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**Three-Dimensional Culture of Oral Progenitor Cells: Effects on small extracellular vesicles production and proliferative function**

**Three-Dimensional Culture: Effects on small extracellular vesicles**

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**Ethical Approval**

Ethical approval (for initial human cell isolation and use) was given by the medical ethics committee of West of Scotland REC 5.

**Conflict of Interests**

All authors declare that no financial relationships exist regarding any of the products involved in this study.

**Background:**

Small extracellular vesicles (SEVs), have a diameter between 30-150nm and play a key role in cell-cell communication. As cells cultured in 3D versus 2D behave differently, this project aimed to assess whether there were differences in SEVs derived from human oral mucosa lamina propria-progenitor cells (OMLP-PCs) cultured in a 3D matrix compared to traditional 2D monolayer cultures.

**Methods:**

OMLP-PCs were cultured in 3D type I collagen matrices or on traditional 2D tissue culture plastic. Cell morphology and viability were assessed by light microscopy, actin staining and trypan blue staining. SEVs secreted by OMLP-PCs were purified and quantitatively analyzed by a BCA assay and nanoparticle tracking analysis (NTA; nanosight™). SEVs were further characterized by flow cytometry. SEV proliferative function was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

**Results:**

Cells cultured in 3D grew well as observed by light microscopy and phalloidin staining with cells branching in three-dimensions (as opposed to the cells grown as monolayers on tissue culture plastic). NTA demonstrated a significantly higher number of SEV-sized particles in the conditioned medium of cells grown in 3D type I collagen matrices versus a 2D monolayer ( $P < 0.01$ ). Like SEVs from 2D culture, SEVs from 3D culture demonstrated a particle size within the expected SEV range. Tetraspannin analysis confirmed that

3D-derived SEVs were positive for typical, expected tetraspannins. Cell proliferation analysis demonstrated that SEVs produced through 3D cell culture conditions, significantly reduced the proliferation of skin fibroblasts when compared to SEVs from 2D monolayers ( $P < 0.05$ ).

**Conclusion:**

3D culture of OMLP-PCs produced typical SEVs but in a greater amount than when the same cells were cultured in 2D. The downstream proliferative potential of the SEVs was influenced by the initial culture methodology. Future work should now assess the potential effects of 3D SEVs on key wound healing activities.

**Key words:** 3D and 2D cell culture, small extracellular vesicles, oral progenitor lamina-propria progenitor cells, collagen

## 1. Introduction

Small extracellular vesicles (SEVs) are the smallest of the cell derived vesicles and are of endosomal origin with a diameter of 30-150 nm and a buoyant floatation density of 1.10~1.20 g/cm<sup>3</sup> in sucrose (1). SEVs secreted from cells both *in vitro* and *in vivo* play a key role in cell-cell communication (2) during both pathological process and physiological process such as tissue repair (3) and stem cell maintenance (4). It is widely accepted that SEVs demonstrate similar biological functions as the cells from which they are derived (5). Existing SEV isolation methods are based on size, structure and membrane proteins, with the SEVs needing to be well characterized to differentiate them from other protein complexes and extracellular vesicles. The characterization of SEVs mainly depends on morphological and landmark protein analysis including the analysis of tetraspanins (CD9, CD63 and CD81; (6)). Low SEV production levels are one of the most critical issues hampering the application of SEVs as therapeutics. Typically, the effective dose of SEVs is around 10-100 µg/mouse whilst the typical yield from 2D monolayer cultures is only around 1 µg/mL (7). Thus, a more effective culture method which could enhance the production and/or function of SEVs would be clinically welcomed.

Whilst many adult stem/stromal cell populations have been reported, oral mucosal lamina propria-progenitor cells (OMLP-PCs) are a novel adult stem cell population isolated from the buccal mucosa that demonstrate significant advantages (ease of isolation, clonal expansion and high potency) over the more commonly studied bone marrow derived mesenchymal stromal cells (MSCs) (8). OMLP-PCs are thought to contribute to the scarless wound

healing ability of the oral mucosa (9). OMLP-PCs are also multipotent (10), immunosuppressive (11) and anti-bacterial (12).

