Biophysical Phenotyping of Mesenchymal Stem Cells along the Osteogenic Differentiation Pathway

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Abstract

Mesenchymal stem cells represent an important resource, for bone regenerative medicine and therapeutic applications. This review focuses on new advancements and biophysical tools which exploit different physical and chemical markers of mesenchymal stem cell populations, to finely characterize phenotype changes along their osteogenic differentiation process. Special attention is paid to recently developed label-free methods, which allow monitoring cell populations with minimal invasiveness. Among them, quantitative phase imaging, suitable for single-cell morphometric analysis, and nanoindentation, functional to cellular biomechanics investigation. Moreover, the pool of ion channels expressed in cells during differentiation is discussed, with particular interest for calcium homoeostasis.
Altogether, a biophysical perspective of osteogenesis is proposed, offering a valuable tool for the assessment of the cell stage, but also suggesting potential physiological links between apparently independent phenomena.

Introduction

Mesenchymal stem cells (MSCs), first discovered in 1974, are adult stem cells that reside in many organs and tissues of the body, including the bone marrow, adipose tissue and umbilical cord (Friedenstein 1976). MSCs are multipotent, and produce cells of mesenchymal lineage, including osteoblasts, chondrocytes, adipocytes and myoblasts (Caplan 1991). They are important in healing and tissue regeneration, have immunomodulatory capacity (Ito et al. 2010; Pileggi et al. 2013; Mehler et al. 2018) and are key to regulation of haematopoietic homeostasis in the bone marrow niche (Pinho and Frenette 2019). MSCs are easily harvested from autologous tissues and are amenable to *in vitro* cell culture, therefore allowing growth of significant cell numbers in the lab - provided the correct cues are employed (Lane et al. 2014). These two factors, alongside their multilineage potential and immunomodulatory properties have made MSCs an attractive target for regenerative medicine and therapeutic applications. However, since their discovery there have been less clinical success stories than first imagined, owing to limitations such as batch-to-batch variability and poor definition of MSCs and their differentiated progeny. Many fundamental and basic mechanisms of MSCs biology have still to be fully understood to harvest the full therapeutic potential of these cells.

Molecular markers for MSCs are a topic of debate, with MSCs from even within the same tissue source expressing heterogenous markers (the minimal criteria for defining MSCs is, however, outlined by (Dominici et al. 2006)).Typically, when MSCs are employed for bone regeneration, the population is most commonly isolated from the marrow and are better described as skeletal stem cells. MSC is, in fact, an inaccurate definition for a highly heterogeneous cell population of cells that can be isolated from various tissues. Skeletal stem cells are a clonogenic population of bone marrow stromal cells that can differentiate into chondrocytes, osteoblasts, adipocytes and haematopoiesis-supporting stroma on the basis of in vivo transplantation studies (Bianco 2013). However, the term MSC is well accepted and we use it throughout this review.

At present, a global ageing population is increasing the demand for musculoskeletal solutions (World Health Organization, 2015). There is currently an unmet, growing need to provide viable bone grafts for clinical use (Giannoudis et al. 2011). A wide range of surgeries require bone grafts, making bone the second most transplanted tissue after blood. Autologous grafts are the current gold standard, although these are in short supply and there are concerns associated with donor-site pain and morbidity (Dimitriou et al. 2011). For bone regeneration, bone marrow derived human MSCs hold great promise as an autologous cell source that can differentiate into mature osteoblasts (Bianco et al. 2001). Tissue-engineered bone grafts could help to fulfil clinical demand and provide a crucial resource for drug screening.

Typically, complex media formulations containing multiple components are employed to stimulate differentiation of MSCs down desired lineages, however these formulations vary in potency and often lead to off target effects (Ghali et al. 2015). For example, MSCs are stimulated to undergo osteogenic differentiation through the use of osteogenic media (OGM), which typically contains a cocktail of dexamethasone, ascorbic acid and glycerophosphate. However, dexamethasone is also a major component of adipogenic stimulating media,

demonstrating these compounds act in a non-specific manner (Ghali et al. 2015). Other common methods to stimulate osteogenesis of MSCs include delivery of supraphysiological doses of growth factors, such as bone morphogenetic proteins (BMP). However, in vivo, these factors are connected to and interact with the physical microenvironment provided by the extracellular matrix (ECM), this specific microenvironment is termed the stem cell niche (Donnelly et al. 2018). The ECM is dynamic and provides both physical and functional cues that cells perceive and respond to through the process of mechanotransduction (Schiller and Fässler 2013). As such, traditional approaches to induce e.g. osteogenesis of MSCs using OGM, which are typically carried out using standard tissue-cultureware completely fail to recapitulate any physical stem cell niche properties. Tissue engineering aims to offer a resolution to this problem; biomaterials (surfaces, scaffolds), biofabrication systems (microfluidics, three-dimensional bioprinting) and biophysical tools offer platforms to both replace damaged and diseased tissue, and to investigate fundamental processes important in stem cell differentiation.

