Two and three-dimensional graphene substrates to magnify osteogenic differentiation of periodontal ligament stem cells

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ABSTRACT

Graphene can induce osteogenic differentiation of stem cells. However, the cellular mechanisms involved in this process remain unexplored. Our objective was to investigate key factors, in both genomic and protein level, involved in the osteogenic differentiation of periodontal ligament stem cells (PDLSCs) in two and three-dimensional graphene substrates. PDLSC were seeded on glass slides (Gl); Gl coated with graphene (2DGp), three-dimensional graphene scaffold (3DGp) and polystyrene scaffold (PS) and cultured with and without osteogenic medium for 28 days. All the substrates allowed stem cell survival and proliferation. 2DGp and 3DGp induced the differentiation of PDLSC into mature osteoblasts at higher levels as compared to Gl and PS. Bone-related gene and proteins (COL I, RUNX2, OCN) were upregulated on graphene regardless the use of osteogenic medium. The high expression of MHY10 and MHY10-V2 on 2DGp and 3DGp suggest that their physical characteristics may play a role in the enhanced differentiation. As the results were boosted by the use of osteogenic medium, we suggest that both chemical and physical properties of graphene act synergistically while ruling osteoblastic differentiation of PDLSC.

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1. Introduction

Graphene is single layer of carbon tightly packed into a two-dimensional honeycomb lattice that combines extreme mechanical strength, high electrical and thermal conductivity, impermeability to gases and optical transparency [1,2]. Chemical vapor deposition (CVD) is a scalable method for production of high quality graphene in large scale that can be transferred to various substrates. Although hydrophobic, CVD-grown graphene is a non-cytotoxic substrate that allow cell attachment and proliferation [3–8]. The material is capable to maintain induced pluripotent stem cells (iPSC) in the...
undifferentiated state [7] and also to induce stem cell differentiation into specific lineages [9]. Substrates coated with graphene-based materials are capable to promote the differentiation of human embryonic stem cells into cardiomyogenic cells [10] and to enhance differentiation of human neural stem cells into neurons [8].

The remarkable effects of graphene in osteogenic differentiation of stem cells support its introduction as an alternative material for bone regeneration [3–6,9,11–15]. Graphene-based materials can accelerate the differentiation towards osteoblast-like cells [3–6]. Mesenchymal stem cells (MSCs) cultured on graphene substrates present early maturation and higher degree of mineralization [4,6,13]. Similar phenomena were also observed with the used graphene oxide nanoribbon (GONR) and reduced GONR (rGONR) grids for MSCs culture. Under stimulation of osteogenic induction medium, these substrates increased the mineralized deposition by 6.4 and 16.3-fold, respectively, as compared to PDMS and glass [3]. Interestingly, graphene-coated polyethylene terephthalate enhances mineralized deposition even without the use of bioactive proteins such as BMP-2 [6]. Moreover, MSCs exhibit accelerated osteogenic differentiation when cultured on graphene sheets as compared to GO and PDMS in the presence of an osteogenic induction medium [5,6]. This behavior might be attributed to the high Young modulus of graphene [6,16]. Although physical properties have an important role on the stem cell fate [17], the role of such intrinsic property of graphene in the enhanced differentiation is still arguable [3,5,6].

While these findings are exciting, the use of flat surfaces in bone research does not take into full account the three-dimensional geometry of corporeal structures. It is well known that cytokine transport, cell signaling and tissue development are affected by the spatial geometry of the microenvironment [18,19]. Recently, three-dimensional graphene constructs (3DGp) were found to enhance neural stem cell proliferation and astrocytes differentiation [20]. Likewise, 3DGp was capable to maintain cell viability and induce osteoblastic differentiation of MSCs. After being cultured in 3DGp for 7 days, MSCs present positive expression of osteocalcin and osteopontin without the use extrinsic chemical inducers [21]. Nonetheless, there is no report regarding the expression profiles of bone-related genes and proteins for osteogenic differentiation on graphene over time. Unveiling these events is a key step towards the applicability of graphene in clinical scenarios for bone regeneration.

