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Original Article

Analysis of the odontogenic and osteogenic potentials of dental pulp *in vivo* using a Col1a1-2.3-GFP transgene

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ABSTRACT Recently, transgenic mice that carry a *Green Fluorescent Protein (GFP)* reporter gene fused to 2.3 kb fragment of rat Col1a1 regulatory sequences *(pOBCol2.3GFPemd)* were generated. In the present study, we have examined the patterns of expression of Col1a1-2.3-GFP during odontoblast differentiation in this transgenic line. We report that Col1a1-2.3-GFP is expressed in newly differentiated odontoblasts secreting predentin and fully differentiated odontoblasts. The pattern of expression of Col1a1-2.3-GFP in odontoblasts is correlated with that of *dentin sialophosphoprotein (DSPP)*. Col1a1-2.3-GFP is also expressed in the osteoblasts and osteocytes of alveolar bone. The pattern of expression of Col1a1-2.3-GFP in osteocytes is correlated with the expression of *Dmp1*. These observations indicate the 2.3 kb rat *Col1a1* promoter fragment has sufficient strength and specificity to monitor the stage-specific changes during both odontoblast and osteoblast differentiation. We also used coronal pulp tissues isolated from postnatal *pOBCol2.3GFPemd* transgenic animals to follow their differentiation after transplantation under the kidney capsule. Our observations provide direct evidence that the dental pulp contains competent progenitor cells capable of differentiating into new generations of odontoblast-like cells which express high levels of Col1a1-2.3-GFP and *DSPP* and secrete tubular containing reparative dentin. We also report that the dental pulp is capable of giving rise to atubular bone-like tissue containing osteocytes expressing high levels of Col1a1-2.3-GFP and *Dmp1*. Our studies indicate that *pOBCol2.3GFPemd* transgenic animals provide a powerful tool for direct examination of the underlying mechanisms and the signaling pathways involved in dentin regeneration and repair, stem cell properties and heterogeneity of the dental pulp.

KEY WORDS: *green fluorescent protein, type I collagen, reparative dentin, pulp, bone*

Introduction

Tooth morphogenesis is regulated by sequential and reciprocal epithelial-mesnechymal interactions (Thesleff and Sharpe, 1997). These interactions are mediated by several signaling pathways (Thesleff and Mikkola, 2002) leading to the formation of tooth buds. Tooth buds progress through cap and bell stages of development generating tooth crowns composed of odontoblasts and ameloblasts secreting dentin and enamel respectively.

Dentinogenesis is regulated by a single layer of highly differentiated post-mitotic odontoblasts originating from the neural crest (NC)-derived cells of the dental papilla (Linde and Goldberg, 1993; Ruch *et al.*, 1995). The differentiation of odontoblasts from the dental papilla occurs independently in each cusp and involves a series of well characterized morphological, molecular and biochemical changes leading to the secretion of dentin (Linde and Goldberg, 1993; Ruch *et al.*, 1995). In developing mouse embryos, odontoblast differentiation begins at the late bell stage (E18) at the tips of the principle cusps, and progresses down the cusp slopes as far as the cervical loops (Linde and Goldberg, 1993; Ruch *et al.*, 1995). Tissue recombination studies have provided evidence that odontoblast differentiation is dependent on inductive signals derived from the inner dental epithelium of the enamel organ and its associated basement membrane (Ruch *et al.*, 1995).

Dentin matrix secreted by odontoblasts is the major component of dental mineralized tissue (Linde and Goldberg, 1993) consisting of inorganic and organic components (Ten Cate, 1998). The inorganic components of dentin consist mostly of hydroxyapatite and water (for reviews see Linde and Goldberg, 1993; Butler, 1995; Butler, 1998). The organic components secreted by odontoblasts consist primarily of collagen fibers and non-collagenous proteins

Abbreviations used in this paper: DSPP, dentin sialophosphoprotein; GFP, green fluorescent protein.

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(NCPs) (for reviews see Linde and Goldberg, 1993; Butler, 1995; Butler, 1998). Type I collagen is the major constituent (approximately 86-90%) of the collagenous proteins in dentin and provides the framework for the deposition of apatite crystal (for reviews see Linde and Goldberg, 1993; Butler, 1995; Butler, 1998). Type III, IV, and V collagens are also detected in dentin matrix but in lesser amounts (Linde and Goldberg, 1993). Previous studies have shown that terminal differentiation of odontoblasts is accompanied by dramatic increases in type I collagen synthesis (Bleicher *et al.*, 1999). The regulatory elements of the rat type I collagen (Col1a1) responsible for Col1a1 expression in bones, teeth and other tissues have been identified (Pavlin *et al.*, 1992; Krebsbach *et al.*, 1993; Bogdanovic *et al.*, 1994; Thomas *et al.*, 1995; Dacic *et al.*, 2001). The NCPs of dentin, although present in small amounts, are of essential functional importance in the mineralization process (Linde and Goldberg, 1993; Steinfort *et al.*, 1994).

The NCPs in dentin include proteins that are also found in bone such as decorin (Steinfort *et al.*, 1994), biglycan (Steinfort *et al.*, 1994), osteonectin (Reichert *et al.*, 1992), osteocalcin (Bronckers *et al.*, 1987), osteopontin (Butler, 1989), bone sialoprotein (Butler, 1989; Linde and Goldberg, 1993; Ritchie *et al.*, 1994), and dentin matrix protein-1 (*Dmp1*) (George *et al.*, 1993; Hirst *et al.*, 1997; MacDougall *et al.*, 1997). However, dentin is characterized by the presence of two dentin-specific NCPs, dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) (for reviews see Veis, 1993; Butler, 1995; Butler, 1998). These two tooth-specific NCPs are encoded by a single gene with a continuous open reading frame and are specific cleavage products of a larger protein, dentin sialophosphoprotein (*DSPP*) (Ritchie and Wang, 1996; MacDougall *et al.*, 1997; Feng *et al.*, 1998) located on human chromosome 4 (MacDougall *et al.*, 1997). DSP is a 95-kDa glycoprotein identified within the dentin matrix and accounts for 5–8% of the dentin extracellular matrix (for reviews see Veis, 1993; Butler, 1995; Butler, 1998). DSP is localized only in dental tissues and its expression is confined to differentiating odontoblasts, with a transient expression in presecretory ameloblasts (for reviews see Veis, 1993; Butler, 1995; Butler, 1998). DPP is synthesized by odontoblasts and secreted through the odontoblastic process at the mineralization front (for reviews see Veis, 1993; Butler, 1995; Butler, 1998). DPP is strongly associated with the mineral phase of dentinogenesis (for reviews see Veis, 1993; Butler, 1995; Butler, 1998). In addition to these extracellular matrix proteins, several signaling molecules such as transforming growth factor beta (TGFβ), bone morphogenic proteins (BMPs), and fibroblast growth factors (FGFs) are found in dentin (Finkelman *et al.*, 1990; Cassidy *et al.*, 1997).

Dentin matrix secreted by odontoblasts can be classified into primary, secondary or tertiary dentin based on the time and circumstances of its secretion (Smith *et al.*, 1995; Tziafas, 1995; Tziafas *et al.*, 2000; Smith and Lesot, 2001). During tooth development, primary dentin is secreted at a relatively fast rate and continues until the completion of root formation (Smith *et al.*, 1995; Tziafas, 1995; Tziafas *et al.*, 2000; Smith and Lesot, 2001). After secretion of primary dentin, the odontoblasts enter a resting state and maintain a basal level of secretory activity and secrete secondary dentin (Smith *et al.*, 1995; Tziafas, 1995; Tziafas *et al.*, 2000; Smith and Lesot, 2001). Physiological secondary dentin is laid down after completion of root formation at a slower rate and continues through the life of the tooth (Smith *et al.*, 1995; Tziafas, 1995; Tziafas *et al.*, 2000; Smith and Lesot, 2001).

Despite their low metabolic activity, the post-mitotic odontoblasts responsible for secretion of primary and secondary dentin survive for the life of the tooth and are able to respond to injuries and pathological conditions. Such responses are seen as localized deposition of tertiary dentin matrix beneath the site of injury. The process of tertiary dentin secretion is also classified as being reactionary or reparative in origin, depending on the severity of the initial injury, and the conditions under which the newly deposited dentin matrix is formed (Smith *et al.*, 1995; Tziafas, 1995; Tziafas *et al.*, 2000; Smith and Lesot, 2001). If the odontoblasts survive the injury, under appropriate conditions they secrete a reactionary dentin matrix (Smith *et al.*, 1995; Tziafas, 1995; Tziafas *et al.*, 2000; Smith and Lesot, 2001). On the other hand, if odontoblasts are irreversibly damaged, they are replaced by a second generation of newly differentiated odontoblast-like cells that give rise to a reparative dentin matrix (Smith *et al.*, 1995; Tziafas, 1995; Tziafas *et al.*, 2000; Smith and Lesot, 2001). Although there is indirect evidence suggesting that reparative dentin is laid down by a new generation of odontoblast-like cells originating from cells within the dental pulp (Sveen and Hawes, 1968; Fitzgerald *et al.*, 1990; Tziafas *et al.*, 2000), the exact origin of the cells responsible for secretion of reparative dentin matrix has not been clearly identified.

