Transplantation of embryonic stem cells improves the regeneration of periodontal furcation defects in a porcine model


Abstract

Objectives: Stem cell-based therapy promises to regenerate lost tissue. Embryonic stem (ES) cells are pluripotent and may provide a virtually unlimited source for transplantation. We investigated whether ES cell transplantation improved the regeneration of furcation defects in a porcine model.

Material and Methods: Experimental periodontitis was induced in the buccal furcations of the bilateral mandibular 2nd premolars of six minipigs. After 4 weeks, the lesions were surgically debrided and implanted with collagen matrix alone (control site) or collagen matrix overlaid with porcine ES cells expressing green fluorescent protein (pES/GFP+) (test site). After 3 months of healing, the clinical parameters were measured again. The treated teeth with adjacent tissue, and part of the major organs, were processed for GFP immunohistochemistry.

Results: We found no obvious teratoma or rejection. The test group had significantly better clinical parameters. Immunohistochemistry (IHC) showed that transplanted pES/GFP+ cells had differentiated to new periodontal ligament and cementum in the test sites. Surprisingly, GFP+ cells were also detectable in the repaired control cementum and remote organs.

Conclusions: We conclude that using ES cells to improve the regeneration of periodontal furcation defects is feasible. More studies are required to assess this potential treatment’s efficacy and safety.

Key words: embryonic stem cells; homing; periodontium; pig; regeneration

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Materials and Methods

Cell culture of GFP-expressing pES cells

The establishment and characterization of GFP-expressing pES (pES/GFP+) cell lines have been published (Yang et al. 2009). Culturing and maintenance of the GFP-expressing pES cells have been described in the studies (Yang et al. 2009, 2010). To maintain the undifferentiated state of the cells, pES/GFP+ colonies were subcultured and replated onto fresh STO feeder layers every 5–7 days. The undifferentiated state of the pES/GFP+ cells was regularly checked using immunocytochemistry (ICC) targeting Oct-4, alkaline phosphatase (ALP), stage-specific embryonic antigen-3 (SSEA-3), stage-specific embryonic antigen-4 (SSEA-4), tumour-related antigen-1-60 (TRA-1-60) and tumour-related antigen-1-81 (TRA-1-81). The activity of ALP was also detected using a Fast Red-based commercial kit (Chemicon, Billerica, MA, USA).

Animal study

The animal study was done following the specifications of an approved animal protocol (LRRIACUC99032) from the Livestock Research Institute, Council of Agriculture (Tainan, Taiwan). The timetable of the animal study is summarized in Fig. 1. Six healthy 5-month-old female miniature pigs (Lanyu GPI-CRC-PGD inbreeding, Lanyu 300) weighing 25–30 kg were obtained and examined for good systemic and oral health. All surgical procedures were done using general anaesthesia with an intravenous injection of zolazepam and tiletamine (Zoletil 50; Virbac Laboratories, Carros, France), and intramuscular injections of 2% Xylazine hydrochloride (HCL) (Rompun; Bayer, Leverkusen, Germany), atropine sulphate (Tai-Yu Chemical Pharmaceutical Co., Ltd, Hsinchu, Taiwan) and a local anaesthetic with 2% lidocaine HCL and 1:80,000 epinephrine (New Stetic S. A., Medellin, Colombia). After clinical assessments of probing depth and attachment level using a periodontal Williams probe (Hu-Friedy, Chicago, IL, USA), intrasulcular incisions were made in the buccal and lingual surfaces of the bilateral mandibular second premolar. After the mucoperiosteal flaps had been raised, an osteotomy was done in the furcation area using water-cooled carbide burs. The created furcation bony defect...
was 4 mm wide, 5 mm deep and 3 mm horizontally into the buccal furcation, and a polyether impression material (Impregum F; 3M, Minneapolis, MN, USA) was inserted into the defect to induce a chronic inflammation, to reduce natural bone repair and to favour formation of biofilm. The flaps were repositioned and sutured using 4.0 absorbable chromic gut sutures (Hu-Friedy). In total, 12 defects were created in six miniature pigs. Four weeks later, the 12 furcation defects were randomly assigned to control or test treatment groups. All the six pigs underwent the same clinical protocol and assessment. The periodontal tissues from four pigs were processed for immunohistochemistry and related research. The furcation tissues and gingival fluid samples were collected from the remaining two pigs for the measurements of inflammatory mediators and cytokines.