Bone marrow (BM) stem cells or BM-derived MSCs are widely used to test the effects of graphene in osteogenesis. BM-derived stem cells are the gold standard for bone research, however they present disadvantages such as the low frequency of nucleated cells obtained from large quantity of sample and the decline in proliferation and differentiation capability with age [22,23]. Alternatively, MSCs from oral sources, such as periodontal ligament stem cells (PDLSCs), can be obtained from both deciduous and permanent teeth. Comparing to the aspiration used to obtain BM stem cells, the procedure performed to obtain PDLSC present several advantages: (a) tooth extraction is considered a minor surgery, (b) it is performed under local anesthesia, (c) no esthetic damage and (d) low cost [19,24,25]. The expression of markers of stemness STRO-1/CD146 by PDLSC is similar to other mesenchymal stem cells [26]. Furthermore, they can also differentiate and generate both soft and hard tissues making them an attractive cell model for tissue engineering research [24,26,27]. Here, we explore the use of two- and three-dimensional graphene as substrates to induce osteogenic differentiation of PDLSC. Our results show that graphene-based substrates permitted PDLSC attachment and proliferation and promoted spontaneous and stimulated osteogenic differentiation. Furthermore, the expression of bone-related and genes and proteins were upregulated in cells cultured in graphene-based substrates.

2. Methods

2.1. Substrate preparation

2DGp was obtained as previously described [28]. Briefly, graphene was coated on copper foils by CVD using a mixture of hydrogen and methane at 1000 °C. The graphene-coated copper foil was etched in 1.5% APC for 8 h. Subsequently, the graphene film was transferred to deionized water for 24 h. The transfer was completed by gently contacting graphene film with glass slide and lifting it out of the water. To remove possible residues from the transfer process, the CVD-grown graphene (2DGp) samples were left in warm acetone for 12 h followed by 3 h of incubation in isopropanol.

CVD was also used to synthesize 3DGp using Ni foam as template (45 mm × 45 mm, density 320 ± 25 g/m², pore size: ~500 μm, Alantum Advanced Technology Materials, China). After the CVD process, the Ni scaffold coated with graphene was placed in a FeCl₃ solution for 72 h at room temperature and rinsed with deionized water for 72 h to remove the etching agents. Raman spectra of graphene substrates were obtained (Raman Microscope CRM 200, Witec, Germany) at room temperature, with an excitation laser source of 532 nm (available in the Supporting Information). The laser power was kept below 0.1 mW to prevent heating of the sample.

Glass slide without graphene (Gl) and a polystyrene scaffold (PS) designed for three-dimensional cell culture (Alvetex® Scaffold 12-well plate format, Reinnervate, United Kingdom) were used as controls.

2.2. Cell culture and characterization

The use of human cells in this study was approved by NUS Institutional Review Board (Approval Number: NUS 2094). Human primary periodontal ligament fibroblasts from single donor were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, USA), supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) [19]. Cells were purified to obtain periodontal ligament stem cells (PDLSCs) by low density seeding method (200 cells/75 cm²), maintained at 70–80% confluence, and harvested (TrypLE, Invitrogen). The isolated PDLSCs were sorted by fluorescence-activated cell sorting analysis (BD Fortessa, BD
Biosciences, Germany) for CD146, CD90 and CD44 (Human Mesenchymal Stem Cell Characterization Kit, Millipore, USA). Results are shown in the Supporting information.

PDLSC were cultured in the same medium used for primary periodontal ligament fibroblasts.

2.3. Osteogenic differentiation of PDLSCs on graphene-based substrates

PDLSC (5 × 10^3 cells, passage 3) were seeded onto the surface of different substrates and left undisturbed for 24 h.

Fig. 1 – Cell proliferation (a) all the substrates were non-cytotoxic and allowed cell proliferation. After 5 days, 3DGp presented the highest proliferation followed by 2DGp and Gl. Osteogenic differentiation (b) and (c) all groups presented higher levels of mineral deposition after 28 days as compared to 14 days. After 28 days, 2DGp and 3DGp under CM presented higher mineralization as compared to Gl and PS with OM (* denotes statistical difference between the groups). (A color version of this figure can be viewed online.)

Fig. 2 – SEM images show that PDLSC could efficiently adhere and proliferate in the substrates after 5 days (scale bar = 50 μm).
Morphology and proliferation of cells were evaluated for 5 days through scanning electron microscopy (SEM, S-3400N, 5.0 kV, Hitachi, Japan). Cell viability was assessed for 5 days using MTS assay (CellTiter 96 AQueous One Solution Assay, Promega, USA).