This review focuses on recent developments to quantify osteogenesis of MSCs. After a brief overview of existing biomolecular methods to assess the differentiation stage, new advancements and biophysical tools will be presented, exploiting different physico-chemical markers of the cell population to finely quantify the onset of the osteogenic phenotype. Particular attention will be dedicated to label-free morphometric and biomechanical indicators (Table 1), other than the role of the main molecular messenger in stem cells, the Calcium ion. All these aspects showed correlations with MSC phenotype modifications, therefore representing valuable non-molecular biomarkers for MSC differentiation.

Aspect	Application	Label-free approach	Reference
Shape	Prediction of osteogenic lineage onset from MSC after administration of chemicals	Phase contrast imaging + analysis software	Matsouka et al. 2013
	Monitoring of the osteogenic potential of MSCs exposure to pulsed electromagnetic field	Brightfield + ImageJ OrientationJ plugin	Petecchia et al. 2015
	Acquisistion of morphological information on MSC samples at different stages of osteo-differentiation	Digital Holographic Microscopy	Petecchia et al. 2017
	Quantification of morphometric indicators of a specific type of MSCs (human dental pulp stem cells)	Phase-retrieval algorithm based on the TIE	Paino et al. 2017
	Investigation of the osteogenic maturation of a conditionally immortalized human fetal osteoblasts line	Combination of TIE and DHM	Bartolozzi et al. 2020
Mechanics	Characterization of the biomechanical properties (Young's Modulus and membrane tether length) of MSCs	Atomic Force Microscopy	Titushkin and Cho 2007
	Description of the mechanical properties of single primary MSCs in attached and suspended states	AFM and optical stretching	Maloney et al. 2010
	Quantification of the mechanical phenotype of single cells (human fetal osteoblasts line)	Single cell nanoindentation	Bartolozzi et al. 2020

Table1. *List of the osteogenic applications of quantitative label-free methods cited in the text.*

Osteogenesis at a glance

First, to understand how such approaches can be used to probe osteogenesis of MSCs, we will consider the osteogenic differentiation pathway. The mature skeleton of mammals is maintained through the balancing activities of the bone-forming osteoblasts, and the bone-resorbing osteoclasts (Gasser and Kneissel 2017). Osteoblasts are part of a group of cells referred to as osteoblast lineage cells that includes mesenchymal progenitors (Panaroni et

al. 2014), preosteoblasts, osteoblasts, bone-lining cells and osteocytes (Long 2012). Osteoblasts, the chief bone-making cells, produce a combination of ECM proteins such as osteocalcin, alkaline phosphatase (ALP) and collagen type I (Alford et al. 2015; Gasser and Kneissel 2017). The ECM of different bone tissue compartments plays an essential role in directing the bone homeostasis and provides structural flexibility to an otherwise overly rigid tissue (Long 2012). The collagen type I rich ECM, known as the osteoid when first deposited and not yet mineralised, serves as a scaffold that is subsequently mineralised through the accumulation of Calcium phosphate in the form of hydroxyapatite (Gasser and Kneissel 2017). This process results in a hard but lightweight nanocomposite material, with a peculiar architecture of intercalated organic and inorganic phases, that provides a microenvironment of specialised mechanical, topographical and chemical milieu (Long 2012). Hence, the ECM also has major influence on the differentiation of MSCs to osteoblasts by both direct cell-ECM interactions (Curry *et al.*, 2016), as well as by modulating growth factor availability (Bonnans et al. 2014).

The differentiation pathway of osteoblasts is generally split into 3 stages: (1) MSC or mesenchymal progenitors, (2) preosteoblasts and (3) osteoblasts. Osteoblasts are often characterised by the expression of mature markers such as osteocalcin, ALP and osteopontin. Preosteoblast refers to a heterogenous compartment of cells transitioning from progenitors to mature osteoblasts (Long 2012). They are commonly considered to express the transcription factor RUNX2 (Runt-related transcription factor-2), then, at a slightly more advanced stage, RUNX2 and osterix (OSX; also known as SP7) (Nakashima et al. 2002).

