UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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ANÁLISE DA ORIGEM DE CÉLULAS PRECURSORAS DE ODONTOBLASTOS DURANTE A DENTINOGÊNESE REPARATIVA

Tese de Doutorado apresentada à Faculdade de Odontologia de Piracicaba, UNICAMP para obtenção do título de Doutor em Clínica Odontológica na Área de Endodontia

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Este exemplar corresponde à
versão final da Tese defendida
pelo aluno e orientada pelo
Prof. Dr. Sergio Roberto Peres Line

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Assinatura do Orientador

PIRACICABA, 2011

FICHA CATALOGRÁFICA ELABORADA POR
MARILENE GIRELLO – CRB8/6159 - BIBLIOTECA DA
FACULDADE DE ODONTOLOGIA DE PIRACICABA DA UNICAMP

Frozoni, Marcos Roberto dos Santos, 1969-
F939a Análise da origem de células precursoras de odontoblastos durante a dentinogênese reparativa / Marcos Roberto dos Santos Frozoni. -- Piracicaba, SP : [s.n.], 2011.

Orientador: Sérgio Roberto Peres Line.
Coorientador: Alexandre Augusto Zaia.
Tese (doutorado) - Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Polpa dental. 2. Parabiose. I. Line, Sérgio Roberto Peres. II. Zaia, Alexandre Augusto. III. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. IV. Título.

Informações para a Biblioteca Digital

Título em Inglês: Analysis of the origin of odontoblast like cells during reparative dentinogenesis
Palavras-chave em Inglês:
Dental pulp
Parabiosis
Área de concentração: Endodontia
Titulação: Doutor em Clínica Odontológica
Banca examinadora:
Alexandre Augusto Zaia [Coorientador]
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João Eduardo Gomes Filho
Franscisco José de Souza Filho
Pedro Duarte Novaes
Data da defesa: 15-12-2011
Programa de Pós-Graduação: Clínica Odontológica

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Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 15 de Dezembro de 2011, considerou o candidato MARCOS ROBERTO DOS SANTOS FROZONI aprovado.

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DEDICATÓRIA

À minha querida e adorável esposa **Daniele** *pela compreensão e respeito a mim dedicados durante todos estes anos e pelo apoio incondicional prestado sob gestos de amor, carinho.*

À minha amada filha que em breve estará entre nós e já é a luz da minha vida.

Aos meus pais **Mônica** *e* **Newton** *(in memorian) que construíram meu caráter, me ensinaram princípios de honestidade, dignidade, responsabilidade, solidariedade, respeito ao próximo, amor, esperança e fé.*

AGRADECIMENTOS

Agradeço a Deus por poder contar sempre com sua ajuda, me dando força e consciência para que pudesse superar os momentos mais difíceis.

Ao meu orientador Prof. Dr. Sergio Roberto Peres Line pela confiança em mim depositada, pelo seu companheirismo, amizade e paciência. Profissional de extrema competência, responsável pelos meus primeiros passos na docência. Meus sinceros agradecimentos pela oportunidade de ser seu aprendiz.

Ao meu co-orientador Prof. Dr. Alexandre Augusto Zaia ser humano ímpar dedicado à endodontia, pela co-orientação neste trabalho e pelo respeito e amizade que sempre me dispensou. Meus sinceros agradecimentos.

À Profª Drª Mina Mina, pela valiosa co-orientação nos laboratórios da Universtiy of Connecticut Health Center – School of Dental Medicine e por me mostrar uma visão crítica sobre ciência.

Ao Prof. Dr. Francisco José de Souza Filho, exemplo de professor, pesquisador e ser humano, o que lhe confere o dom de conquistar o respeito e a admiração de quantos dele se acercam.

À Profª. Drª. Brenda Paula Figueiredo A. Gomes, exemplo de competência e dedicação, pelo estímulo e oportunos ensinamentos pessoais e profissionais.

Ao Prof. Dr. Waldocyr Simões que me despertou para o universo da iniciação científica, pela relação de amizade e respeitosa confiança.

Aos professores da área de Endodontia da Faculdade de Odontologia de Piracicaba (FOP) – UNICAMP, pelo respeito e dedicação que sempre a mim dispensaram em todos os momentos de dúvidas e necessidades.

Aos colegas do Programa de Pós-Graduação em Endodontia da Faculdade de Odontologia de Piracicaba (FOP) - UNICAMP, pela inesquecível amizade e agradável convívio em todos os nossos momentos de alegrias e dificuldades.

Aos colegas de docência da Faculdade de Odontologia de Araras: Prof. Dr. Cid Alonso Manicardi, Prof. Dr. Homero Cassonato Jr., Prof. Samuel Henrique Camara De Bem e Prof. Alex Corrarelo, por estes longos anos juntos, dedicados a fazer o melhor possível para levar o conhecimento e educação aos nossos alunos.

À CAPES, pelo financiamento, através de bolsa de estudos, do meu estagio de dourado no exterior (PDEE), realizado na University of Connecticut Health Center.

Aos meus sogros Antonio Benedito Bonfante e Marilda Carandina Bonfante, meus segundos pais, pelos bons momentos passados juntos, respeito, paciência e consideração com que sempre me trataram.

Aos meus irmãos Isabela, Marco Antônio e Celso Antônio por estarmos conseguindo juntos, apesar das dificuldades, darmos continuidade aos ensinamentos de nossos pais.

Aos amigos de São Sebastião da Grama, que desde a infância sempre me dedicaram carinhosa amizade, grande respeito e momentos inesquecíveis.

A todos aqueles que de forma direta ou indireta colaboraram por mais esta realização de um sonho.

Paciência, dedicação, perseverança e,

acima de tudo, vontade inabalável

de se atingir a meta.

Ludwig van Beethoven

RESUMO

Na polpa saudável, os odontoblastos pós-mitóticos responsáveis pela secreção de dentina primária e secundária sobrevivem durante a toda a vida do dente e são responsáveis pelas respostas às injúrias externas através da produção de dentina terciária focalmente abaixo do local da agressão. Os odontoblastos sobreviventes à injúria secretam uma matriz de dentina reacional, entretanto, os irreversivelmente danificados, são substituídos por uma segunda geração de células com as mesmas características morfofisiológicas. Estas células são derivadas do recrutamento, proliferação e diferenciação de células pulpares que possuam propriedades de células tronco, para formar uma nova matriz de dentina reparativa como parte do processo de reparo do complexo dentino pulpar. O mecanismo de reparo que segue após uma injúria dental é fundamental para a sobrevivência da polpa e envolve uma série de processos que precisam ser completamente esclarecidos mais precisamente a origem das células precursoras da nova geração de odontoblastos que irão secretar dentina reparativa. Embora evidências sugiram que esta nova geração de odontoblastos tenha origem de células do tecido conjuntivo pulpar, a exata origem destas células ainda não está completamente esclarecida. O objetivo deste estudo foi utilizar camundongos transgênicos que expressam *green fluorecent protein* (GFP) em todas as células do corpo, com exceção dos eritrócitos, e a técnica cirúrgica de parabiose para unir dois camundongos isogênicos, um GFP e outro não-GFP de maneira a formar um par parabiótico, que possa compartilhar a mesma circulação sanguínea cruzada (células marcadas do camundongo GFP passam para a corrente circulatória do camundongo não-GFP). Após a parabiose foi realizada uma exposição pulpar, devidamente capeada, nos molares dos camundongos não-GFP para estimular a produção de dentina terciária reparativa e conseqüentemente a diferenciação de novos odontoblastos. Os animais foram sacrificados e os molares dos camundongos não-GFP, processados para análise em microscopia de fluorescência. Foi observada a presença de células GFP (células verdes ao microscópio de

fluorescência), originárias do sangue periférico (SP) de camundongos GFP, participando do processo de dentinogênese reparativa nos molares de camundongos não-GFP. Este estudo sugere a primeira evidência da participação de células troco mesenquimais do sangue periférico (CTM-SP) no processo de diferenciação de novos odontoblastos durante a dentinogênese reparativa.

Palavras Chave: Células tronco da polpa dental, proteína fluorescente verde, nova geração de odontoblastos, parabiose, dentina reparativa.

ABSTRACT

In the healthy pulp, the post-mitotic odontoblasts responsible for secretion of primary and secondary dentin survives during the tooth life and are able to respond to injuries with the production of tertiary dentin focally beneath the site of the injury. If the odontoblast survives the injury it secrets a reactionary dentin matrix but if it is irreversibly damaged, it is replaced by a second generation of odontoblast-like cells, with the same morphophysiological profile. Those cells are derived from recruitment, proliferation, and differentiation of pulp cells, which can have stem cell properties to form a new reparative dentin matrix as part of repair process in the dentin-pulp organ. The repair mechanism that follows a tooth injury is critical to the pulp survival and involves a series of processes that need to be completely understood more precisely the origin of the odontoblast-like cells that will secrete reparative dentin. Although evidences suggesting that odontoblast-like cells originate from cells within dental pulp connective tissue the exact origin of these odontoblast-like cells is not clearly defined. The aim of this study was to use transgenic mice expressing green fluorescent protein (GFP) in all body cells except erythrocytes and parabiosis surgical technique for joining two inbred mice, a GFP and other non-GFP to form one parabiotic pair, who can share the same cross-circulation (labeled cells from GFP mice move into the bloodstream of non-GFP mice After parabiosis a pulp exposure, properly capped, was performed on the molar of the recipient non-GFP mice to stimulate the production of tertiary reparative dentin and hence differentiation of odontoblast-like cells. The animals were sacrificed and the molars of the non-GFP mice processed for fluorescence microscopy. It was observed the presence of GFP cells (green cells to the fluorescence microscope), originating from peripheral blood (PB) from GFP mice, participating in the reparative dentinogenesis process in the molars of non-GFP mice. This study suggests the first evidence of the participation of mesenchymal stem cells from PB (MSC-PB) in the differentiation process of odontoblast-like cells during reparative dentinogenesis.

Key Words: Dental pulp stem cells, green fluorescence protein, odontoblast-like cells, parabiosis, and reparative dentinogenesis.

SUMÁRIO

INTRODUÇÃO

O desenvolvimento do dente envolve uma série de interações recíprocas entre o epitélio oral e células mesenquimais da crista neural, estas células mesenquimais encapsuladas pelo órgão de esmalte formam a papila dental que formará a polpa dental. A última divisão das células periféricas da papila dental que estão em contato com a membrana basal, membrana esta que está entre as células do epitélio interno do órgão de esmalte e as células periféricas da papila dental, dá origem a duas populações de células filhas: os pré-odontoblastos e as células da camada sub odontoblástica (células de Höhl). Moléculas sinalizadoras expressas no epitélio dentário e capturadas na membrana basal controlam a diferenciação dos pré-odontoblastos em odontoblastos (Ruch *et al.,* 1995). Estes odontoblastos produzem os componentes da matriz extracelular encontrada na dentina e estão implicados na mineralização dentinária (Mitsiadis & Rahiotis, 2004). Depois da secreção da dentina primária circumpulpar durante a dentinogênese, a secreção da dentina secundária, a partir da erupção dentária e formação completa da raiz, continuará ao longo de toda a vida do dente em média de deposição lenta (Baume *et al*., 1980).

Muitas injúrias como cárie, atrição, abrasão, erosão, trauma, procedimentos e materiais restauradores podem acometer o dente durante sua vida (Nanci *et al*., 2008). A habilidade do complexo dentino-pulpar em responder a tais injúrias pela formação de tecido mineralizado tem sido a longo tempo reconhecida (Searls *et al.,* 1967, Bergenholtz, 1981, Trowbridge, 1981 e Langeland, 1987). Diferente das dentinas fisiológicas primárias e secundárias, que são formadas ao longo de toda a borda da polpa, a dentina terciária é focalmente secretada pelos odontoblastos em resposta à uma injúria externa (Murray *et al.,* 2000 e Nanci *et al*., 2008). A dentina terciária é a mais comum e conhecida característica do reparo pulpar e é dividida em reacional e reparativa (Tziafas, 1995, Smith *et al*., 1995, Tziafas *et al*., 2000, Smith & Lesot, 2001, Mitsiadis e Rahiotis, 2004).

Frente a uma agressão mais superficial a resposta da polpa se dá pela deposição de dentina reacional que é secretada pelos odontoblastos pré-existentes que sobreviveram à injúria (Tziafas *et al*., 2000 e Mitsiadis & Rahiotis, 2004). Após uma agressão mais intensa, que leva à morte dos odontoblastos originais, uma seqüência de eventos inflamatórios e de reparo ocorrem na área de degeneração odontoblástica (Kim, 1990 e Chiego, 1992) e, em um estado metabólico apropriado do complexo dentina-polpa, uma nova geração de odontoblastos se diferencia a partir de células pulpares e inicia a secreção de dentina reparativa (Smith *et al*., 1995, Tziafas, 1995, Bjørndal & Darvann, 1999, Tziafas *et al*., 2000 e Smith & Lesot, 2001)

Uma cascata de sinais moleculares provenientes de interações entre moléculas da matriz extracelular da polpa, secretados por odontoblastos funcionais ou fibroblastos pulpares (Tziafas, 1995, Yoshiba *et al*., 1996), regula a proliferação, migração e diferenciação de células pulpares em uma nova linhagem de odontoblastos e estimulam a secreção de matriz orgânica por parte destas novas células (Smith *et al*., 2001).

Moléculas bioativas tais como: TGFβ (transforming grow factor), BMPs (bone grow factor), FGFs (fibroblast grow factor), e IGFs (insulin grow factor) se originam da matriz dentinária pré-existente e são liberados depois de uma injúria (Ruch *et al*., 1995, Lesot *et al*., 1994 e Tziafas *et al*., 2000), provendo sinais quimiotáticos que recrutam células inflamatórias da corrente vascular pulpar e células indiferenciadas da polpa para o local da agressão, estimulando a angiogênese e iniciando a reação de reparo tecidual (Grant *et al*., 2002, Pelosi *et al*., 2002, Larrivée *et al*., 2005 e Bailey *et al*., 2006).

