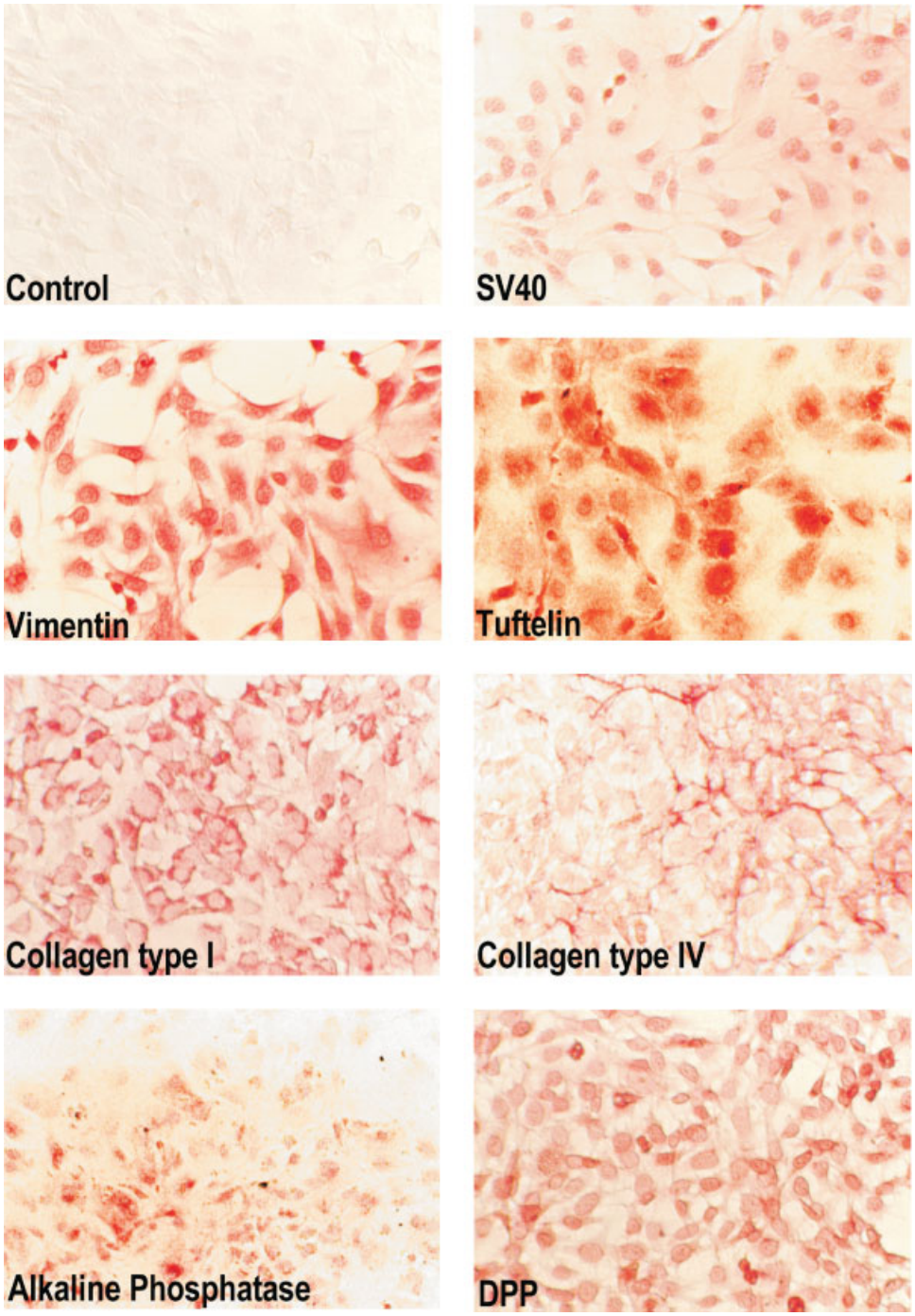

Fig. 4. Immunocytochemical staining of DPM cells cultured under differentiation conditions. Antibodies used are labeled in each picture. The control represents cells stained with secondary antibody alone. The samples used for SV40, vimentin and tuftelin were grown for 5 days while the rest of the samples were grown for 24 days. DPP, dentin phosphoprotein; 100× magnification.

