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Cyclic uniaxial compression of human stem cells seeded on a bone biomimetic nanocomposite decreases anti-osteogenic commitment evoked by shear stress☆

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ABSTRACT

Objective: Chemical supplementation of culture media to induce differentiation of adult stem cells seeded on a scaffold may mask other differentiation triggers such as scaffold stiffness, chemical composition or mechanical stimulation. However, stem cells can be differentiated towards osteoblasts without any supplementation given an appropriate osteogenic scaffold and an adequate mechanical stimulation.

Materials and Methods: Electrospun meshes of poly-lactic-co-glycolic acid and amorphous calcium phosphate nanoparticles (PLGA/aCaP) in a weight ratio of 60:40 were seeded with human adipose-derived stem cells (ASCs) and cultured in DMEM. After two weeks of static cultivation, they were either further cultivated statically for another two weeks (group 1), or placed in a Bose® bioreactor with a flow rate per area of 0.16 mL cm^{-2} min^{1} (group 2). Furthermore, group 3 was also cultivated under perfusion, however, with an additional uniaxial cyclic compression. Stiffness of the scaffolds was assessed as a function of time. After a total of four weeks, minimum stem cell criteria markers as well as typical markers for osteogenesis, endothelial cell differentiation, adipogenesis and chondrogenesis were analyzed by quantitative real-time PCR, cell distribution within the scaffolds by histology and protein expression by immunohistochemistry.

Results: Dynamic conditions (perfusion ± uniaxial cyclic compression) significantly upregulated gene and protein expression of PPAR-γ-2 compared to static cultivation, while osteogenic markers were slightly downregulated. However, the compression in the perfusion bioreactor favored osteogenesis compared to mere perfusion as indicated by upregulation of ALP, Runx2 and collagen I. This behavior was not only attributed to the compressive load, but also to the significant increase in stiffness of the scaffold. Furthermore, CD105 was significantly upregulated under compression.

Conclusions: Although an osteogenic electrospun composite material with an organic (PLGA) and an inorganic phase (aCaP nanoparticles) was used as scaffold, the dynamic cultivation as realized by either perfusion alone or an additional compression did not upregulate typical osteogenic genes when compared to static cultivation. In contrast, there was a significant upregulation of the adipogenic gene PPAR-γ-2. However, this anti-osteogenic starting point evoked by mere perfusion was partially reversed by an additional compression. Our findings exemplify that bone tissue engineering using adult stem cells should consider any other differentiations that may be triggered and overwhelm the desired differentiation, although experimental conditions theoretically provide cues to achieve it – like an osteogenic scaffold and mechanical stimulation.

1. Introduction

Recently, the differentiation of stem cells towards a desired phenotype has gained much attention in regenerative medical approaches (Hu et al., 2016). Besides chemical cues (Zuk et al., 2001), mechanical stimuli have been reported to assist stem cells in their

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differentiation behavior towards different cell types. For example, static compressive load has been applied to the C2C12 pluripotent mesenchymal cell line and genes relevant for osteogenesis and chondrogenesis were upregulated, while genes important for adipogenesis were downregulated (Yanagisawa et al., 2007). Moreover, dynamic compression of rabbit adipose-derived stem cells (ASCs) supported chondrogenesis as assessed by a typical marker gene, Sox9 (Chen et al., 2015). Furthermore, hMSCs exposed to shear stress responded by an increase in BMP-2, bone sialoprotein and osteopontin without chemical supplementation (Yourek et al., 2010). There are many more studies, all including the application of mechanical stimulation of stem cells, leading to osteogenesis (Thompson et al., 2012; Sittichokechaiwut et al., 2010; Brunelli et al., 2017; McCoy and O'Brien, 2010; Nii et al., 2013), to chondrogenesis (Chen et al., 2015; Thorpe et al., 2010; Mathieu and Loboa, 2012), to adipogenesis (Mathieu and Loboa, 2012; Huang et al., 2010) or endothelium cell differentiation (Bai et al., 2010). Noteworthy to mention, also the absence of load realized by microgravity experiments, has a remarkable influence on the gene expression, like chondrogenic commitment in adipose-derived stem cells (Yu et al., 2011) with enhanced collagen II/I gene expression (Wuest et al., 2015).

Besides mechanical stimulation of stem cells, another important factor is the substrate stiffness (Engler et al., 2006; Discher et al., 2005; J.W. Zhang et al., 2017; T. Zhang et al., 2017; Andrea et al., 2016; Diederich et al., 2013; Teong et al., 2017; Mullen et al., 2015; Murphy et al., 2012; Keogh et al., 2010; Olivares-Navarrete et al., 2017; Chen et al., 2010). For instance, Zhang and coworkers examined polydimethylsiloxane-based matrices with different degrees of stiffness and examined ASCs' differentiation along the osteogenic and adipogenic pathways (J.W. Zhang et al., 2017; T. Zhang et al., 2017). They reported a preferred osteogenic differentiation on harder matrices (mimicking stiffness of osteoids), while ASCs turned towards adipocytes on softer substrates (J.W. Zhang et al., 2017; T. Zhang et al., 2017). For collagen-glycosaminoglycan, scaffolds of 0.5 kPa stiffness facilitated chondrogenesis of MSCs, while scaffolds with a stiffness of 1.5 kPa directed the MSCs towards the osteogenic lineage; stiffness was varied by different crosslinking treatments (Murphy et al., 2012). However, optimum stiffness to promote osteogenesis cannot simply follow the advice “the harder the better”, but must take into account that early osteogenesis as characterized by upregulation of Runx2 occurs with decreasing stiffness, while maintenance of mature osteoblasts requires stiffer matrices (Olivares-Navarrete et al., 2017). Furthermore, trends may be the other way round with accelerated formation of bone matrix for lower Young's moduli, as reported for three-dimensional Zr-Si organic-inorganic scaffolds seeded with human ASCs (Koroleva et al., 2015). In addition to stiffness, the topography and chemistry of respective scaffolds may influence the material-driven differentiation, as shown for calcium phosphate ceramics (T. Zhang et al., 2017; J.W. Zhang et al., 2017) or for polymeric foams (Viswanathan et al., 2015).