Estudos têm identificado várias potenciais populações de células na polpa dentária adulta, capazes de dar origem a uma nova geração de odontoblastos durante a dentinogênese reparativa. Incluindo: células da camada sub-odontoblástica (Kitamura *et al*., 2001 e Harada *et al*., 2008), células indiferenciadas em volta de vasos pulpares, conhecidas como pericitos, vistos perto e distantes do local do estímulo podendo se proliferar e migrar para a área danificada e participar no processo de reparo da polpa (Smith & Lesot, 2001, Shi

& Gronthos, 2003, Iohara *et al*., 2006 e Lovschall *et al*., 2007), populações de células progenitoras competentes, similares a células indiferenciadas da papila dental, derivadas da crista neural, que dentro do estroma do tecido conjuntivo pulpar, poderiam se diferenciariam em odontoblastos (Fitzgerald, 1979, Baume *et al*., 1980, Yamamura, 1985, Stanley, 1989, Goldberg e Lasfargues, 1995 e Tziafas *et al*., 2002). Alguns pesquisadores têm demonstrado que fibroblastos perto de vasos sanguíneos poderiam se diferenciar em odontoblastos secretores de matriz de dentina reparativa (Bjørndal & Darvann *,* 1999 e About & Mitsiadis, 2001).

De fato, o mecanismo da dentinogênese reparativa ainda não está completamente caracterizado bem como, a origem, recrutamento e diferenciação de células pulpares em uma segunda geração de odontoblastos responsáveis pela secreção da matriz de dentina reparativa (Braut *et al*., 2003, Mitsiadis & Rahiotis, 2004, Mina & Braut, 2004 e Harada *et al*., 2008).

Algumas questões que devem ser esclarecidas são, se todas as células da polpa ou apenas uma específica sub-população podem responder a estímulos externos e secretar dentina reparativa e se estas células precursoras da nova geração de odontoblastos estão dentro do tecido conjuntivo pulpar desde o desenvolvimento do dente ou podem ter origem externa, como por exemplo estar em constante fluxo na corrente circulatória como células tronco mesenquimais do sangue periférico (CTM-SP). E se em um apropriado estado metabólico podem migrar da micro-circulação da polpa para o tecido conjuntivo pulpar e estimuladas por fatores de crescimento e sinais moleculares ativos, dar origem a uma segunda geração de odontoblastos no mecanismo de reparo do órgão dental. (Kardos *et al*., 1998, Kitamura *et al*., 2001, Tziafas *et al*., 2000, Braut *et al*., 2003, Murray *et al*., 2003, Mina & Braut, 2004 e Tziafas, 2004).

A célula tronco mesenquimal (CTM) é caracterizada por ser uma célula indiferenciada, de origem não hematopoiética, que pode gerar células filhas por um período limitado de vida, possui morfologia fibroblastóide e se aderem à superfície plástica de discos de cultura celular (Friedenstein *et a*l., 1970). A medula óssea é a principal fonte de CTM sendo que dentro deste tecido existem

duas classe de células tronco (Friedenstein *et al*., 1968), células tronco hematopoiéticas (CTH) que são precursoras de todos os tipos de células sanguíneas e uma segunda classe que são as CTM propriamente ditas (Bonnet, 2003) que têm a capacidade de se diferenciar *in vivo* e *in vitro,* em células do tecido conjuntivo, incluindo adipócitos, osteócitos, condrócitos e miócitos além de células de outras linhagens incluindo neuroectodérmica e endodérmica (Gregory *et al*., 2005).

As CTM também podem ser encontradas no tecido adiposo (Fraser *et al.*, 2006), pulmão (Griffiths *et al*., 2005), coração (Beltrami, *et al*., 2003), cordão umbilical (Bobis *et al*., 2006) e SP (Mendrone Junior, 2009). As CTM-SP podem ser facilmente isoladas e apresentam um grande potencial proliferativo e capacidade de diferenciação (Tondreau *et al.,* 2005).

As CTM-SP estão em constante fluxo na corrente circulatória e um importante modelo para se estudar estas células é o modelo de parabiose que envolve a união cirúrgica de dois animais, que passarão a compartilhar a mesma circulação sanguínea, permitindo que células circulantes sanguíneas (CCS) de um animal migrem para o SP do outro animal (Finerty, 1952, Wright *et al*., 2001, McKinney-Freeman & Goodell, 2004 e Bailey *et al*., 2006). A análise de citrometria de fluxo revela que células derivadas de animais parabióticos são detectadas nos parceiros 3 dias após a cirurgia e estão completamente equilibradas (40% a 50% da circulação total) dentro de uma semana, a circulação cruzada nos pares parabiontes é estável por mais de nove semanas (Wright *et al*., 2001 e McKinney-Freeman *&* Goodell, 2004).

As CCS de um parceiro parabiótico, podem migrar, via corrente circulatória, e serem encontradas, participando da regeneração de tecidos, no outro parceiro parabiótico, como fibras musculares esqueléticas danificadas por toxinas (Jackson *et al*., 2004 e Palermo *et al*., 2005), em modelos de injúria no fígado (Lagasse *et al*., 2000), tecidos cardíacos (Orlic *et al*., 2003) ou células vasculares endoteliais no processo de angiogênese (Grant *et al*., 2002, Pelosi *et al*., 2002, Larrivée *et al*., 2005 e Bailey *et al*., 2006).

A exposição pulpar e o capeamento direto usando um material adequado, podem induzir a um processo de dentinogênese reparativa. Muitos modelos de exposição pulpar e capeamento direto têm sido usados no estudo do reparo do complexo dentino pulpar (ex.: humanos, ratos, ferretes e cães) (Smith, 2002). Uma vez que nestes modelos a exposição pulpar resulta em perda da camada de odontoblastos logo abaixo do local da injúria, a formação de uma dentina reparativa requer o recrutamento e a diferenciação de células progenitoras de uma segunda geração de odontoblastos (Tziafas *et al*., 2000, Smith & Lesot, 2001 Tziafas *et al*., 2002, Tziafas, 2004, e Yasuda *et al*., 2008).

Recentemente, Simon *et al.* (2008) desenvolveram um interessante modelo para estudar a regeneração dentinária e pulpar em molares de camundongos, este modelo tem potencial para ser extremamente informativo em estudos de sinalização molecular, origem, recrutamento, e diferenciação de células em odontoblastos e pode ser usado para verificar se CCS estão envolvidas no processo de reparo pulpar e secreção de dentina reparativa.

Enquanto estudos *in vitro* são válidos em delinear o processo celular e molecular que toma lugar na dentinogênese terciária e podem racionalizar o desenho de experimentos *in vivo*, eles não podem substituí-los completamente, uma vez que, em experimentos *in vivo,* estão presentes fatores sistêmicos que influenciam amplamente o espectro do processo de reparo (Tziafas *et al*., 2000).

Recentemente foram desenvolvidos camundongos transgênicos os quais a proteína fluorescente verde (green fluorescent protein) (GFP) esta sob controle de promotores específicos. Estes camundongos transgênicos expressam GFP em células do corpo dependendo do promotor que foi utilizado (Okabe *et al*., 1997, Kalajzic *et al*., 2002a).

A análise da camada de odontoblastos da polpa dentária de camundongos GFP, sob controle do promotor do colágeno tipo I (Kalajzic *et al*., 2002a) ou sob controle do promotor da ß actina (Okabe *et al*., 1997), usando cortes não corados, fixados em solução tamponada de formalina a 10% e infiltrados com parafina, sob a ótica de microscopia de fluorescência mostrou que a proteína GFP é fortemente expressa no citosol das células

odontoblásticas completamente diferenciadas (Frozoni 2011). Por tanto, estes camundongos GFP, são um interessante modelo para monitorar o recrutamento e diferenciação da nova geração de células formadoras de dentina reparativa no processo de reparo pulpar após uma injúria externa (Braut *et al*., 2003 e Mina & Braut, 2004).

Diante de uma incompleta definição da origem de células que dão origem a uma segunda geração de odontoblastos na dentinogênese reparativa, será utilizado neste estudo o modelo de parabiose associado ao modelo de exposição pulpar, para a análise da participação de CCS, oriundas de camundongos GPF, no processo de dentinogênese reparativa em molares de camundongos não-GFP após exposição e capeamento pulpar.

Analysis of reparative dentinogenesis in pOBCol3.6GFPtpz transgenic mice

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Running title: Tertiary dentinogenesis in mice

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

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Acknowledgements

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

Abstract

Aim To examine the feasibility of using the pOBCol3.6GFPtpz 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 molars on 12-16 week old pOBCol3.6GFPtpz transgenic mice in CD1 and C57/Bl6 genetic background. Direct pulp capping on exposed teeth were performed using mineral trioxide aggregate (MTA) followed by restoration with 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. Maxillary arch was isolated, fixed and processed for histological and epifluorescence analysis to examine reparative dentinogenesis.

Results Analysis of teeth immediately after pulp exposure showed absence of odontoblasts expressing 3.6-GFP at the injury site. Evidence of reparative dentinogenesis was present at 4 weeks in pOBCol3.6GFPtpz mice in CD1 background and at 8 weeks in pOBCol3.6GFPtpz mice in C57/Bl6 background. The reparative dentin in both genetic background contained newly formed atubular-mineralized tissue resembling a dentin bridge and/or osteodentin that was lined by cells expressing 3.6-GFP as well as 3.6-GFP expressing cells embedded within the atubular matrix.

Conclusion The pOBCol3.6GFPtpz transgenic animals provide a unique model for direct analysis of cellular and molecular mechanisms of pulp repair and tertiary dentinogenesis in vivo. Our study also shows the effects of the capping material and the genetic background of the mice in the sequence and timing of reparative dentinogenesis.

Introduction

Dentin matrix secreted by odontoblasts is the major component of dental mineralized tissue consisting of inorganic and organic components (Linde & Goldberg, 1993). The organic components secreted by odontoblasts consist primarily of collagen fibers and non-collagenous proteins (NCPs). Type I collagen is the major constituent (approximately 86-90%) of the collagenous proteins in dentin and provides the framework for the deposition apatite crystal (Linde & Goldberg 1993, Butler 1995, Butler 1998). Previous studies have shown terminal differentiation of odontoblasts is accompanied by dramatic increases in type I collagen synthesis (Bleicher *et al.,* 1999).

Dentin matrix 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 & Lesot 2001). During tooth development, primary dentin is secreted until the completion of root formation (Smith et al. 1995, Tziafas 1995; Tziafas *et al*. 2000, Smith and Lesot 2001). Secondary dentin is secreted throughout life at a much slower rate and results in a decrease of the pulp chamber size (Lisi *et al*. 2003, Qin *et al*. 2007).

Unlike primary or secondary dentins that are formed along the entire pulp–dentin border, tertiary dentin is secreted focally by odontoblasts in response to injury (Searls 1967, Ruch *et al*. 1995, Murray *et al.* 2000, Mitsiadis & Rahiotis 2004, Nanci 2008). Tertiary dentin is divided into reactionary and reparative dentin (Bergenholtz 1981, Trowbridge 1981, Langeland 1987, Smith *et al.* 1995, Tziafas *et al.* 2000). Reactionary dentin is secreted by pre-existing odontoblasts that survive the injury/insult (Bergenholtz 1981, Trowbridge 1981). Following more intense injury/insult that leads to odontoblast death, newly differentiated odontoblast-like cells secrete reparative dentin (Langeland 1987, Trowbridge 1981, Smith *et al.* 1995, Tziafas *et al.* 2000). Reparative dentinogenesis involves a cascade of inflammatory and healing events that occur rapidly following the

odontoblasts degeneration (Tziafas 1995, Smith & Lesot 2001) and the formation of a new generation of odontoblast-like cells (Batouli *et al.* 2003).

Potential populations of cells in dental pulp capable of giving rise to the new generation of odontoblast-like cells during reparative dentinogenesis are numerous and include the cell-rich layer of Höhl adjacent to the odontoblasts, undifferentiated mesenchymal cells, pulp fibroblasts (Sloan & Smith 2007, Sloan & Waddington 2009, Balic & Mina 2010) and putative post-natal mesenchymal stem cells (MSC) (Gronthos *et al*. 2000, Gronthos *et al*. 2002, Miura *et al.* 2003).

The process of reparative dentinogenesis and cells giving rise to odontoblast-like cells have been the subject of intense investigation using a variety of *in vivo* and *in vitro* approaches and various animal models including mice (Bergenholtz 1981, Kardos *et al.* 1998, Braut *et al.* 2003, Liu H *et al*. 2006, Sloan & Smith 2007, Harada *et al.* 2008, Simon *et al*. 2008, Sloan & Waddington 2009).

Transgenic mouse lines in which GFP expression is under the control of tissue- and stage-specific regulatory elements of genes involved in dentinogenesis and osteogenesis have provided valuable tools for examining the stepwise progression and differentiation of progenitors into odontoblasts and osteoblasts (Uribe *et al.* 2011, Balic *et al.* 2010, Balic & Mina 2011, Boban *et al.* 2010). Our previous studies in pOBCol3.6GFPtpz transgenic mice in which 3.6-kb fragments of rat type I collagen promoter drive the expression of GFP have shown that early polarizing odontoblasts could be identified by the expression of pOBCol3.6GFP, late polarizing odontoblasts by the expression of pOBCol2.3GFP and functional odontoblasts secreting pre-dentin by the expression of DMP1-GFP transgenes (Balic *et al.* 2010, Balic & Mina 2011).

Together these previous studies showed that pOBCol3.6GFPtpz animal provide a unique model to examine odontoblast differentiation from a progenitor population. Therefore, in the present study we have used the pOBCol3.6GFPtpz animal to gain insight into the sequence of events during reparative dentinogenesis.

