A feasibility study for the analysis of reparative dentinogenesis in pOBCol3.6GFPtpz transgenic mice

M. Frozoni1,2, A. Balic2, K. Sagomonyants2, A. A. Zaia1, S. R. P. Line3 & M. Mina2

1Division of Endodontics, Department of Restorative Dentistry, Piracicaba Dental School, University of Campinas, São Paulo, Brazil; 2Division of Pediatric Dentistry, Department of Craniofacial Sciences, School of Dental Medicine, University of Connecticut Health Center, Farmington, CT, USA; and 3Department of Morphology, Division of Histology and Embryology, Piracicaba Dental School, University of Campinas, São Paulo, Brazil

Abstract


Aim To examine the feasibility of using the pOBCol3.6GFPtpz [3.6-green fluorescent protein (GFP)] transgenic mice as an in vivo model for studying the biological sequence of events during pulp healing and reparative dentinogenesis.

Methodology Pulp exposures were created in the first maxillary molar of 12–16-week-old 3.6-GFP transgenic mice with CD1 and C57/Bl6 genetic background. Direct pulp capping on exposed teeth was performed using mineral trioxide aggregate followed by restoration with a light-cured adhesive system (AS) and composite resin. In control teeth, the AS was placed in direct contact with the pulp. Animals were euthanized at various time points after pulp exposure and capping. The maxillary arch was isolated, fixed and processed for histological and epifluorescence analysis to examine reparative dentinogenesis.

Results Analysis of teeth immediately after pulp exposure revealed absence of odontoblasts expressing 3.6-GFP at the injury site. Evidence of reparative dentinogenesis was apparent at 4 weeks in 3.6-GFP mice in CD1 background and at 8 weeks in 3.6-GFP mice with C57/Bl6 background. The reparative dentine with both groups contained newly formed atubular-mineralized tissue resembling a dentine bridge and/or osteodentine that was lined by cells expressing 3.6-GFP as well as 3.6-GFP expressing cells embedded within the atubular matrix.

Conclusion This study was conducted in a few animals and did not allow statistical analysis. The results revealed that the 3.6-GFP transgenic animals provide a unique model for direct analysis of cellular and molecular mechanisms of pulp repair and tertiary dentinogenesis in vivo. The study also shows the effects of the capping material and the genetic background of the mice in the sequence and timing of reparative dentinogenesis.

Keywords: adhesive system, dentine bridge, green fluorescent protein, mineral trioxide aggregate, odontoblast-like cells, reparative dentinogenesis.

Received 8 November 2011; accepted 3 March 2012

Introduction

Transgenic mouse lines in which green fluorescent protein (GFP) expression is under the control of tissue- and stage-specific regulatory elements of genes involved in dentinogenesis have provided valuable tools for examining the stepwise progression and differentiation of progenitors into odontoblasts (Braut et al. 2003, Mina & Braut 2004, Balic & Mina 2005, 2011, Balic et al. 2009, 2010). Previous studies using 3.6-GFP transgenic mice in which GFP expression is under control of the rat 3.6-kb collagen type I promoter fragments showed that this transgene is first activated at low levels in cells in early stages of polarization (Balic et al. 2010). The expression of the transgene is significantly upregulated in more advanced stages of odontoblast differentiation. In fully differentiated odontoblasts, the expression of the transgene extends into the odontoblast processes (Mina & Braut 2004).

Additional experiments using 2.3-GFP and DMP1-GFP transgenic mice showed that the 2.3-GFP transgene was activated at a later stage of polarization, just before or at the time of formation of secretory/functional odontoblasts, and DMP1-GFP was activated in secretory/functional odontoblasts producing predentine (Balic & Mina 2005, 2011, Balic et al. 2010). Together, these observations indicated that activation and subsequent expression of these transgenes provide valuable markers for the identification of cells at different stage of odontoblast differentiation.

Therefore, in this study, 3.6-GFP transgenic animals were used to gain insight into the sequence of events during reparative dentinogenesis.