**pES/GFP**+ cell transplantation

Four weeks after the initial surgery, and 1 day before the cell transplantation surgery, 1 x 10⁶ pES/GFP**+** cells were transferred onto a square collagen matrix cut to 5 x 5 mm (CollaTape; Zimmer Dental, Carlsbad, CA, USA) immersed in medium for ES cells. The collagen membrane was used as a vehicle for pES/GFP**+** cells, not for "guided tissue regeneration." Before the second surgery, the clinical assessments of probing depth and attachment level were recorded again. The intrasulcular incisions were done both buccally and lingually to expose the furcation defects. The defects and roots were scaled and the root was planed with an ultrasonic scaler and curettes. The right and left sides were randomly assigned to control treatment with collagen matrix alone (n = 6) or to test treatment with pES/GFP**+** cells on collagen matrix (n = 6). The matrix with or without cells were tucked into the lesions. The flaps were replaced and sutured using 4.0 absorbable sutures and left for 12 weeks of healing. The mandibles of four pigs were dissected and split into two halves for in vivo imaging analysis (IVIS 50; Xenogen Corp., Alameda, CA, USA) to detect fluorescence levels. After IVIS analysis, the teeth and associated tissue samples from the experimental area were harvested and fixed with 4% formaldehyde for immunohistochemical (IHC) analysis. The samples were subsequently decalcified with buffered 10% edetic acid (pH 8.0) and embedded in paraffin. Sections 4-μm thick were deparaffinized and stained with H&E or processed for IHC staining. After the same clinical assessments, we collected gingival crevicular fluid samples from the remaining two pigs for enzyme-linked immunosorbent assays (ELISAs) of four proinflammatory cytokines (IL-1β, IL-6, TNF-α and IL-12). The furcation tissues were excavated for Western blotting of three inflammation mediators (phospho-p38, phospho-ERK1/2, NF-κB p65). Maxillary premolars were used as healthy and unoperated controls. In our pilot study, we unexpectedly found that GFP**+** cells were also detectable in the control site. We suspected that the ES cells had a “homing” property, as do mesenchymal stem cells (Belema-Bedada et al. 2008, König et al. 2010). Therefore, we also removed parts of the major organs to detect whether GFP**+** cells had migrated to distant organs from the experimental sites.

**Immunohistochemistry to identify pES/GFP**+ derived cells and their molecular phenotypes

Paraffin-embedded tissues were cut into 4-μm sections and deparaffinized and rehydrated with serial xylene and ethyl alcohol. After quenching endogenous peroxidase activity, the slides were rinsed three times for 5 min each time in phosphate-buffered saline. After the slides had been blocked for 20 min, they were incubated with primary antibodies for GFP (Novus Biologicals, Littleton, CO, USA) at a concentration of 1 μg/ml overnight at 4°C. The following day, the slides were incubated with biotin-conjugated secondary antibodies and streptavidin-horse-radish peroxidase following the manufacturer’s instructions (Abcam, Cambridge, MA, USA). Finally, peroxidase activity was detected with an AEC chromagen kit (Zymed, San Francisco, CA, USA) and then counter-stained with Mayer’s haematoxylin. Rabbit IgG isotype control (Novus Biologicals, USA) was used in IHC as negative control. To further confirm the pES/GFP**+** cells really differentiated to periodontal tissues, we also performed IHC of periodontal ligament (PDL) markers (periostin; asporin) and cementoblasts/osteoblasts markers (cementum attachment protein (CAP); osteopontin; osteocalcin; RUNX2) on the same tissues. The antibodies for periostin, asporin (also known as PDL-associated protein 1), osteocalcin, and RUNX2 were from Abcam. The antibody for osteopontin was from Lab Vision (Fremont, CA, USA). The antibody for CAP was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Statistical analysis**

The clinical data are means ± SD mm. Wilcoxon signed rank test was used to identify any significant differences (at the level of p < 0.05) between the test and control groups. StatView 4.5 (Abacus Concepts, Berkeley, CA, USA) was used for all statistical analysis.