Material and methods

Transgenic mice

All animal procedures performed in this study were in accordance with Animal Care Committee (ACC) guidelines from University of Connecticut Health Center (UCHC). The pOBCol3.6GFPtpz mouse is a transgenic reporter animal in which green fluorescent protein (GFP) expression is under control of the rat 3.6-kb collagen type I promoter fragments (Kalajzic *et al*. 2002a). The Col1a1-3.6-GFP (referred as 3.6-GFP) fragment is expressed in low but detectable levels in young/functional odontoblast and in high levels in fully differentiated odontoblasts as well as osteoblast and osteocytes (Mina & Braut 2004). Col1a1-3.6-GFP is expressed at high levels in the entire layer of fully differentiated odontoblasts secreting dentin and pre-dentin and extends to the odontoblast processes. Therefore pOBCol3.6GFPtpz transgenic reporter mouse is a powerful model to study odontoblast-like cells differentiation and pulp tissue repairing mechanism.

Pulp exposure

To stimulate the reparative dentinogenesis, dental pulp exposure was performed in the maxillary first right molar of thirty pOBCol3.6GFPtpz mice (12 to 16 weeks old) in CD1 and C57/Bl6 background as described by Simon *et al.* (2008), with some modification. Mice were anesthetized and a cavity was drilled with a carbide burr (diameter 0.04 mm) on the occlusal surface of the molar (class I cavity), in the mesial half of the crown, centered 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 of the tip diameter with a 2% taper, this approach enabled to control the pulp exposure size to approximately 150 μm (size of the tip of the file) (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. Mineral trioxide aggregate was placed in contact with the pulp using a probe tip, and condensed gently with a sterile paper point # 35 (Dentsply Maillefer, Ballaigues, Switzerland). Subsequently, the cavity was sealed with light-cured composite resin (Z100® 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 MTA or without capping material immediately analyzed after exposure.

Tissue isolation and analysis

To analyze 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 analyzed 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 hours. Samples were then decalcified for 7 days in 15% EDTA, 0,5% of formalin (pH 7.5) at 4ºC.

Serial cross sections of 7µm were placed onto ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA, USA) deparaffinized 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 Col3.6GFPtpz (referred to as 3.6-GFP) signal and in GFPmcherry filter to avoid the detection of any GFP auto fluorescence. The images were overlaid to eliminate tissue auto fluorescence using user-defined computation program AxioVision Rel 4.7 software (Carl Zeiss, Thornwood, NY). After epifluorescence analysis the same or adjacent sections were washed in PBS,

processed for hematoxylin and eosin (H&E) staining using standard protocols and analyzed 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 dentin and odontoblasts at the pulp exposure site in transgenic animals in both CD1 (Fig. 2a, b) and C57/Bl6 background (data not shown). Epifluorescence analysis showed the expression of 3.6-GFP transgene in odontoblast layer covering the dental pulp except at the injury site (Fig. 2b) confirming the destruction of the odontoblasts at this location. Histological and epifluorescence analysis of pulps capped with MTA or AS, 7 days after pulp exposure showed no evidence of the formation of organized dentin matrix or reparative dentinogenesis in transgenic animals in both CD1 (Fig. 2c-f) and C57/Bl6 background (data not shown). However, in transgenic animals in CD1 background (but not in C57/Bl6) the exposure site underneath the MTA and AS contained cells expressing high and low levels of 3.6-GFP (Fig. 2d, f). The number of 3.6-GFP expressing cells in teeth capped with AS (Fig. 2f) were lower than those in teeth capped with MTA (observatory anlysis) (Fig. 2d). At both time points small pieces of dentin (referred to dentin chips) were frequently observed in the dental pulp (Fig. 2a-f). These chips consisted of tubular dentin matrix surrounded by numerous odontoblasts expressing high levels of 3.6-GFP transgene (Fig. 2a-f). In transgenic mice in C57/Bl6 background there was no cell expressing 3.6-GFP under the exposure site (data not shown) until 7 days after injury.

Analysis of reparative dentinogenesis 4 and 8 weeks after pulp exposure

Histological analysis 4 weeks after injury showed a clear evidence of reparative dentinogenesis in pulps capped with MTA in transgenic animals in CD1 (but not 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 osteodentin, extending to the center of the pulp (Fig. 3a). This matrix was lined with cells expressing 3.6-GFP and contained 3.6-GFP expressing cells embedded within the matrix (Fig. 3b). The second pattern of reparative dentinogenesis was the formation of a well-defined dentin bridge that appears to seal the exposure site (Fig. 3c). This dentin bridge contained atubular matrix lined with cells expressing high levels of 3.6-GFP but did not contain significant number of 3.6-GFP expressing cells embedded within the matrix (Fig. 3d). After 4 weeks dentin chips although still present in the pulp were devoid of cells expressing 3.6-GFP transgene (Fig. 3b, d).

Histological and epifluorescence analysis of samples capped with AS, after 4 weeks of pulp exposure, in transgenic animals in CD1 background showed no evidence of reparative dentinogenesis (dentin bridge or osteodentin) (Fig. 3e). However, in these animals there was a thickened layer of dentin indicating extensive reactionary dentinogenesis over a new globular calcified dentin (Fig. 3e, f). This new reactionary dentin was lined with odontoblasts expressing high levels of 3.6-GFP transgene (Fig. 3F). Transgenic animals in C57/Bl6 background capped with MTA four weeks after exposure showed no evident osteodentin or dentin bridge. In these animals there were islands of focal dentin matrix lined by a few cells expressing high levels of 3.6-GFP signal (Fig. 4a, b). In transgenic animals in C57/Bl6 background capped with AS, there was no evidence of reparative dentinogenesis or islands of dentin matrix deposition (data not shown).

After 8 weeks of pulp exposure histological and epifluorescence analysis of pulp from transgenic animals in C57/Bl6 background capped with MTA showed the formation of an extensive atubular matrix, resembling osteodentin (Fig. 4c). This matrix also extended from the injury site to the center of the pulp obliterating

the exposure as well as the pulp chamber (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). At 8 weeks after exposure in animals capped with AS, there was evidence of reactionary dentinogenesis characterized by thickened dentin secreted by pre-existing odontoblasts expressing 3.6-GFP (Fig. 4e, f). There also were regions of matrices resembling reparative osteodentin under the exposure site, lined with new secretory cells expressing 3.6-GFP (Fig. 4e, f). The extent and frequency of reparative dentinogenesis in teeth treated with AS was significantly lower than that in teeth treated with MTA in both backgrounds.

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 originating from dental pulp cells secrete reparative dentin at the injury site (Trowbridge 1981, Langeland 1987, Smith *et al.* 1995, Tziafas *et al.* 2000). Although the formation of reparative dentinogenesis is well studied, the origin of the cells giving rise to odontoblast-like cells and cellular and molecular events leading to this reparative process are still not well understood. Previous *in vitro* studies have shown that pOBCol3.6GFPtpz animal provide a unique model to examine odontoblast differentiation from a progenitor population (Balic *et al.* 2010). Therefore, in the present study we have used the pOBCol3.6GFPtpz transgenic mice 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, we used a pulp exposure on maxillary first molars of pOBCol3.6GFPtpz transgenic mice using protocol described before (Simon *et al*. 2008) with some modifications. Utilization of this transgenic animals allowed us to gain a better insight into many aspects of this reparative process

including destruction of odontoblasts after pulp exposure, presence of dentin chips at the healing pulp, the fate of the pre-existing odontoblasts around these chips, recruitment of progenitors into the injury site and their subsequent differentiation.

Our observations showed that the pulp exposure protocol leads 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. Our observations also showed no evidence of dentin bridge formation in pulps capped with MTA or AS in transgenic animals in CD1 and C57/Bl6 background 7 days after pulp exposure that are consistent with previous results (Simon *et al.* 2008). Interestingly our results showed appearance of 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 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) that using a cavity model in rats molar demonstrated that cells committed to odontoblasts lineage were arranged along injury site 2-5 days after the odontoblast death. Based on previous observations (Balic *et al.* 2010), our results show that these cells at the site of injury expressing 3.6-GFP transgene are in early stages of polarization.

The numbers of 3.6-GFP expressing cells in pulps under MTA were higher as compared to AS (observatory analysis). The increased number of 3.6-GFP underneath MTA most likely is related to its characteristics such high pH, calcium release, biocompatibility (Parirokh & Torabinejad 2010), good seal capacity avoiding micro leakages (Barrieshi-Nusair & Hammad 2005) and ability to promote cell proliferation (Paranjpe *et al.* 2010). Previous studies have also shown that MTA up-regulated the expression of *Runx2*, *osteocalcin*, *alkalin*

phosphatase and *DSPP*, indicating the MTA promote the differentiation of pulp cells into odontoblast-like cells (Paranjpe *et al*. 2010).

In the present study reparative dentinogenesis and dentin bridges were observed 4 weeks after pulp exposure in pulps of the transgenic animal in CD1 background and 8 weeks after pulp exposure in pulps of the transgenic animal in C57/Bl6 background capped with MTA. These observations indicated the delayed process of dentine repair and regeneration in C57/Bl6 strains. However, despite the delayed process of reparative dentinogenesis, the reparative response of the pulp in both strains was similar.

We observed two different patterns of reparative dentinogenesis. One was a bulky atubular calcified tissue under the capping material that extended into the center of the pulp resulting in the obliteration of the pulp chamber. In this atubular matrix we detected 3.6-GFP expressing cells surrounding the matrix and embedded in the matrix. These atubular structures with their cellular contents resemble the osteodentin described in the literature (Sloan & Smith 2007, Sloan & Waddington 2009) and 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).

Another 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 dentin 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). Our study showed that the calcified bridge was lined with 3.6-GFP expressing cells. Previous studies have demonstrated that cells lining the dentin bridge under MTA were immunoreactive for Nestin and osteopontin and for dentin sialoprotein (Kuratate *et al.* 2008, Andelin *et al.* 2003) suggesting that they are odontoblasts.

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 secreting dentin- and bone-like tissues difficult. The distinction between these cell type and calcified matrix is an important issue in the light of more recent observations that have provided clear evidence for osteogenic and dentinogenic potential of dental pulp cells (Balic & Mina 2010, Ogawa *et al*. 2006, Takamori *et al.* 2008).

Transgenic animals in both backgrounds capped with AS showed evidence for reactionary dentinogenesis and very limited reparative dentinogenesis. Reactionary dentinogenesis in these teeth consisted of a thickened dentin over a globular calcified dentin narrowing the exposure site. The extensive amount of reactionary dentin in teeth capped with AS is due to short and long term inflammation in the dental pulp caused by the acid primer (Dominguez *et al*. 2003, de Souza Costa *et al.* 2001) that stimulates pre-existing odontoblast to secrete reactionary dentin over the dentin walls.

The reparative dentinogenesis in teeth capped with AS is in agreement with Akimoto *et al.* 1998 that reported new dentin bridge formation directly adjacent to the Clearfil system interface. This limited reparative dentin could be explained by the fact that self-etching AS such Clearfill® 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).

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

Conclusion

The data provided by this study has demonstrated the feasibility of using pOBCol3.6GFPtpz transgenic report mice either in CD1 or C57/Bl6 background in combination with the pulp exposure protocol as a powerful *in vivo* model for analysis of pulp healing process, and biological events of cells differentiation involved in reparative and reactionary dentinogenesis. This opens new opportunities for the use of others transgenic animals in which GFP coding sequences is under the control of tissue-specific regulatory elements (i.e., DSPP) and animals with regulatory elements for progenitor or stem cells markers (i.e., α-SMA) for better understanding of various aspects of tertiary dentinogenesis.

Tables

Table 1. Number of animals analyzed at each time point with different treatments.

Time of analysis after pulp exposure	pOBCol3.6GFPtpz transgenic mice in CD1 background		pOBCol3.6GFPtpz transgenic mice in C57/Bl6 background	
Immediately (no capping)	2		2	
	MTA	**AS**	**MTA**	**AS**
1 week	2	2	2	2
4 weeks	4	2	4	2
8 weeks	-	-	4	2

Figures

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 center of the first molar (M1). (c) Small cavity (arrow) on center of the occlusal surface. (d) Endodontic hand file used to mechanically expose the pulp. (e) Pulp exposure (arrow). (f) Probe used to apply the MTA. (g) Pulp capping with MTA. (h) Cavity restoration with resin composite.

