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Nanodiamond modified copolymer scaffolds affects tumour progression of early neoplastic oral keratinocytes

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ABSTRACT

This study aimed to evaluate the tumorigenic potential of functionalising poly(LLA-co-CL) scaffolds. The copolymer scaffolds were functionalised with nanodiamonds (nDP) or with nDP and physisorbed BMP-2 (nDP-PHY) to enhance osteoinductivity. Culturing early neoplastic dysplastic keratinocytes (DOK^{Luc}) on nDP modified scaffolds reduced significantly their subsequent sphere formation ability and decreased significantly the cells' proliferation in the supra-basal layers of *in vitro* 3D oral neoplastic mucosa (3D-OT) when compared to DOK^{Luc} previously cultured on nDP-PHY scaffolds. Using an *in vivo* non-invasive environmentally-induced oral carcinogenesis model, nDP scaffolds were observed to reduce bioluminescence intensity of tumours formed by DOK^{Luc} + carcinoma associated fibroblasts (CAF). nDP modification was also found to promote differentiation of DOK^{Luc} both *in vitro* in 3D-OT and *in vivo* in xenografts formed by DOK^{Luc} alone. The nDP-PHY scaffold had the highest number of invasive tumours formed by DOK^{Luc} + CAF outside the scaffold area compared to the nDP and control scaffolds. In conclusion, *in vitro* and *in vivo* results presented here demonstrate that nDP modified copolymer scaffolds are able to decrease the tumorigenic potential of DOK^{Luc}, while confirming concerns for the therapeutic use of BMP-2 for reconstruction of bone defects in oral cancer patients due to its tumour promoting capabilities.

1. Introduction

Extensive efforts have been made in bone tissue engineering (BTE) to combine osteogenic growth factors with scaffolds in order to control their release, thus regenerating bone defects with minimal side effects [1]. Bone morphogenetic proteins (BMPs) are members of the large TGF-β superfamily, which play a crucial role in regulating cell proliferation, apoptosis, differentiation and

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organogenesis [2]. Clinical trials using large amounts of BMP-2 in an absorbable collagen sponge were carried out in several orthopaedic approaches. These ranged from spinal fusions to long bone fractures and more recently augmenting the alveolar ridge, cleft reconstructions and sinus lift surgeries [3–5]. The use of BMP-2 was reported advantageous over the 'gold standard', autologous bone graft, by shortening the operating time and decreasing hospitalisation costs. However, its off-label use even in oral and maxillofacial surgeries came with several side effects [6–8]. Those side effects were attributed to uncontrolled release of high doses of the embedded growth factor. Cancer has been an alarming adverse effect reported by studies on spinal surgeries using BMP-2, while some studies showed a link between use of BMP-2 and carcinogenesis; others did not find this association [9,10]. Nevertheless, the observed increase in cancer incidence, while low and uncertain, remains a real concern for the use of recombinant BMP-2 (rhBMP-2). In addition, several studies expressed concern and had implications for the safe delivery of BMP-2 when reconstructing bone defects after extensive surgeries for treating oral squamous cell carcinomas (OSCC) [11,12]. *In vitro* and *in vivo* investigations of the effects of BMP-2 on OSCC cells showed an increase in the invasive potential and poor survival rate after exposure to BMP-2 [11,12]. Moreover, this association was also observed for gastric and breast cancer cells [13]. The association between BMP-2 and OSCC was also indicated from patient studies. In a cohort of 149 patients with oral, head and neck squamous cell carcinoma analysed retrospectively; tumours with higher BMP-2 expression were found to have higher rates of local failure and local recurrence [14]. In normal human keratinocytes *in vitro*, BMP-2 was shown to modulate the expression of molecules related to Wnt signalling which may be affected by the fate of keratinocytes, such as their proliferation, migration and differentiation [15]. Also, epithelial to mesenchymal transition in human skin would healing was shown to be modulated by BMP-2 in human normal keratinocytes *in vitro* [16].

Nanodiamond (nDP), a carbon derived nanoparticle of about 4–5 nm in diameter with low chemical reactivity and unique physical properties [17,18] have emerged as potentially useful materials in bone regeneration. As a stable colloid, they were used to deliver osteogenic molecules such as BMP-2 for promoting bone formation [19]. Cell viability assays showed that nDP are not toxic to a variety of cell types [20]. They have been tested for their cytotoxicity in suspensions and it was reported that higher concentrations affect cell proliferation and metabolic activities in macrophages [21]. Nano-sized materials demonstrated controversial results during long term implantations in murine models [22], but assessment of carcinogenicity of nDP is an unexplored area, especially in the context of biomaterial modifications.