Recently, transgenic mice in which green fluorescent protein (GFP) expression is under the control of the rat 3.6 kb and 2.3 kb Col1a1 promoter fragments were generated (Kalajzic *et al.*, 2002). Analysis of these animals indicated that the 3.6-kb promoter directed strong expression of GFP mRNA and protein in bone and other unmineralized type I collagen-expressing tissues, while the 2.3 kb promoter directed expression of GFP mRNA and protein only in bone and tendon (Kalajzic *et al.*, 2002). Furthermore, it has been shown that in primary osteoblast cultures derived from either neonatal calvaria or bone marrow stromal fibroblasts the expression of Col1a1-3.6-GFP and Col1a1-2.3-GFP identify cells at different stages of osteoblast differentiation (Kalajzic *et al.*, 2002). In these cultures, expression of Col1a1-3.6-GFP occurs prior to the appearance of mineralized nodules and is correlated with the appearance of pre-osteoblasts expressing alkaline phosphatase and type I collagen. On the other hand, expression of Col1a1-2.3-GFP is seen after mineralization and is correlated with the appearance of differentiated osteocytes expressing bone sialoprotein and osteocalcin (Kalajzic *et al.*, 2002).

In the present study, we have examined the patterns of expression of Col1a1-GFP during odontoblast differentiation in transgenic mice containing a 2.3 kb (pOBCol2.3GFPemd) (Kalajzic *et al.*, 2002) rat Col1a1 promoter fragment. To determine whether or not this promoter fragment has sufficient strength and specificity to monitor the stage-specific changes in α1(I) collagen expression during odontoblast differentiation, the pattern of expression of Col1a1-2.3-GFP was also correlated with the patterns of expression of *DSPP* and *Dmp1*. Furthermore, using pulps isolated from Col1a1-2.3-GFP transgenic animals the differentiation potential of dental pulp

and the ability to give rise to new generations of odontoblasts secreting a reparative-like dentin matrix were examined.

Results

Localization of Col1a1-2.3-GFP during Molar Tooth Development in pOBCol2.3GFP *Transgenic Mice*

No detectable levels of Col1a1-2.3-GFP expression were detected in the dental tissues during the initiation, early bud and cap stages of molar tooth development (E10-15) (Fig. 1 A-C, and data not shown). Expression of Col1a1-2.3-GFP became evident at the bell stage (E18) of tooth development (Fig. 1 D-F). At this stage Col1a1-2.3-GFP expression was not detected in dental papilla, pre-odontoblasts, or polarizing odontoblasts. However, low but detectable levels of Col1a1-2.3-GFP were expressed in a limited group of young odontoblasts at the tip of the mesio-lingual cusp of the first mandibular molar engaged in the secretion of a thin layer of predentin (Fig. 1 E,F). At this stage of development, Col1a1-2.3-GFP expression was not detected in other cusps of the first molars or in the second molars that contain less differentiated odontoblasts (not shown).

As tooth development progressed continual increases in the levels of Col1a1-2.3-GFP expression were observed in odontoblasts. During the late bell stage of tooth development (E19) high levels of Col1a1-2.3-GFP expression were expressed in odontoblasts at the tips of both cusps of the first molars (Fig. 2 A,B).

At the secretory stage of crown formation during postnatal growth (P1-P5), Col1a1-2.3-GFP was expressed at high levels in the entire layer of odontoblasts covering the dental pulp (Figs. 1 G-I; 2 E,F), and its expression extended into the odontoblast processes (Fig. 1 G-I). Very low but detectable levels of Col1a1-2.3-GFP were also expressed in some but not all cells in the dental pulp

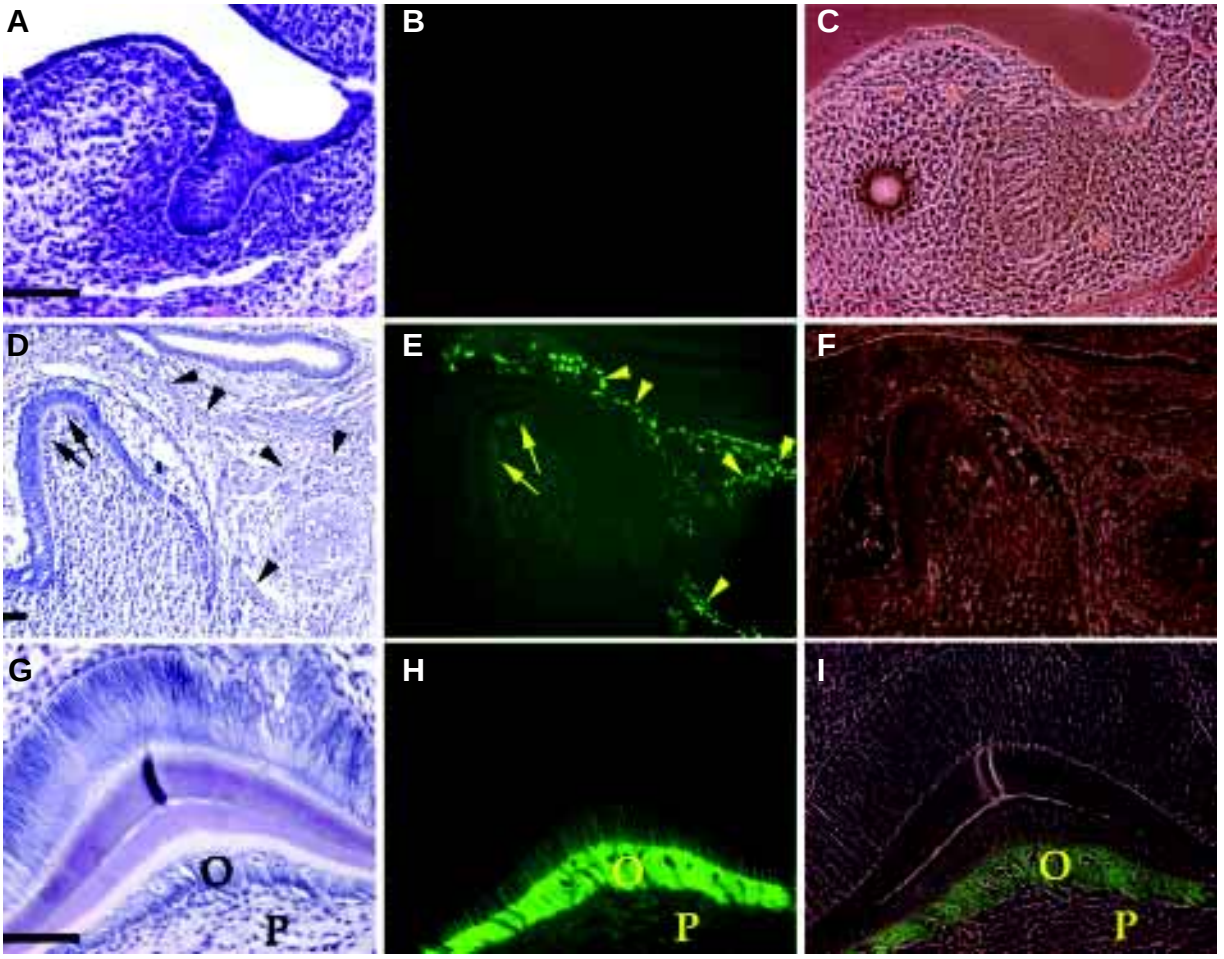

Fig. 1. Col1a1-2.3-GFP expression during molar tooth development from late bud stage to postnatal day 5 in pOBCol2.3-GFP heterozygous transgenic mice. *Bright-field (A,D,G), epifluorescence (B,E,H) and dark-field (C,F,I), images of sections through developing teeth at E13 (A-C), E18 (D-F), and P5 (G-I). In each stage of development, sections were first processed for epifluorescence and dark-field analysis. The same section was then processed for Hematoxylin - Eosin staining and viewed under bright-field.* **(A-C)** *AT E13, Col1a1-2.3-GFP is not expressed in either the epithelial or mesenchymal components of the tooth germ.* **(D-F)** *At E18, very low but detectable levels of Col1a1-2.3-GFP expression are detected in the functional odontoblasts (indicated by arrows in E) located at the tip of the mesio-lingual cusp of the first mandibular molar. Col1a1-2.3-GFP is also expressed in the osteocytes and osteoblasts (indicated by arrowheads in E) of the developing alveolar bone.* **(G-I)** *At P5, Col1a1-2.3-GFP is expressed at high levels in terminally differentiated odontoblasts (O). Col1a1-2.3-GFP expression extends into the odontoblast processes at the cuspal tip. Low levels of Col1a1-2.3-GFP expression were observed in the dental pulp (P). Scale bar in all pictures,100 μm.*