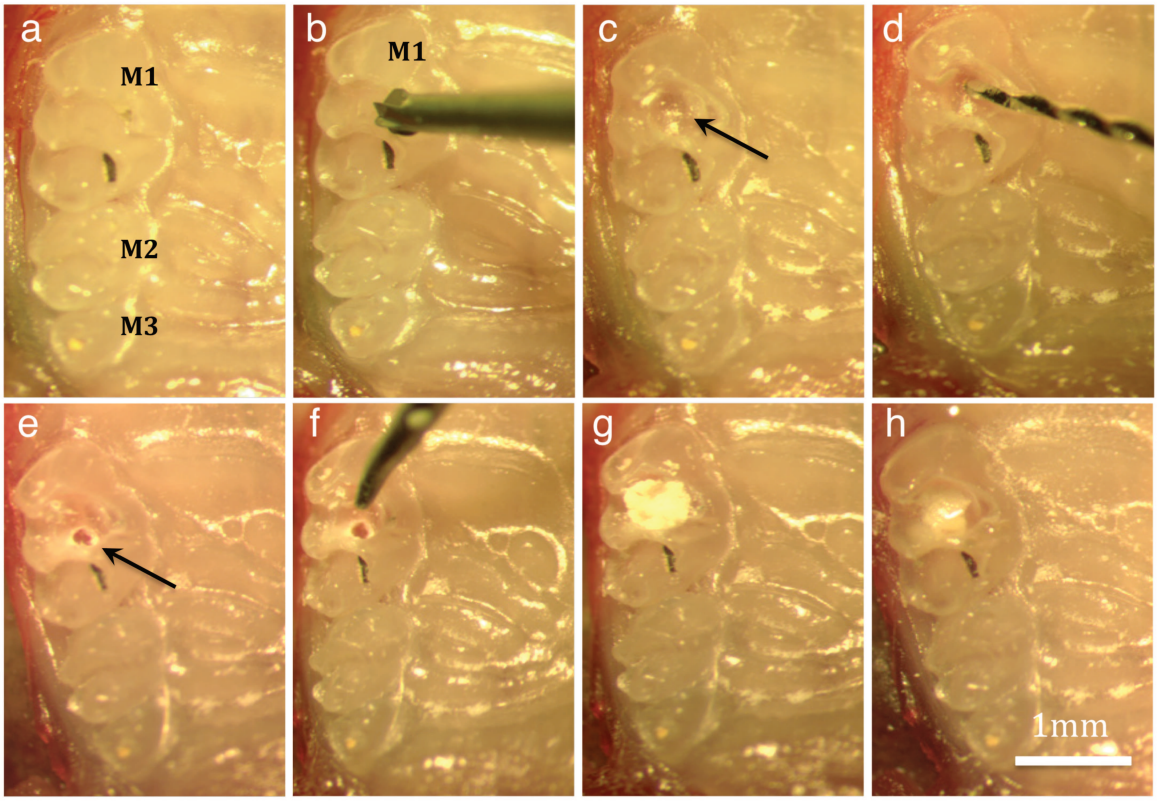


Figure 2. Histological sections stained with H & E (a, c, e) and epifluorescence analyses of adjacent sections (b, d, f) of teeth from pOBCol3.6GFPtpz transgenic mice in CD1 background immediately after pulp exposure (a and b) and 7 days after exposure (c-f). (a and b) show the absence of odontoblasts (indicated by dashed rectangle) and dentin at the exposure site (indicated by stars). Dentin chips in the exposure site are marked by an asterix. Note the expression of 3.6-GFP in the odontoblasts layer around pulp-dentin complex (dashed arrows) and around a dentin 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 MTA. E and F represent images from a tooth capped with 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-dentin. Full arrows mark expression of 3.6-GFP in odontoblast around dentin chips. Abbreviations: d=dentin; p=pulp; (*)= dentin chips; (dashed arrow)= original odontoblasts ★= exposure site. Scale bar= 100µm.

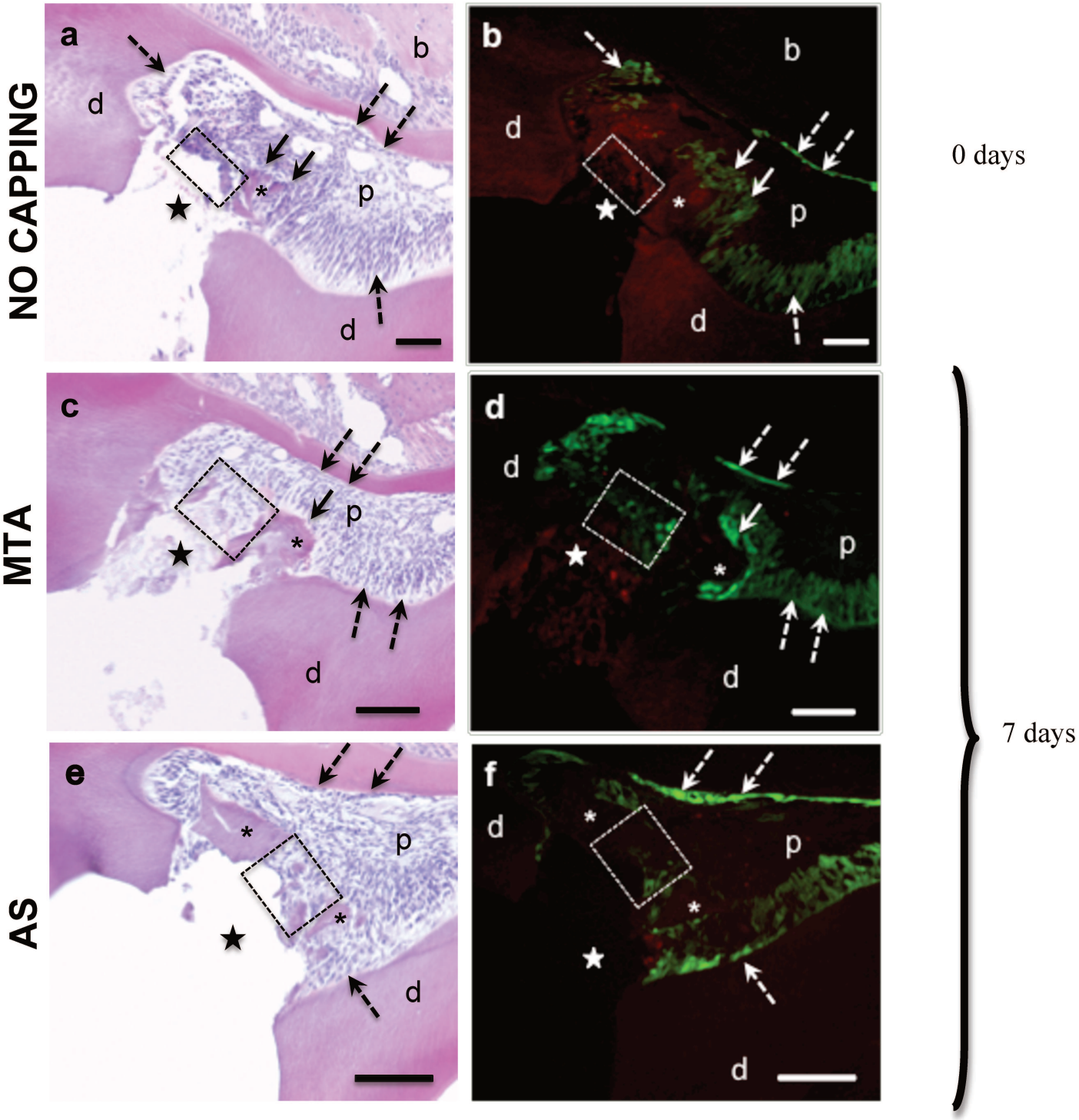

NO CAPPING
MTA
AS
a
b
c
d
e
f
b
d
p
0 days
7 days

Figure 3. Histological sections stained with H & E (a, c, e) and epifluorescence analyses of adjacent sections (b, d, f) of teeth from pOBCol3.6GFPtpz transgenic mice in CD1 background 4 weeks after pulp exposure. (a - d) are representative images of sections from teeth capped with MTA. (e and f) are representative images of adjacent section from a tooth capped with AS. (a and b) show the formation of newly synthesized matrix (outlined with dashed lines) that extends into the pulp at the exposure site. This matrix is atubular and contains 3.6-GFP expressing cells lining its surrounding (indicated by arrowheads) and 3.6-GFP expressing cells embedded in the matrix (indicated by full arrows) resembling osteodentin. (c and d) show the formation of a well-defined dentin bridge (outlined with dashed lines). Note the clear expressing of 3.6-GFP (indicated by arrowheads) in cells lining this bridge. Note the presence of dentin chips (indicated by asterix) in images (a-d), dentin chips at this time point are devoid of 3.6-GFP expressing odontoblast. (e and f) show the thickened layer of reactionary dentin (rd) over a globular calcified dentin (gd) at the borders of the pulp close to the exposure site. Note the high expression of 3.6-GFP in original odontoblasts underneath the reactionary dentine around the pulp (indicated by dashed arrows). Abbreviations: d=dentin; gd=globular calcified dentin, p=pulp; rd=reactionary dentin; (*)= dentin chips; (dashed arrow)= original odontoblasts; ★= exposure site. Scale bar= 100µm.

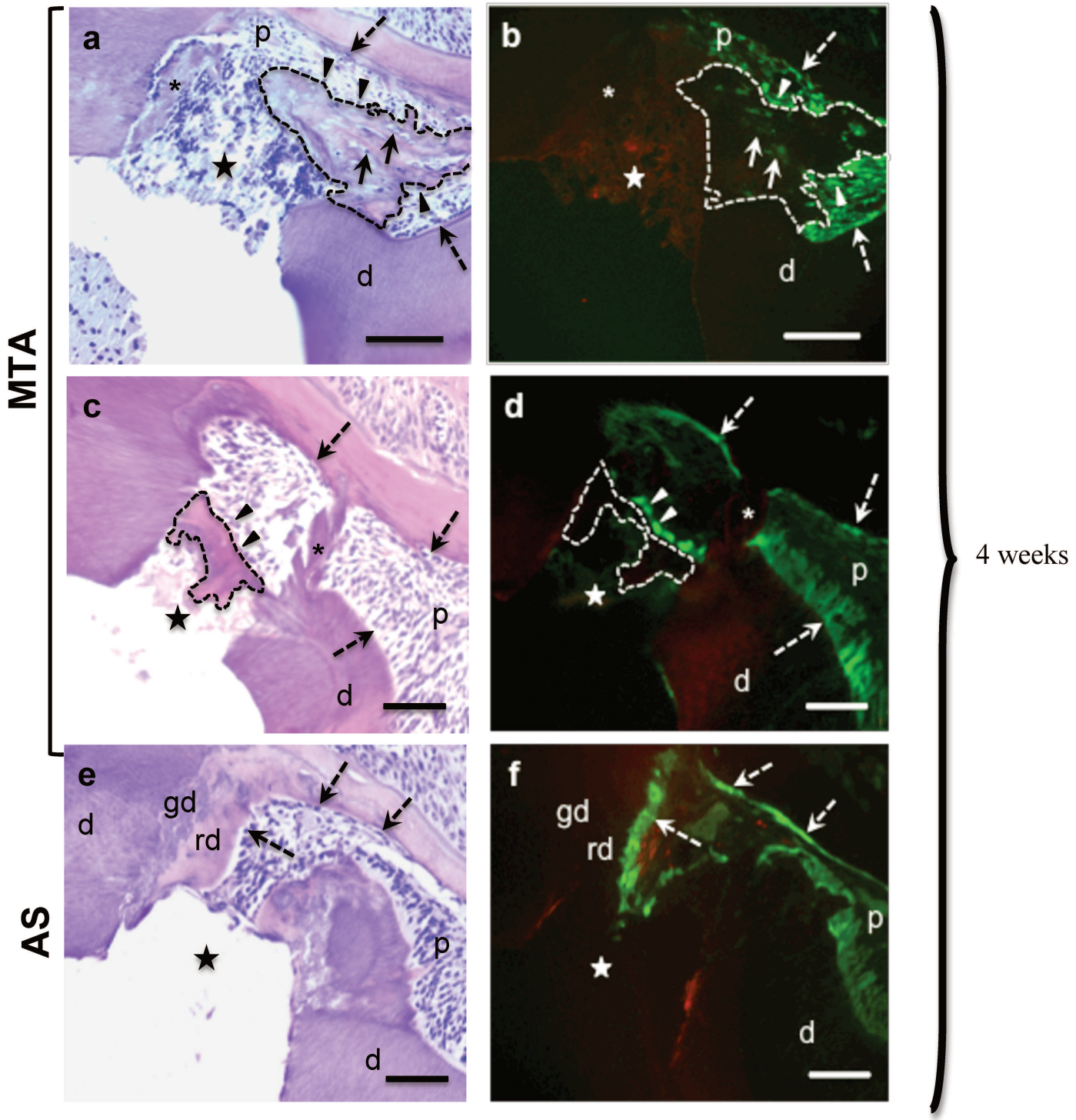
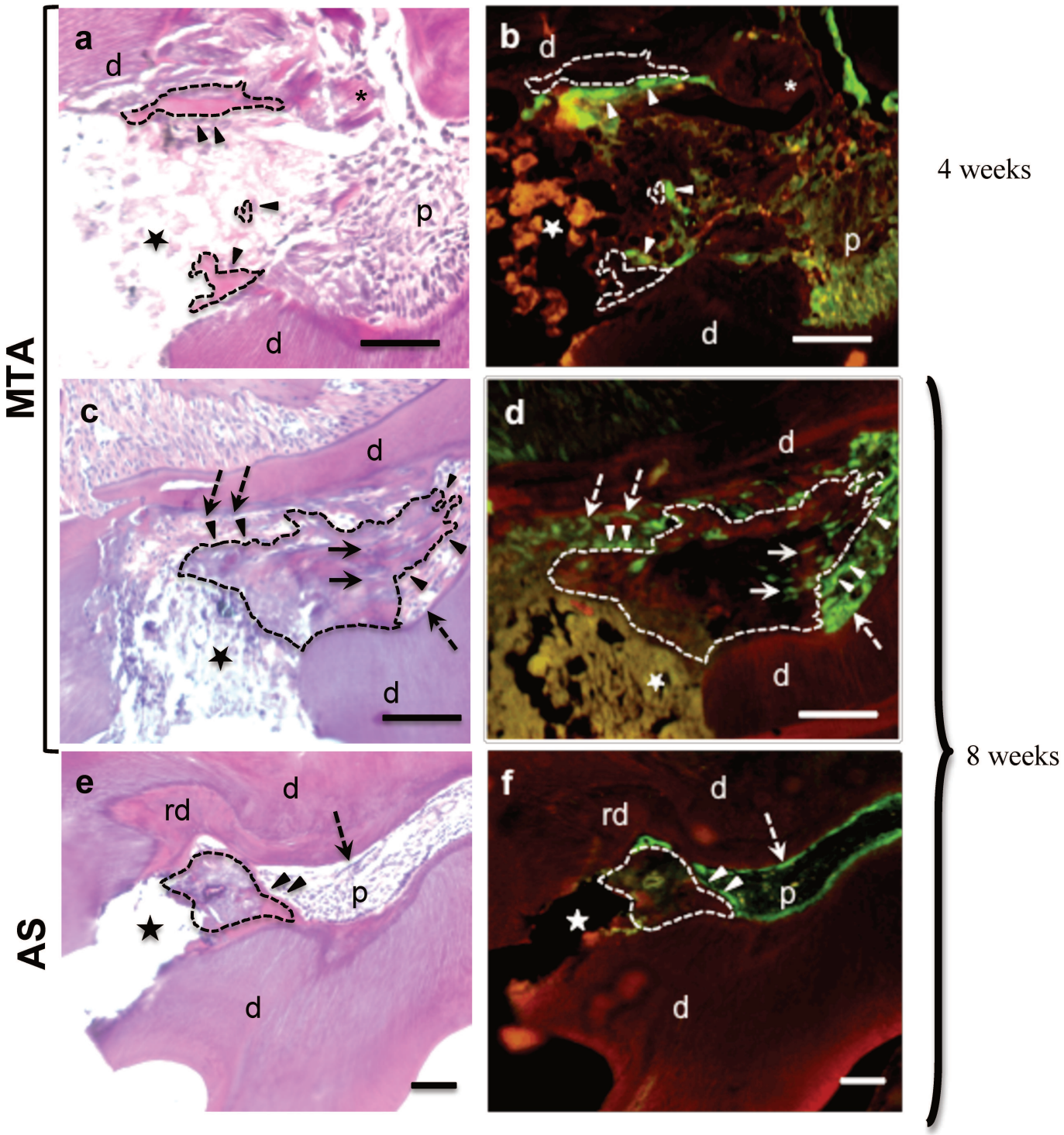