Hence, if mechanical stimulation of a cell-seeded scaffold is examined, not only the direct effect on the cells has to be considered, but also simultaneous changes in stiffness during the mechanical stimulation process. For example, a 2.65-fold change in stiffness after 6 weeks of cultivation was reported for a dehydrothermally treated collagen-glycosaminoglycan scaffold seeded with MC3T3 pre-osteoblastic cells – even without mechanical stimulation (Keogh et al., 2010). Application of mechanical stimulation might *a fortiori* influence the stiffness. However, reports on cell-loaded scaffolds cultivated in perfusion bioreactors (McCoy and O'Brien, 2010) often do not address such changes of stiffness during the time window of dynamic stimulation and cultivation (McCoy and O'Brien, 2012); rather focusing on a “net” outcome in terms of an optimum tissue engineered construct.

Here, we examined the impact of a perfusion regimen with or without additional compression on the scaffold stiffness of a well-established bone biomimetic nanocomposite intended at osteogenesis and seeded with human ASCs. Changes in gene expression of ASCs caused by mechanical stimulation and simultaneous changes in stiffness were assessed under static and dynamic conditions. The nanocomposite was a 60:40 w/w PLGA: amorphous calcium phosphate (aCaP) nanoparticles (Loher et al., 2006). Good adhesion to and proliferation within PLGA/aCaP nanocomposite has been reported for human ASCs in vitro under static cultivation (Buschmann et al., 2012; Gao et al., 2014) and under perfusion and compression (Baumgartner et al., 2015). *In vivo* performance of PLGA/aCaP and bone formation was successful in sheep (Schneider et al., 2011) and in rabbits (Schneider et al., 2009).

We hypothesized that

(i) shear stress as induced by perfusion cultivation of human ASCs seeded on a bone biomimetic PLGA/aCaP nanocomposite enhances typical osteogenic gene expression compared to static cultivation (Yourek et al., 2010), while downregulating other genes such as relevant for adipogenesis, chondrogenesis or endothelium cell differentiation.
(ii) an additional cyclic compression regimen (Chen et al., 2017) together with the shear stress evoked by the perfusion increases the scaffold stiffness, promoting even more osteogenic differentiation of human ASCs on PLGA/aCaP compared to mere perfusion.

2. Materials and methods

2.1. Cell isolation

Human ASCs were isolated from fat tissue with the consent of the patient according to Swiss (KEK-ZH: StV 7–2009) and international ethical guidelines (ClinicalTrials.gov Identifier: NCT01218945) as reported in (Buschmann et al., 2013). The extraction procedure was performed following Zuk *et al.* Zuk et al. (2001). ASCs were characterized according to established procedures (Buschmann et al., 2012; Gronthos et al., 2001). Of the 30 isolated primary ASC lines (Buschmann et al., 2013), one was selected based on findings in a previous study concerning its differentiation capacity; it was shown to differentiate easily towards the endothelial cell (EC) phenotype and moderately to well towards osteoblasts (OBs) (Gao et al., 2014). Hence, it seemed a challenge to direct this cell line towards osteogenesis without chemical supplementation. The fat for these primary cells had been received from a 29-year old woman by abdominal liposuction.

2.2. Multilineage cell differentiation

Lineage specific differentiation of ASCs towards the OB, the EC, the adipogenic and the chondrogenic cell lineage were achieved using cell culture media supplementation according to Zuk et al. (2001). Von Kossa and Alizarin red staining were used to semi-quantitatively evaluate osteogenic differentiation extent, CD31 immunohistochemical staining to see the endothelial cell differentiation, Alcian Blue staining in order to evaluate the ability of the ASCs for chondrogenesis and Oil Red O staining to verify adipogenic differentiation as reported previously (Hess et al., 2017).

2.3. Scaffold materials

Clinically approved PLGA (85:15) was received from Boehringer Ingelheim. The aCaP nanoparticles (Ca/P = 1.5) were prepared by flame spray pyrolysis as described by Loher *et al.* Loher et al. (2005) using calcium-2-ethylhexanoic salt (synthesized with calcium hydroxide from Riedel de Haen, Ph. Eur. and ethylhexanoic acid from Sigma-Aldrich) and tributyl phosphate (Sigma-Aldrich, 98%). PLGA/aCaP nanocomposites were prepared according to (Schneider et al., 2008). To combine the two components, the aCaP nanoparticles were dispersed in chloroform (Riedel de Haen, Ph. Eur.) containing 5 wt% (referring to the latter on added polymer) Tween20 (Polysorbate20, Fluka, Ph. Eur.) using an ultrasonic bath (Bandelin Sonorex Digitec). PLGA (6 wt% in