**Material and methods**

**Transgenic mice**

All animal procedures were performed in accordance with Animal Care Committee (ACC) guidelines from University of Connecticut Health Center (UCHC). To stimulate the reparative dentinogenesis, dental pulp exposure was performed in the maxillary first right molar of thirty 3.6-GFP mice (12–16 weeks old) with CD1 (outbred) genetic background and C57/Bl6 (inbred) background as described by Simon et al. (2008), with some modification. C57/Bl6 is the most widely used inbred strain used for genetic studies because of the isogenicity (having the same genotype) within a strain. Mice were anaesthetized and a cavity was prepared with a carbide bur (diameter 0.40 mm) on the occlusal surface of the molar (Class I cavity), in

*Figure 1* Procedure of pulp exposure, capping and restoration. (a) Occlusal view of the first (M1), second (M2) and third (M3) molars. In all images, the palatal side of the arch is on the right. (b) Position of the carbide bur on the centre of the first molar (M1). (c) Small cavity (arrow) on centre of the occlusal surface. (d) Endodontic hand file used to mechanically expose the pulp. (e) Pulp exposure (arrow). (f) Probe used to apply the mineral trioxide aggregate (MTA). (g) Pulp capping with MTA. (h) Cavity restoration with resin composite.
the mesial half of the crown, centred on the labial-palatal aspect of the tooth until the pulp was visible through the transparency of the dentine floor of the cavity. A pulp exposure was subsequently created mechanically using an endodontic hand file with 0.15-mm diameter tip with a 2% taper; this approach controlled the pulp exposure size to approximately 150 μm (size of the file tip) (Fig. 1). Exposed pulps were capped using mineral trioxide aggregate (MTA Angelus®; Angelus S/A, Paraná, Brazil) mixed with sterile water following the manufacturer’s recommendations. MTA was placed in contact with the pulp using a probe tip and condensed gently with a sterile paper point (size 35) (Dentsply Maillefer, Ballaigues, Switzerland). Subsequently, the cavity was sealed with light-cured composite resin (Z100/C210 3M, São Paulo, Brazil), associated with a two-step self-etching adhesive system (AS) (Clearfill SE Bond; Kuraray, Okayama, Japan) (Fig. 1). As a control group, pulp exposures were directly capped with AS overlaid with light-cured composite resin without any MTA or without capping material analysed immediately after exposure.

**Tissue isolation and analysis**

To analyse the sequence of reparative dentinogenesis, animals were euthanized after pulp exposure at different periods of time by intracardiac perfusion with 10% buffered formalin as described before (Palermo et al. 2005). The number of mice analysed in each time point for each genetic background is shown in Table 1. After perfusion, maxillary arches were isolated, cleaned from soft tissue, trimmed and fixed in 10% formalin solution for additional 24 h. Samples were decalcified for 7 days in 15% EDTA, 0.5% of formalin (pH 7.5) at 4°C and then embedded in paraffin following the standard protocols. Serial cross-sections of 7 μm were placed onto ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA, USA) deparaffinized with xylene, rehydrated and processed for epifluorescence analysis. To visualize GFP signal, deparaffinized sections were mounted with glycerol/PBS (50%: 50%). The fluorescence signal in these sections was examined in at least 20 sections through the region of injury and repair from each animal using an Axio Observer.Z1 for epifluorescence microscope (Carl Zeiss, Thornwood, NY, USA). Sections were examined in GFPtpz filter to allow the detection of 3.6-GFP signal and in GFPhcerry filter to avoid the detection of any GFP autofluorescence. The images were overlaid to eliminate tissue autofluorescence using user-defined computation program AxioVision Rel. 4.7 software (Carl Zeiss). After epifluorescence analysis, the same or adjacent sections were washed in PBS, processed for haematoxylin and eosin (H&E) staining using standard protocols and analysed by light microscopy.

**Results**

**Analysis of reparative dentinogenesis immediately and 7 days after pulp exposure**

Histological and epifluorescence analyses of teeth immediately after pulp exposure showed absence of dentine and odontoblasts expressing 3.6-GFP transgene at the pulp exposure site in transgenic animals with both CD1 (Fig. 2a,b) and C57/Bl6 background (data not shown) confirming the destruction of the odontoblast at this location. Analysis of pulps capped with MTA or AS, 7 days after pulp exposure, showed no evidence of reparative dentinogenesis in transgenic animals with both CD1 (Fig. 2c–f) and C57/Bl6 background (data not shown). However, in transgenic animals with CD1 background (but not with C57/Bl6), the exposure site underneath the MTA and AS contained cells expressing high and low levels of 3.6-GFP (Fig. 2d,f).