Osteoblasts are generated from MSCs through two distinct processes *in vivo*: intramembranous or endochondral ossification. *In vivo* the two processes result in identical progeny, but intramembranous ossification is limited to certain parts of the skull and clavicle, whereas endochondral ossification produces osteoblasts in the rest of the skeleton (Long 2012). During intramembranous ossification, MSCs condense and directly differentiate into osteoblasts. However, during endochondral ossification, MSCs condense to first form chondrocytes (cartilage cells) and perichondral cells (Kronenberg 2003). During bone development, chondrocytes initially proliferate, then exit the cell cycle and undergo hypertrophy (a substantial increase in cell size); only then do osteoblasts differentiate from the perichondral cells (L. Yang et al. 2014). These differentiation processes of MSCs to osteoblasts require the activity of specific transcription factors that are expressed and function at distinct timepoints (J. Yang et al. 2014). MSCs can give rise to osteoblasts, chondrocytes and adipocytes (Fig.1). Osteogenesis requires the expression and activation of RUNX2, considered the master regulator of osteogenesis. It regulates the expression of many osteoblast genes, and acts throughout induction, proliferation and maturation of osteoblasts (Long 2012). Next, OSX is activated downstream of RUNX2; OSX is a zinc finger transcription factor, and conditional knockout studies in mice have demonstrated it is required for osteoblast differentiation (Nakashima et al. 2002). Finally, ATF4 (activating transcription factor 4), which has important roles in mature osteoblasts, directly regulates expression of osteoblast-derived molecules osteocalcin, and RANKL (receptor activator of nuclear factor -kB ligand), promotes osteoclast differentiation and function (Yu et al. 2005; Elefteriou et al. 2006). A subset of osteoblasts then become osteocytes, once entombed in the bone matrix (Long 2012).

These transcription factors are regulated by a range of developmental signals, and biomaterial strategies have been used to investigate environmental stimuli that recapitulate aspects of the in vivo niche (Dalby et al. 2018, Donnelly et al. 2018). For example, mature osteoblasts require the formation of large, super-mature, adhesions (greater than 5 um long), this leads to increased intracellular tension, which is linked to osteogenesis (Biggs et al. 2009). This mature adhesion, high intracellular tension morphology has been demonstrated to lead to tensile forces in the nucleus that can lead to changes in nuclear shape (Ingber 2003a; Ingber 2003b; Wang et al. 2009), consequently affecting chromosomal arrangements and ultimately gene expression (Dalby et al. 2007a; Dalby et al. 2007b; McNamara et al. 2012; Tsimbouri et al. 2013).

Signalling from cell adhesion is primarily through the MAPK pathway (mitogen-activated protein kinases), downstream is the MAPK extracellular signal-related kinase 1 (ERK1; also known as MAPK3)/ERK2. ERK1/2 is known to be a key regulator of growth and proliferation, and also has a key role in phosphorylation of transcription factors that control MSC phenotype (Ge et al. 2007). For osteogenesis, formation of supermature adhesions and increased intracellular tension leads to an increase in ERK1/2 activity, leading to the phosphorylation of both RUNX2 and PPARy (Biggs et al. 2009; Ge et al. 2016). PPARy is the master regulator of adipogenesis, adipocytes are low-adherent cells, and therefore exhibit low intracellular tension. When RUNX2 is activated upon phosphorylation, osteoblast commitment is initiated, whilst at the same time phosphorylation of PPARy leads to its deactivation, and adipogenesis is suppressed (Ge et al. 2016).