to be present. Transcripts for IGFBP2 are present at all time points samples of DPM differentiation tested. IGFBP3 is expressed at later stages of differentiation while IGFBP6 appears to be expressed mostly under proliferation conditions. No expression of IGFBP1, IGFBP4, and IGFBP5 was apparent (not shown).

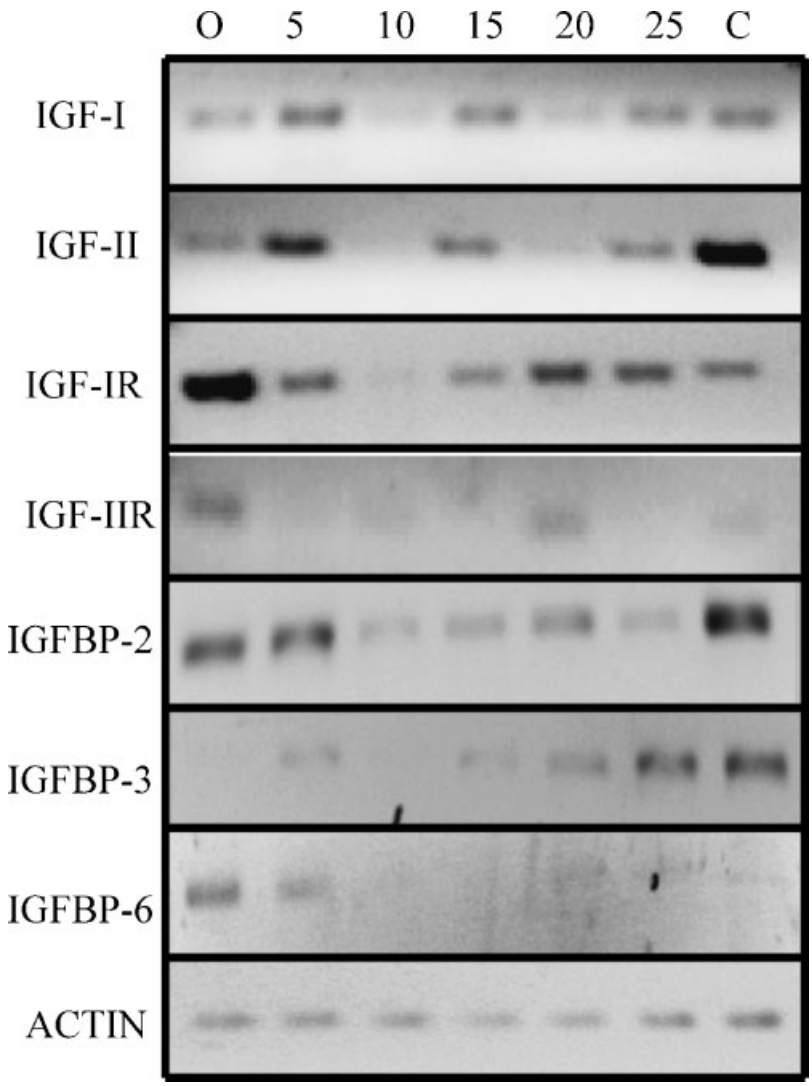

Fig. 5. Expression of members of the IGF system determined by RT-PCR. DPM cells were grown for different periods of time, RNA was extracted and cDNA prepared for RT-PCR. C = control cDNA which was prepared from 2-day postnatal mouse mandibular molars mRNA.

The Effect of IGF-I and IGF-II on DPM Cell Proliferation and Differentiation

To find out if IGFs have any effect on DPM cells, and at what concentrations, we first determined their effect on cell proliferation. The data shown in Figure 6 indicates that at concentrations of 50 ng/ml, both IGF-I and IGF-II significantly increased proliferation of DPM cells. Higher concentration of IGFs did not result in higher proliferation rates.