In recent years, it has been reported that paracrine factors derived from stem cells are the main facilitators in driving tissue repair. For example, factors derived from bone marrow stromal cells can promote macrophage and endothelial cell migration/recruitment and thus enhance wound healing (13), and paracrine factors derived from adult stem cells also play a key role in myocardial protection and neovascularization processes thus enhancing the reparative process of infarcted hearts (14). It has now been reported that the paracrine signalling molecules which mediate such repair processes include SEVs (14). For example, SEVs can enhance fibroblast proliferation (15, 16) and migration towards a wound (17), prevent myofibroblast formation via SEV-derived microRNAs (18) and stimulate wound angiogenesis (15, 19). *In vivo* studies have reported that SEVs can significantly increase wound healing rates and reduce scar widths (20). Hence, due to their functions, ease of storage and simplified delivery mechanisms SEV therapies are being actively developed and could replace current cell therapeutic approaches (21).

It is well reported that cells cultured in a 3-dimensional (3D) matrix perform differently compared to cells cultured on traditional 2-dimensional (2D) tissue culture plastic (22). 3D cell culture involves the use of a scaffold or matrix that influences the survival, growth and differentiation of cells; mimicking *in vivo* conditions within the body (23). For example, fibroblasts play a key role in the skin wound healing process (24). Compared to traditional monolayer cultures, 3D cell cultures provide a more complex physical environment with

cells acquiring a markedly different geometry and demonstrating altered migration and matrix remodelling capacities (25).

Studies investigating SEVs from 2D versus 3D culture conditions have demonstrated that 3D culture conditions can still promote the production of SEVs (22). Specifically, it has been reported that SEVs derived from human MSCs cultured under 3D conditions can improve functional recovery after traumatic brain injury and, compared to 2D SEVs, provided a better outcome in spatial learning (22). This suggests that 3D cell culture might be an advantageous method to produce functional SEVs.

However, what is clear to date is that there have been very few studies assessing the effects of 3D (*in vivo*-like) environments on SEV efficacy and therapeutic potential and it is not yet known whether SEVs isolated from cells after 3D culture are functionally different from SEVs isolated from traditional 2D cell culture. Hence, in this study, the yield of SEVs isolated from oral progenitor 2D and 3D cultures was compared as well as their function in a simple *in vitro* proliferative assay.

## **2. Materials and Methods**

### **2.1 Cell culture**

OMLP-PCs and skin fibroblasts were isolated and expanded by members of the Regenerative Biology Group (School of Dentistry, Cardiff University) as previously described (8). OMLP-PCs were cultured in basal medium (Dulbecco's Minimal Essential Medium (DMEM) supplemented with 100µg/mL



L-Glutamine, 1% (v/v) Antibiotics/Antimycotics) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (SCM; Serum Containing Medium). Every 5-7 days, cells were passaged at around 80% confluency by the addition of 0.05% (v/v) Trypsin (Gibco) and then re-seeded at a density of  $2 \times 10^3$  cells/cm<sup>2</sup>. Cells in culture were fed with SCM every 2-3 days.

## **2.2 Imaging of cells**

Brightfield images of cells were captured using a Nikon Eclipse TS100 microscope equipped with a Cannon LA DC 58E digital camera. Confocal images were captured using a Leica SP5 confocal laser scanning microscope.

## **2.3 Collection of 2D Conditioned Medium**

OMLP-PC conditioned medium (CM) was collected from monolayers of cells that had been maintained for at least 2 passages in SCM made up with SEV depleted FBS (SEV-FBS) in place of regular FBS. SEV-FBS was produced by ultracentrifugation of FBS at 100,000xg for 18-20 hrs at 4°C in a Beckman Coulter XP100 ultracentrifuge with a 70Ti fixed angle rotor before being passed through a 0.2µm and then a 0.1µm filter (MerkMillipore; Massachusetts, USA). Cells were seeded at a density of 4000 cells/cm<sup>2</sup> and left in culture for 48 hrs until 50-60% confluent. Medium was then removed and replaced with fresh SEV-depleted SCM and cells returned to culture for 72 hrs until 85-95% confluent before the CM was collected. The collected CM was centrifuged at 500xg for 7 mins to pellet any dead cells. To remove any remaining debris or microvesicles the supernatant collected was next centrifuged at 10,000xg for 7

mins then collected again and filtered through a 0.2µm filter. CM was either used immediately for vesicle isolation or stored at -80°C until required.