Osteogenic differentiation induction medium (OM) was used to promote osteoblastic differentiation. Cells were treated with OM [100 nM dexamethasone (Sigma–Aldrich, USA), 5 mM β-glycerophosphate (Sigma–Aldrich), 50 μg/ml ascorbate phosphate (Sigma–Aldrich) and 10% FBS (Invitrogen)] in DMEM (Invitrogen) for 28 days. Culture medium (CM), as described in Section 2.2, was used as control. Mineral deposit formation was quantitatively assessed after 14 and 28 days with alizarin red S staining. Cells were washed by phosphate buffered saline (Invitrogen) and fixed with 4% paraformaldehyde (room temperature, 20 min). After washing with deionized water, cells were stained with 40 mmol/L of alizarin red (Sigma–Aldrich) in distilled water (pH of 4.2 maintained with ammonium hydroxide) in 37°C incubator for 30 min. Samples were treated with 10% cetylpyridinium chloride solution (Sigma–Aldrich) at room temperature for 15 min and the absorbance was measured by microplate reader (Infinite M200, Tecan, Germany) at a wavelength of 540 nm. Negative controls were substrates devoid of cells. Here, and throughout this work, statistical analyses were performed with Mann–Whitney test ($\alpha = 5\%$, SigmaStat 2.0, SPSS, USA).

Gene expression was obtained based in the ΔΔCq method for calculating relative gene expression from quantification cycle (Cq) values obtained by quantitative real-time PCR (qPCR) analysis. The PDLSCs from each group were harvested and total RNA was isolated (Purelink RNA Mini Kit, Invitrogen). Following, cDNA synthesis was performed (iScript RT Supermix, Bio-Rad, USA) and the expression of the target genes [collagen type I (COL I), Runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), myosin heavy chain 10 (MYH10), myosin heavy chain 10 transcript variant 2 (MYH10-V2)] and reference genes GAPDH and β-actin were quantitatively assessed. The oligonucleotide primer sequences shown in Table S1 in SI. The mRNA expression levels of target genes were normalized according to previously described [29]. Our data were normalized using β-actin as there was no statistical difference in the expression of the reference genes for any of the conditions tested (target gene, substrate and time point).

Protein expressions of COL I, OCN and RUNX2 were evaluated after 14 and 28 days. Briefly, samples were fixed with 4% paraformaldehyde for 20 min and incubated with primary antibodies COL I (1:200), OCN (1:300) and RUNX2 (1:200) (Abcam, United Kingdom) overnight at 4°C. The secondary antibody labeled by FITC was used and incubated at 37°C for one hour. The specimens were counterstained with DAPI (Invitrogen). Fluorescent stains were imaged on a confocal microscope (FV1000 camera, Olympus Optical, Japan). Protein expression was not imaged in PS due to strong background staining.

3. Results and discussion

All the substrates allowed cell attachment and proliferation (Figs. 1a and 2). After 5 days, 3DGp presented the higher proliferation as compared to PS (Fig. 1a). This difference might be related by the bigger surface area available for cell spreading in this substrate. There was no difference between Gl and 2DGp for any period evaluated. SEM images showed that PDLSCs efficiently adhered on all experimental substrates after 1 day of culture. After 5 days the substrates were covered by cells (Fig. 2).

Previous studies showed that graphene holds potential to sustain osteogenic differentiation of BM and BM-derived MSCs [5,6,9,14,21]. As PDLSC can be obtained from naturally exfoliated teeth, which is one of the only disposable postnatal human tissues, the interest towards stem cells from oral sources for regenerative dentistry and medicine has increased [24,27]. Our results show that graphene-based
substrates induce the differentiation of PDLSC into functional osteoblasts, capable of secreting mineralized matrix (Fig. 1b and c). Remarkably, after 28 days, 2DGp and 3DGp under CM presented higher levels of mineralization as compared Gl and PS with OM, exposing the capability of graphene to promote spontaneous osteogenic differentiation of PDLSC. Nonetheless, these phenomena were enhanced with the use of chemical inductors for osteogenic differentiation. These can be attributed to the capability of graphene to adsorb typical osteogenic inducers such as dexamethasone and β-

Fig. 4 – Expression of bone-related markers on PDLSC cultured on 3DGp and PS. With exception of the expression of RUNX2 obtained for PS + OM (7 days), all the groups were significantly higher than the control (PS + CM = dashed line). For all the genes, 3DGp + CM presented higher expression than PS + OM for all time points evaluated. The use of OM increased all the expressions in 3DGp in comparison to its 3DGp + CM (*"within the bar: similar to Gl + CM; " denotes statistical difference between the groups). (A color version of this figure can be viewed online.)