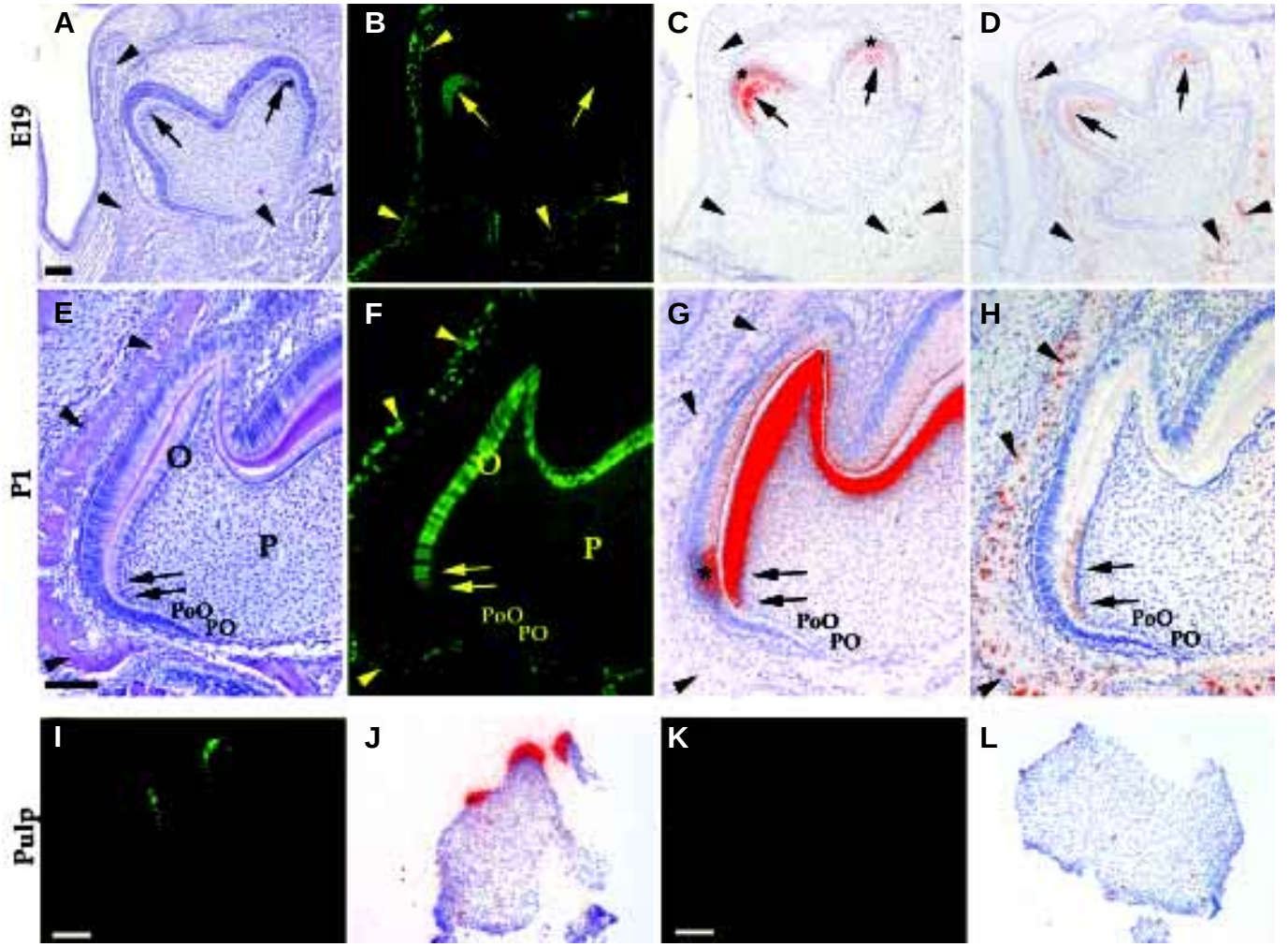

Fig. 2. Correlation of Col1a-2.3-GFP, *DSPP* and *Dmp1* expression in the developing molars of pOBCol2.3GFP transgenic mice. *Bright-field (A,E), epifluorescence (B,F) and pseudo-colored bright-field (C,D,G,H) images of the adjacent frontal sections through molars isolated from E19 (A-D) and P1 (E-H).* **(A)** *A hematoxylin and eosin-stained section through the mandibular first molar at E19.* **(B)** *An epifluorescence image of the section shown in (A). High levels of Col1a1-2.3-GFP expression can be seen in functional odontoblasts at the tips of both cusps of the first mandibular molar (indicated by arrows). Col1a1-2.3-GFP is also expressed in osteoblasts and osteocytes (indicated by arrowheads) of the developing alveolar bone.* **(C)** In situ *hybridization analysis of the section adjacent to that in (B) with a* DSPP *riboprobe. Similar to Col1a1-2.3-GFP,* DSPP *is expressed in functional odontoblasts at the tip of the cusps of the first mandibular molar (indicated by arrows).* DSPP *is also expressed in the pre-secretory ameloblasts (indicated by *) opposing the* DSPP*-expressing odontoblasts.* DSPP *is not expressed in the osteoblasts and osteocytes of the developing alveolar bone (indicated by arrowheads).* **(D)** In situ *hybridization analysis of the another adjacent section with a* Dmp1 *riboprobe, showing low but detectable levels of* Dmp1 *expression in the functional odontoblasts at the tips of the cusps of the first mandibular molar (indicated by arrow).* Dmp1 *is also expressed in the osteocytes of the developing alveolar bone (indicated by arrowheads).* **(E)** *A hematoxylin & eosin-stained section through the maxillary first molar at P1.* **(F)** *An epifluorescence image of the section shown in (E). High levels of Col1a1-2.3-GFP are expressed by the entire layer of odontoblasts covering the dental pulp and in the osteoblasts and osteocytes of the developing alveolar bone (indicated by arrowheads). Col1a1-2.3-GFP is not expressed in undifferentiated pre-odontoblasts (PO) or polarizing odontoblasts (PoO). Low by detectable levels of Col1a1GFP expression is also detected in the dental pulp (P). Functional odontoblasts are indicated by arrows.* **(G,H)** In situ *hybridization analyses of two adjacent sections hybridized with* DSPP *(G) and* Dmp1 *(H) riboprobes.* DSPP *is expressed at high levels by the entire layer of odontoblasts covering the dental pulp (G) and in a group of early secretory ameloblasts (indicated by *) located at the cervical loop opposing the* DSPP*-expressing functional odontoblasts (indicated by arrows).* DSPP *in not expressed by undifferentiated pre-odontoblasts (PO) and the osteoblasts and osteocytes of the developing bone (indicated by arrowheads). Low but detectable levels of* Dmp1 *are expressed in the functional odontoblasts (indicated by arrows) at the cervical loop, and in the osteocytes (indicated by arrowheads) of the developing alveolar bone. Scale bar in all pictures, 100 μm. Abbreviations: P, dental pulp; PO, pre-odontoblasts; PoO, polarizing odontoblasts.* **(I,K)** *Epifluorescence and (J, L)pseudo-colored bright-field images of the sections through isolated pulps after mechanical separation (I,J) and after removal of the pulp horns (K,L).* **(J,L)** *Pseudo-colored images of sections hybridized with the* DSPP *riboprobe. High levels of Col1a1-2.3-GFP (I) and* DSPP *(J) are expressed in remaining odontoblasts at the tips of the pulp horns. Note the lack of strong Col1a1-2.3-GFP (K) and* DSPP *(L) expression in isolated tissue after removal of the pulp horns. Also note the low by detectable levels of Col1a1-2.3-GFP expression in the remaining pulp tissue (K). Scale bars in all pictures, 100 μm.*

(Figs. 1 H,I; 2 F,I,K). Odontoblasts secreting secondary dentin (P13) expressed high levels of Col1a1-2.3-GFP expression (not shown).

Between E18-P13, in addition to its expression in differentiating and differentiated odontoblasts, Col1a1-2.3-GFP was expressed at high levels in the osteoblasts and osteocytes within the developing alveolar bone (Figs. 1 D-F; 2 A,B,E,F).