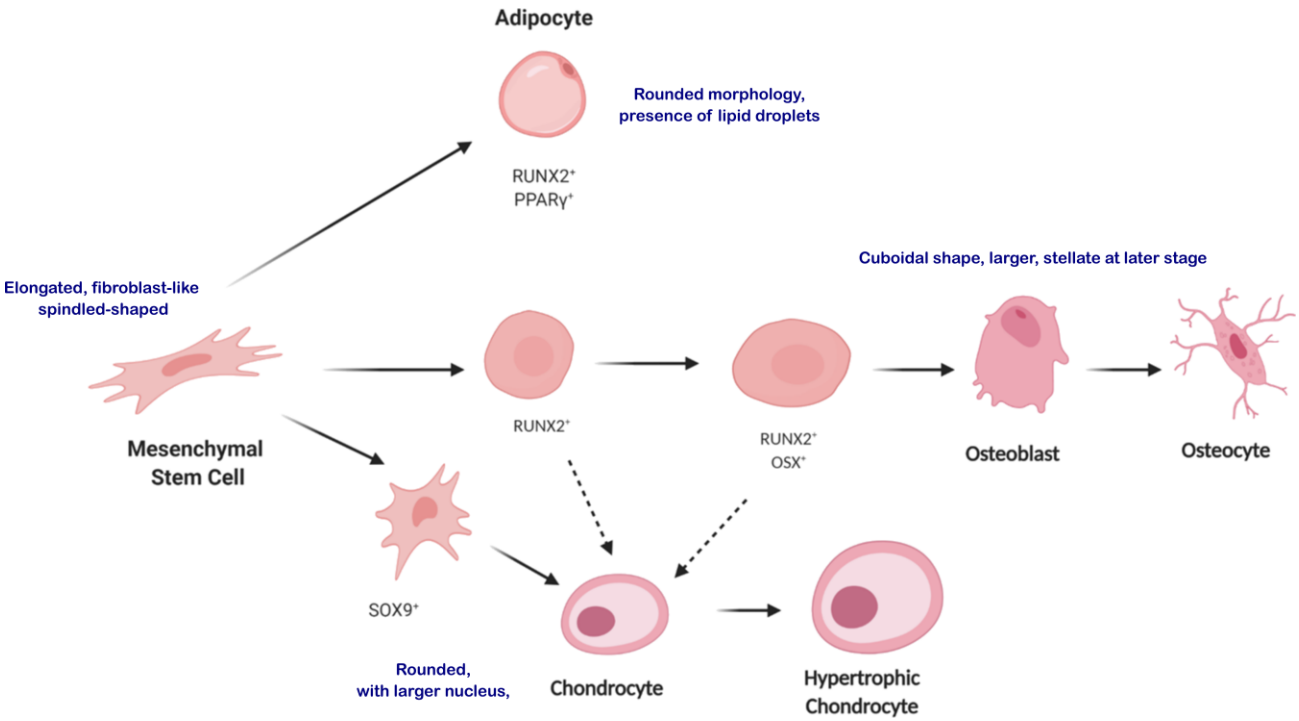

Fig.1 *Stages of osteoblast lineage differentiation. MSCs can give rise to osteoblasts, chondrocytes and adipocytes. MSCs that give rise to osteoblasts and chondrocytes initially express the transcription factor SOX9. For osteoblasts, initial steps are characterized by the*

expression of RUNX2 and OSX, ultimately leading to the development of mature osteoblasts. A subset of osteoblasts then become osteocytes once entombed in the bone matrix. For adipogenesis, the transcription factor PPARy is activated whilst RUNX2 is subsequently inactivated. In blue morphological observations are reported, associated to each differentiation path. Figure modified from Long 2012. Created with BioRender.com

MSC morphology during osteogenic differentiation

The interest in studying cell morphology relies on the fact that structure and function in cells are tightly connected, and a progressive change in shape and size is observed during a differentiation process (Rangamani et al. 2013; Schakenraad et al. 2019; Cutiongco 2020). The tight connection between cell state and its shape allows consideration of morphological parameters as potential markers for assessing cell phenotype. To date, cell morphometric phenotyping has been demonstrated successfully in several applications, ranging from cancer (Wu et al. 2015; Domura et al. 2017) to hematopathology (Ford 2013).

Several complementary microscopy approaches can be used to provide a qualitative description of the changes in cell shape and size during differentiation, such as fluorescence microscopy, high resolution scanning electron microscopy (Sonowal et al. 2013) and high contrast phase imaging (Briggs et al. 2009). Undifferentiated MSCs appear as elongated spindle-shaped, showing a swirling morphology when reaching higher confluence [Wang et al. 2018]. While entering a specific differentiation pathway, MSCs show a broad range of changes. Osteogenic MSCs become cuboidal and stellate, larger and more polygonal, while adipogenic cells appear globular (with certain cells exhibiting evident fat droplets from early stages) and chondrogenic MSCs tend to aggregate and exhibit a high ratio of nucleus to cytoplasm (Zhang et al. 2018).

The basic qualitative description can be extended by a more quantitative measurement of morphometric indicators, automatically performed using general purpose (such as ImageJ (Rueden et al. 2017) or Gwyddion (Nečas and Klapetek 2012)) or more specialized (e.g. CellProfiler (McQuin et al. 2018)) image processing software. An example is represented by the work of Matsouka et al. (2013), where the authors investigated the possibility of predicting the onset of osteogenic differentiation of MSCs, using phase contrast images of different time points after the beginning of the chemical induction procedure. Several features were extracted from the images, and their changes in time correlated with the expression of specific osteogenic markers. A Ridge regression machine learning approach was developed, and it demonstrated to be effective in the prediction of ALP activity and calcium deposition.