Aliphatic polymers have been commonly used in the biomedical field and also approved by the Food and Drug Administration in several clinical products [23]. Copolymer scaffolds synthesised from L-lactide (LLA) and ε-caprolactone (ε-CL), (poly[LLA-co-CL]), have been comprehensively validated as BTE scaffolds with encouraging cyto-compatibility and osteoconductivtiy outcomes both *in vitro* and *in vivo* [24–26]. Modifying these copolymer scaffolds with nDP improved their mechanical properties and enhanced their wettability which positively affected their osteoconductive potential [27,28]. A modality of delivering BMP-2 to bone defects using poly(LLA-co-CL) scaffolds modified with nDP was recently developed in an attempt to reduce side effects from conventional high dose burst delivery. It proved successful physisorption of BMP-2 onto the nDP; sustained low amounts of bioactive BMP-2 released up to 70 days, and enhanced osteogenic differentiation of human mesenchymal stem cells, in addition to accelerated bone formation in a rat mandible critical-sized defect [29]. This construct carries two stimulating factors, nanodiamond particles and BMP-2, which makes a testing of its biocompatibility on one hand side and long term adverse effects on the other hand crucial. Biomaterials implanted, such as those for the purpose of tissue regeneration have been reported on their potential of inducing foreign body carcinogenesis and are usually tested in long term 2 year rodent assays or half a year in transgenic mice [30]. Although this phenomenon is rarely encountered in humans [31], it cannot be overlooked and every new biomaterial is required to rigorously undergo adverse effect testing for regulatory reasons [32].

An *in vivo* environmentally-induced oral carcinogenesis model to screen the tumorigenic potential of BTE scaffolds has been recently reported by our research group to successfully and reliably monitor the scaffolds by bioluminescence (BLI) in an attempt to surpass the limitations of the long term rodent assays [33]. The model has been developed using early neoplastic dysplastic oral keratinocytes (DOK) and the poly(LLA-co-CL) scaffolds. The DOK are derived from a tongue dysplasia (premalignant oral mucosa lesion) that progressed after 11 years into a well-differentiated OSCC [34]. They were reported to be partly transformed but non-tumorigenic in NUDE mice and our previous research on non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice showed that DOK at a low density were tumorigenic only when co-inoculated with carcinoma associated fibroblasts (CAF) [35]. The use of dysplastic cells instead of normal keratinocytes was carried to gain advantage over the time spent to reproduce mutagenic events in tumour models with experimental setting from normal cells [36]. We therefore chose to use the early neoplastic cells, DOK, as a screening tool to evaluate the tumour promoting potential of scaffolds, providing a faster alternative to the long 'lifetime' *in vivo* models [33].

In this current investigation the developed model was used along with several *in vitro* functional tumorigenicity assays to evaluate the effect of functionalising poly(LLA-co-CL) scaffolds with nDP or with nDP and physisorbed BMP-2.

The authors postulate that the functionalised poly(LLA-co-CL) scaffolds with nDP will have an effect on the differentiation of DOK owing to nDP's inherent functional groups that were shown to enhance the differentiation of other type of cells [28].

2. Materials and methods

2.1. Scaffold fabrication and modification with nDP and nDP + BMP-2

Poly(LLA-co-CL) was synthesised from 75 mol % L-lactide and 25 mol % ε-caprolactone as previously described [26,37] confirmed by proton nuclear magnetic resonance (Bruker Avance 400, Billerica, MA, USA). The purified copolymer had a number average molecular weight of ≈100,000 Da and a molar mass dispersity ≈1.3 determined by size exclusion chromatography (Polymer Laboratories, U.K.). The porous poly(LLA-co-CL) scaffolds were prepared by solvent casting particulate leaching and a disc-shaped scaffold (diameter ≈ 12 mm/thickness ≈ 1.3 mm) for *in vitro* experiments and (diameter ≈ 6 mm/thickness ≈ 1.3 mm) for *in vivo* experiments [26]. Porosity was >83% measured by micro computed tomography, (Skyscan 1172, Bruker) (40 kV and 2.4 μm voxel).

Detonation diamond purified by acid (Gansu Lingyun Corp., Lanzhou, China) was milled for dispersion using a method previously described [38] producing a narrow size distribution at ~5 nm particle diameter (measured by dynamic light scattering in water) and low agglomeration of the diamond particles. Briefly, scaffolds were modified with the nDP solution (2 wt %, i.e. 20 mg/ml) by a vacuum technique: 0.5 ml nDP solution and one scaffold were put in a glass beaker and perfused in vacuum (Oerlikon Leybold, TRIVAC