## **2.4 Fabrication of 3D type I collagen lattices and collection of conditioned medium**

3D type I collagen lattices were set up in 60mm bacteriological grade plates using 2.4mL 2x DMEM (0.48mL 10x DMEM, 0.12mL 1M NaHCO<sub>3</sub>, 0.048mL 100x Glutamine, 0.048mL 100x Non-Essential Amino Acids, 1.68mL Sterile H<sub>2</sub>O and 0.06mL 1M NaOH), 0.6mL SEV-FBS, 0.6mL 0.1M NaOH, 1.8mL rat tail type I collagen (1.7mg/mL; First Link) and 5x10<sup>5</sup> OMLP-PCs made up in 0.6mL of SEV-depleted SCM. Lattices were left to polymerize for 1 hr at 37°C then 6mL of low concentration SEV-depleted FBS media (3% FBS) was added on the top of the lattice. To produce CM for larger scale SEV isolation, 150mm plates were utilized with all reagents/cells scaled up accordingly and CM was processed as above.

## **2.5. Cell cytoskeleton staining**

Phalloidin staining was undertaken according to the manufacturer's instructions. In brief, lattices were washed with Phosphate Buffered Saline (PBS) before fixing with 4% (v/v) paraformaldehyde at room temperature. After 30 mins the lattice was washed twice with PBS and stained with 1nM Phalloidin-Atto-594 (Sigma-Aldrich) for 30 mins. The lattice was then washed 3 times with PBS and the nuclei counterstained with 10µM Hoechst 33258.

Confocal images were captured using a Leica SP5 Laser scanning confocal microscope.

## **2.6. Assessment of OMLP-PC viability**

To assess the viability of OMLP-PCs in 2D and in 3D lattices,  $5.4 \times 10^5$  OMLP-PCs were seeded into either 6 well plates or a 60mm diameter type I collagen lattice. After 72 hrs cells in 2D were trypsinised and resuspended in SCM. Also, after 72 hrs the collagen matrix was degraded with 1mg/mL collagenase for 1 hr at 37°C. Following digestion, cells were pelleted by centrifugation at 350xg for 5 mins. Viable cell counts were then undertaken by mixing equal volumes of cells and 0.05% (w/v) trypan blue and then counting on a haemocytometer under a microscope.

## **2.7. OMLP-PC SEVs isolation**

SEVs were isolated from CM using a commercially available kit (ExoSpin; Cell Guidance Systems; Cambridge, UK) involving a two stage SEV purification protocol (precipitation and size exclusion column). If frozen, CM was thawed rapidly in a 37°C water bath before adding a half volume of 'buffer A' (e.g. 5mL buffer added to 10mL CM) and incubated overnight at 4°C. CM/buffer was centrifuged at 16,000xg using a Beckman Coulter Optima LE 80-K ultracentrifuge for 2 hrs to pellet any EVs. Pelleted material was collected in 1mL of PBS before passing through an ExoSpin Midi column. Fractions 7-13 were collected as these contain the SEVs. Fractions 7-13 were then

concentrated, using a 100KDa molecular weight cut off filter resulting in OMLP-PC SEVs.

## **2.8 BCA assay**

SEV protein concentration was determined using a MicroBCA Protein Assay (ThermoFisher Scientific) according to manufacturer's protocol. A standard curve was produced with a serial dilution from 2000µg/mL Bovine Serum Albumin (BSA). SEVs were diluted between 1:5-1:10 so that they would fit on the scale. SEV protein concentrations were calculated based on absorbance values and the standard curve.

## **2.9 Nanoparticle Tracking Analysis**

Nanoparticle tracking analysis (NTA) (NanoSight LM10 HS microscope, NanoSight Ltd, Amesbury, UK) was used to analyze the size and concentration of SEVs. The SEVs were diluted in ddH<sub>2</sub>O at 1:100 for testing. Under the action of Brownian Motion, the particle movement trails were recorded and captured by the camera connected to NTA system. The NTA system was used to analyze the results with the particle detection threshold set to 2 and the screen gain to 10.