The tight connection between cell morphology and differentiation state is mainly associated with underlying changes in cytoskeletal organization. In fact, in undifferentiated MSCs, actin cytoskeleton is organized as thin, parallel microfilaments spread across the cytoplasm, which confer to the cells the typical fibroblastic spindle shape morphology (Rodriguez et al. 2004). Over the course of several days, differentiation and actin reorganization take place, leading to lineage-specific cell shape. When acquiring the adipogenic fate, MSCs reduce focal adhesions and disrupt the acting network, resulting in a rounded cell morphology (Kawaguchi et al. 2003). When differentiating towards osteoblasts, MSCs increase actin polymerization

to form robust, crisscrossed patterns of actin cytoskeleton, and their shape appears larger and cuboidal (Yourek et al. 2007). When moving towards chondrogenic fate, actin microfilaments mostly localise close to the cell membrane as a predominantly cortical structure, and cells acquire round shape (Langelier et al. 2000). Following this idea, Treiser and co-workers (2010) designed a predictive approach based on fluorescence images of actin-stained cellular samples. For each cell, 43 cytoskeletal features were identified, describing whole cell and individual filament morphology (in terms of shapes, intensities, textures, and spatial distributions). Using a dimensionality reduction approach (Multidimensional Scaling), key combinations of the morphology descriptors were reduced into 3 dimensions, which were used to classify cell subpopulations grown either in basal or in lineages conditioning media. Validation of lineage prediction was performed through a Support Vector Machine. It showed high accuracy, specificity, and sensitivity of the unsupervised classification approach in distinguishing osteogenic differentiation, demonstrating excellent performance in anticipating long-term cellular behaviors from very early stage culture, days to weeks before they were experimentally detected with concurrent biomolecular methods.

The possibility for a cell to acquire a differentiated morphology is tightly linked with the ability to create adhesion structures, pivoting on them to sustain the energy stored in the resulting three dimensional tensegrity structure (Ingber 2003a). Morphological markers are expected to be particularly effective while monitoring the role on differentiation potential of a specific substrate chemistry (Petecchia 2017), morphology (McNamara 2010) or mechanics (Mao 2016). This approach was recently exploited to quantify the efficacy of different clinically relevant materials for the differentiation towards the osteoblast lineage (Long et al. 2019). Here the author stained the cell membrane and analyzed the evolution in time of specific indicators: aspect ratio (Ar), circularity (C) and roundness (R). In fact, combination of these three features can describe typical cell geometries derived from MSC differentiation: elongated spindle shape could be associated to Ar~4, C~0.2, R~0.3 combination; polygonal and cuboidal cell morphologies to Ar~3, C~0.36, R~0.32 combination; while stellate cell morphologies to the Ar~2, C~0.22, R~0.51 combination. Using these descriptors to quantify the differentiation efficiency, Long et al. evaluated the effect of different titanium-based substrates, showing that incorporation of micro- and nano-roughness on titanium materials improves the rate at which MSCs differentiate to osteoblasts. Notably, improved differentiation correlates with a rapid adoption by the MSCs of a stellate morphology, typical of mature osteoblasts or even osteocytes. Similar approaches have been used in other studies, looking into the role of substrate chemistry and nanostructured organization on the osteogenic differentiation (Loye et al. 2018, Banik et al. 2016, Lavenus et al. 2011, Dalby et al. 2007c). The use of single cell morphological markers to quantify the differentiation stage of MSCs demonstrated to be effective to highlight differences between substrates, particularly outperforming as an early indicator of longer-term fate commitment. The forecasting power of MSCs shape in predicting differentiation lineage was previously highlighted, demonstrating that MSCs adipocytic or osteogenic fate is tightly associated to shape- and RhoA (Rho Kinase)-mediated signals, which can act at an earlier stage factor (McBeath et al. 2004).

