

## Two- Versus Three-Dimensional In Vitro Differentiation of Human Pulp Cells Into Odontoblastic Cells

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The spatial organization of the pulp cells may modify the cytodifferentiation process. The purpose of this study was to compare the two- versus three-dimensional cell culture systems for differentiation of human odontoblastic cells in vitro. Pulpal cores from freshly extracted human third molars were cultured in vitro in a perfusion device on two types of membranes: polyester membrane (two-dimensional [2D] cell culture) and nylon mesh (three-dimensional [3D] cell culture). The cells were incubated with minimum essential medium containing (a) substitute serum, (b) 10% fetal calf serum (FCS), (c) 10% fetal calf serum + 2 mM  $\beta$ -glycerophosphate ( $\beta$ GP), and (d) 10% fetal calf serum + transforming growth factor (TGF) $\beta$ 1. Immunohistochemistry was used to evaluate the expression of collagen I, osteonectin, and nestin. Small differences were observed between 2D and 3D cell culture systems. This was particularly evident in the 10% FCS group.  $\beta$ -Glycerophosphate in the 3D system seems to stimulate the osteogenic cell phenotype, as a considerable induction of osteonectin is observed.

**Keywords** Human, Odontoblast, Cytodifferentiation, Three Dimension, Culture.

### INTRODUCTION

Odontoblasts are postmitotic cells synthesizing the organic matrix of dentin. Since odontoblasts cannot be cultured in vitro by traditional cell culture methods, several innovative culture systems have been proposed [1–3]. For research purposes, dental papillae of neonatal rodents have been extensively used with success [1]. However this system is not suitable to evaluate the interactions between restorative materials used in common dentistry and pulpal cells. Pulp slices from extracted human teeth cultured for 30 days [3] or young rodent teeth [2] cultured up to 28 days behaved similar to pulpal cells in vivo, thus offering a nice

model for studying the factors affecting pulp repair. However, the disadvantage of this system is that reparative dentin secretion cannot be studied; reparative dentin implies differentiation of pulp cells into odontoblasts, and this process is absent from the slices since all the preexisting odontoblasts remain intact.

In addition to that culture system, classical two-dimensional (2D) cell culture systems have been used. Pulp stem cells have been cultured in presence of various chemicals in order to induce the differentiation of pulp cells into odontoblastic cells in vitro. These cells exhibit an elongated shape and secrete several proteins that are specific to odontoblasts such as dentin sialoprotein (DSP). Differentiation of odontoblastic cells may be induced by  $\beta$ -glycerophosphate [4], calcium hydroxide [5], dexamethasone [6], ascorbic acid [7], and several growth factors [8, 9]. These well-established systems may reproduce the physiological and molecular events of human dentin production in vitro [10]. In spite of these in vitro models, a three-dimensional (3D) cell culture system might be useful and necessary for pulp tissue engineering [11–13] or for cytotoxicity testing [14]. This opens new fields of research based on a biological approach of dental therapy. As a matter of fact, human pulp fibroblasts have been grafted onto synthetic scaffolds [11] in order to replace dental pulp after root canal therapy [12, 13]. Furthermore, the newly used pulp chamber with 3D cell cultures has been found to be a good in vitro model for testing cytotoxicity of restorative materials [14], since this system is less sensitive than conventional 2D systems.

The purpose of the present study was to compare a 2D and a 3D culture system for in vitro differentiation of human pulp cells into odontoblastic cells.

### MATERIALS AND METHODS

#### Reagents

All materials used for culture media preparation were purchased from Gibco BRL (Life Technologies, Inc., Grand Island,

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NY) unless otherwise stated. Minimum essential medium (MEM) was supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin (Biowhittaker, Gagny, France) and 0.25  $\mu\text{g}/\text{ml}$  amphotericin B (Fungizone).

### Teeth

Normal immature third molars freshly extracted during treatment of 16- to 18-year-old patients were used after their informed consent.

### Antibodies

Rabbit polyclonal antibodies against the type I collagen were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Mouse monoclonal antibody to osteonectin was obtained from Takara Biochemicals (Takara Shuzo Co. Ltd., Shiga, Japan). Preparation and characterization of the polyclonal antibodies against nestin have been already described [15] and the specificity of these antibodies to human odontoblasts has been detailed [16].