The effect of IGFs on gene expression was analyzed at different days of differentiation under serum-less conditions (TFF-BSA media) by measuring expression of genes associated with the odontoblast phenotype using real time PCR The results shown in Figure 7 indicate that IGF-I increased the expression of vimentin after 10 days in culture but IGF-II had no effect. After 20 days in culture both IGF-I and IGF-II resulted in a slight inhibition. There was a statistically significant increase in the expression of tuftelin with IGF-I after 10 days while IGF-II decreased the expression of tuftelin mRNA at the same time. After 20 days in culture, tuftelin expression was slightly reduced in the treated samples. Both IGF-I and IGF-II increased the expression of Collagen Type-I and ONC after both, 10 and 20 days in culture. Osteopontin and osteocalcin

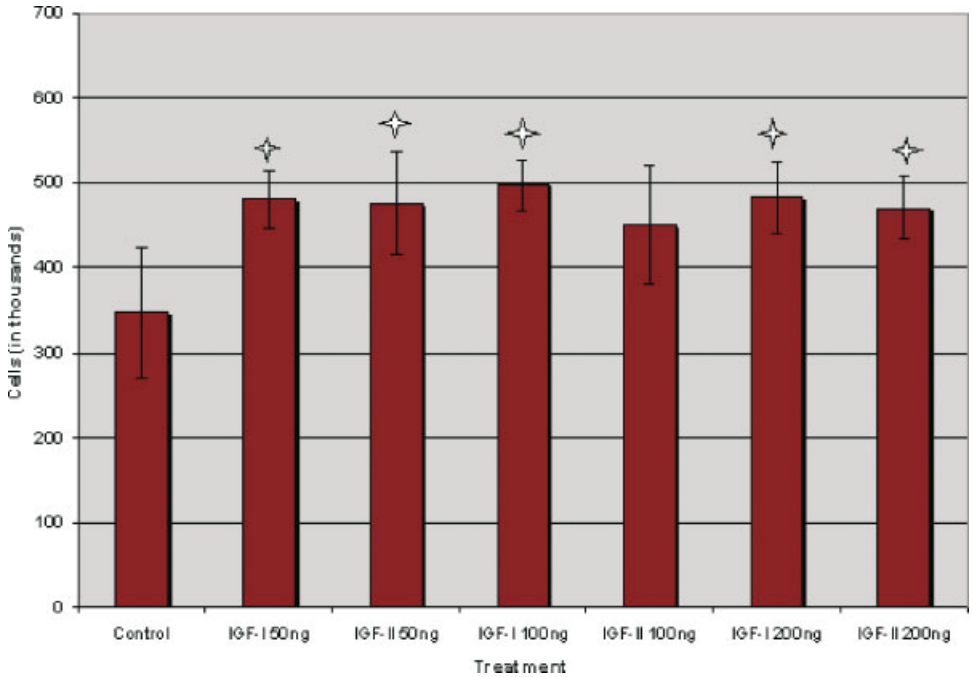

Fig. 6. Effect of IGF-I and IGF-II on DPM cell proliferation. Cells were grown in TFF + BSA serum-free media in the presence of different concentrations of IGFs for 24 h; **P*-value <0.01, ***P*-value <0.001. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

transcripts were increased at 10 days in culture, but slightly decreased at 20 days. Transcripts for dentine-associated genes like DMP-1 was significantly decreased while DSPP transcripts were inhibited on cells treated with IGFs after 10 days in differentiation and no expression was seen after 20 days even in the control.