Mesenchymal cells have been largely studied for their colony-forming ability and differentiation potential, but they also present peculiar collective mechanisms of migration

and cadherin-driven organization (Theveneau 2012). The spatial pattern of a mesenchymal population and the local polarization of the culture could provide additional information on the stage and activity of MSCs. This concept was exploited to monitor the osteogenic potential of MSCs exposure to pulsed electromagnetic fields (Petecchia et al. 2015). In this paper, the authors acquired brightfield images of MSC populations and quantified the local orientation of cells using a dedicated ImageJ plugin (OrientationJ, Rezakhaniha 2012). MSCs grown in sparse cultures showed remarkable morphological variability. This diversity was reduced when cells started interacting, and reached an organized and well oriented layer at higher confluency levels. Noticeably, this peculiar orientation at higher density was rapidly lost upon osteogenic differentiation, and the typical rounded and non-oriented organization of osteoblasts emerged. This mechanism was efficiently caught by the orientation analysis: undifferentiated cells were characterized by a uniform orientation distribution, while differentiated cells presented a broader distribution, with no leading orientation peak.

Quantitative phase imaging

Morphology-based approaches to MSCs assessment along the osteogenic pathway are extremely versatile and suitable to finely quantify the differentiation stage. Nevertheless, obtaining high quality images of micron-sized transparent objects, such as single cells, is a challenging microscopy task. One possible approach to overcome this issue is to use chromophores to label specific structures or compartments of the cell, and image the sample under fluorescence or brightfield microscopy. While providing sharp and neat images, the introduction of labels in the sample is typically invasive, and not suitable to monitor the time course evolution of a differentiating MSC over days or even weeks. Moreover, standard label-free microscopy techniques to enhance the contrast, such as phase contrast or differential interference contrast (DIC), are fundamentally qualitative and difficult to quantify with simple algorithms (Su 2013). Recently, a new class of instruments has emerged as a promising tool for single cell label-free imaging, quantitative phase imaging (QPI) (Ferraro 2011). Cells are poorly observable with standard methods, because they are thin and do not scatter much of the incident light, resulting in a very mild deformation of the intensity recorded by the camera. QPI extends standard techniques by collecting not only the intensity of the electromagnetic field, but also the change in phase due to the light passing through the sample. The resulting phase image carries information on the thickness of the cell in each pixel, convoluted with the refractive index, and can be represented as a three-dimensional surface (Popescu and Park 2015). The technology behind the implementation of QPI is nowadays entering the maturity phase, and a growing number of established applications in biology and medicine are emerging (Park 2020).

The adoption of QPI techniques in the context of single cell phenotyping provides a twofold advantage. First of all, phase images are well suited to perform an efficient segmentation of single cells, that normally is the trickiest processing step with traditional images (Loewke 2018). In other words, QPI is an optimal technique to extract 2D morphometric indicators from cellular samples and feed them to any of the previously reported data mining approaches. Moreover, the information encoded in the image contains reference to the thickness of the sample, from which a completely new set of features can be extracted, such as volume, surface roughness or average thickness, to list some.

QPI methods are roughly divided in two families, whether they are based on interferometry or not. The most relevant implementation belonging to the former class is Digital Holographic Microscopy (DHM) (Lee et al. 2013). In short, with DHM, the illumination light (a coherent source) is split before reaching the sample so that one half of the beam goes through the sample (sample beam) and is combined afterwards with the half beam which did not traverse the sample (reference beam). The resulting interference pattern carries the information about the phase jump experienced by the sample beam. The signal can be acquired with a standard CCD camera and digitally unwrapped to obtain the encoded quantitative volumetric information, thus allowing to reconstruct a three-dimensional representation of the cellular sample based on a single exposure (Merola et al. 2013). Petecchia et al. (2017) exploited this approach to acquire morphological information on MSC samples at different stages of osteo-differentiation. More than 40 DHM-powered geometrical 2D and 3D parameters were analysed and quantified, concentrating on those that correlated the most with the differentiation stage. Interestingly, the authors showed that 2D features (such as area and roughness) are highly affected by the characteristics of the substrate (in their case nanostructured TiO_2 with respect to glass and flat TiO_2). Conversely, 3D-like features (such as the average thickness) were affected more by the time spent in culture than by the growth substrate.

While DHM provides a highly effective microscopy tool, theoretically able to acquire images with a vertical resolution of about 1/100th of the wavelength (i.e. less than 1nm), its implementation requires dedicate optics and high mechanical stability, and existing commercial instruments are still quite expensive (Paturzo 2018). With different features, non-interferometric quantitative phase microscopy has been recently suggested as a cheaper alternative, based on the numerical solution of the Transport of Intensity (TIE) equation (Zuo 2014). This approach only requires a point monochromatic light source (coherent in space but not necessarily in time) and the reconstruction is based on the acquisition of a vertical stack of brightfield images (Barty et al. 1998; Kou et al. 2010). In practice, the TIE approach can be implemented on any standard microscope, as long as the focal position can be precisely controlled (Sbrana et al. 2017). A phase-retrieval algorithm based on the TIE was successfully applied to quantify morphometric indicators of a specific type of MSCs (human dental pulp stem cells) (Paino et al. 2017) and more recently Bartolozzi et al. investigated the osteogenic maturation of a conditionally immortalized human fetal osteoblasts line (hFOB 1.19) (Bartolozzi et al. 2020). In this paper TIE and DHM were used in combination, to provide both quantitative and qualitative details while monitoring the change of the cell shape towards maturation.