Our analyses of developing maxillary and mandibular arches of pOBCol2.3GFP transgenic mice indicate that the 2.3 kb fragment of rat Col1a1 regulatory sequences drive expression of GFP in differentiating and differentiated odontoblasts and osteocytes.

Correlation of Col1a1-2.3-GFP, DSPP *and* Dmp1 *expression in the Developing Maxillary and Mandibular Arches in* pOBCol2.3GFP *Transgenic Mice*

To be able to more closely correlate the pattern of expression of Col1a1-2.3-GFP with different stages of odontoblast and osteoblast differentiation, the pattern of expression of Col1a1-2.3-GFP was compared with the patterns of expression of mRNAs for *DSPP* and *Dmp1*.

Our *in situ* hybridization analyses showed that *DSPP* and *Dmp1* are not expressed in dental tissues at early stages of tooth development (data not shown). The earliest detectable signals for *DSPP* and *Dmp1* appeared at the late bell stage of tooth development (E19). At this stage of development, high levels of *DSPP* (Fig. 2C) and low but detectable levels of *Dmp1* (Fig. 2D) were expressed in the limited group of functional odontoblasts at the tips of cusps of the first mandibular molars. At E19 hybridization signal for *DSPP* (but not *Dmp1*) was also detected in the presecretory ameloblasts opposing the functional odontoblasts expressing *DSPP* and *Dmp1* (Fig. 2C). At E19 *Dmp1* (but not *DSPP*) was also expressed in osteocytes (but not osteoblasts) of the alveolar bone (Fig. 2D).

During the secretory stages of tooth development (P1-P5), high levels of *DSPP* expression were detected in the entire layer of odontoblasts covering the dental pulp (Fig. 2G), and in the limited group of pre-ameloblasts located at the cervical loops (Fig. 2G). Hybridization of the adjacent section with *Dmp1* riboprobe revealed its restricted expression in the functional odontoblasts located at the cervical loops (Fig. 2H). *Dmp1* was not expressed by fully differentiated odontoblasts (Fig. 2H). Between E18-P13 high levels of expression of *Dmp1* (but not *DSPP)* were detected in osteocytes within the developing alveolar bone (Fig. 2 D,H and data not shown). At all these stages no signal above background was detected in sections hybridized with sense riboprobes for *Dmp1* and *DSPP* (not shown).

Our *in situ* hybridization analyses are consistent with results reported by others (D'Souza *et al.*, 1997; Begue-Kirn *et al.*, 1998) and show that *Dmp1* and *DSPP* are co-expressed in functional odontoblasts engaged in the secretion of unmineralized predentin. Fully differentiated odontoblasts associated with mineralized dentin express high levels of *DSPP* but no detectable levels of *Dmp1*. In contrast to *DSPP*, *Dmp1* is expressed in osteocytes of the alveolar bones (D'Souza *et al.*, 1997; Kamiya and Takagi, 2001; Toyosawa *et al.*, 2001).

A comparison between the expression patterns of *DSPP* and *Dmp1* with that of Col1a1-2.3-GFP in adjacent sections indicates a close correlation between their time of appearance and expression in functional odontoblasts engaged in secreting predentin (E19, P1). There is also a close correlation between the patterns of expression of Col1a1-2.3-GFP and *DSPP* in differentiated odontoblasts, and a close correlation between the expression of Col1a1-2.3-GFP and *Dmp1* in osteocytes in the alveolar bone. These observations indicate that the 2.3 kb rat Col1a1 promoter fragment has sufficient strength and specificity to monitor the stage-specific changes during both odontoblast and osteoblast differentiation.

Differentiation Potential of Dental Pulp in pOBCol2.3GFP *Transgenic Mice and Localization of Col1a1-2.3-GFP in Pulp Cells after Transplantation*

Our *in vivo* observations indicate that expression of Col1a1-2.3-GFP is a good marker for the appearance of functional and terminally differentiated odontoblasts and that the expression of the Col1a1-2.3-GFP transgene can be used to examine the progression of progenitor cells into odontoblasts.

Therefore, using pulps isolated from 5-day-old transgenic animals, the odontogenic potential of the cells within the dental pulp was examined. As the first step, freshly isolated pulps from pOBCol2.3GFP transgenic animals were processed for epifluorescence and *in situ* hybridization analyses using the *DSPP* riboprobe. These experiments indicated that, after mechanical isolation a small layer of odontoblasts expressing high levels of Col1a1-2.3-GFP and *DSPP* remained at the tips of the isolated pulp horns (Fig. 2 I,J). To ensure the absence of odontoblasts in the isolated pulp prior to transplantation, the pulp horns were removed and the remaining pulp tissues, free of odontoblasts as determined by the lack of high levels of expression of Col1a1-2.3-GFP and *DSPP* (Fig. 2 K,L), were used for transplantation**.** Epifluorescence analysis of this tissue confirmed the presence of low by detectable levels of Col1a1-2.3-GFP in the remaining dental pulp (Fig. 2K). Fifty-four pulps free of odontoblasts were grafted under the kidney capsule of host animals. Explants were harvested at 7 days (n=20), 10 days (n=25) and 14 days (n=9).

Histological examination of 7-day-old transplanted tissues revealed the presence of eosinophilic and unmineralized osteoid-like tissues in all explants (data not shown). Cartilaginous islands with differentiated chondrocytes (Fig. 3A) were also present in some of the 7-day-old explants. The presence of cartilage in these explants was confirmed by Alcian-blue staining (Fig. 3A).

In addition, all 7-day-old transplanted tissues (Fig. 3 A-D), as well as 10-day-old (Fig. 3 E-H) and 14-day-old (not shown) transplanted tissues contained mineralized islands with osteocyte-like cells embedded within the atubular-mineralized matrix (Fig. 3 A-C, E,G). The presence of mineralized islands in these explants were confirmed by various methods (Fig. 4 A-F) including Alizarin red-S staining of undeclacified sections (Fig. 4 E,F) in additional explants (n=21). These mineralized islands were morphologically similar to previously described atubular osteotypic matrices. There was also growth in the mineralized islands in 10-day-old explants (Fig. 3 E,G) as compared to 7-day-old explants (Fig. 3 A-D). In addition, the surface of all of the mineralized islands in 10-day-old explants and some (but not all) 7-day-old explants were lined with elongated and polarized cells (Fig. 3 A-D, E,G). Mineralized matrices associated with polarized cells contained tubular structures (Fig. 3 B,G), contained very few if any osteocyte-like cells (Fig. 3 A, 3B, 3E-H) and were well demarcated from the atubular osteotypic matrices (Fig. 3E).

