STEM CELL CULTURE

Making bone via nanoscale kicks

Causing nanoscale vibrations in bone-marrow stromal cells embedded in a soft collagen gel induces the cells to undergo osteogenic differentiation and mineralization via mechanosensitive signalling pathways.

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The role of mechanical forces in guiding skeletal-tissue morphogenesis has been well appreciated since Julius Wolff postulated in 1892 that healthy bone adapts to mechanical loads[1]. Applications of Wolff's law quickly found their way into clinical settings, despite poor understanding of how bone cells remodel bone architecture in response to load. Indeed, for decades physical therapists have treated patients through the application of forces, manual therapy or electromagnetic waves (such as ultrasound or extracorporeal shock-wave therapy) to improve skeletal function after injury, or to treat skeletal disorders. More recently, vibrating plates that shake the whole body at a frequency of 30 Hz have become commercially available to complement treatment for osteoporosis[2]. And in orthopaedic surgery, Wolff's law underpins the concept of distraction osteogenesis, a surgical technique involving the stretching of the two ends of a fractured bone to accelerate bone-healing rates.

To tissue engineers who aim to create bone tissue with human-derived bone-marrow stromal cells (hBMSCs) as a starting material, mechanical loading is appealing as an alternative to cell stimulation with expensive growth factors. In order to apply forces to cells, bioreactors that can apply stretch, fluid shear stress and compression to cells adherent to 2D surfaces or embedded in 3D scaffolds have served to demonstrate that mechanical load indeed promotes osteogenic differentiation of progenitor cells and mesenchymal stem cells in vitro. However, most studies have reported the combined effects of soluble cues and mechanical load on osteogenic differentiation, and only a handful of papers have suggested that mechanical load in the absence of osteoinductive soluble factors (such as dexamethasone and bone morphogenetic proteins) may be sufficient to prime cells towards the osteogenic lineage[3,4]. Additional evidence that mechanical cues can drive stem cell differentiation emerged from studies that investigated how cell-generated forces control lineage commitment. These studies demonstrated that by changing cell shape[5] and matrix stiffness[6], hBMSCs alter myosin activity and the RhoA/ROCK (Ras homolog gene family, member A/Rho-associated, coiled-coil containing protein kinase) pathway, which direct lineage commitment in the absence of soluble factors. However, such experiments were performed on cells grown on planar substrates, and hence it remains unclear whether mechanical load is sufficient to drive osteogenic differentiation in hBMSCs embedded in a 3D matrix. Reporting in *Nature Biomedical Engineering*, Matthew Dalby and colleagues now show that nanovibrations can on their own induce osteogenic differentiation of primary hBMSCs embedded in a collagen hydrogel[7], as occurs in 2D cultures[8].

By making use of the reverse piezoelectric effect, Dalby and co-authors built a bioreactor in which an applied voltage drives nanodisplacements of piezoelectric ceramic elements, causing a connected plate to vibrate. The nanovibrating plate is magnetically bonded to a six-well plate containing monolayers of hBMSCs or collagen type-I hydrogels loaded with cells (Fig. 1). Computational finite-element modelling and laser interferometry confirmed that nanovibrations with amplitude of 25 nm at a frequency of 1,000 Hz were transmitted homogeneously through the well plate. These nanovibrations were sensed by the adherent hBMSCs, as indicated by upregulated gene expression for osteogenic gene markers, including osterix, alkaline phosphatase and osteopontin. Because low-stiffness substrates such as collagen are non-permissive to osteogenic differentiation, one would not expect these results. Yet by using rheology, finite-element modelling and laser interferometry,