### METHODS

Culture of dental pulp cells was performed as previously described [10, 16]. Briefly, confluent cultures were collected by trypsinization (0.2% trypsin and 0.02% ethylenediamine tetraacetic acid, EDTA) and subcultured for 1 month with a continuous perfusion system (Minucells and Minutissue GmbH, Bad Abbach, Germany) at a 0.3 ml medium/h rate (Figure 1).

The MEM used was supplemented with one of the following: (a) growth factor-free serum substitute, (b) 10% fetal calf serum (FCS), (c) 10% fetal calf serum + 2 mM  $\beta$ -glycerophosphate ( $\beta\text{GP}$ ), and (d) 10% fetal calf serum + transforming growth factor ( $\text{TGF}$ ) $\beta$ 1 (5 ng/ml). The cells were cultured, in the pulp chamber device (Figure 2), on two types of membranes: polyester membrane (2D cell culture) and nylon mesh (3D culture system) (Advanced Tissue Sciences, La Jolla, CA).

The culture medium was continuously perfused to mimic pulp clearance and also to remove the by-products of the cells that may accumulate over 1 month and modify the culture conditions. The perfusion rate was calculated from a previous comparative study [13], which showed a statistically significant influence of the perfusion rate on the outcome of cytotoxicity studies. After 1 month of cell culture, the cells were fixed with 70% ethanol for 1 h at 4°C and processed for immunohistochemistry.

The cells were permeabilized for 15 min with 0.5% Triton X-100 in phosphate-buffered saline (PBS). Primary antibodies were diluted in PBS containing 0.1% bovine serum albumin (BSA). The incubation with primary antibodies was performed overnight at 4°C at the following concentrations: anti-collagen I antibodies were used at 40  $\mu\text{g}/\text{ml}$ , and anti-onectin at 50  $\mu\text{g}/\text{ml}$ . Anti-nestin antibodies were diluted 1:1500 in PBS. The immunostaining was revealed using the labeled streptavidin-biotin kit (LSAB; Dako Corporation, Carpinteria, CA) according to the manufacturer's instructions. Glycergel was used as a mounting medium (Dako Corporation, Carpinteria, CA). Controls were performed by incubations with unrelated primary antibodies.

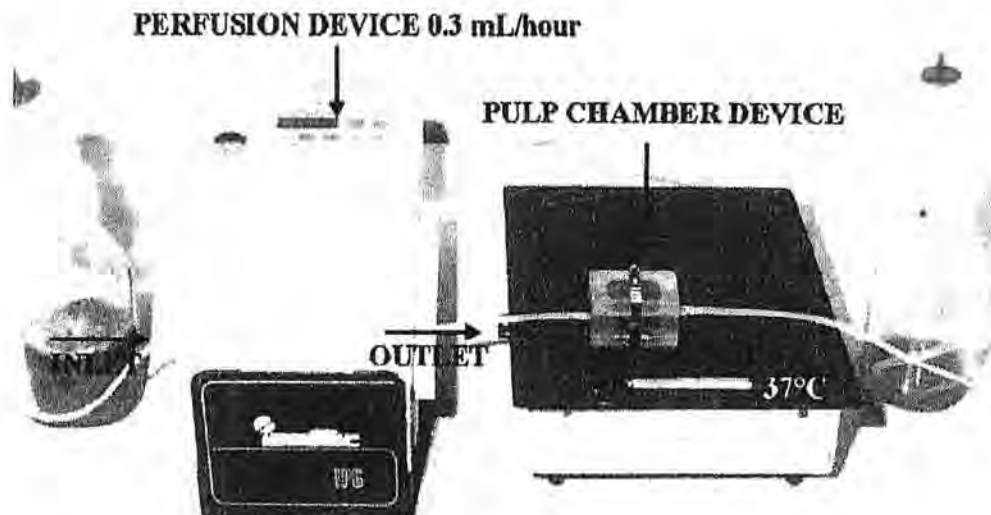
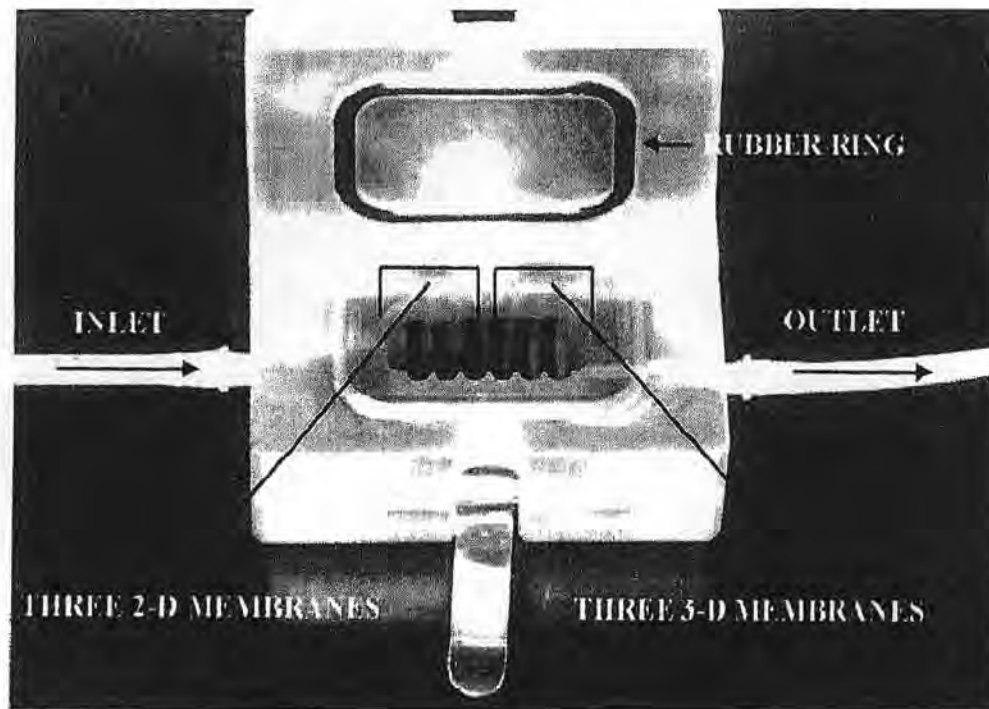