Fig. 5 – RUNX2 protein expression. After 14 days, the expression of RUNX2 (green) can be detected for 2DGp even without the use of OM. After 28 days under OM, the expression on 2DGp is stronger as compared to Gl. 3DGp present positive expression throughout the structure regardless the use of OM for both time point evaluated (nuclei were stained with DAPI (blue); scale bar = 50 μm). (A color version of this figure can be viewed online.)
glycerophosphate \cite{3,5} Similar trends were reported for BM stem cells cultured on CVD-grown graphene and MSC from umbilical cord blood cultured on graphene oxide or reduced graphene oxide substrates \cite{3,5}. Nonetheless, the cells used on those studies failed to deposit minerals without the use of inducers contrarily to what we have observed for PDLSC here (Fig. 1b and c). Collectively, our data show that 2DGp and 3DGp are not only capable to amplify, but also to induce the deposition of mineralized matrix by PDLSC.

To shed a light in the possible cellular mechanisms involved in these enhanced differentiation, we have analyzed, separately for two (Gl and 2DGp) and three-dimensional substrates (3DGp and PS), the expression of genes and proteins related to different stages of osteogenic differentiation.

Graphene-based substrates presented high gene and protein expression of RUNX2 irrespective of the presence of OM (Figs. 3–5). RUNX2 is a member of the RUNT-domain family of transcription factors that activates COL I and OCN \cite{30,31}. It is essential for osteoblastic differentiation and skeletal morphogenesis acting as a scaffold for nucleic acids and regulatory factors involved in skeletal gene expression \cite{32}. Furthermore, RUNX2 can upregulate the expression of OCN, which is the bone marker expressed in late stages of osteoblastic differentiation \cite{32,33}. Immunofluorescence analysis for RUNX2 (Fig. 5) showed higher expression in cells on 2DGp after both 14 and 28 days in comparison to Gl. Such expressions were only observed for Gl with the use of OM. Notably, RUNX2 was evenly expressed throughout the 3DGp after 14 days regardless the presence OM (Fig. S3 in SI).

Collagen is the main component of bone matrix and it is the most abundant osteoblast-specific noncollagenous proteins. COL I gene is expressed during the developmental sequence of osteoblast differentiation and play critical roles in regulation cell differentiation, bone matrix formation and mineralization \cite{31,34}. Cells cultured on 2DGp under CM presented a modest increase in the expression of COL-I as compared to the expression observed using OM. This can be due to the lack of ascorbic acid in CM. Ascorbic acid, is important for collagen type I formation and osteoblastic differentiation \cite{35}. In fact, more collagen is formed in animals receiving adequate ascorbic acid than in those deprived of it \cite{35}. The increase of COL I expression on 2DGp with the use of OM (Figs. 3 and 6), corroborate the findings of Lee et al. that graphene may act as a concentrator of ascorbic acid \cite{5}.

The combination of ascorbic acid and type I collagen upregulates OCN gene expression and increases ALP activity in PDLSC \cite{36}. Thus, it is reasonable that both gene and protein expressions of OCN (Figs. 3 and 7) are not remarkably high on 2DGp with CM, following the trend observed for COL I. This is of high interest once that the matrix secreted on 2DGp without OM may present dissimilarities in comparison to that obtained with OM or to the one that naturally occurs in the body. Nonetheless, more investigation is needed to characterize the matrix obtained.