MTA
AS
a
b
c
d
e
f
p
d
gd
rd
4 weeks

Figure 4. Histological sections stained with H&E (a, c, e) and epifluorescence analyses of sections (b, d, f) of teeth from pOBCol3.6GFPtpz transgenic mice in C57/Bl6 background 4 and 8 weeks after pulp exposure. (a and c) are images of a section through a pulp capped with MTA after 4 weeks. Note the presence of focal islands of matrix (outlined with dashed lines) in close contact with the capping material containing a few cells-expressing 3.6-GFP around them (indicated by arrowheads). (c and d) are images from a tooth capped with MTA after 8 weeks. Note the formation of newly synthesized matrix (outlined by dashed lines) that extends into the pulp from the exposure site. This matrix resembling osteodentin is atubular and contains 3.6-GFP expressing cells lining its surrounding (indicated by arrowheads) and 3.6-GFP expressing cells embedded in the matrix (indicated by full arrows). (e and f) are images from a tooth capped with AS after 8 weeks. Note the thickened layer of reactionary dentin (rd) over a calcified dentin at the borders of the pulp close to the exposure site. Note the high expression of 3.6-GFP in original odontoblasts underneath the reactionary dentin and around the pulp (indicated by dashed arrows). Also note the presence of a small osteodentin (outlined by dashed lines). Abbreviations: d=dentin; p=pulp; pd=thickened pre-dentin; (dashed arrow)=original odontoblasts; (*)= dentin chips; ★= exposure site. Scale bar= 100µm.


a
d
*
p
d
b
d
*
p
d
4 weeks
MTA
c
d
d
d
d
d
d
8 weeks
AS
e
rd
d
p
d
f
rd
d
p
d

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Analysis of the Contribuition of Circulating Blood Cells in Reparative Dentinogenesis Using Parabiosis Model in Mice

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Acknowledgements

We thank all individuals who provided valuable input and technical assistance in various aspects of these studies including Dr. Hector Leonardo Aguila, Dr. Ivo Kalajzic, Dr. Tatjana Barisic-Dujmovic, Dr. Christian Jacome-Galarza and Dr. Anamaria Balic. The authors deny any conflicts of interest. This study was supported in part by a grant from AAE Foundation (American Association of Endodontics), by a grant from CAPES (Brazilian Federal Agency for Support and

Evaluation of Graduate Education) (n°. 3422/09-7) and a grant from NIH (National Institute of Health) (n°. DE016689).

Abstract

Introduction: The aim of this study was to analyze the contribution of circulating progenitor cells (CPC) to odontoblast-like cells during reparative dentinogenesis. **Methods:** Parabiosis was established between C57/Bl6-TgN(ACTbEGFP)10sb/J transgenic mice (GFP+) and C57/Bl6 wild type mice (GFP-) to ensure blood cross circulation. Reparative dentinogenesis was stimulated by pulp exposures and capping on the first maxillary molar in the C57/Bl6 wild type. Histological sections of injured molars from C57BL/6 mice were analyzed by epifluorescence microscopy to examine the contributions of circulating CPC-GFP+ cells from C57/Bl6-TgN(ACTbEGFP)10sb/J donor mice to reparative dentinogenesis. **Results:** After 4 and 8 weeks of pulp injury there were GFP+ cells participating in the exposed molar reparative dentinogenesis. **Conclusion:** The present study gives the evidence of the possible contribution of CPC from peripheral blood (PB) in the reparative dentinogenesis after pulp injury.

Key words

Dental pulp, green fluorescence protein, odontoblast-like cells, parabiosis, reparative dentinogenesis.

Introduction

The ability of the dentin–pulp complex to respond to injuries by hard tissue formation has long been recognized (1, 2). Unlike primary or secondary dentins that are formed along the entire pulp–dentin border, tertiary dentin is secreted focally (3, 4). Tertiary dentin is divided into reactionary and reparative dentin (5, 6). Reactionary dentin is secreted by pre-existing odontoblast following a mild injury, (6, 7). Reparative dentin is secreted by newly differentiated odontoblast-like cells after intense injuries that lead to odontoblast death (5, 7,). Reparative dentinogenesis involves a cascade of inflammatory and healing events that occur rapidly following the degeneration of odontoblasts (8, 9) and the formation of a new generation of odontoblast-like cells (10). Stem cells Studies have identified several potential populations of cells in post-natal dental pulp, capable of giving rise to a new generation of odontoblast-like cells during reparative dentinogenesis including cells from the cell-rich layer of Höhl adjacent to the odontoblasts (11, 12), a population of competent progenitor cells, similar of the neural crest derived cells of the dental papilla (13, 14), a unique population of mesenchymal stem cells (MSCs) in postnatal dental pulp, referred to as dental pulp stem cells (DPSC) (15, 16) and cell population residing around blood vessels (17, 18). However, there is still little known of the molecular mechanisms involved in dental healing and, most notably, the origin, of the cell population giving rise to odontoblast-like cells secreting reparative dentin matrix (6, 12, 19, 20).

There are several possible mechanisms that could account for generation of new cells during regeneration. One possibility is that the regeneration of the lost cell type is regulated by resident stem cells also referred to as tissue-specific stem /progenitors cells residing in the specific niches in a given organ (21). A second possibility is the involvement of the circulating precursors, perhaps of the mesenchymal or hematopoietic lineages in this process. In this possibility cell

from circulation, enter damaged tissues and differentiate into specific cell type that has been lost (21, 22).

The present study was designed to examine the contributions of the circulating progenitor cells (CPC) to odontoblast-like cells differentiation during reparative dentinogenesis. To do so, we created parabiotic pairs of a genetically marked mouse in which the GFP expression is under the control of chicken ß-actin promoter (one partner) (23) and a wild type mouse (other partner) this parabiotic couple shared anastomosed circulatory system (24). Mice were left parabiosed for 2 weeks for the cross circulation establishment, and then pulp exposures were created in the maxillary first right molar of the GFP- to stimulate reparative dentinogenesis.

Material end Methods

Transgenic mice

All animal procedure performed in this study were in accordance with Animal Care Committee (ACC) guidelines from University of Connecticut Health Center (UCHC). Wild type C57/Bl6 (referred as GFP-) and C57/Bl6-TgN(ACTbEGFP)10sb/J transgenic mice (referred as GFP+) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). In GFP+ transgenic mice the Enhanced Green Florescent Protein (EGFP) is under the control of a chicken ß-actin promoter and a cytomegalovirus (CMV) enhancer and therefore the GFP is expressed uniformly in all cells except in erythrocytes.

Parabiosis

Parabiosis, establishes a common blood circulation by surgical pairing two individuals of parabionts. 12 to 16 weeks animals were paired based on the technique described before (25) (Fig. 1). Briefly, animals with the average weight of 20g were anesthetized with 100-mg/kg of ketamine and 10-mg/kg of xylazine.

The surgical site was shaved and sterilized with 5% povidine-iodine solution. Incisions were made from the hip to the angle of the mandible, and the skin was freed of attached tissue. The aligned mice were sutured with discontinuous stitches by using absorbable silk suture material and joined with 9-mm wound clips at the dorsal side. Buprenorphine hydrochloride (0.2 –0.5 mg/kg) was administered daily for the first 2 days after surgery. The wound clips were removed after 2 week.

Pulp exposure

To stimulate the reparative dentinogenesis, 2 weeks after parabiosis, dental pulps of the maxillary first molars of the GFP- mice were exposed as described before (26, 27) (Fig. 1). Mice were anesthetized and a cavity was prepared with a carbide burr (diameter 0.04 mm) on the mesial half of the occlusal surface of the molar until the pulp was visible through the cavity floor A pulp exposure was subsequently created mechanically using an endodontic hand file with 0.15 mm of the tip diameter with a 2% taper, this approach enabled to control the pulp exposure size to approximately 150 μm (tip size of the file). Exposed pulps were capped using mineral trioxide aggregate (MTA Angelus®; Angelus S/A, Paraná, Brazil) mixed with sterile water following the manufacturer's recommendations. Mineral trioxide aggregate were placed in contact with the pulp using a probe tip, and condensed gently with a sterile paper point # 35 (Dentsply Maillefer, Ballaigues, Switzerland). Subsequently, the cavity was sealed with light-cured composite resin (Z100® 3M, São Paulo, Brazil), associated with a two-step adhesive system (AS) (Clearfill® SE Bond, Kuraray, Okayama, Japan) (27)

Flow Cytometry

To verify cross circulation between the parabionts, blood was collected from tails of the GFP- mice, 2 and 10 week after pairing (Fig. 1) and analyzed by flow cytometry (FACS) as described before (25). Blood was collected into FACS tubes

with 500μl of 8μM EDTA in PBS. After collection, 2ml of Dextran (2%) was added; blood was sedimented, the supernatant discharged and remaining red blood cells lysed with 0,5 ml of ammonium chloride (ACK lysing buffer, Invitrogen™, Grand Island, NY, USA). Cells were then washed in 500μl of staining medium, resuspended in 500μl of staining medium containing 5 μg/mL of propidium iodide (PI) and stained with CD45-APCcyan (1:100) antibody for 30 minutes at 4°C. Between 100,000 to 300,000 cells were used for analysis using a BD-LSR II multicolor analyzer (Becton-Dickinson, San Jose, CA, USA). Data were processed using FlowJo 7.6 software (Tree Star Inc, Ashland, OR, USA) in the institutional Flow Cytometry Facility at UCHC.

Tissue isolation and analysis

To analyze the sequence of reparative dentinogenesis and the contribution of CPC-GFP+ in this process, 4-8 weeks after pulp exposures (Fig. 1) animals were euthanized by intracardiac perfusion with 10% of buffered formalin solution. After perfusion, maxillary arches were isolated, cleaned from soft tissue, trimmed, and fixed in 10% formalin solution for additional 24 hours. Samples were decalcified for 7 days in 15% EDTA plus 0,5% of formalin (pH 7.5) at 4ºC. Serial cross sections of 7µm were placed onto ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA, USA) deparaffinized and processed for epifluorescence analysis. To visualize GFP signal deparaffinized sections were mounted with glycerol/PBS (50%: 50%). The fluorescence signal 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 using GFPtopaz and GFPmcherry filters. The images were overlaid to eliminate tissue auto fluorescence using user-defined computation program AxioVision Rel 4.7 software (Carl Zeiss, Thornwood, NY). After epifluorescence analysis the same sections were washed in PBS, processed for hematoxylin and eosin (H&E) staining using standard protocols and analyzed by light microscopy.

TRAP Staining.

Odontoclast/osteoclasts were identified using a tartrate-resistant acid phosphatase (TRAP) staining using naphtol AS BI phosphate method as described before (28). Sections were incubated in the staining solution for 1h at 37°C, washed with water, and then stained with hematoxylin.

Results

GFP expression in transgenic mice

Histological and epifluorescence analysis of teeth from GFP+ mice showed a high levels of GFP expression in the dental pulp cells and in the entire layer of a fully differentiated odontoblasts and odontoblastic process (Fig. 2A-C) confirming the uniform expression of GFP in all cells including odontoblasts.

Analysis of the contribution of circulating progenitor cells to the reparative dentinogenesis

Parabiotic surgery proved to be a very traumatic procedure and a high rate of mortality occurred between 3 to 5 days after parabiosis. Parabiotic surgeries were performed in 20 couples of GFP+ and GFP- animals and analysis was performed in the 6 parabiont partners that survived. Flow cytometry analysis of peripheral blood (PB) from the wild-type recipient parabiont showed the presence of approximately 54.3% and 59.8% of GFP+ cells after 2 (n=4) and 10 weeks (n=2) of parabiosis respectively (Table 1). A High percentage of the donor-derived GFP+ cells in the circulatory system of the wild type recipient mice expressed the hematopoietic antigen CD45 (Table 2) conformining a full blood chimerism (Table 1).

Epifluorescence analysis of the section through maxillary molars of the recipient partner 4 weeks after pulp exposure (6 weeks after parabiosis) showed

donor-derived GFP+ cells within the pulp and in the surrounding alveolar bones (Fig. 3). In the tooth there was an influx of GFP+ cells in the recipient animals through the apical foramen (Fig. 3).