Figure 1. The perfusion system: an automatic device that allows constant renewal of the culture medium at 0.3 ml/h within the pulp chamber system.



**Figure 2.** Polycarbonate pulp chamber system: This device contains simultaneously three 2D and three 3D membranes. The inlet is on the left side and the outlet on the right side.

## RESULTS AND DISCUSSION

When the serum substitute was used, no difference between the 2D and the 3D cell culture system was found (Table 1 and Figure 3A). The cells expressed all of the proteins weakly. This means that in absence of growth factors, spatial arrangement of the cells does not influence the ability of the cells to express specific proteins. Even in the absence of growth factors, whatever their spatial arrangement is, the pulp stem cells can secrete weakly proteins specific to odontoblasts after 1 month of culture.

No difference was observed when using a serum substitute or MEM + 10% FCS on the 3D system. On the other hand, no protein was detected when using MEM + 10% FCS on the 2D system. This may indicate that FCS is not important to in vitro cell differentiation when working with a 3D cell culture system.

**TABLE 1**

Comparison of the immunohistochemical staining on 2D and 3D membranes.

	2D Coll I	3D Coll I	2D OSN	3D OSN	2D Nestin	3D Nestin
Serum substitute	+	+	+	+	+	+
10% FCS	0	+	0	+	0	+
10% FCS + $\beta$ GP	+	+	+	++	+	+
10% FCS + TGF $\beta$ 1	++	+	++	++	++	++

It might also suggest that spatial arrangement is of prime importance when working with FCS alone because no protein was detected with the 2D cell culture system while all of them were weakly expressed with the 3D cell culture system (Table 1). The use of a higher concentration of FCS seems to be of prime importance in obtaining development of dental papilla in vitro similar to that obtained in vivo [17]. Fetal calf serum contains growth factors in unknown concentrations, probably varying from a batch to another. The use of  $\beta$ GP or TGF $\beta$ 1 gives much better results to obtain in vitro differentiation of odontoblastic cells.