**Results**

**Defect preparation, cell transplantation and clinical outcome**

The timetable of in vivo experimental procedure was summarized in Fig. 1. The periodontal furcation defects on both sides were created to a defined configuration (Fig. 2). The pES/GFP**+** cells we used were between 43 and 45 passages. The results of ICC demonstrated the cells expressed ES cell markers: Oct 3/4, ALP, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 (Fig. 3). This indicated that the cells were in an undifferentiated state. None of the pigs treated with pES/GFP**+** cells showed post-operative adverse effects; their physiological activities were normal after the surgery. No neoplasms were found during the autopsies and post-mortem organ examinations. The clinical parameters for each pig were listed in Table S1. The clinical data indicated that there were no significant differences between control and test teeth after the furcation defects had been created (Fig. 4a,b). Twelve weeks after open debridement and pES/GFP**+** cell transplantation, the test group had significantly better clinical results for probing depth.

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Cell tracking using IVIS and immunohistochemistry

IVIS indicated that the fluorescence levels from test teeth were about twice as high as those from control teeth (Fig. 5). Histological observation showed obvious root (dentin) resorption in the control furcation areas. Irregularly shaped cellular cementum without PDL fibre had been deposited on the resorbed dentinal surface. In the test furcation area, the mandible, a thick layer of reparative cementum (demarcated by multiple reversal lines) with collagen fibrils emanating from its surface were anchored to the adjacent alveolar bone. The fibres appeared to be consistent with Sharpey’s fibres oriented perpendicularly to the tooth root (Fig. 6). Inflammatory cells infiltration was obvious in the furcation areas of both groups. IHC staining for GFP showed that many cells in the newly formed cementum and PDL in test teeth were positive for GFP expression; however, the isotype control was negative. Unexpectedly, the cementocytes in the control teeth were also immunoreactive for GFP (Fig. 6). The staining suggested that the repaired and regenerated tissue was derived from the transplanted pES/GFP- plus cells. To further confirm the pES/GFP+ cells really differentiated to periodontal tissues, we performed IHC of PDL and cementoblasts/osteoblasts markers on the same tissues. The IHC results on the same furcation area as Fig. 6 showed that all the cementocytes and a portion of PDL cells was immunoreactive for osteocalcin. Osteopontin was detectable in the cementum and a portion of PDL cells, but not in dentin. The reversal lines were well demarcated for the osteopontin staining. Cementum and PDL exhibited positive staining of CAP. Dentin was negative for CAP staining. The expression of RUNX2 was localized in the nuclei of PDL cells and a few cementocytes. Most of the osteoblasts in the adjacent alveolar bone were positive for RUNX2 staining. The expression patterns of peristin and asporin were very similar and limited to the PDL (Fig. 7). In summary, the IHC results confirmed that the pES/GFP+ cells had differentiated to cementocytes and PDL cells.

Detection of pES/GFP-derived cells in distant organs

We also examined whether there were GFP+ cells in the remote organs. Some of the osteoblasts, blood cells and paravascular cells in endosteal spaces of the alveolar bone were immunoreactive for GFP (Figure S1). Some organs including lung, urinary bladder, colon, small intestine, liver, stomach, salivary glands, gingiva, anus and trachea contain GFP+ cells. The GFP+ cells were detectable in the whole epitheliums of bronchi, bronchioles, gingiva and urinary bladder. In some other tissues, such as salivary glands, anus, stomach, colon, small intestine and trachea, only a subpopulation of epithelial cells express GFP (Figure S2). Distinguishable staining of GFP was not observed in other examined organs. These results suggested that pES/GFP+ derived cells might migrate to remote tissue, especially tissue with a high turnover rate, via blood vessels.