Since the presence of IGFs resulted in changes in the phenotype expressed by these cells, we wanted to determine if it had also an effect on the mineralization of the extracellular matrix secreted by these cells. Cells were grown in serum-less differentiation media treated with 50 ng/ml of IGF-I or IGF-II and samples collected at intervals of 5 days for Von Kossa staining As can be seen in Figure 8, in the presence of both IGF-I and IGF-II, Von Kossa positive stain can be seen as early as 10 days, with more prominent nodules clearly visible after 15 days in culture. In contrast, cells grown in the absence of IGFs are Von Kossa negative. In these cells, Von Kossa positive nodules are not seen until 30 days in culture (Fig. 2).

DISCUSSION

The effects of IGFs on bone have been extensively studied. IGFs are thought to be mediators of the effect of growth hormone (GH) in the deposition of new bone during growth in vertebrates by regulation of genes important for mineralization. Similarities between tooth and bone suggest the possibility that IGFs could regulate tooth development in similar ways to that in bone. During tooth development, the expression of IGF-I in the rat incisor can be found in the apical loop, secretory ameloblasts, secretory odontoblasts and maturation ameloblasts [Joseph et al., 1993, 1994, 1996]. IGF-IR was immunohistochemically localized at early stages of development to the outer and inner enamel epithelium, a few cells of the stellate reticulum and a few cells of the dental papilla in contact with the enamel organ. IGF-I receptor was also present in DPM and in some cells of the dental follicle at late stages of tooth development [Bellone et al., 1990]. The presence members of the IGF system in the developing tooth and, more importantly, its receptor in late stages of tooth development emphasizes their importance. In order to better understand the effects of IGFs on odontoblast differentiation, we established an odontogenic cell line from *H-2K^b*-tsA58 transgenic mice capable of differentiating and producing a mineralized extracellular matrix in vitro.