$\beta$ GP has already been successfully used to produce human dentin in vitro [10].  $\beta$ GP is metabolized by alkaline phosphatase and the combination in vitro of the phosphorus released from  $\beta$ GP and calcium contained in the medium permits the formation of nodules of mineralization. This permits an in vivo rapid induction of mineralization when used for direct pulp capping [18] or apical barrier formation [19]. The addition of  $\beta$ GP to the medium containing 10% FCS greatly modifies the results of the study. All of the proteins tested were weakly expressed when 2 mM  $\beta$ GP was added to the medium. In the 3D system, osteonectin reactivity was much stronger in presence of  $\beta$ GP. This is in favor of a 3D cell culture system when using  $\beta$ GP as a mineralization inductor. When direct pulp cappings are performed with calcium hydroxide, it has been shown that the calcium incorporated during hard tissue formation does not come from the calcium hydroxide itself but rather from the pulp blood flow (20). Therefore, in the same way,  $\beta$ GP probably does not only act as a source of phosphorus but could be involved in the odontoblast cells differentiation. This has



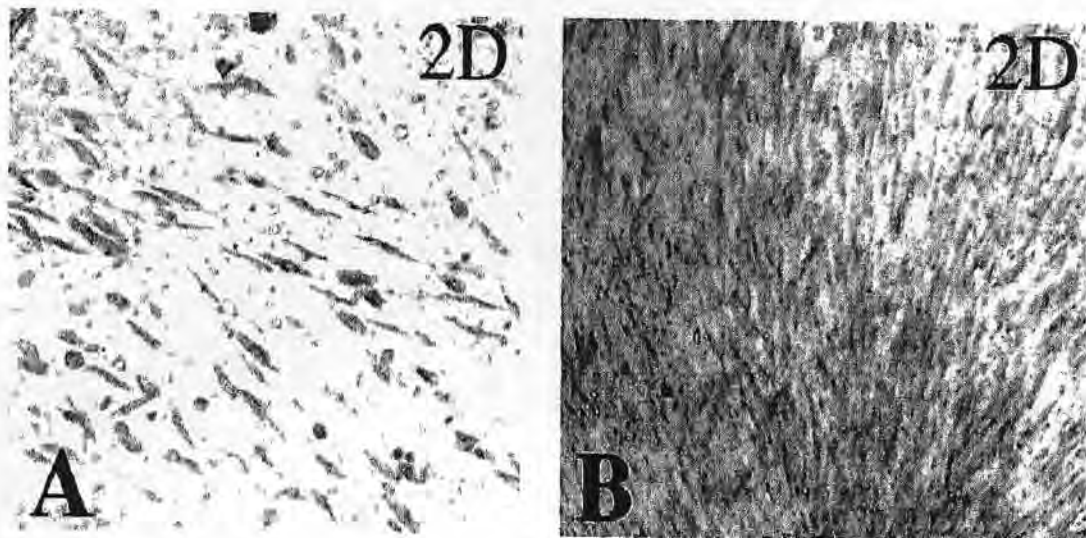


Figure 3. Immunohistochemical detection of osteonectin in the 2D culture system in the presence of either replacement serum (A) or 10% FCS/TGF $\beta$ 1 (B).

been suggested in earlier studies on cultured human pulp cells where the addition of  $\beta$ GP to pulp cells cultivated with 10% FCS induced the odontoblastic differentiation while in the absence of  $\beta$ GP no odontoblastic differentiation was observed [10, 21]. Although growth factors could be present in the serum, the addition of  $\beta$ GP seems to be determinant in this differentiation process even if the mechanism remains to be elucidated.

When TGF $\beta$ 1 was added to the medium, no difference was found between the 2D and the 3D cell culture systems except for collagen type I that was more expressed in the 2D culture system. Nestin and osteonectin were strongly expressed in both 2D and 3D systems (Table 1 and Figure 3B).

Under the culture conditions of this study, mineralization nodules were not observed. This may be due to the fact that the medium flow disturbs the crystal growth and renders their formation impossible. When working with growth factors to induce *in vitro* cytodifferentiation the 3D cell culture system does not bring any advantage over the 2D system.

In conclusion, the 3D system brings advantages over the 2D system only when working with 10% FCS. When TGF $\beta$ 1 is added to the medium the 3D system does not bring any advantage. Therefore we can recommend either the use of a 3D system when working with 10% FCS or the use of  $\beta$ GP associated to TGF $\beta$ 1 to induce differentiation of odontoblastic cells and to allow them to secrete dentin.

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