Despite the lack of statistical analysis, the observations in transgenic mice with CD1 background showed that the number of 3.6-GFP expressing cells in teeth capped with AS (Fig. 2f) was lower than in teeth capped with MTA (Fig. 2d). Small pieces of dentine were frequently observed in the dental pulp (Fig. 2a–f). These chips consisted of tubular dentine matrix surrounded by numerous odontoblasts expressing high levels of 3.6-GFP transgene (Fig. 2a–f).

<table>
<thead>
<tr>
<th>Time of analysis after pulp exposure</th>
<th>3.6-GFP transgenic mice with CD1 background</th>
<th>3.6-GFP transgenic mice with C57/Bl6 background</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immediately (no capping)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>1 week</td>
<td>MTA 2</td>
<td>MTA 2</td>
</tr>
<tr>
<td></td>
<td>AS 2</td>
<td>AS 2</td>
</tr>
<tr>
<td>4 weeks</td>
<td>MTA 4</td>
<td>MTA 4</td>
</tr>
<tr>
<td></td>
<td>AS 2</td>
<td>AS 2</td>
</tr>
<tr>
<td>8 weeks</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

AS, adhesive system; GFP, green fluorescent protein; MTA, mineral trioxide aggregate.
Analysis of reparative dentinogenesis 4 and 8 weeks after pulp exposure

Histological analysis 4 weeks after injury revealed clear evidence of reparative dentinogenesis in pulps capped with MTA in transgenic animals with CD1 (but not with C57/Bl6 background). In these animals, two patterns of reparative dentinogenesis were observed. One was the formation of an extensive atubular matrix in close contact with MTA, resembling an osteodentine, extending to the pulp (Fig. 3a). This matrix was lined with cells expressing 3.6-GFP under the matrix (indicated by dashed arrows). The second pattern of reparative dentinogenesis was the formation of a well-defined dentine bridge that appeared to seal the exposure site (Fig. 3b). This dentine bridge contained atubular matrix lined with cells expressing high levels of 3.6-GFP but did not contain a significant number of 3.6-GFP expressing cells embedded within the matrix (Fig. 3c). After 4 weeks, dentine chips, although still present in the pulp, were devoid of cells expressing 3.6-GFP transgene (Fig. 3d). In teeth capped with AS with CD1 background, no evidence of reparative dentinogenesis (dentine bridge or osteodentine) was evident after 4 weeks (Fig. 3e). However, in these animals, there was a thickened layer of dentine indicating extensive reactionary dentinogenesis over a new globular calcified dentine (Fig. 3f). This new reactionary dentine was lined with odontoblasts expressing high levels of 3.6-GFP transgene (Fig. 3f).

Transgenic animals with C57/Bl6 background capped with MTA 4 weeks after exposure showed no evident of osteodentine or dentine bridge. In these animals, there were islands of focal dentine matrix lined by a few cells expressing high levels of 3.6-GFP signal (Fig. 4a,b). In transgenic animals with C57/Bl6 background capped with AS, there was no evidence of reparative dentinogenesis or islands of dentine matrix deposition (data not shown).

Analysis of teeth in transgenic animals with C57/Bl6 background after 8 weeks of pulp exposure capped with MTA revealed the formation of an extensive atubular matrix, resembling osteodentine (Fig. 4c). This matrix

Figure 2  Histological sections stained with H&E (a, c, e) and epifluorescence analyses of adjacent sections (b, d, f) of teeth from 3.6-green fluorescent protein (GFP) transgenic mice with CD1 background immediately after pulp exposure (a and b) and 7 days after exposure (c–f). (a and b) show the absence of odontoblasts (indicated by dashed rectangle) and dentine at the exposure site (indicated by stars). Dentine chips in the exposure site are marked by an asterix. Note the expression of 3.6-GFP in the odontoblasts layer around pulp-dentine complex (dashed arrows) and around a dentine chip (indicated by full arrows). Also note the absence of cells expressing 3.6-GFP underneath the exposure site (indicated by dashed rectangle). (c and d) represent images from a tooth capped with mineral trioxide aggregate (MTA). (e and f) represent images from a tooth capped with adhesive system (AS). Note the expression of 3.6-GFP in cells in close contact with the capping materials in d and f (marked by dashed boxes). Also note that the number of cells expressing 3.6-GFP in the tooth capped with MTA (dashed box in d) is higher than in the tooth capped with AS (dashed box in f). Dashed arrows mark expression of 3.6-GFP in the odontoblast layer around pulp-dentine. Full arrows mark expression of 3.6-GFP in odontoblast around dentine chips. d = dentine; p = pulp; (*) = dentine chips; (dashed arrow) = original odontoblasts; star = exposure site. Scale bar = 100 μm.
also extended from the injury site into the pulp (Fig. 4c) and was lined with cells expressing 3.6-GFP and contained 3.6-GFP expressing cells entrapped within the new matrix (Fig. 4d). In teeth capped with AS, there was evidence of reactionary dentinogenesis characterized by thickened dentine secreted by pre-existing odontoblasts expressing 3.6-GFP (Fig. 4e,f) and reparative dentinogenesis (matrix lined with new secretory cells expressing 3.6-GFP) under the exposure site (Fig. 4e,f). Despite the lack of statistical analysis, the extent and frequency of reparative dentinogenesis in teeth treated with AS was lower than in teeth treated with MTA.