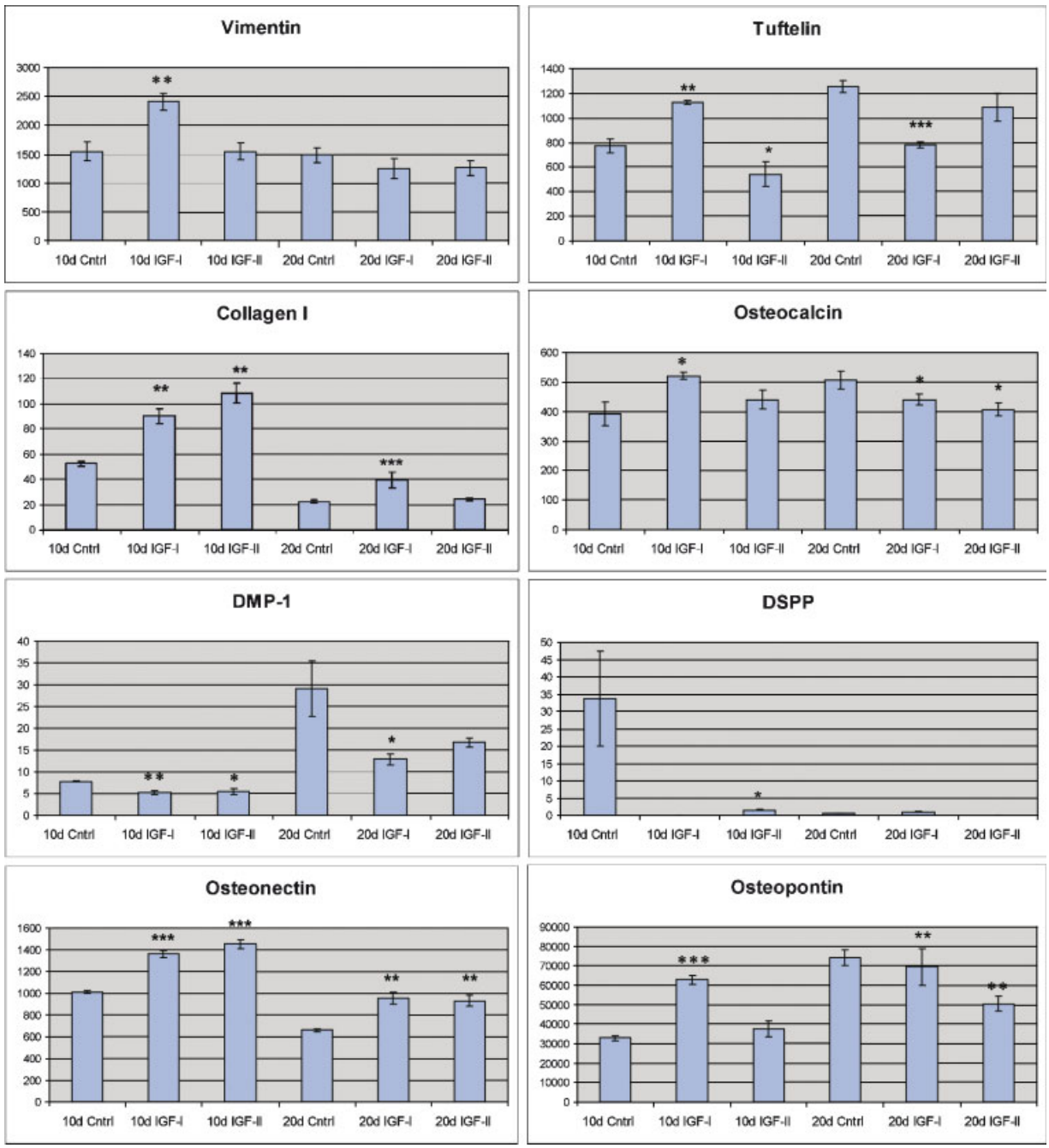

Fig. 7. Graphical representation of the real-time RT-PCR results from DPM cells grown in TFF + BSA serum-free media treated with IGF-I, IGF-II or untreated controls for 10 and 20 days. The results are normalized to GAPDH and the Y-axis represents values relative to a Standard curve; **P*-value < 0.05, ***P*-value < 0.01, ****P*-value < 0.001. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Availability of cell lines that can differentiate in a controlled environment in vitro is important to understand the process of tissue differentiation leading to tooth development. As such, the availability of dental papillae mesenchymal cells that can differentiate to odontoblast-like cells and produce a dentin mineralized matrix is of tremendous value to understand the process of dentinogenesis. DPM cells in primary culture have been used in many studies [Nakashima, 1991; Seux et al., 1991; Kuo et al., 1992; MacDougall et al., 1995; Satoyoshi et al., 1995;