Histological analysis of the first maxillary molars of the recipient partner 4 weeks after the pulp exposure showed no evidence of calcified dentin bridge formation. However there were dentin chips at the periphery of the exposure site (Fig. 4A). Epifluorescence analysis of the same section showed the presence of scattered GFP+ cells in the pulp tissue. A few larger GFP+ cells were associated with dentin chips (Figure 4B, C).

A clear evidence of a new matrix resembling reparative dentin at the exposure site was observed in histological analysis 8 weeks after injury (10 weeks after parabiosis) (Fig. 4D). This matrix extended into the pulp, appearing atubular (Fig. 4D, G). This matrix was also surrounded by a thin layer of less calcified matrix resembling pre-dentine (Fig. 4D, G). Interestingly, epifluorescence analysis showed a large number of GFP+ cells around and in close vicinity of the new matrix (Fig. 4E, H). Since the GFP+ cells associated with the new matrix were large the possibility that they cells represent osteoclasts/odontoclasts was examined. TRAP staining showed a very few if any osteoclast/odontoclast activity in the GFP+ cells around the new matrix formation (Fig. 5A-C). As a control for TRAP staining was used the alveolar bone of the same sections of the exposed molars. In the control TRAP staining showed intense TRAP activity in GFP+ cell around the bone marrow lacunae (Fig. 5D-F).

Discussion

In the present study to address the possibility of a circulatory cells contribution to the regenerating of the dentin/pulp complex, we created pairs of genetically marked mice and wild type mice that began to share anastomosed circulatory system. Reparative dentinogenesis was stimulated by pulp exposures on the

maxillary molars of the wild type mice. Parabiosis using a GFP+ parabiont has been used to investigate various contributions of circulatory cells in dermal fibroblast/myofibroblast progenitors (25), non-bone marrow progenitors (29), endothelial progenitors (30) and bone marrow stem cells (31).

Our recent studies using pulp exposure in transgenic mice showed that during reparative dentinogenesis cells underneath the exposure site and in contact with capping materials activated 3.6-GFP transgene and participate in the secretion of osteodentin that was lined with odontoblast-like cells expressing high levels of 3.6-GFP and contained cells expressing 3.6-GFP transgene embedded within the matrix. However, in this studies we could not identify the origin of cells in which 3.6-GFP was activated (27).

The parabiosis model and pulp exposure allowed us to investigate the possible contribution of circulatory cells primarily hematopoietic cells from donor in reparative dentinogenesis in the recipient wild type partner. Our observation showed that a blood chimerism between two parabionts was established by 2 weeks and maintained up to 10 weeks, which is in agreement with previous observations (24, 32). FACS analyses also showed that the majority of the GFP+ cells were of hematopoietic origin.

Our observations showed the absence of the formation of dentin bridge by 4 weeks after pulp exposure that is consistent with previous observations (26, 27). Epifluorescence analysis of the same sections showed an influx of GFP+ through the apical foramen towards the injured pulp and the presence of a few GFP+ cells scattered in the pulp chamber. Some of them were large cells with elongated cytoplasm closely associated with dentin chips, suggesting an odontoclastic action. Odontoclasts are mononucleated or multinucleated cells that are mainly involved in the resorption of dental hard tissues, which presumably originate from circulating hematopoietic progenitor cells, that arrive in the pulp chamber via blood circulation (33).

Analysis of sections 8 weeks after pulp exposure showed a clear evidence of a new matrix deposition in agreement with a previous study (27). A pre-dentin-

like tissue surrounded this new fibrous matrix. There were numerous GFP+ cells in close contact with the newly synthesized matrix. TRAP staining, considered to be a specific histochemical marker for odontoclast/osteoclasts differentiation (33, 34) was detected only in a very few GFP+ cells lining this matrix excluding the possibility that those cells were odontoclast/osteoclasts. GFP+ cells around the new matrix could represent cells within the mononuclear phagocyte system that includes the pre-monocytes and their precursors, the monocytes, and the macrophages derived from peripheral blood (35) in the tissues. However these cells are expected to be present in the dental pulp predominantly during pulpits with intense inflammatory infiltrate (36) and in the initials steps of healing pulp process (37).

It also remained possible that GFP+ cells around the new matrix are odontoblast-like cells with a secretory activity. Since there is evidence indicating that cells of hematopoietic origin do not contribute to the regeneration of the cells giving rise to skeletal tissue (38), the GFP+ cells in the present study might have originated from mesenchymal stem cells (MSC) derived from PB. Although the amount of MSC obtained from PB is lower as 1.2 to 13 per million of mononuclear cells (39), it has been demonstrated that allogenic PB-MSC enhance bone regeneration in the rabbit bone defects (40) and contribute to the formation of bone-like tissue into the dental pulp, suggesting PB-MSC could differentiate in odontoblast-like cells and to produce mineralized tissue (41).

It is consensual that MSC do not express CD45 (42, 43). Our FACS analyses sowed 96.5% of GFP+ cells; in the peripheral blood of the GFP- mice were CD45+ indicating that the small fraction of the CD45- (3.5%) population is the potential progenitor for odontoblast-like cells around the newly synthesized matrix. However, further immunocytochemical experiments with DSPP antibody are necessary to verify the GFP+ phenotype around the new matrix as odontoblasts.

Tables

Table 1. Percentage of the expression of GFP and CD45 in the peripheral blood of the wild type recipient mice determined by FACS at different time points after parabiosis. Values represent means ± SE from at least 2-4 individual experiments.

Weeks after parabiosis	% GFP -	% GFP +	% CD45 -	% CD45 +
4 (n=4)	45.7 ± 2.1	54.3 ± 4.1	3.5 ± 0.8	96.5 ± 0.8
8 (n=2)	40.2 ± 3.0	59.8 ± 2.1	4.3 ± 0.8	95.8 ± 0.8

Figures

Figure 1. Schematic figure showing the outline of the experiments performed in the present study. Two weeks after parabiosis between the GFP+ (green mice) and GFP- (black mice), blood sample were collected from the tails of the recipient (GFP- black mice) for FACS analysis and a pulp exposure was performed in the first maxillary molar of recipient mice to stimulate reparative dentinogenesis. Four and 8 weeks after the pulp exposure, the couples were separated; blood samples were collected from the GFP- mice to analyze long term of cross circulation. At these time points GFP- mice were sacrificed and processed for various analyses.

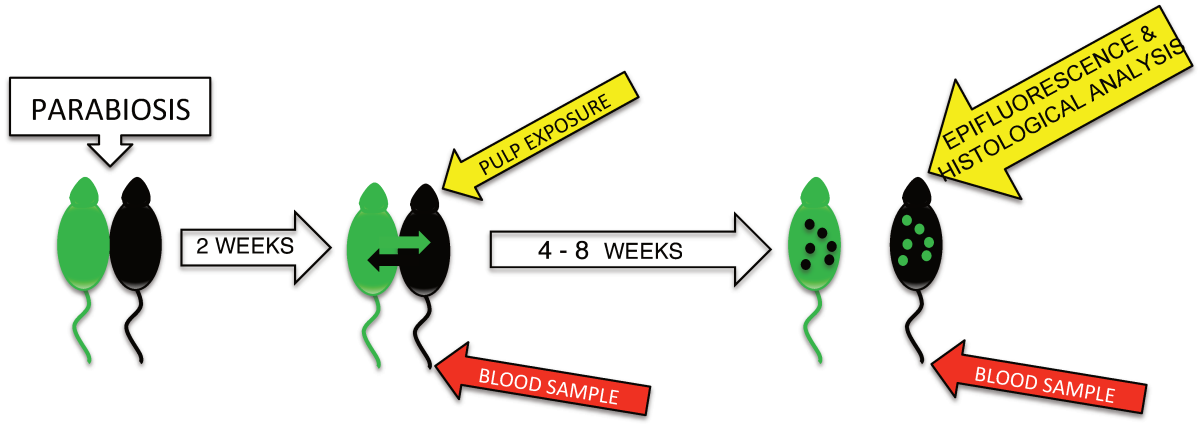


Figure 2. Images of sections through the non-exposed first maxillary molar of a C57/Bl6-TgN(ACTbEGFP)10sb/J transgenic (GFP+ mice). (A) Intact odontoblast layer (dashed arrow) and pulp cells (full arrow). (B) Expression of GFP in intact odontoblast layer (dashed arrows) and pulp cells (full arrows). (C) Overlay of both images, A and B. Abbreviations d=dentin; p=pulp. Scale bars= 100µm.

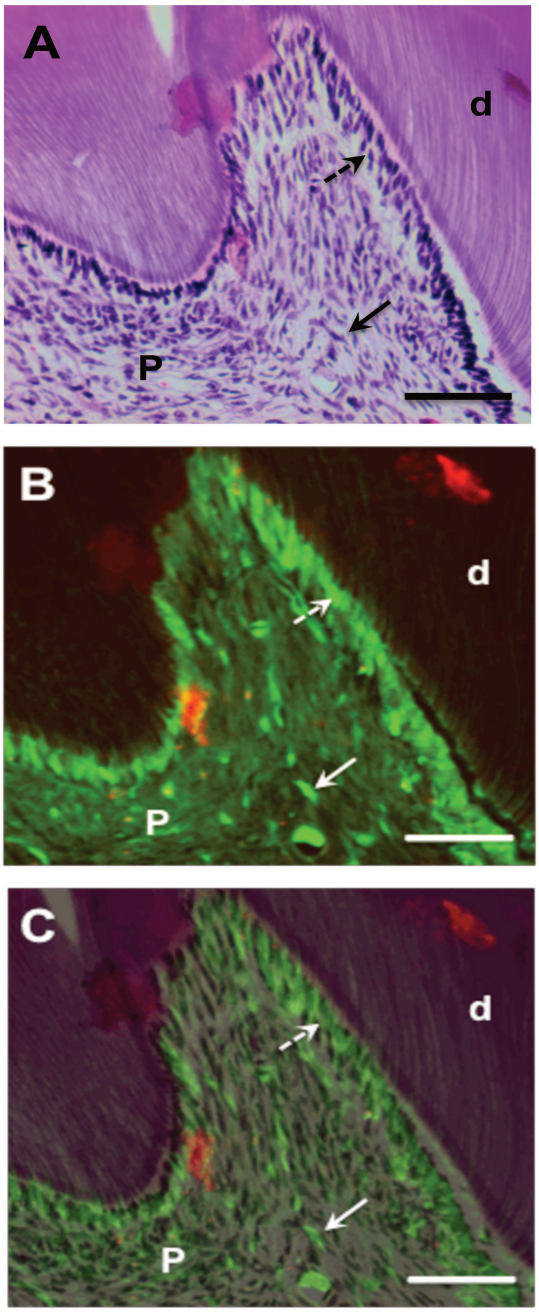


Figure 3. Images of sections through the upper first molar from the GFP- mice, 4 weeks after pulp exposure. Note the GFP+ cells penetrating trough the apical foramen (full arrows) and in the dental pulp and in the surrounding alveolar bone. Abbreviations d=dentin; p=pulp; b=alveolar bone; ★= pulp exposure site. Scale bars= 100µm.