Discussion

It has been well documented that after an intense injury that leads to destruction and eliminates the pre-existing odontoblast layer, odontoblast-like cells originate from dental pulp cells and secrete reparative dentine at the injury site (Sloan & Smith 2007, Waddington et al. 2009, Simon et al. 2011). Although the formation of reparative dentinogenesis has been studied, the origin of the cells giving rise to odontoblast-like cells and cellular and molecular events leading to this reparative process is not well understood. Previous studies have shown that 3.6-GFP animals provide a unique model to examine odontoblast differentiation from a progenitor population (Mina & Braut 2004, Balic et al. 2010). Therefore, in this study, 3.6-GFP transgenic mice were used to gain insight in vivo into the sequence of events during reparative dentinogenesis.

In this study, to stimulate the differentiation of odontoblast-like cells from progenitor or stem cell population, exposure on maxillary first molars of 3.6-GFP transgenic mice was used as described before (Simon et al. 2008) with some modifications. Utilization of transgenic animals allowed a better insight into many aspects of this reparative process including...
destruction of odontoblasts after pulp exposure, presence of dentine chips at the healing pulp, the fate of the pre-existing odontoblasts around these chips, recruitment of progenitors to the injury site and their subsequent differentiation and the formation of different patterns of tertiary dentine.

The observations revealed that the pulp exposure protocol led to complete destruction of odontoblasts as evident by the absence of cells expressing 3.6-GFP transgene in the pulp tissue under the exposure site immediately after injury. The results revealed no evidence of dentine bridge formation in pulps capped with MTA or AS in transgenic animals with CD1 and C57/B6 backgrounds 7 days after pulp exposure that are consistent with previous results (Simon et al. 2008).

Interestingly, results demonstrated the 3.6-GFP expressing cells in the pulp tissue underneath the site of injury 7 days after pulp exposure. The appearance of 3.6-GFP transgene in these cells is before the formation of calcified tissue and is related to the activation or up-regulation of 3.6-GFP transgene in cells in close proximity to the injury and/or in cells recruited to the site of injury from a distance. These findings on early appearance of cells committed to dentinogenic lineage at the injury site are in agreement with results reported by others (Harada et al. 2008, Ishikawa et al. 2010) who used rat molars to demonstrate that cells committed to odontoblasts lineage appeared at the injury site 2–5 days after odontoblast death. Based on previous observations (Balic et al. 2010), the results show that these cells at the site of injury expressing 3.6-GFP transgene are in early stages of polarization.

Despite the lack of statistical analysis, the observation revealed that the numbers of 3.6-GFP expressing cells in pulps under MTA were higher than those under AS which is most likely related to the properties of MTA such as its high pH, calcium release and biocompatibility (Parirokh & Torabinejad 2010). MTA also is known for its good sealing capacity (Barrieshi-Nusair & Hammad 2005), its ability to promote cell proliferation (Paranjpe et al. 2010) and ability to up-regulate the gene expression (Runx2, osteocalcin, alkaline phosphatase...
and DSPP), and the differentiation of pulp cells into odontoblast-like cells (Paranjpe et al. 2010).