Fig. 6 – COL I protein expression. After 14 and 28 days, the expression of COL I (green) in 2DGp with or without OM is similar to its Gl counterpart can be detected for 2DGp even without the use of OM. For 3DGp, the use of OM increased the expression of COL I for both time points evaluated (nuclei were stained with DAPI (blue); scale bar = 50 \mu m). (A color version of this figure can be viewed online.)
Notably, the proteins were harmoniously expressed throughout 3DGp (Figs. S3–S5 in Supporting information). The high expression of RUNX2, COL-I and OCN in 3DGp in absence of OM may be attributed to the spatial arrangement of the material. Three-dimensional culture systems have strong chondrogenic potential and present higher COL-I mRNA expression than two-dimensional ones [37]. In fact, stem-cell populations are established in three dimensional anatomic locations that regulate how they participate in tissue generation, maintenance and repair [24,38]. Thus, cells placed within three-dimensional scaffolds exhibit high attaching and cloning efficiencies, fast proliferation and longer life span [19,24,39]. The ability of cells in 3DGp to express high levels of COL-I and OCN when deprived of OM (Figs. 6 and 7) may be further explained by the fact that cells cultured in three-dimensional environments respond better to hormones and exhibit lower requirements for serum and growth factors [39].

Physical characteristics of the substrates play an important role in cell differentiation. Stem cells present lineage commitment with extreme sensitivity to tissue-level elasticity [17,40,41]. Soft substrates (PET and PDMS) coated with graphene presented similar OCN expression and calcium deposition as compared to stiffer substrates (glass and Si/SiO$_2$) [6]. To further explore the role of physical characteristics of graphene in the osteogenic events we evaluated the gene expression of MYH10 and MYH10-V2 (Fig. 8). MYH10 is a cytoskeletal protein tightly associated with mitochondrial DNA [42] which changes as the substrate rigidity increases and its polarization is suppressed in cells on soft substrates. The inhibition of MYH blocks all elasticity-directed lineage specification. It is involved in cell force signaling pathway and encodes a member of the myosin superfamily. Myosins are actin-dependent motor proteins with diverse functions including regulation of cytokinesis and cell motility [17].

In our study, significant increases of MYH10 and MYH10-V2 were observed for 3DGp in comparison to PS irrespective of the use of OM (Fig. 8). PS has a low elastic modulus (~3–3.5 GPa) as compared to graphene (~1–2.4 TPa [16,43]). Thus, the substrate stiffness might be one of the factors creating exogenous forces that could stimulate the mechanoregulatory circuit modulating the cytoskeletal tension [17,44]. In fact, intrinsic material properties were already pointed as a driving force for spontaneous osteogenic differentiation of MSCs in titanium nanotubes in the absence of biochemical stimulation [45]. The elastic modulus of glass used here (~68 GPa) was higher than PS but still 15 times lower than graphene. As it was suggested that graphene, when used as a coating, only slightly increased Young’s modulus of substrate [5], the high expressions of MYH10 and MYH10-V2 could also be correlated to the flexibility for out-of-plane deformation of graphene [6] and the presence surface characteristics such as wrinkles and ripples [3]. Nonetheless, the substrate stiffness

![Image](image-url)
directly regulates stem cell lineage specification and may enhance osteoblastic differentiation by upregulating the expression of RUNX2, OCN and COL I as we have observed here in for 2DGp and 3DGp (Fig. 4).

We need to highlight that the three-dimensional scaffolds used (3DGp and PS) differ not only by the material that they are made of but also in geometrical features (e.g. superficial area, pore size). The latter are well recognized to have effects on stem cell differentiation [24]. Hence, one should not extrapolate the findings from the genetic analysis and mineralization deposition to favor 3DGp over PS as the latter was used merely as a control for cell differentiation and assays.

4. Conclusion

As PDLSC can be obtained from human disposable tissues without major surgical procedures, the potential of graphene to promote their osteogenic differentiation have significant impact in regenerative dentistry and medicine. In this study, graphene acted as non-cytotoxic substrate capable to induce osteogenic differentiation of PDLSC. We showed that the material, in both two and three-dimensional arrangements, increases the genomic and protein expression of RUNX2, key transcription factors involved in the early stages of osteogenesis, and OCN, a late bone marker in osteoblasts. Most remarkably, graphene increased the gene expression of MHY10 and MHY10-V2. As these findings were improved with the use of OM, we can suggest that both physical and chemical characteristics of graphene work synergistically while enhancing differentiation. Further understanding of the extension of graphene’s physical properties on the mechanisms ruling osteoblastic differentiation of stem cells will broaden the avenue to use the material as a substrate for bone tissue regeneration.

Conflict of Interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Stem cell and graphene characterization, cross-section of 3DGp showing RUNX2, COL I and OCN expression. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbon.2015.05.071.

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