Discussion

In this swine study, we found that transplanting ES cells improved periodontal regeneration in furcation defects. A portion of the regenerated cementocytes and PDL cells were differentiated from transplanted ES cells. There were no adverse effects or tumour formation in the major organs of the pigs. ES-derived cells were detected in areas with a high turnover rate and distant from surgical sites. This limited study demonstrates the feasibility of using ES cell transplantation to regenerate periodontal tissue.
Currently, the most documented clinical approach for regenerating periodontal tissue in furcation defects is “guided tissue regeneration.” Quantitative analysis of clinical outcomes after guided tissue regeneration suggests that it is a predictable procedure to treat specific configurations of furcation defects, but that it offers limited benefits when treating other types of defects (Villar & Cochran 2010). More predictable treatment protocols suited for all configurations of furcation defects are needed. Others (Lang et al. 1998, Liu et al. 2008, Zheng et al. 2009, Suaid et al. 2011, 2012) have shown that transplanting various types of postnatal stem cells is promising for regenerating periodontal tissue. However, these cells have limited proliferation and differentiation capacities, and many of them are difficult to obtain in the grownups who have less available and usable deciduous or wisdom teeth. Pluripotent cells, including ES and iPSC cells, are able to proliferate indefinitely in vitro and represent a potentially unlimited source of stem cells in regenerative medicine. Published information on the feasibility of ES cells transplantation to regenerate periodontal tissue is limited. A few in vitro studies (Inanc et al. 2009, Elcin et al. 2010) have reported that ES cells, when co-cultured with adult PDL fibroblasts or on tooth root surfaces, can be guided to differentiate towards fibroblastic and osteoblastic lineages. There are no published animal studies that explore the potential of using ES cells to treat periodontal furcation defects. Our findings clearly indicated that transplanting ES cells yielded some regeneration in the furcation area. ES cells would be an excellent source for regenerating periodontium if their major drawbacks could be overcome.

The risk of tumorigenesis is a major obstacle to ES cell therapy. ES cells can generate teratoma in immunocompromised animals (Solter 2006). The development of teratoma is related to the number of cells transplanted, grafted sites and the differentiated status of the cells (Laflamme et al. 2007, Fong et al. 2010). We found no teratoma in any of the pigs within the limited observation time. We hypothesize that teratomas did not develop because we used healthy and immunocompetent pigs as recipients, while most other studies used immunocompromised mice (Fong et al. 2010). It is reported that although ES cells may have a fragile immune privilege, they still represent novel targets of attack by elements of the immune system (Wu et al. 2008). A recent in vivo study reported that susceptibility to natural killer cells might constitute a common feature of pluripotent cells. Teratoma growth is reduced after natural killer cells are activated (Dressel et al. 2010). In our short-term porcine study, immunorejection seemed not to be a problem because pES/GFP⁺-derived cells still existed in the recipient teeth after 3 months of healing, and the teeth were still functioning as well as the control teeth were. We did not give our pigs any immunosuppressant drugs. The results of ELISA and Western blot for pro-inflammatory cytokines and signalling pathways suggested that transplantation of allogeneic ES cells was not responsible for the inflammation. We did not clean the teeth for the pigs during the experimental time. The created furcation involvement were not completely close after treatment. Plaque accumulation was most plausible for the inflammation. Although none of our pigs had teratoma, this issue should not be overlooked before clinical application of ES cells. In future studies, we will try to guide the differentiation of pES/GFP⁺ cells to periodontium-associated cells before transplanting ES cells into periodontal defects to reduce tumorigenic risk. Recently, subpopulations of tooth-associated

Fig. 3. Expression of Oct-4, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 (ES cell markers) of the pES/GFP⁺ colonies was determined using biotin-streptavidin-peroxidase based immunocytochemistry staining. The chromogen is 3-Amino-9-Ethylcarbazole (AEC) and appears as red. The activity of ALP was detected using a Fast Red commercial kit (Chemicon). pES, porcine embryonic stem cells; GFP, green fluorescent protein. Scale bar = 50 μm.
stem cells expressing ES cells markers have been successfully isolated (Kerkis et al. 2006, Siqueira da Fonseca et al. 2009, d’Aquino et al. 2011). Some of the cells have great renewal ability and pluripotency. Teratoma formation has never been observed for these cells. It would be interesting to test whether these post-natal stem cells with ES cell characteristics can differentiate to periodontium-associated cells since they are safer than ES cells regarding the risk of teratoma formation.