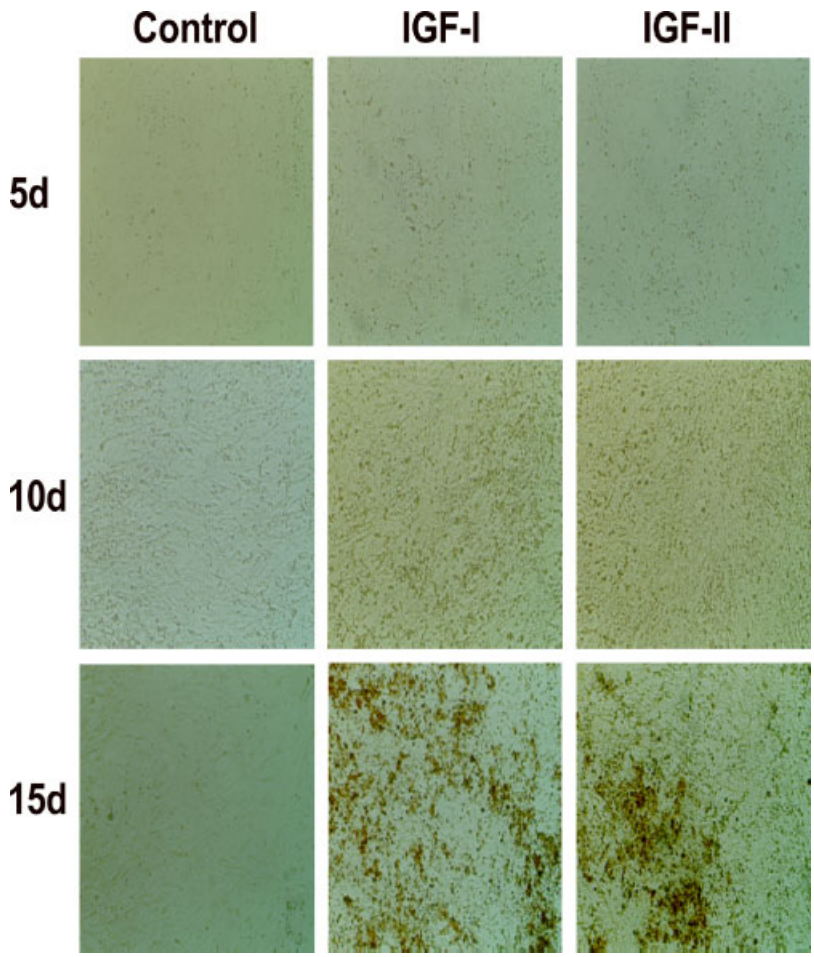

Fig. 8. Von Kossa staining of DPM cells cultured for 5, 10, and 15 days under differentiation conditions in serum-less TFF-BSA media. The cells were treated with IGF-I, IGF-II or untreated controls, 40× magnification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Bégue-Kirn et al., 1998b]. Unfortunately, cells in primary cultures become senescent after a relatively short period and new animals need to be sacrificed to obtain new cells. The alternative is to use immortalized cell lines (using chemical or viral transformations), but sometimes the normal physiology of these cell lines is altered and they might lose their ability to differentiate in vitro. The use of transgenic mice with conditionally immortalized genes provides a good and simple solution since cells derived from these animals maintain the capability to differentiate in vitro after inactivation of the immortalizing gene. In this study, we demonstrate that DPM cells derived from the Immortomouse can be grown indefinitely while under permissive conditions and when placed under differentiation conditions they differentiate to odontoblast-like cells and deposit a mineralized extracellular matrix resembling dentin.

When Immortomouse-derived DPM cells were grown in the presence of ascorbic acid and β-glycerophosphate, after 20 days of culture the cells start arranging into nodules, a typical indication for initiation of mineralization, and by 30 days they show a strong Von Kossa positive staining. Immunohistochemical analysis showed filamentous-like structures between the cells after 25 days in differentiation conditions. These filaments were immunoreactive for Collagen Type-IV antibody. Collagen Type-IV has been found in the basement membrane dividing pre-ameloblast cells from pre-odontoblast cells in the developing tooth in vivo and our results suggest that DPM cells contribute to the formation of the basement membrane by

synthesizing and secreting this protein. RNA transcripts for Collagen Type-IV were also detected at early stages of differentiation conditions. Cranial neural crest cells populate odontogenic mesenchymal tissues including the DPM cells [Chai et al., 2000] therefore the mesenchymal nature of the Immortomouse-derived DPM cells was demonstrated by the expression of vimentin, a mesenchymal marker. Vimentin transcripts were detected under permissive culture conditions and continue at all stages of differentiation. Collagen Type-I, the most abundant protein in bone and dentin is also expressed by these cells.

The presence of transcripts for other proteins associated with mineralization like AP, osteocalcin and DMP-1 were detected 5 days after the cells were placed under differentiation conditions. Transcripts for osteopontin and ONC were detected at all stages including under permissive conditions. Our cells were also immunoreactive to DPP and tuftelin. Transcripts for *dspp*, which encodes for DPP and Dental sialoprotein (DSP), were also found in cells differentiating for 25 days. DSPP is an important marker for odontoblast differentiation and its presence in these cells supports their odontoblast phenotype. Tuftelin, although initially thought to be an enamel organ epithelial marker, it has been localized at earlier stages of odontoblast differentiation [Diekwisch et al., 1997; Zeichner-David et al., 1997]. Taken all together these results indicate that the Immortomouse-derived DPM cell line is a suitable line to study the effect of IGFs on dentin formation.