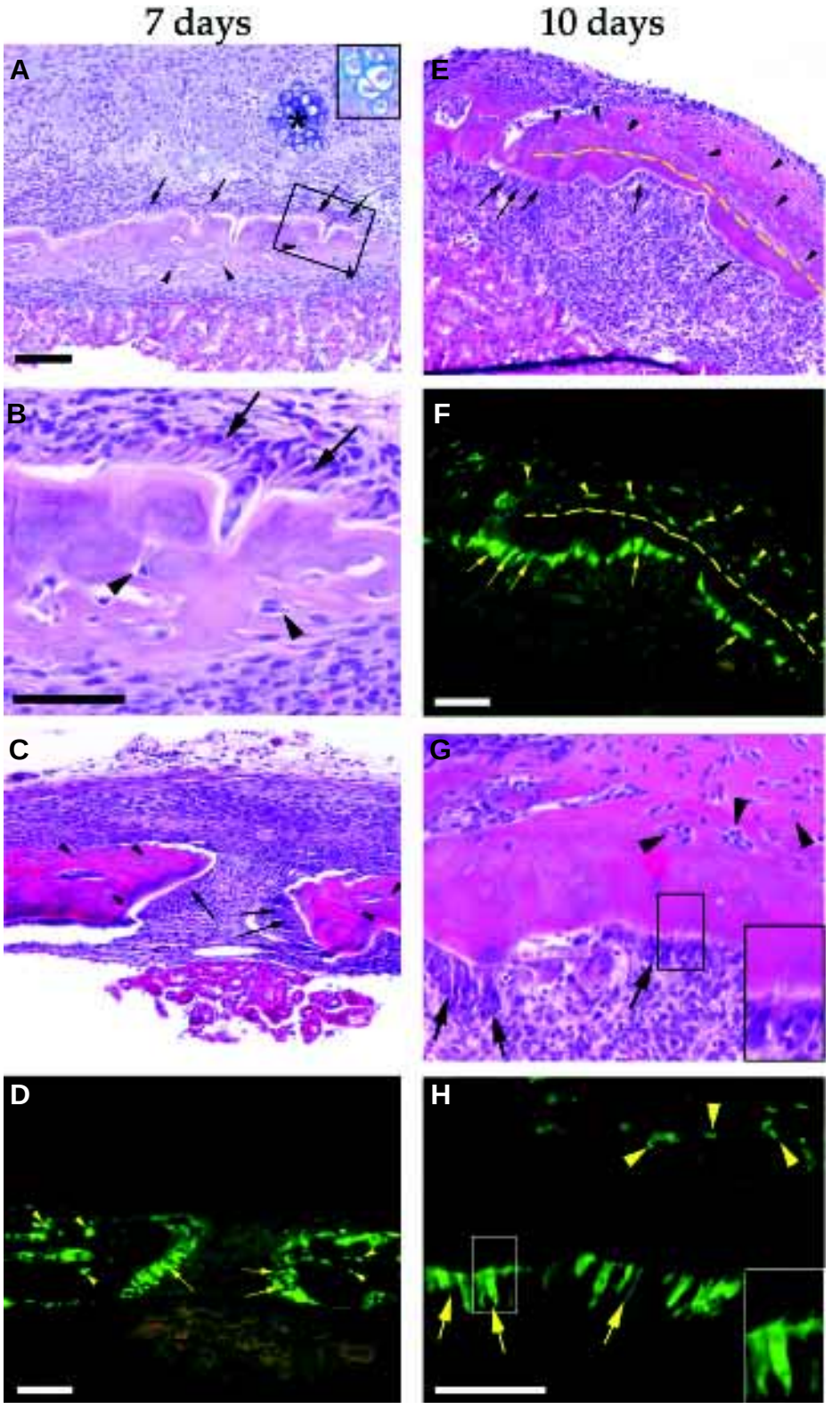

Fig. 3. Analysis of differentiation potentials of pulp from pOBCol2.3GFP transgenic mice after transplantation under the kidney capsule. *Bright-field (A,B,C,E,G), and epifluorescence (D,F,H) images of sections through coronal portions of pulp isolated from mandibular first molars of* pOBCol2.3GFP *transgenic mice transplanted for 7 (A-D) and 10 (E-H) days under the kidney capsule of host mice.* **(A)** *A hematoxylin and eosin section through a 7-day old explant showing the formation of cartilaginous island (indicated by *) and mineralized islands. The inset is the higher magnification of cartilaginous tissue in adjacent section stained with Alcian blue which stains the cartilage protoeglycan blue. The mineralized islands contain both osteocyte-like cells surrounded by mineralized matrix (indicated by arrowheads) and elongated cells lining the surface of the mineralized matrix (indicated by arrows).* **(B)** *A higher magnification of the area indicated in (A) showing the elongated and polarized morphology of the cells lining the surface of the mineralized matrices (indicated by arrows) and osteocyte-like cells surrounded by mineralized matrix (indicated by arrowheads).* **(C,D)** *Bright-field and epifluorescence images of the same section through another 7-day old explant showing the formation of mineralized islands containing osteocyte-like cells surrounded by mineralized matrix (indicated by arrowheads) and elongated cells lining the surface of the mineralized matrix (indicated by arrow). High levels of Col1a1-2.3-GFP are expressed in both osteoblast-like cells (indicated by arrowheads) and elongated cells (indicated by arrows). Bright-field* **(E)** *and epifluorescence* **(F)** *images of the same section through pulp transplanted for 10 days showing the formation of mineralized island containing osteocyte-like cells surrounded by mineralized matrix (indicated by arrowheads) and elongated cells lining the surface of the mineralized matrix (indicated by arrows). High levels of Col1a1- 2.3-GFP are expressed in both cell types (F). Note that the demarcation (indicated by dashed line) between the osteotypic-like and the tubular matrices.* **(G)** *A stained section through another pulp transplanted for 10 days showing the formation of tubular containing matrix lined with odontoblast-like cells (indicated by arrows). Arrowheads indicate the osteocyte-like cells. The inset is the higher magnification of region indicated by the rectangle showing the tubular-containing matrix.* **(H)** *Epifluorescence image of section in (G) showing the expression of Col1a1-2.3-GFP in odontoblast-like cells (indicated by arrows) and osteocyte-like cells (indicated by arrowheads). Note that the expression of Col1a1-2.3-GFP extends into the odontoblast-like processes within the tubular-containing matrix associated with odontoblast-like cells shown in the inset, which is a higher magnification of the region indicated by the rectangle. Scale bars in all pictures, 100 μm.*

Epifluorescence analysis showed that in contrast to low to undetectable levels of Col1a1-2.3-GFP expression in freshly isolated dental pulp (Fig. 1K), 7-to 14- day old explanted pulps contained cells expressing high levels of Col1a1-2.3–GFP (Fig. 3 D,F,H and data not shown). Col1a1-2.3-GFP was expressed in osteocyte-like cells embedded within the osteotypic matrices and elongated polarized cells lining the tubular-containing matrices (Fig. 3 D,F,H). In the 10-day old explants, the expression of Col1a1-2.3-GFP extended into the tubular-containing matrices (Fig. 3H). The expression of Col1a1-2.3-GFP in elongated polarized cells and in tubular containing matrices in the 10-day old explanted tissue resembled the patterns of expression of Col1a1-2.3-GFP in odontoblasts and odontoblast processes *in vivo* (compare Figs. 1H, 3H) suggestive of odontoblast-like phenotype of these elongated cells. Examination of 10-day old explants revealed the presence of small population of osteocyte-like cells associated with osteotypic matrices devoid of Col1a1-2.3-GFP expression. On the other hand, all the odontoblast-like cells lining the tubular-containing matrices expressed high levels of Col1a1-2.3-GFP and did not contain cells devoid of Col1a1-2.3-GFP expression.

Localization of DSPP *and* Dmp1 *in Pulp Cells expressing Col1a1-2.3-GFP after Transplantation*

The expression of Col1a1-2.3-GFP in explanted tissues shows that explanted pulps isolated from pOBCol2.3GFP transgenic mice contain progenitor cells capable of giving rise to osteocyte- and odontoblast-like cells. Our *in vivo* studies indicated that fully differentiated odontoblasts express high levels of *DSPP* but no detectable levels of *Dmp1* (Fig. 2 E-F). On the other hand, osteocytes in the developing alveolar bone express high levels of *Dmp1*, but no detectable levels of *DSPP* (Fig. 2 A-D, E-H). Therefore, based on these observations the osteoblastic vs. odontoblastic phenotype of the differentiated cells in 7- and 10-day old explants were further distinguished by examination of the patterns of expression of *DSPP* and *Dmp1* in adjacent sections to those processed for epifluorescent analysis (n=15). In these studies fully differentiated odontoblasts were characterized by the high levels of expression of *DSPP*, Col1a1-2.3-GFP, and the lack of expression of Dmp1, whereas osteocytes by the high levels of expression of *Dmp1*, Col1a1-2.3-GFP, and the lack of expression of *DSPP*.

In situ hybridization analysis of the 7- and 10-day old explants showed high levels of *DSPP* expression by most but not all cells expressing Col1a1-2.3-GFP (Fig. 5 A,B,D,E). In these explants *DSPP* expression was not limited to elongated odontoblast-like cells lining the surface of the mineralized tissues and was also detected in osteocyte-like cells embedded within mineralized matrices (Fig. 5 B,E). Hybridization of adjacent sections with *Dmp1* riboprobe also revealed its expression in most but not all cells expressing Col1a1-2.3-GFP (Fig. 5 C,F). Similar to the patterns of expression of *DSPP*,