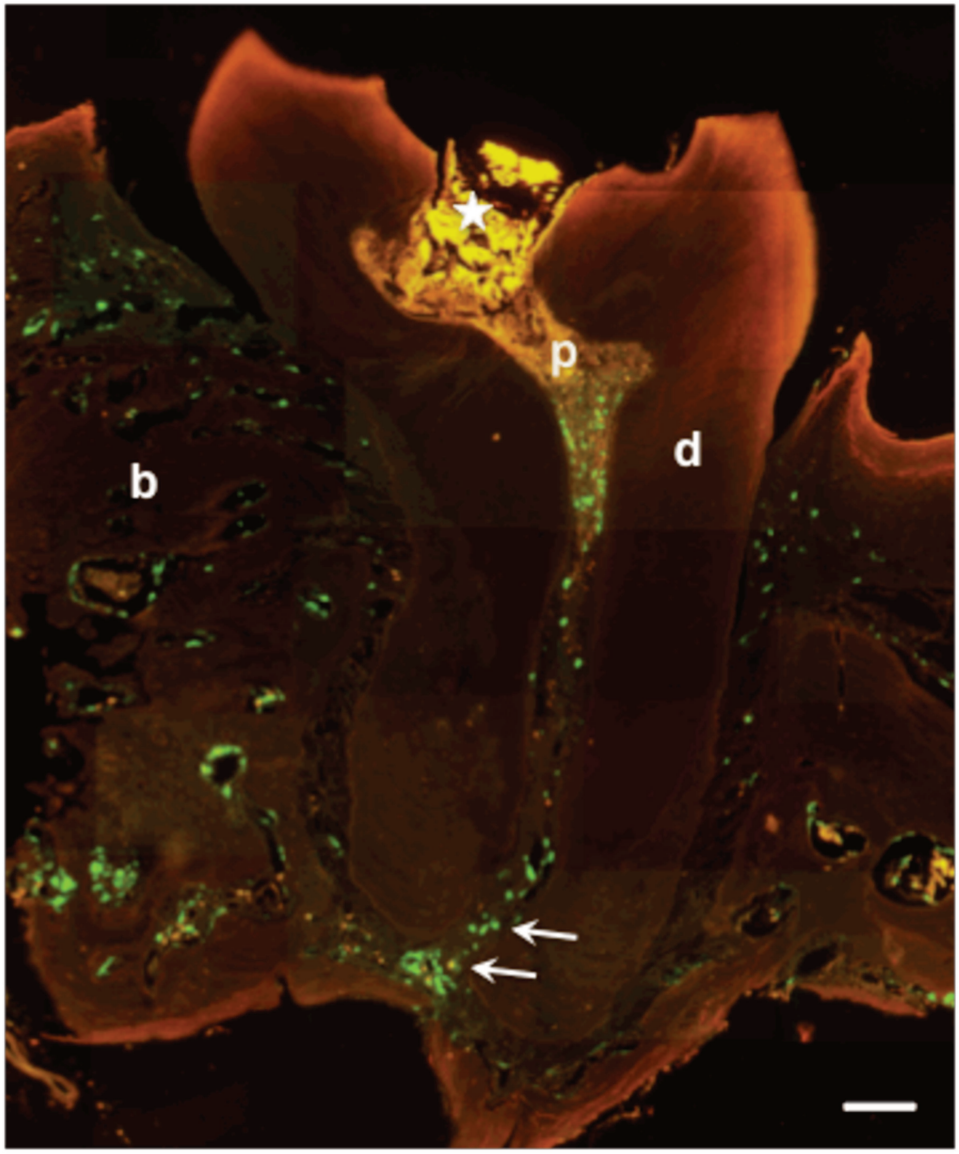
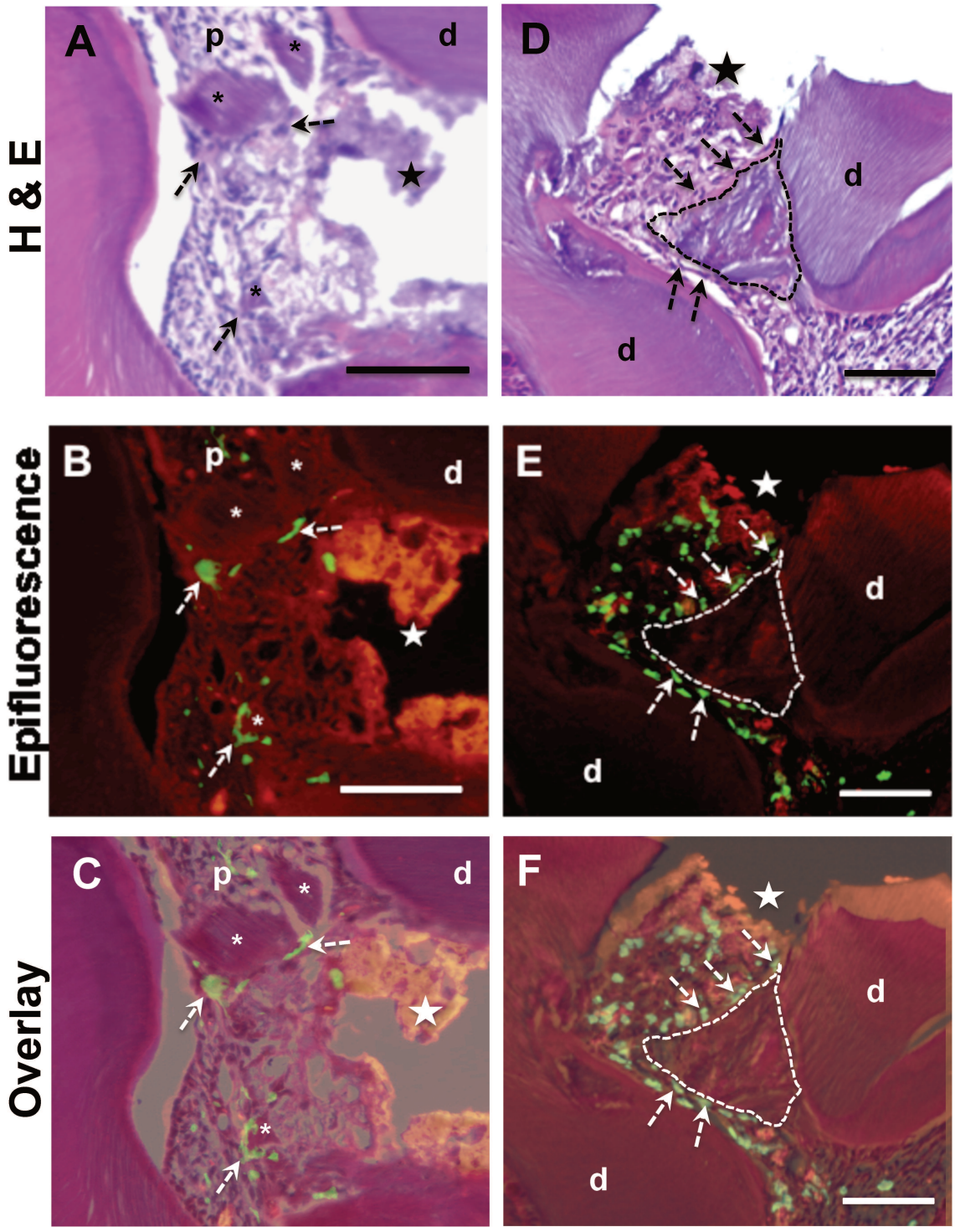


Figure 4. Images of sections through the exposed first maxillary molar of a C57/Bl6 wild type recipient mice (GFP- mice). (D-C) Images of sections after 4 weeks of pulp exposure. (A) Note no evidence of dentin bridge formation and the presence of dentin chips (*) at the periphery of the exposure site surrounded by elongated cells (dashed arrows). (B) Note the expression of GFP in the cells in the pulp and around the dentin chips (indicated by dashed arrows). (C) Overlay of both images, A and B. (D-F) Images of sections after 8 weeks of pulp exposure. (D) Note the formation of reparative dentin (indicated by dashed lines) underneath the exposure site that extends into the pulp surrounded by cells (dashed arrows). (E) Note the GFP+ expressing cells in the dental pulp and in some cells around the reparative matrix (dashed arrows). (F) Overlay of both images, D and E. (G, H) Higher magnification of D and E. (G) A pre-dentin-like tissue surrounded the reparative dentin (arrowheads). (H) Note the GFP+ expression in some cells (dashed arrows) in close contact with the pre-dentin-like tissue (arrowheads) lining the reparative matrix. Abbreviations d=dentin; p=pulp; ★= pulp exposure site. Scale bars= 100μm.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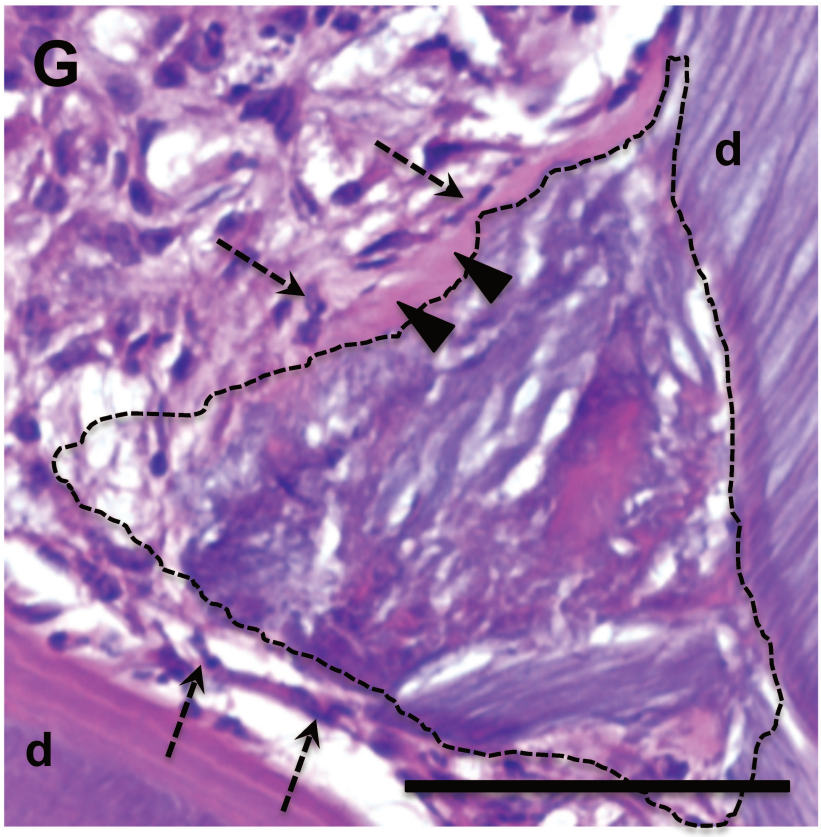
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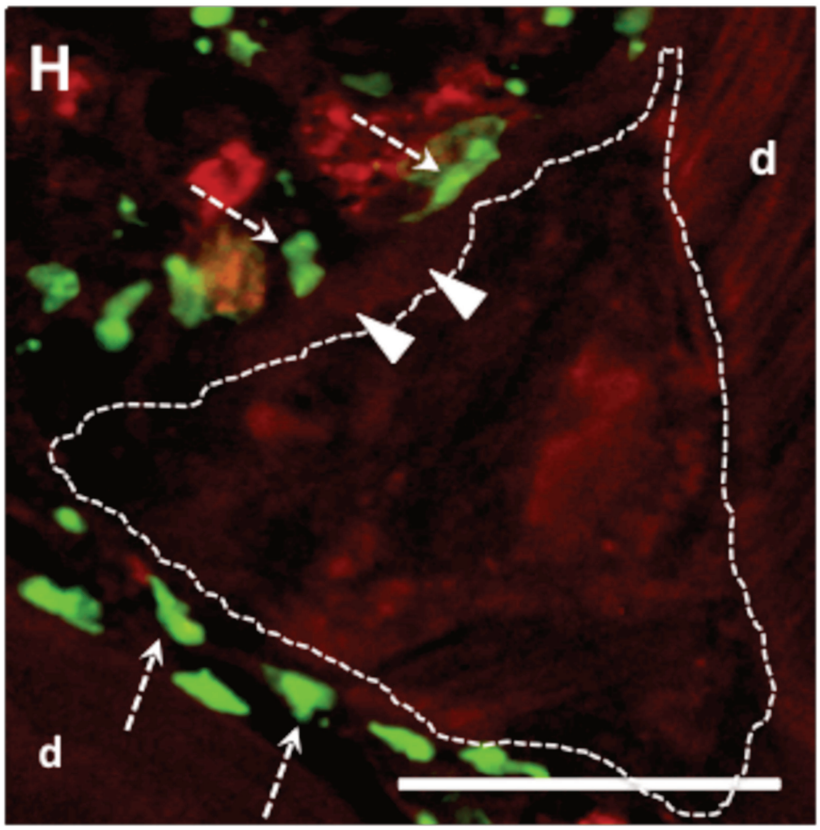
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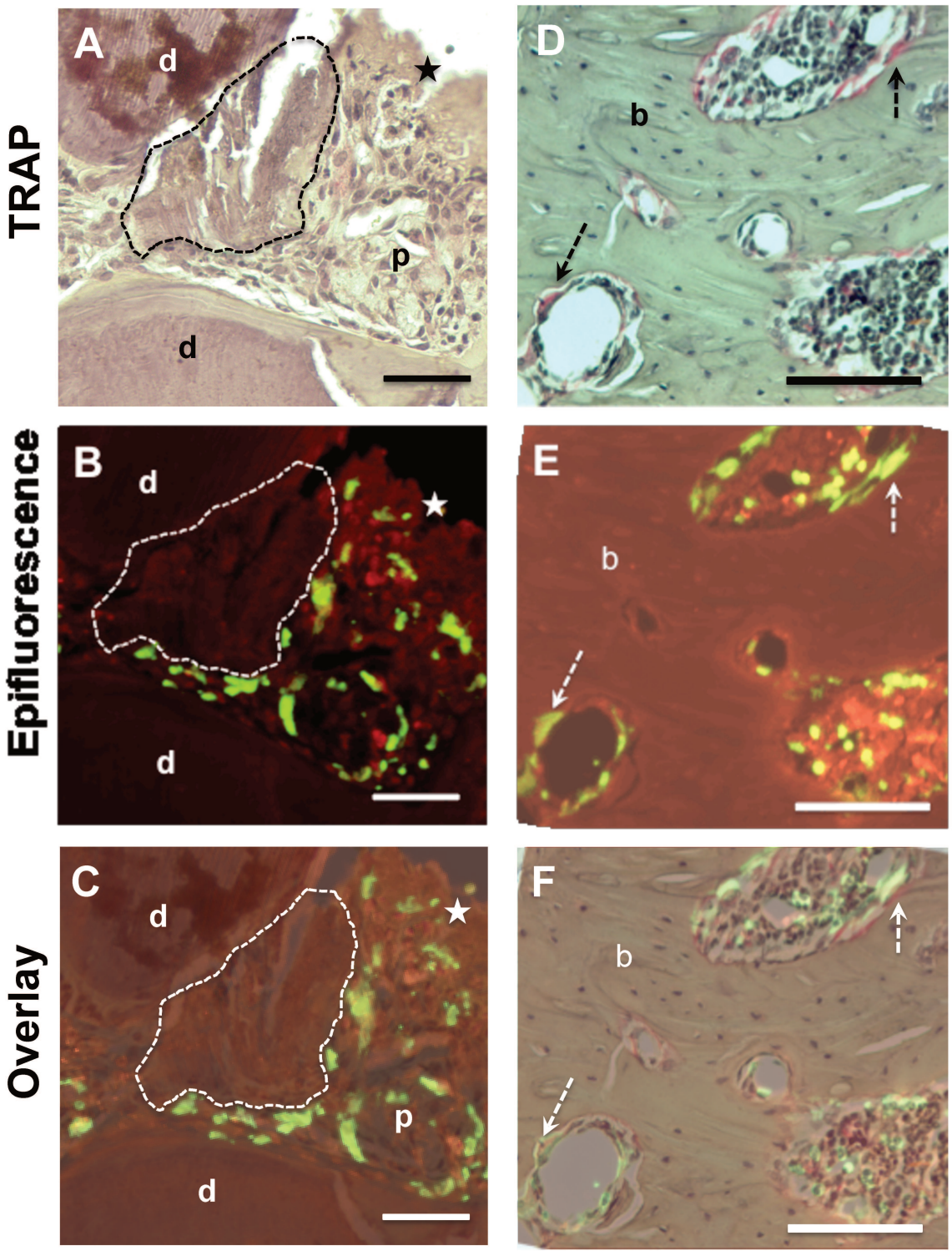
Figure 5. (A-C) Images of sections through the upper first molar from the GFP- mice 8 weeks after pulp exposure. The reparative matrix at the exposure site is indicated by dashed line. (A) Image of a section stained for TRAP and counter stained with hematoxylin. Note no TRAP activity was detected in the pulp cells. (B) Image of the same section analyzed by epifluorescence to visualize the GFP+ cells. Note the GFP+ expressing cells around the reparative dentin. (C) Overlay of both images A and B. (D-F) Alveolar bones of the same section in A and B used as a control for TRAP activity. (D) Image of a section stained for TRAP and counter stained with hematoxylin. Note TRAP activity (in red) in most cells within the bone marrow lacunae (dashed arrows). (E) Epifluorescence analysis of the same section in D. Note cells expressing GFP+ within the bone marrow lacunae (dashed arrows). (F) Overlay of both images, D and E. Note majority of cells expressing GFP+ are also stained for TRAP (dashed arrows). Abbreviations d=dentin; p=pulp; b= alveolar bone; ★= pulp exposure site. Scale bars= 100μm.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CONSIDERAÇÕES GERAIS

É sabido que após uma intensa injúria ao órgão dental que leva á destruição da camada de odontoblastos, uma nova geração de odontoblastos se origina a partir de células indiferenciadas da polpa e secretam dentina reparativa focalmente abaixo do local da injúria (Trowbridge, 1981, Langeland, 1987, Smith *et al.* 1995, Tziafas *et al.* 2000).