## **2.10. FACS analysis**

25µg of SEVs isolated from either 2D or 3D cultures, were bound to 20µL of CD63 coated Dynabeads (Thermo Fisher, USA) in a 100µL final volume overnight at 4°C made up in 0.1% (w/v) sterile filtered BSA (Isolation buffer). Following an overnight incubation, beads and SEVs were captured using a Dynal® MPC-L magnet for 1 min. The supernatant was removed and beads washed in 300µL of isolation buffer. Tubes were then placed back on the magnet and the supernatant removed before resuspending the beads in 100µL of isolation buffer for staining. Either the IgG control or test antibodies were added; IgG1 FITC (50µg/mL), IgG1 PE (50µg/mL), CD81 FITC (50µg/mL), CD63 FITC (50µg/mL) or CD9 PE (50µg/mL) (all from Miltenyi, Germany). Primary antibodies were incubated at 4°C in the dark for 30mins. After this incubation beads were washed twice with isolation buffer and finally resuspended in 400µL of isolation buffer for analysis. Samples were analyzed on a FACSCanto II Flow cytometer (BD Biosciences) equipped with a 488nm and 535nm laser excitation source. A minimum of 10,000 events were recorded per sample. Fluorophore-conjugated immunoglobulins were used as controls. All data was analyzed using the software package FlowJo version vX0.7.

### **2.11. MTT assay**

Skin fibroblasts were seeded at a density of  $1 \times 10^3$  cells/well (100µL SCM) into test wells of a 96-well plate with 4 blank wells containing medium only as a control group. Cells were allowed to adhere for 24h in SCM. After 24 hrs culture, the medium was changed to serum free medium to growth arrest the

cells. After a further 24hrs SEVs (from either 2D or 3D culture) were added at concentrations 0-100µg/mL. Plates were incubated for a further 3 or 5 days. On the day of assessment, 20µL of 5mg/mL MTT (dissolved in PBS and 0.22µm filtered) was added into each well and incubated for 4 hrs at 37°C. After 4 hrs the solution containing MTT and medium was carefully removed and 100µL Dimethyl Sulphoxide was added followed by an incubation for 30 mins at 37°C. The absorbance of the solution was read in a plate reader (540 nm). Each replicate was set up as  $n=6$  with each experiment repeated at least 3 times.

## **2.12. Statistical Analysis**

SPSS was used to analysis data produced and present data as the mean  $\pm$  standard error of the mean (SEM) or standard deviation (SD). After determining equal variances and normal distribution, one or two-way analysis of variance (ANOVA), Bartlett's test and the Kolmogorov-Smirnov test were used respectively to compare the mean. Unpaired two-tailed Student's t-tests was used to demonstrate the statistical significance, with confidence limits set at 95 % and P-values of  $< 0.05$  considered significant.

### **3. Results**

#### **3.1 Evaluation of cells cultured in 2D and 3D environments**

OMLP-PCs were seeded into type I collagen lattices and cultured over a period of 120 hrs. Over this time the cells reorganized the lattice such that its diameter was reduced (Fig. 1A). Cell morphology within these lattices demonstrated that the cells were viable and they were observed to spread out and taken on stellate morphology (Figs. 1B-E). Assessment of cell viability and number demonstrated no significant differences between cells cultured in 2D monolayers versus 3D lattices over a 72 hr period (Figs. 2A&B;  $P>0.05$ ).

#### **3.2 SEV production in 2D monolayers versus 3D type I collagen lattices**

To investigate any differences in the yield of SEVs produced in 2D versus 3D conditions, the particle concentration of the purified SEVs was determined by NTA. As demonstrated in Fig. 3A, the concentration of SEVs derived from 3D was significantly higher than from 2D monolayers ( $P<0.01$ ). Figs. 3B&C demonstrate the modal particle size for both 2D- and 3D-derived SEVs. 3D derived SEVs demonstrated a modal size of 118.1nm whilst 2D SEVs demonstrated a modal size of 92.9 nm, both within the expected range for SEVs. SEVs are also known to be enriched in tetraspannins; CD9, CD81 and CD63 (6). To analyse tetraspannin expression on EVs, vesicles were captured on CD63 coated beads and then levels of tetraspannins determined by flow cytometry. As shown in Fig. 4, SEVs produced by both 3D and 2D culture of OMLP-PCs were positive for CD9, CD63 and CD81.