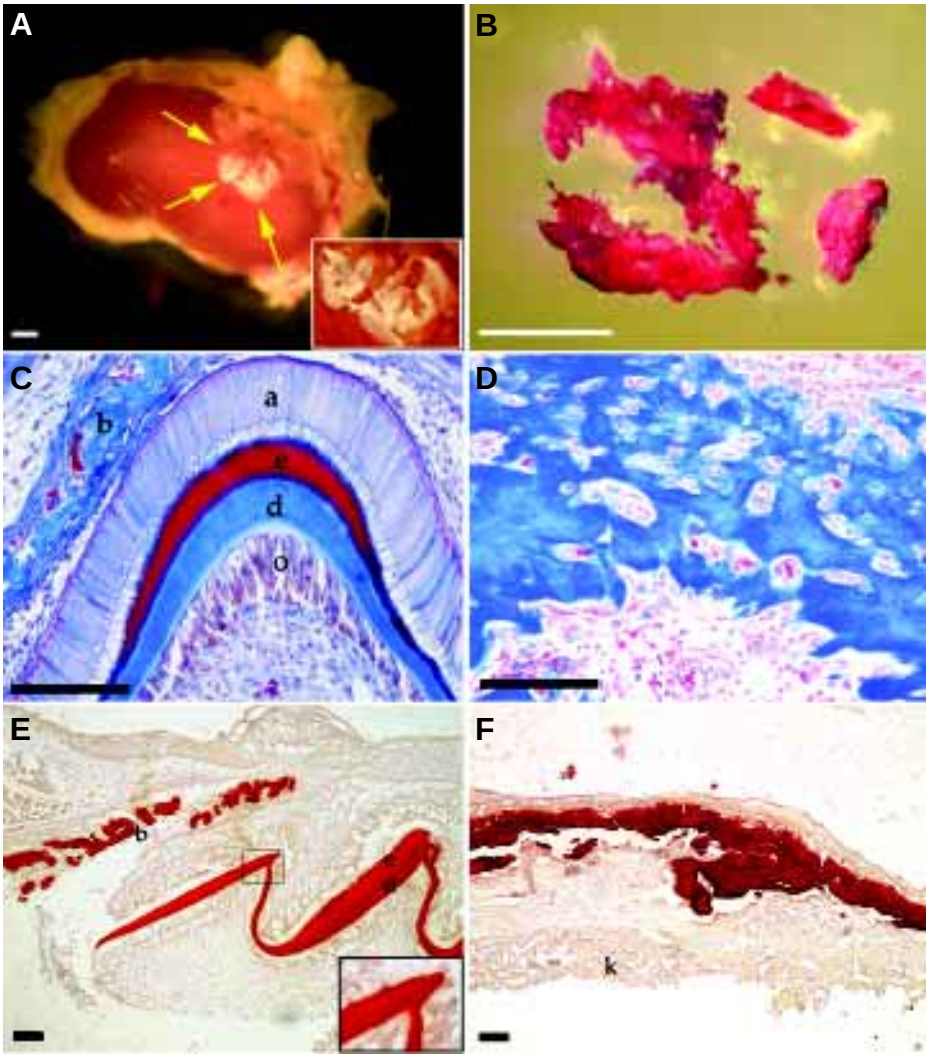

Fig. 4. Formation of mineralized tissue in transplanted pulps. (A) *Image of a kidney containing multiple 10-day-old mineralized transplanted pulp tissue (indicated by arrows). The inset is a higher magnification of mineralized transplanted pulp tissue.* **(B)** *Whole mount staining of 10-day-old mineralized transplanted pulp tissue with Alizarin red/ Alcian blue solution showing the extensive amount of mineralized tissue in the explant that is stained with Alizarin red.* **(C,D)** *Decalcified sections through the first maxillary molar at P5 (C) and pulp transplanted for 10 days under the kidney capsule (D), stained with Mallory's aniline blue collagen stain. Note the intense blue staining in bone (b) and dentine (d) that contain collagen in the maxillary first molar at P5. Also note the presence of blue-stained collagen-containing matrices in the transplanted tissue after 10 days.* **(E,F)** *Undecalcified sections through the first maxillary molar at P5, and pulp transplanted for 10 days under the kidney capsule stained with Alizarin red S. Note the intense red staining in all mineralized tissues including bone (b), dentine (d) and enamel (e) in the first maxillary molar at P5 (E). Also note that the lack of red staining in the unmineralized layer of pre-dentin shown in the inset in (E) which is a higher magnification of the cuspal region indicated by the rectangle. Alizarin red S staining shows significant mineralization in the transplanted tissue after 10 days (F). Scale bars in Figures A,B, 1 mm; C-F, 100 μm. Abbreviations: a, ameloblasts; b, bone; d, dentin; e, enamel; k, kidney; o, odontoblasts.*

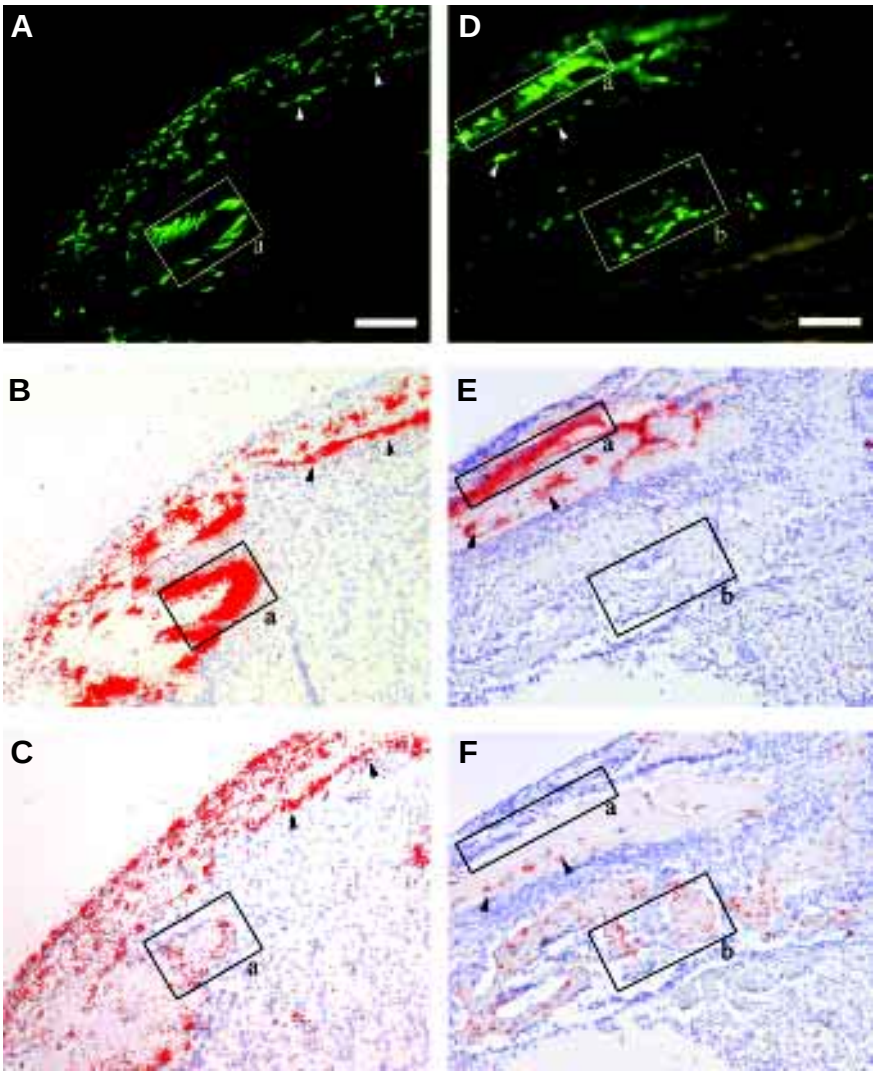

Fig. 5. Correlation of Colla1-2.3-GFP, *DSPP* and *Dmp1* expression in transplanted pulps. *Epifluorescence* **(A,D)** *and pseudo-colored bright-field* **(B,C,E,F)** *images of sections through coronal pulps isolated from mandibular first molars of* pOBCol2.3GFP *transgenic mice transplanted for 7 (A-C) and 10 (D-F) days under the kidney capsule. Representative areas in the sections are highlighted for comparative purposes in A-C (a) and in D-F (a,b). (A–C) Rectangular regions (a) indicate areas of elongated and polarized cells expressing Colla1-2.3-GFP and* DSPP *but low expression of* Dmp1, *suggestive of polarized odontontoblast-like phenotype of early odontoblast differentiation. Compare this to Fig. 2 C,D of developing odontoblasts in early stages of tooth development. (D-F) Rectangular regions (a) indicate an area of polarized odontoblast cells expressing high levels of Colla1-2.3-GFP and* DSPP, *but undetectable levels of* Dmp1. *Compare this to Fig. 2 G,H of fully differentiated odontoblasts. In (D-F), the rectangular regions (b) indicate areas of bone with osteocytes in lacunae which express Colla1-2.3-GFP and* Dmp1, *but undetectable levels of* DSPP, *characteristic of bone. In all figures arrowheads indicate cells expressing high levels of Colla1-2.3-GFP,* DSPP *and* Dmp1. *Scales bars in all figures, 100* μm.

expression of *Dmp1* was seen in both osteocyte-like and odontoblasts-like cells expressing Col1a1-2.3-GFP (Fig. 5 C,F).

Close comparisons between the patterns of Col1a1-2.3-GFP expression with those of *DSPP* and *Dmp1* in adjacent sections from these explants revealed the presence of three groups of cells associated with mineralized matrices.