We unexpectedly found that there were also pES/GFP+-derived cells in the control sites and distant organs, especially in tissue that had been remodelled or that had a high turnover rate. “Stem cell homing” seems a plausible explanation for this finding. The migration of circulating cells through the blood to specific organs and to bone marrow niches is termed “homing”. The chemokine stromal derived factor-1 (SDF-1) has a major role in the homing and engraftment of hematopoietic stem and progenitor cells to the bone marrow, and together with its receptor, CXCR4, functions in stem cell seeding of the bone marrow during embryonic development (Lapidot et al. 2005). Our data suggested that pES/GFP+-derived cells might migrate to remote organs via the blood stream after the periodontal surgery. This finding is consistent with a recent study (Su et al. 2010) that used immunocompetent mice to investigate the early homing behaviour of ES cell-derived mesenchymal cells.

Our study has some limitations: short-term and small-sized sampling. Although the results of all assays were consistent among the pigs, we will increase the sample size and observe the results for a longer time in future studies. In the wound healing process after cell transplantation, host cells mobilized by secreted factors from transplanted cells can contribute to the regeneration as well. In future studies, additional control groups carrying non-stem cells are needed to clarify the relative significance of transplanted cells. As iPS cells have similar biological characteristics and elicit fewer ethical concerns than do ES cells, we will develop porcine iPS cells and determine whether they have the same regenerative effects for furcation defects as ES cells do. For future clinical applications and to reduce the risk of tumour formation, we will guide the differentiation of ES cells to periodontium-associated fibroblastic cells before transplantation. The homing property of the ES-derived cell will be further clarified and characterized. A non-invasive detection system, such as positron emission tomography, may be considered for whole body screening of tumour formation during the experiment.

In summary, transplanting ES cells contribute to the formation of

![Fig. 4. Clinical parameters of the furcations at three different time points. (a) -4 weeks indicate the probing depth at baseline. The middle columns indicate the data measured just before the embryonic stem (ES) cells were transplanted. +12 weeks indicate the data measured before the pigs were killed. (b) The attachment levels were measured at different time points, as described in (a).](image1)

![Fig. 5. An ex vivo bioluminescent image of an excised pig mandible showing substantial cell engraftments at the test tooth. Scale bar = 10 mm.](image2)

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new periodontal apparatus, which, in turn, leads to a better clinical outcome. There is no significantly adverse effect. Homing of ES cells to wound healing sites and distant organs with a high turnover rate is suspected. Our study shows the feasibility of using ES cells to improve the regeneration of periodontal furcation defects. More studies are required to assess the efficacy and safety of this potential treatment.

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References

Clinical Relevance

Scientific rationale for the study: Compared with the other types of stem cells, embryonic stem (ES) cells are more unlimited in cell source for transplantation. We aimed to test whether ES cells transplantation improved periodontal regeneration.

Principal findings: Transplanting porcine ES cells improved periodontal regeneration in furcation defects of miniature pigs. The regenerated cementocytes and PDL cells were partially derived from transplanted ES cells and their differentiated derivatives have a fragile immune privilege but still represent novel targets of immune attack.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Clinical parameters of the miniature pigs.

**Figure S1.** Immunohistochemistry of GFP in the mandibular tissue sample consisted of alveolar bone and mucosa. The green arrow heads indicate cells immunoreactive for GFP. Scale bars = 50 μm.

**Figure S2.** H&E staining and IHC of GFP on urinary bladder, salivary gland, lung, anus, and trachea. The green arrow heads indicate cells positive for GFP expression compared with isotype control staining. Scale bars = 100 μm for upper two rows of panels. Scale bars = 50 μm for the other panels.

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