In this study, reparative dentinogenesis and dentine bridges were observed in pulps capped with MTA, after 4 weeks of exposure with transgenic animals with CD1 background and 8 weeks after exposure with C57/Bl6 background. These observations indicated the delayed process of dentine repair and regeneration with C57/Bl6 strains. The differences in the timing of reparative dentinogenesis with the CD1 and with C57/Bl6 backgrounds are consistent with the influences of genetic backgrounds on the expression of various diseases and repair processes (Everett 2011).

Two different patterns of reparative dentinogenesis were observed. One was a bulky, atubular calcified tissue under the capping material that extended into the pulp resulting in the obliteration of the pulp tissue under the capping material that extended into the pulp chamber. In this atubular matrix, 3.6-GFP expressing cells surrounding the matrix and embedded in the matrix were detected. These atubular structures with their cellular contents resemble the osteodentine described in the literature (Sloan & Smith 2007, Waddington et al. 2009) and were similar to the structures reported by others after transplantation of pulp tissue under the kidney capsule (Braut et al. 2003) and after reimplantation of rat molars (Zhao et al. 2007, Ishikawa et al. 2010).

The other pattern of reparative dentinogenesis was a well-defined calcified bridge along the exposure site underneath the MTA. This finding is consistent with previous publications, which reported the secretion of reparative matrix forming a dentine bridge 14 days after pulp exposure and MTA capping in rats molars (Andelin et al. 2003, Kuratate et al. 2008, Simon et al. 2008, Shahravan et al. 2011). This study showed that the calcified bridge was lined with 3.6-GFP expressing cells. Previous studies have demonstrated that cells lining the dentine bridge under MTA were immunoreactive for nestin, osteopontin and for Dentine sialoprotein (DSP) (Andelin et al. 2003, Kuratate et al. 2008).

Previous studies have shown the expression of 3.6-GFP transgene in cells in both dentinogenic and osteogenic lineage, making the distinction between odontoblasts- and osteoblasts-like cells secreting dentine- and bone-like tissues difficult. The distinction between these cell type is important in the light of more recent observations that have provided clear evidence for osteogenic and dentinogenic potential of dental pulp cells (Ogawa et al. 2006, Takamori et al. 2008, Balic et al. 2010).

Transgenic animals with both backgrounds capped with AS had evidence of extensive reactionary dentinogenesis consisting of a thickened dentine over a globular calcified dentine narrowing the exposure site. The extensive amount of reactionary dentine in teeth capped with AS is because of short- and long-term inflammation in the dental pulp caused by the acid primer (de Souza Costa et al. 2001, Dominguez et al. 2003) that stimulates pre-existing odontoblast to secrete reactionary dentine over the dentine walls.

The reparative dentinogenesis in teeth capped with AS is in agreement with Akimoto et al. (1998) who reported new dentine bridge formation directly adjacent to the Clearfil system interface. This reparative dentine could be explained by the fact that self-etching AS such Clearfil® SE Bond used in this study contains a phosphoric acid monoester, in the acid primer, with a pH higher than others acid primers and produces a substantially milder effect on pulp tissue (Akimoto et al. 1998, Koliniotou-Koumpia & Tziafas 2005).

Consistent with results described before (Simon et al. 2008), the results revealed the presence of dentine chips at the periphery of the injury deeply impacted into the pulp as a consequence of the endodontic file penetration. The chips, although present at all time points, were lined with surviving odontoblast expressing 3.6-GFP up to 7 days but not thereafter. Previous results have shown that this dentine debris can stimulate the formation of hard tissue (Jaber et al. 1991). However, a deep impaction of dentine chips can decrease the rate of healing and bridge formation (Domínguez et al. 2003).

**Conclusion**

The data provided by this study demonstrated the feasibility of using 3.6-GFP transgenic report mice either with CD1 or C57/Bl6 background in combination with the pulp exposure protocol as a powerful in vivo model for the analysis of pulp healing process and biological events involved in reparative and reactionary dentinogenesis. This opens new opportunities for the use of other transgenic animals in which GFP coding sequences are under the control of tissuespecific regulatory elements (i.e. dentine sialophosphoprotein (DSPP)) and animals with regulatory elements for progenitor or stem cells markers (i.e. z-SMA) for better understanding of various aspects of tertiary dentinogenesis.
Acknowledgements

We would like to thank many individuals including Ms. Barbra Rodgers and all the people who helped with laboratory techniques used in this experiment. This study was supported by grants from NIH (R01-DE016689) to MM and CAPES (3422/09-7) to MF.

References