The first group was characterized by expression of high levels of Col1a1-2.3-GFP and *DSPP* but low to undetectable levels of *Dmp1* (indicated by the a in Fig. 5 A-F). This group of cells displayed elongated morphology, lining the surface of the tubular-containing matrices. The morphology of the cells, their associated mineralized matrices, and the expression patterns of *DSPP*, *Dmp1,* and Col1a1-2.3-GFP in these cells resembled those in differentiated odontoblasts *in vivo* suggestive of their odontoblast-like phenotype. These observations indicate that cells within the coronal portion of the pulp, devoid of any odontoblasts, are capable of giving rise to new generations of odontoblast-like cells that express *DSPP* and secrete tubular reparative dentin.

The second group was characterized by expression of high levels of Col1a1-2.3-GFP, and *DSPP*, but low to undetectable levels of *Dmp1* (indicated by arrowheads in Fig. 5 A-F). The co-expression of these three markers was seen in both osteocyte-like cells as well as elongated cells lining the surface of the mineralized matrix. These observations suggest that osteocyte-like cells depositing the atubular osteotypic matrices may be similar to functional odontoblasts *in vivo*.

The third group was characterized by expression of high levels of Col1a1-2.3-GFP and *Dmp1* but undetectable levels of *DSPP* (indicated by "b" in Fig. 5 D-F). The patterns of expression of these three markers resembled their patterns of expression in osteocytes of the developing alveolar bone *in vivo*, indicating the osteogenic potential of the dental pulp.

Discussion

Our tissue distribution studies indicate that expression of Col1a1-2.3-GFP in developing maxillary and mandibular arches of pOBCol2.3GFPemd transgenic mice is restricted to differentiating and differentiated odontoblasts and osteoblasts. Our observations also show a correlation between the expression of Col1a1-2.3-GFP with that of *DSPP* and *Dmp1* in odontoblasts and osteocytes, respectively.

Our analysis of the patterns of expression of Col1a1-2.3-GFP in developing teeth of pOBCol2.3GFP transgenic mice indicates that Col1a1-2.3-GFP is not expressed in dental tissues at early stages of tooth development or in pre-odontoblasts or polarizing odontoblasts. Col1a1-2.3-GFP is first expressed in the functional odontoblasts secreting predentin and its high levels of expression is maintained in terminally differentiated odontoblasts. These observations are consistent with our observations in the developing incisors (Braut *et al.*, 2002) and indicate that in developing teeth expression of Col1a1-2.3-GFP closely follows the expression of α1(I) collagen during odontoblast differentiation and for the most part (except for the developing pulp) are consistent with immunostaining for CAT protein in developing teeth of transgenic line that carries a CAT reporter gene fused to 2.3 kb of

rat Col1a1 regulatory sequences (Pavlin *et al.*, 1992; Thomas *et al.*, 1995). Although previous studies did not detect expression of Col1a1-2.3-CAT in the developing pulp, our studies show low but detectable levels of Col1a1-2.3-GFP in some but not all pulp cells.

Comparison of expression patterns of Col1a1-2.3-GFP with *DSPP* and *Dmp1* shows that the expression of Col1a1-GFP directed by the 2.3 kb fragment of the rat Col1a1 promoter closely follows the patterns of expression of *DSPP* in differentiating and differentiated odontoblasts.

Our analyses also show that Col1a1-2.3-GFP is expressed in osteoblasts and osteocytes of alveolar bone. In the alveolar bones, the expression of Col1a1-2.3-GFP in osteocytes is correlated with the expression of *Dmp1*.

The patterns of expression of Col1a1-2.3-GFP in odontoblasts as well as osteoblasts indicate that the 2.3 kb promoter fragment of Cola1-2.3-GFP has sufficient strength and specificity to monitor the stage-specific changes during odontoblast and osteoblast differentiation. These observations indicate that expression of Col1a1-2.3-GFP in these animals provides a useful marker for following the progression of progenitor cells into both odontogenic and osteogenic lineages.

Differentiation Potential of Dental Pulp

The ability of the dentin-pulp complex to respond to a variety of pathological conditions and injury by localized deposition of a tertiary dentin matrix has long been recognized**.** In the case of mild injury to the tooth, the surviving post-mitotic odontoblasts at the site of injury, responsible for the secretion of primary and secondary dentin, are stimulated to up-regulate their synthetic and secretory activities to secrete a reactionary dentin matrix with tubular structures similar to and continuous with those in physiological dentin (Smith *et al.*, 1995; Smith and Lesot, 2001). It has been suggested that factor(s) and signaling molecules similar to those involved in physiological dentinogenesis, including TGFbs and their associated proteoglycans, IGF-I and II, members of BMP family and FGF family of signaling molecules, are involved in mediating the increased activities of odontoblasts during reactionary dentinogenesis (for recent reviews Tziafas *et al.*, 2000; Smith and Lesot, 2001). These factor(s) and signaling molecules are thought to be sequestered within the dentin matrix and released during dentin decalcification (for recent reviews Tziafas *et al.*, 2000; Smith and Lesot, 2001).

On the other hand, in pathological conditions that cause death and destruction of odontoblasts, the damaged dentin is replaced by reparative dentin secreted by a new generation of odontoblast-like cells (Tziafas, 1995; Tziafas *et al.*, 1995; Tziafas *et al.*, 2000). It has been suggested that reparative dentinogenesis *in vivo* results in the secretion of tubular dentin that may show discontinuity in tubular structure and possibly reduction in dentin permeability. Therefore, in contrast to reactionary responses, reparative dentinogenesis represents a more complex sequence of biological process, which is dependent on multiple factors, including the presence of responsive progenitor cells as well as the appropriate inductive molecular signals for induction of differentiation of the new generation of odontoblasts.

The underlying cellular and molecular mechanisms regulating the sequence of events leading to reactionary dentinogenesis have been the subject of intense investigation using a variety of *in vitro* and *in vivo* approaches (for recent reviews see Linde and Goldberg, 1993; Tziafas *et al.*, 2000; Smith and Lesot, 2001). These studies have provided valuable but indirect information regarding the dentinogenic capacity of dental pulp cells. Although the available evidence suggests that reparative dentin most likely is deposited by a new generation of odontoblast-like cells originating from the underlying pulpal cells, the exact origin of the precursor cells giving rise to new odontoblasts and the signaling pathways involved in their overt differentiation have not been clearly identified. Problems with previous studies are numerous and include the lack of clear identification of progenitor cells giving rise to a new generation of odontoblasts and the lack of full characterization of the differentiated cells originating from the progenitor cells.

In the present study we have used odontoblast-free coronal pulp tissues isolated from pOBCol2.3GFPemd transgenic animals and followed their differentiation after transplantation under the kidney capsule. The phenotype of the differentiated cells originating from dental pulp were characterized by upregulated expression of Col1a1-2.3-GFP, expression of *DSPP*, and *Dmp1* and the morphology of the cells and their associated mineralized matrices.

Our results provide direct evidence that cells within the coronal portion of the pulp, devoid of any odontoblasts, are able to give rise to a new generation of odontoblasts secreting tubule-containing reparative dentin. We also show that tubular matrices are lined with elongated and polarized odontoblasts-like cells that express high levels of Col1a1-2.3-GFP and *DSPP*. *DSPP*-expressing cells associated with tubular matrices reveal well-developed odontoblasts process extending into tubular structures.

Our observations also show the formation of atubular osteotypic matrices in explanted dental pulps. Although these matrices morphologically resemble the matrix of woven bone, the expression of Col1a1-2.3-GFP and *DSPP* in cells embedded in the atubular matrix are suggestive of an odontoblast-like phenotype. However, unlike *DSPP*-expressing cells associated with tubular-containing matrices, the *DSPP*-expressing cells within the osteotypic matrices lack odontoblast process.

The ability of the dental pulp to give rise to new generations of odontoblast-like cells secreting reparative dentin suggests that populations of competent progenitor cells similar to the embryonic dental papilla reside within the dental pulp isolated from postnatal mice. It is well documented that the differentiation of odontoblasts from the NC-derived dental papilla is dependent on inductive signals derived from the basement membrane (Ruch *et al.*, 1995). Results reported by others have suggested that the differentiation of odontoblast-like cells elaborating tubular matrix from dental pulp is seen only after transplantation of pulp tissue within its surrounding tooth structure (Inoue and Shimono, 1992), or after recombination with epithelial root sheath (Thomas and Kollar, 1989). Isolated pulp tissue implanted in a variety of others sites, including kidney and the anterior chamber of the eye give rise to an osteotypic matrix but not reparative dentin (Zussman, 1966; Luostarinen and Ronning, 1977; Yamamura, 1985; Takei *et al.*, 1988; Inoue and Shimono, 1992).

Our observations show that dental pulps implanted under the kidney capsule in the absence of dental epithelium or exogenous signaling molecules, give rise to both osteotypic matrix and reparative dentin. These observations suggest that reparative dentinogenesis in the dental pulp may be mediated by inductive signals residing within the extracellular matrix between dental pulp cells and/or signals derived from the atubular osteotypic matrix. This

possibility suggests a role for osteotypic matrix during reparative dentinogenesis that is similar to the roles of the basement membrane during physiological dentiongenesis *in vivo*.