Existem diferentes mecanismos que podem contribuir para a geração de novas células durante um processo regenerativo. Uma possibilidade é que a regeneração do tipo de célula perdida é regulada por células-tronco residentes em nichos específicos em um determinado órgão (Tanaka & Reddien, 2011). Uma segunda possibilidade é o envolvimento de células progenitoras circulantes na corrente sanguínea, talvez das linhagens mesenquimais ou hematopoiéticas. Estas células migrariam da circulação, entrariam nos tecidos danificados e se diferenciariam em tipos celulares específicos que se perderam (Tanaka & Reddien 2011, Zaidi & Nixon 2007). Estudos sobre células-tronco identificaram várias populações de células com potencial de dar origem a uma nova geração de células semelhantes aos odontoblastos durante a dentinogênese reparadora (Lovschall *et al.,* 2007, Iohara *et al.,* 2006, Batouli *et al.,* 2003, Miura *et al*., 2003, Tziafas *et al*., 2002, Goldberg & Lasfargues 1995, Harada *et al*., 2008, Kitamura *et al.,* 2001)

O fato é que a origem das células precursoras de novos odontoblastos durante a dentinogênese reparativa ainda não foi completamente elucidada (Harada *et al.,* 2008, Mitsiadis & Rahiotis 2004, Braut *et al*., 2003, Mina & Braut 2004). Uma das hipóteses a ser testada neste estudo é a possibilidade de células indiferenciadas circulantes no sangue periférico (SP) (células tronco mesenquimais (CTM) ou células tronco hematopoiética (CTH)) poderiam migrar, e em um favorável estado metabólico da polpa dental, se diferenciar em células

precursoras da nova geração de odontoblastos durante o processo de dentinogênese reparativa.

Para se testar a hipótese mencionada foi usado, neste experimento, o modelo de camundongos transgênicos *Green fluorescente protein* (GFP). Este tipo de camundongo transgênico têm a proteína GFP inserida em seu DNA em associação com promotores específicos, e uma vez este promotor sendo expresso pelo célula do camundongo, a proteína GFP (verde ao microscópio de fluorescência) também será expressa (Uribe *et al.,* 2011, Balic *et al*., 2010, Balic & Mina 2011, Boban *et al*., 2010).

Antes de se realizar o experimento com o objetivo de testar a participação de células sanguíneas na dentinogênese reparativa (*Analysis of the Contribuition of Circulating Blood Cells in Reparative Dentinogenesis Using Parabiosis Model in Mice*) foi necessário se avaliar a formação de dentina reparativa em camundongos transgênicos e a cronologia dos eventos celulares neste processo.

Para isto, foi realizado um outro experimento (*Analysis of reparative dentinogenesis in pOBCol3.6GFPtpz transgenic mice)* usando um modelo para se estimular a formação de dentina reparativa através da exposição e capeamento pulpar em molares de camundongos (Simon *et al.,* 2008). Este modelo foi usado em camundongos transgênicos pOBCol3.6GFPtpz (Kalajzic *et al.,* 2002a) que têm a proteína GFP associada ao promotor do colágenos tipo I, ou seja, toda vez que o colágeno tipo I é sintetizado a proteína GFP também e expressa. Neste tipo de camundongo a proteína GFP é expressa em toda célula odontoblástica e em seu processo celular dentro do túbulo dentinário (Mina & Braut, 2004). Foi usado como material capeador nas exposições pulpares realizadas, o MTA e no grupo controle o sistema adesivo foi colocado em direto contato com a polpa, os dois grupos foram restaurados com resina composta.

Este experimento preliminar foi realizado em duas linhagens diferentes de camundongos transgênicos pOBCol3.6GFPtpz (GFP para o colágeno tipo I), uma foi a linhagem CD1 (camundongo branco) e a outra foi a linhagem C57/Bl6

(camundongo preto). Foram usadas duas linhagens diferentes de camundongos com o objetivo de se avaliar o diferente comportamento da polpa dental destes animais durante o processo de dentinogênese reparativa.

Foi observado através de análises histológicas e de epifluorescência que imediatamente após a exposição pulpar (dentes não capeados) houve uma completa destruição da camada de odontoblastos logo abaixo do local da exposição (Simon *et al.,* 2008), pré-requisito para que haja a diferenciação de novas células odontoblásticas e produção de dentina reparativa (Trowbridge, 1981, Langeland, 1987, Smith *et al.,* 1995, Tziafas *et al.,* 2000).

Após 7 dias de exposição pulpar e capeamento ainda não havia a formação de dentina reparativa no local da exposição pulpar, mais havia a presença de células, expressando GFP, logo abaixo ao local da injúria (Harada *et al.,* 2008, Ishikawa *et al.,* 2010), significando uma atividade de sínteze de colágeno tipo I por parte desta células, dando origem aos primeiros eventos da dentinogênese reparativa.

Após 4 semanas de exposição e capeamento pulpar houve a formação de dentina reparativa em camundongos da linhagem CD1 capeados com MTA, com presença de células GFP+ em volta e incluída na nova matriz de dentina reparativa (Sloan & Smith, 2007, Sloan & Waddington, 2009, Braut *et al*, 2003, Zhao *et al.,* 2007, Ishikawa *et al.,* 2010). Em camundongos capeados diretamente com o sistema adesivo (sem MTA) não houve a formação de dentina reparativa mais sim de dentina reacional produzida pelos odontoblastos pré-existentes da polpa dental (Dominguez *et al.,* 2003, de Souza Costa *et al.,* 2001).

Em camundongos C57/Bl6 o processo de dentinogênese só ocorreu 8 semanas após a exposição pulpar e capeamento com MTA ou com sistema adesivo. Comprovando assim um processo tardio de produção de dentina reparativa nesta linhagem de camundongo. Uma vez caracterizado o processo de dentinogênese em camundongos, foi realizado o experimento para se

analisar a participação de células do sangue na produção de dentina reparativa usando o modelo de parabiose.

O modelo de parabiose consiste em se unir cirurgicamente dois camundongos que passam a dividir a mesma circulação sanguínea cruzada, células de um camundongo circulam na corrente sanguínea do outro camundongo parabiótico à partir da primeira semana de parabiose, perdurando por até 9 semanas, atingindo até 50% de circulação cruzada entre o par parabiótico (Aicher *et al.,* 2007, Purhonen *et al.,* 2008, Goldman *et al.,* 2009, Boban *et al.,* 2008).

A parabiose foi realizada usando um camundongo transgênico C57BL/6-TgN(ACTbEGFP)10sb/J (referido com GFP+) que tem a proteína GFP associada ao promotor da β-actina, e é expressa em todas as células do corpo inclusive células sanguíneas com exceção dos eritrócitos e um camundongo não transgênico C57/Bl6 (referido com GFP-).

Em nosso exerimento, foram detectadas, através de análise de citometria de fluxo (FACS), mais de 50% de células GFP+ no sangue do camundongo GFP-, à partir da segunda semana de parabiose, e este chimerismo perdurou por até 10 semanas de parabiose atingindo até 59% de circulação cruzada. Esta análise de FACS comprova o sucesso da cirurgia de parabiose em promover circulação sanguínea cruzadas entre os parceiros parabióticos.

Após 2 semanas de parabiose os camundongos eram novamente anestesiados e uma exposição e capeamento pulpar era realizado no molar superior direito do camundongo GFP-, com o intúito de se estimular a produção de dentina reparativa neste animal. Depois de 4 ou 8 semanas da exposição pulpar os camundongos parabióticos eram sacrificados, separados e o molar do camundongo GFP- era processado para análise histológica e de epifluorescência.

Como mostrado no experimento preliminar, e confirmado neste estudo, após 4 semanas de exposição e capeamento pulpar, não houve a formação de barreira dentinária abaixo da exposição pulpar. Mais havia a clara presença de

células GFP+ (vindas do SP), logo abaixo do local da exposição, na polpa dental do camundongo GFP-, em sua maioria estavam associadas a pequenos fragmentos de dentina impactados dentro do tecido pulpar, muito provavelmente estas células estavam exercendo um função clástica no processo de dentinogênese reparativa, o que é consistente com outros estudos na literatura (36, 37). Depois de 8 semanas de exposição pulpar houve a formação de uma evidente barreira dentinária abaixo do local da injúria, o que foi consistente com o estudo preliminar aqui desenvolvido (Frozoni 2011). Esta nova barreira dentinária estava circundada por um tecido semelhante a pré-dentina e possuia células GFP+, com o citoplasma alongado, em íntimo contato com este tecido semelhante à pré-dentina.

Uma vez que morfologicamente estas células eram semelhantes à células clásticas (Domon *et al.,* 1997, Sahara *et al.*,1996). Realizou-se análise para atividade de osteoclastos/odontoclastos através de histoquímica para TRAP (Domon *et al.*, 1997, Nilsen & Magnusson, 1979). Nenhuma das células GFP+ em íntimo contato com a dentina reparativa foi positiva para TRAP, excluindo-se a possibilidade destas células serem osteoclastos/odontoclastos.

Outra possibilidade é que as células GFP+, em contato com a dentina reparativa nos molares dos camundongos GFP-, possuam atividade secretória e estejam participando da produção de dentina terciária reparativa no processo de reparo dental. Embora sejam necessários mais experimentos para se caracterizar especificamente a linhagem de células sanguíneas participantes do reparo dental, este experimento sugere evidências que CTM circulantes na corrente sanguínea participam no processo de dentinogênese reparativa sendo uma das prováveis sub-populações capazes de se diferenciar em uma nova geração de odontoblastos durante o processo de reparo dental.

CONCLUSÃO

Os achados deste estudo dão suporte as seguintes conclusões:

Experimento 1 - ***Analysis of reparative dentinogenesis in pOBCol3.6GFPtpz transgenic mice.***

- O modelo de exposição pulpar é efetivo em destruir os odontoblastos abaixo local da exposição pulpar

- Após 7 dias da exposição e capeamento pulpar ocorreu uma diferenciação de células que passaram a sintetizar colágeno tipo I e a expressar 3.6-GFP

- No camundongo CD1 em 4 semanas após a exposição pulpar já temos a formação de dentina reparativa, tanto ponte de dentina quanto osteodentina, produzida por células recém diferenciadas da polpa dental

- No camundongo C57/Bl6 a formação de dentina reparativa ocorreu após 8 semanas de exposição pulpar sugerindo um processo de dentinogênese tardio

Experimento 2 ***-Analysis of Reparative Dentinogenesis Using Parabiosis Model in Mice***

- Após 4 semanas de exposição e capeamento pulpar não houve evidência de formação de dentina reparativa em camundongos C57/Bl6 e as células GFP originárias do sangue periférico estavam associadas com fragmentos de dentina sugerindo uma ação clástica

- Após 8 semanas de exposição e capeamento pulpar houve evidência de formação de dentina reparativa e as células GFP originárias do sangue periférico estavam associadas com a nova matriz dentinária sugerindo uma ação secretora

- As células GFP originárias do sangue periférico associadas à dentina reparativa foram negativas para a atividade de TRAP excluindo a possibilidade de serem odontoclastos

- Novos experimentos devem ser realizados para se identificar especificamente as células do sangue periférico que participam da dentinogênese reparativa

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ANEXOS

ANEXO 1

UNICAMP

CEEA/Unicamp

Comissão de Ética na Experimentação Animal
CEEA/Unicamp

CERTIFICADO

Certificamos que o Protocolo nº **1710-1**, sobre **"Efeito da origem de células formadoras de dentina reparativa e polpas dentais de camundongos GFP"**, sob a responsabilidade de **Prof. Dr. Sérgio Roberto Peres Line / Marcos Roberto dos Santos Frozoni**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **13 de março de 2009**.

CERTIFICATE

We certify that the protocol nº **1710-1**, entitled **"Study of odontoblast-like cells in GFP dental pulp mice"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **March 13, 2008**.

Campinas, 16 de março de 2009.

Profa. Dra. Ana Maria A. Guaraldo
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ANEXO 2

International Endodontic Journal
The Official Journal of the British Endodontic Society and the European Society of Endodontology

Submission Confirmation

Thank you for submitting your manuscript to *International Endodontic Journal*.

Manuscript ID:	IEJ-11-00639
Title:	Analysis of reparative dentinogenesis in pOBCol3.6GFPtpz transgenic mice
Authors:	Frozoni, Marcos Balic, Anamaria Sagomonyants, Karen Zaia, Alexandre Line, Sergio Mina, Mina
Date Submitted:	08-Nov-2011