Relevance of Ca^{2+} in MSC osteogenesis

Calcium is the most abundant mineral in the body and accumulates principally in the skeleton, where it has a structural function, and from where it can be recruited to be delivered to body fluids, serum, muscle, heart, brain and other body compartments. Ca^{2+} is fundamental for bone cell metabolism, so that even a modest expected or unexpected, endogenous or exogenous stimulus is very likely to cause a change in the concentration of intra- or extra-

cellular Calcium and the activation of downstream signalling pathways. During their maturation, bone cells become progressively embedded and then immobilized in the ECM whose conspicuous inorganic component consists mostly of CaP in the form of hydroxyapatite. A significant remodeling takes place continuously in bone tissue, with the old osteoblasts replaced by new cells which are stimulated to enter the osteogenic process in relation to the microenvironment created by the osteoclasts controlling the process of bone resorption. In playing their role, osteoclasts release a multitude of stimulating factors in the surroundings, including Calcium. Interestingly, a compartmentalization of extracellular Calcium functional to the optimization of cell activity is established, with concentration ranging from 8-40 mM along the osteoclast reabsorbing edge, down to less than 2mM at the rear of the cell (Silver et al. 1988). It is well established that each cell type is endowed with a sophisticated molecular network finalised to construct a versatile Calcium signalling system. Moreover, in the cytosol of MSCs, preosteoblasts and mature osteoblasts, a spatial and temporal pattern of Ca^{2+} concentration exists, and is useful to encode information for cell activity.

There is an increasing evidence that many pathways of MSCs osteogenesis are finely regulated by intracellular Calcium concentration including protein secretion, exocytosis and gene expression. The combination of growing MSCs and engineered scaffolds loaded with Ca^{2+} has been widely exploited in recent years. It represents a very powerful tool to find novel biomimetic materials for bone regeneration, but also to assess the multiple influences of Ca^{2+} in MSCs osteogenesis. Engineered biomimetic materials, recapitulating physico-chemical cues of the native tissue, have a well assessed osteogenicity, osteoconductivity, and osteoinductivity. Nevertheless it is still largely unclear which intracellular metabolic pathways they activate (Szpalski et al. 2012). MSCs grown on 3D gelatin scaffolds in a medium containing high concentration of Calcium show enhanced expression of osteogenic genes and cytokine secretion through a mechanism involving cooperation between Calcium, BMP-2 and its effect on SMAD signalling. Interestingly, this cooperation is differently regulated by Calcium throughout the various stages of the differentiation process. During the first days of differentiation, increased Calcium levels antagonize BMP-2 signalling, while on longer term the presence of Calcium is synergistic with BMP-2 to the consolidation of specific osteogenic pathways (Aquino-Martinez et al. 2017). Barradas and collaborators assessed that high levels of extracellular (6.8 mM) Ca^{2+} added to MSCs growth medium significantly enhanced transcription of bone related extracellular matrix proteins, osteocalcin, osteopontin and bone sialoprotein (BSP). Furthermore, a threefold up-regulation of BMP-2 was also observed. The authors went beyond the evidence that MSCs growing in high-Calcium culture medium develop an osteoblastic phenotype, and they tried to identify the signal transduction pathways linking extracellular Ca^{2+} and osteogenic gene transcription. The authors found that MEK1/2 is essential for BMP-2 expression probably via c-Fos expression, thus identified as an early Ca^{2+} response gene (Barradas et al. 2012).