Our observations indicate that the formation of reparative dentin in explanted dental pulps is indirect and is preceded by the formation of atubular osteotypic matrix (also called fibrodentin) and is consistent with the results reported by others (reviewed by Tziafas *et al.*, 2000; Smith and Lesot, 2001). Previous studies have suggested that deposition of an atubular osteotypic matrix, although a non-specific response of the pulp, plays a critical role in the initiation of reparative dentinogenesis (reviewed by Tziafas *et al.*, 2000; Smith and Lesot, 2001). Therefore, it is possible that during reparative dentinogenesis, osteotypic matrix may provide the source of the inductive signaling molecules.

Our transplantation studies indicate that pulp cells, in addition to giving rise to odontoblasts, can give rise to chondrocytes and osteoblasts secreting cartilage-and bone-like matrices respectively. The formation of bone-like matrices in explanted tissue in this study is supported by the high levels of expression of Col1a1-2.3-GFP and *Dmp1* but not *DSPP* in the osteocyte-like cells embedded within bone-like mineralized matrices and reveal the osteogenic potential of the dental pulp. The osteogenic potential of the dental pulp in the present study is consistent with the previous reported osteogenic potentials of dental sac (Yoshikawa and Kollar, 1981) and adult pulps (Zussman, 1966; Takei *et al.*, 1988). The presence of a small population of osteocyte-like cells associated with bone-like matrices in the explants that did not express Col1a1-2.3-GFP indicate the contribution of the host cells to the formation of the osteocyte- and bone-like matrices. However, odontoblast-like cells lining the reparative dentine did not contain Col1a1-2.3-GFP negative cells indicating that all odontoblasts have originated from cells within the transplanted pulp. These observations are suggestive of osteo-inductive potential of explanted dental pulp capable of inducing bone-like tissues in cells recruited from the host.

The multiple developmental potentials of the dental pulp in our study are consistent with previous observations in various experimental animals (Yamamura, 1985; Nakashima, 1989; Rutherford *et al.*, 1993; Rutherford *et al.*, 1994; Tziafas, 1995) and suggest that the dental pulp from postnatal mice, similar to human dental pulp (Gronthos *et al.*, 2000; Gronthos *et al.*, 2002), contains stem cells.

It is clear from our experimental data that dental pulp isolated from postnatal mice contains competent precursor cells capable of differentiation into a new generation of odontoblast-like cells secreting reparative dentin. However, it is still unclear whether this ability is shared by all pulpal cells or only a specific sub-population of pulp cells. Dental pulp is a vascularized fibrous connective tissue. The cellular components consist of odontoblasts, fibroblasts, macrophages, endothelial cells and lymphocytes (Ten Cate, 1998). Odontoblasts are present as a single layer lining the periphery of the pulp. However, fibroblasts are the most numerous cell type in the pulp and are localized mainly in the “cell-rich zone” of the coronal portion of the pulp (Ten Cate, 1998). In addition, it is thought that pulp also contains a group of “undifferentiated” mesenchymal cells from which the connective tissue of the pulp is derived (Rutherford, 1995; Ten Cate, 1998; Tziafas *et al.*, 2000). The cellularity of the pulp is age-dependent and diminishes with age (Rutherford, 1995; Ten Cate, 1998; Tziafas *et al.*, 2000). The low levels of Col1a1-2.3-GFP expression in the pulp in pOBCol2.3GFPemd transgenic animals provide an exciting and unique opportunity to gain insight into possible heterogeneity of the cells within the dental pulp population. Together these observation show that cells within the coronal portion of the pulp, devoid of any odontoblasts, are capable of giving rise to odontoblast-like cells secreting tubular-containing reparative dentin and atubular osteotypic matrices. We also show the ability of dental pulp to give rise to osteocyte-like cells that secrete bone-like matrices and chondrocytes secreting cartilage-like matrices.

Materials and Methods

Tissue Isolation

Maxillary and mandibular arches were isolated from pOBCol2.3GFPemd transgenic heterozygous mice at different stages of embryonic development (E10-21) and postnatal growth (P1, P5, and P13). Tissue fragments were fixed in 4% paraformaldehyde/PBS, decalcified in 15% EDTA, processed and embedded in paraffin using standard protocols. Serial sections of 6-7μm were placed onto ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA, USA). Selected sections were processed for hematoxylin and eosin (H,E) staining using standard protocols. Adjacent sections were deparaffinized and processed for epifluorescence and *in situ* hybridization analysis.

Epifluorescence Analysis

To visualize Col1a1-2.3-GFP expression, deparaffinized sections were mounted with glycerol/PBS. Col1a1-2.3-GFP fluorescence in the tissue sections was then visualized using a Nikon E600 microscope equipped with FITC and Texas Red dual fluorescent filter cube and photographed using a Spot RT TM digital camera (Diagnostic instruments Inc, Sterling Heights, MI). The dual fluorescent filter allows the yellow-green GFP signal to be distinguished from the light red autofluorescent background located in the marrow space and decalcified bone. The digitized images from bright-field, epifluorescence, and dark-field microscopy were assembled with Adobe PhotoShop 6.0 software (Adobe Systems, San Jose, CA, USA).

In Situ *Hybridization Analyses*

The patterns of expression of *DSPP* and *Dmp1* in the developing arches were examined by *in situ* hybridization to tissue sections using ^{32}P-labeled RNA probes as previously described (Mina *et al.*, 2002). A 1445 bp fragment of mouse *DSPP* cDNA corresponding to nucleotides 1442-2887 (accession # NM-010080) (kindly provided by Dr. M.MacDougall) in Bluescript was digested with *EcoR1* or *Sac1* and transcribed with T3 or T7 RNA polymerase for antisense and sense probes respectively (D’Souza *et al.*, 1997). A 623 bp fragment of mouse *Dmp1* cDNA corresponding to nucleotides 146-769 within the 5’ translated region of the cDNA (accession # U65020, kindly provided by Dr. M.MacDougall) in Bluescript was digested with *Kpn1* or *Sac1* and transcribed with T3 or T7 RNA polymerase for antisense and sense probes respectively (D’Souza *et al.*, 1997). For comparative analyses of the patterns of expression, serial sections were used so that expression patterns for *DSPP*, *Dmp1*, and Col1a1-2.3-GFP could be compared within the same animal and explant. Slides were left to develop between 2 days to 2 weeks. Following hybridization and development of the emulsion, sections were counter-stained with Hematoxylin. Photographs were taken under dark-field or bright-field illumination with a Spot RT TM camera. Images were digitized and processed as described in previous section. The silver grains in the dark-field image were selected, colored red, then superimposed onto the bright-field image. Images in Figs. 2 and 5 are PhotoShop pseudo-colored superimpositions of the *in situ* hybridization signals and bright-field images.

Isolation of Pulp Tissues and Grafting under the Kidney Capsule

The coronal portions of the pulps from first mandibular molars were isolated from 5-day-old pOBCol2.3GFPemd heterozygous mice. Pulp

tissues were prepared for transplantation under the kidney capsule of host mice (Brody *et al.*, 1998). The explants were harvested after 7, 10, and 14 days and processed for paraffin embedding. Serial sections were obtained and processed for hematoxylin and eosin staining. Alternate sections were also processes for epifluorescence and *in situ* hybridization analysis as described above. Selected decalcified sections were also processed for staining with Alcian blue and Mallory's anilin blue collagen staining (Sheerman and Hrapchak, 1980)

Histochemical Staining of Mineral

The formation of mineralized islands in transplanted tissue, were confirmed by Alizarin red-S staining of undecalified explants. Explants were harvested, fixed overnight in 4% paraformaldehyde/PBS, washed in cold PBS two times for 15 min each, infiltrate overnight with 30% soucrose in PBS, and embedded in Cryomatrix medium (Thermo Shandon, Pittsburgh, PA). Five μm sections were obtained according to the CryoJane^{R} user manual (Instrumedics Inc, Hackensack, NJ) and stained with 2% Alizarin red-S (Ph:4.3) according to standard protocol (Sheerman and Hrapchak, 1980). Some of the explants were fixed in 95% ethanol for 1 day followed by 100% acetone for 12 hours. Explants were stained for 12 hours in Alizarin red/ Alcian blue solution (0.015% Alcian blue, 0.005% Alizarin red in 1% acid alcohol). Explants were then cleared at room temperature in 1% KOH solution for 1 day and processed through an increasing series of glycerol/1% KOH solutions and stored in 80% glycerol/1% KOH.

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