### **3.3 Functional assessment of SEVs derived from cells cultured in 2D and 3D**

To investigate the effects of SEVs derived from OMLP-PCs cultured in monolayer and 3D conditions on cellular proliferation, skin fibroblasts were cultured with/without SEVs before an MTT assay was undertaken. After 3 days in culture there was no significant difference in the proliferation of the skin fibroblasts when they were exposed to either 2D or 3D SEVs (Fig. 5;  $P>0.05$ ). However, after 5 days there was a significant, dose dependent, increase in cell growth on exposure to the 2D SEVs ( $P<0.05$  at  $100\mu\text{g/mL}$  and  $P<0.01$  at  $50\mu\text{g/mL}$ ). However, such an effect was not observed for the 3D SEVs (which failed to drive cell proliferation ( $P>0.05$ )).



#### 4. Discussion

It is well reported that cells cultured in 2D and 3D generally perform differently, as 3D cell culture allows cells to grow in a complex, immersive physical structure which is similar to the environment *in vivo* (26). SEVs, which contain varying cargos, can work as the messengers among cells and it could be these vesicles that are key in driving differential cell behavior in 2D and 3D environments. Hence, the aim of this study was to compare SEVs from OMLP-PCs cultured in 2D and 3D (type I collagen) environments.

OMLP-PCs survived well with the 3D collagenous environment, spreading out and reorganizing the lattice. The particles derived and collected from both 2D and 3D conditions were confirmed as SEVs by NTA analysis and flow cytometry - particles demonstrated a modal size of between 30-130nm and positivity for the tetraspannins (CD9, CD81 and CD63) as would be expected for SEVs (27). NTA analysis demonstrated that the concentration of SEVs derived from 2D cultures was lower than that derived from 3D cultures however, there was no significant difference in modal size between them. This suggests that utilization of 3D cell culture systems could increase SEVs yield potentially offering a route to wider scale up if optimized further. In support of the findings in this paper, the yield of SEVs derived from 3D MSCs has also been reported to be increased when such cells were cultured in 3D environments (28). Furthermore, according to the research of Reka which utilizes bioreactors, 3D cell culture can potentially increase SEV yield by up to 20 times when compared to 2D monolayer cultures (28).

An MTT assay demonstrated that SEVs derived from 2D cell culture were functionally different to SEVs derived from 3D cultures in increasing fibroblast proliferation. This indicates that the culture environment plays an important role in the functionality of the secreted EVs. Unlike Zhang et al. (22), in this paper it was observed that 2D SEVs drove an increase in cell proliferation whereas 3D culture derived SEVs did not. A key difference is that these SEVs were produced from cells as a part of a static 3D cell culture system which may influence the cells ability to be exposed to oxygen and nutrients and to clear out waste metabolites. Furthermore, as there are differing cargos that constitute the SEV make up (29), it would be expected that a different cell population (in this case OMLP-PCs) would result in different SEV cargo loading and hence this would lead to a different functionality of such entities. Also, in the experiments reported here both frozen and fresh CM was utilized for SEV isolation. The work of Mijung et al (30) reports that the best storage condition for exosomes (SEVs) is in a frozen state. Hence, the fact that SEVs were either used fresh or from frozen stocks in the studies presented here suggests that little difference would be observed,

Interestingly, the findings reported here demonstrated that 2D-derived SEVs can stimulate cell proliferation whilst 3D (more *in vivo*-like)-derived SEVs do not. This actually may represent a distinct advantage typical of the anti-scarring nature of the oral mucosa in that fibroblast number can be effectively controlled, something not observed during normal fibrosis/scarring. Further investigations are therefore warranted to determine the future potential of 3D-derived SEVs as anti-scarring therapies.

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