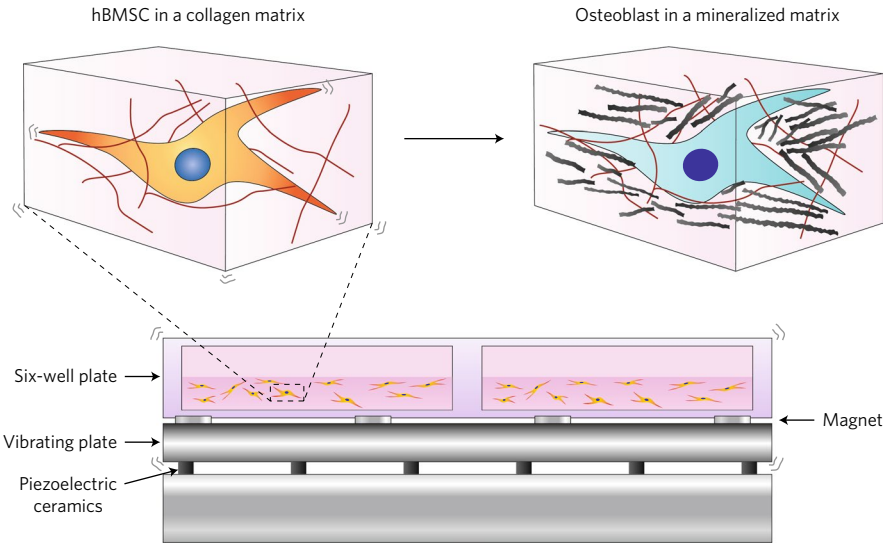

Fig. 1 | Nanovibration-induced osteogenic differentiation and mineralization of human bone-marrow stromal cells (hBMSCs). Cells embedded in a collagen gel (gel fibres, red) can be mechanically stimulated via nanovibrations generated by piezoelectric ceramic elements. Because of the viscoelastic properties of the collagen matrix, the nanovibrations are transmitted to the cells, triggering the differentiation of the hBMSCs (orange) into osteoblasts (blue), which deposit mineral matrix (black).

the authors found that the viscoelastic properties of the collagen gel allow the transfer of high-frequency vibrations to the cells in the 3D environment. Vibration amplitudes at 1,000 Hz measured at the edge of the well were 35 nm, compared with 24 nm at the centre. Under these conditions, nanovibrated hBMSCs adopted an osteogenic phenotype and exhibited mineralization via the generation of what appeared to be calcium phosphate, the mineral form found in bone.

To study the mechanisms underlying nanostimulation, Dalby and co-authors first tested whether actomyosin contractility mediated nanovibration-induced osteogenic differentiation, given the established role of RhoA/ROCK signalling and myosin-2 activity in mesenchymal stem cell differentiation on 2D substrates[5,6]. The authors show that the inhibition of ROCK signalling and myosin-2 activity by small molecules (Y27632 and blebbistatin) indeed downregulated gene-expression levels of early- and mid-stage bone markers. These findings suggest that 3D osteogenesis via nanovibration is a mechanotransductive process involving cytoskeletal tension. However, the authors note that the impact of cytoskeletal inhibition on gene expression was not as dramatic as reported for 2D osteogenesis, which leads to the hypothesis that alternative mechanisms may play a more prominent role in nanovibrated 3D matrices. The authors found a clue for such an alternative mechanism in their cytoskeletal-inhibition data; blocking ROCK and myosin-2 activity also hampered nanovibration-regulated gene expression of the mechanosensitive ion channels Piezo 2 and TRPV1 (transient receptor potential vanilloid subfamily member 1). These findings motivated the authors to further investigate the role of these ion channels on nanovibrated cells and 3D osteogenesis. Given that TRPV1 was the most mechanoresponsive protein to nanostimulation, the authors used a metabolomics approach in combination with inhibition studies for TRPV1 and its downstream effector PKC (protein kinase C). They found that 3D osteogenesis by nanovibration was mediated by the TRPV1-PKC-Wnt/β-catenin axis. Although the authors did not demonstrate a direct link between β-catenin and osteogenic markers, it is well established that β-catenin promotes osteogenesis by directly stimulating the expression of RUNX2, a master transcriptional regulator of bone formation[9]. Overall, Dalby and colleagues' findings suggest that, in addition to adhesion and cytoskeletal tension, mechanosensitive ion channels play a significant role in the sensing of nanovibration and in controlling osteogenic differentiation in soft 3D matrices.

Although Dalby and co-authors propose that TRPV1-PKC-Wnt/β-catenin is a central pathway activated by nanovibration, their data also indicate that this is not a linear pathway, but rather three nodes within a regulatory network that is affected by many other factors. Still, knowledge of the complete regulatory networks underpinning nanovibrated osteogenesis is not necessarily a prerequisite for translating the technology to clinical settings. Analogous to the many applications of Wolff's law in the clinic, the application of nanovibration to generate osteogenic-tissue intermediates could have a significant impact, even without full comprehension of the underlying mechanisms. As pointed out by the authors, 3D mineralization of hBMSCs via nanovibrations doesn't require the use of additional chemicals, growth factors or bioactive scaffolds, sophisticated culture ware, or dedicated bioreactors. Because one bioreactor can generate 60 ml of mineralizing osteoblasts in a collagen matrix, nanovibration technology could be considered as a cost-effective way to generate large volumes of implantable mineralized tissue intermediates. Furthermore, the bioreactor does not come in direct contact with the cell-embedding hydrogel, which implies that this technology can be readily applied in a good-manufacturing-practice workflow without the need to meet the regulations of the Food and Drug Administration for new scaffolds or induction chemicals. Therefore, from an economic and regulatory viewpoint, only a few barriers may need to be overcome to bring this technology from the laboratory to the clinic. Ultimately, the clinical impact of this technology will depend on whether nanovibrated osteogenic-tissue intermediates will remain viable and continue to mature after implantation in a patient's bone defect. ❐

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