The presence of transcripts for SV40 T-Ag under differentiation conditions was somehow unexpected although it has been reported that small levels of the SV40 T-Ag transcripts are not that uncommon [Jat et al., 1991]. Jat et al. [1991] noted that the H-2Kb promoter is active in a wide variety of tissues at various levels and expression can be increased above basal levels in most cells by exposure to IFN. However, the SV40 T-Ag gene product is only functional at the permissive temperature of 33°C and is degraded at the non-permissive temperature of 39.5°C.

The pattern of expression of members of the IGF system in our DPM cells in vitro was determined. The presence of IGF-I, IGF-II, and IGF-I receptor transcripts at proliferation and all differentiation stages, and IGF-IIR under proliferation conditions, underlines their significance in these cells. The presence at all time points of the IGFBP2, an IGF inhibitor [Ross et al., 1989], suggests the possibility that the DPM cells regulate, in an autocrine manner, the action of IGFs. The transcripts for IGFBP3 are only seen after the cells are induced to differentiate. This binding protein is thought not only to be an IGF inhibitor responsible for inhibiting cell growth [Villaudy et al., 1991] but also to have IGF-independent activities as inducer of apoptosis [Rajah et al., 1997]. We also detected the presence of IGFBP6 under proliferation conditions. This protein binds IGF-II with very high affinity and it is thought to be a potent IGF inhibitor.

When these cells were treated with IGFs in serum-less differentiation conditions we observed that the process of mineralization started earlier than in untreated controls suggesting that IGFs accelerate the expression of some genes associated with this process. To test this hypothesis we measured the relative levels of expression of genes associated with odontoblast differentiation and mineralization using real-time RT-PCR. The levels of expression of the mesenchymal marker vimentin of cells treated with IGFs compared to untreated controls was only significantly increased by IGF-I at 10 days in culture. The relative small change of vimentin transcripts suggests that IGFs did not change the DPM cells mesenchymal phenotype throughout differentiation. The RNA transcripts for Collagen Type-I and ONC, two important genes for mineralization, were significantly increased by both IGFs at 10 days in differentiating conditions. The increased induction of these two genes was maintained at 20 days. Transcripts for osteocalcin, osteopontin and tuftelin were significantly increased at 10 days, but only for cells treated with IGF-I, while IGF-II increased, but not statistically significantly, transcripts for osteocalcin and osteopontin. Proteins encoded by these transcripts are known to be important for mineralization therefore it is possible that the increase of transcripts for these genes at early stages of differentiation is associated with the observed early mineralization. After 20 days in differentiating conditions only the transcripts for Collagen Type-I and ONC were increased. Interestingly, dentine related genes such as DSPP and DMP-1 were inhibited or significantly down regulated by IGFs in these cells. It is important to note the discrepancy between

the expression of DSPP at 25 days in Figure 3 and at 10 days in Figure 7. This difference is probably due to the different growth conditions used in these experiments. The data presented in Figure 3 was obtained when DPM cells were grown in differentiating media containing 10% fetal calf serum. The data presented in Figure 7 represents results obtained with cells grown under differentiation conditions without serum, in the TFF-BSA medium. These conditions are needed to determine the real effect of exogenous IGFs since serum contains IGFs. Never the less, what is relevant to this study is that in either condition, these cells express DSPP which is considered a marker for odontoblasts.

These results are in agreement with our previous results, using tooth organs maintained in in vitro culture conditions, where IGFs also inhibited expression of DSPP and DMP-1 [Catón et al., 2005] while inducing enamel specific genes like amelogenin and ameloblastin [Takahashi et al., 1998].

Interestingly, the presence of IGFs accelerated the production of a mineralized extracellular matrix while inhibiting the expression of dentin-associated genes believed to participate in the process of dentin mineralization. This suggests that the mineralized matrix produced by these DPM cells in the presence of exogenous IGFs resembles more a bone-like mineralized matrix than a dentin-like mineralized matrix.

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