Later on, Viti and collaborators using a Ca-PO_4 substrate triggered osteogenic differentiation thus confirming that Calcium and Phosphate ions dissolution from engineered scaffolds is the main source of bioactivity of bone biomaterials (Viti et al. 2016). The authors in this paper offer information on the biomolecular mechanisms involved in Ca-PO_4 driven stem cell osteogenesis. Based on MSC gene and microRNA expression profiles, and relying on literature knowledge, they assigned a key role to the Calcium-driven BMP-signalling pathway and assessed the involvement of genes belonging to SMAD and RAS families in early

phosphate-driven pathways. Furthermore, they found a reduction of miR-138, miR-140 and miR-193 in agreement with an ongoing process of intramembranous ossification. It is noteworthy that the combination of Ca^{2+} and PO_4^{3-} triggers osteogenic differentiation through BMPs/SMAD and RAS signaling pathways, which, conversely, are often modulated by dexamethasone addition to the culture medium.

According to Shih and collaborators (2014), substrate matrix containing Ca-PO_4 moieties stimulates MSCs to enter osteogenic differentiation, but through the activation of a previously unknown metabolic mechanism which involves phosphate-ATP-adenosine. Extracellular PO_4^{3-} , easily released from the hydroxyapatite particles of the matrix, first would enter the cell through the SLC20a1 Na^+/PO_4^{3+} antiporter and then the mitochondria, where it serves as substrate for synthesis of ATP. Back to cytosol, ATP would be secreted and rapidly metabolized in adenosine, which, once bound to A2b receptors, would commit MSC osteogenesis mainly increasing synthesis of RUNX2, a pivotal transcription factor. Even if the focus of this pathway is centred on PO_4^{3-} metabolism, a relevant role is ascribed also to Ca^{2+} released from CaP, in that it would contribute to drive a dynamic environment which favours cell maturation (Shih et al. 2014).The combination of artificial bones made of b–tricalcium phosphate and BM-MSC in culture is considered an efficient approach for the realization of good bone for transplantation. In this combined culture BM-MSCs, while undergoing osteogenesis, assume calcium taking it from the growth medium. Interestingly a strong inverse correlation between Calcium concentration of the medium and the osteocalcin content of the bone construct has been observed, thus suggesting that the measure of [Ca^{2+}] of the conditioned medium can be adopted as a nondestructive reliable marker of the ongoing MSC osteogenesis and a suitable strategy to select good artificial
bone for implants (Tanikake et al. 2017). Overall Ca^{2+}, with its double nature of biological signal carrier or stage-specific marker, positively influences MSCs osteogenesis, regulating the process from its very early stages.

Investigating the role of Ca^{2+} in MSC osteogenesis

The level of cytosolic Calcium concentration is generally maintained by various transport processes occurring through the plasma membrane, and by Ca^{2+} released from internal stores. In undifferentiated MSCs, Ca^{2+} flux through the plasma membrane is mediated by several players, namely voltage-gated Ca channels (VGCC), store-operated Ca^{2+} channels (SOC), Na-Ca exchangers (NCXs) and a Ca^{2+} ATPase pump (PMCAs) (Kawano et al. 2002; Heubach et al. 2004; Li et al. 2005; Zahanic et al. 2005)**.** On the other side major contributors to Ca^{2+} release from intracellular stores are the inositol 1,4,5, trisphosphate receptors (InsP3), while ryanodine receptors (RyR), don't seem to be active in undifferentiated MSCs [Fig. 2].

Several types of ionic channels and transporters are responsible for the flow of Calcium ions into the MSCs. Their concerted action has an important role in promoting the onset of the differentiation process toward osteoblastic phenotype (Heubach et al. 2004; Li et al. 2005; Petecchia et al. 2015).

At least two members of the family of Ca^{2+} channels which mediate Ca^{2+} influx in response to membrane depolarization (VGCC), the L-type and T-type VGCC, are expressed and play an active role in MSCs. Taking advantage of electrophysiological approaches, several groups initially focussed on the L-type VGCC, so called because of their long lasting current during

cell depolarization, and characterized by high sensitivity to 1,4-dihydropyridines. (Li et al. 2005; Heubach et al. 2004; Zahanic et al. 2005).The results did not fully agree with later research; indeed Calcium entry through L-VGCC was estimated to give just a small contribution with respect to the ponderous entry mediated from store operated Calcium channels (Kawano et al. 2003; Zahanic et al. 2005) and overall it was stated that MSC osteogenesis do not require L-VGCC activity. Conversely according to other studies L-type VGCC impact on MSC osteogenesis turned out to be significant. Barradas and collaborators (2012) verified that blockage of L-type VGCC with nifedipine produced a significant downregulation of BPM-2 expression which usually promotes osteogenesis. Currently, ERK pathway and Wnt-beta-catenin signalling have been recognized as downstream effectors of osteogenic differentiation mediated by Ca^{2+} entry through L-type VGCC (see for review Tan et al. 2019).

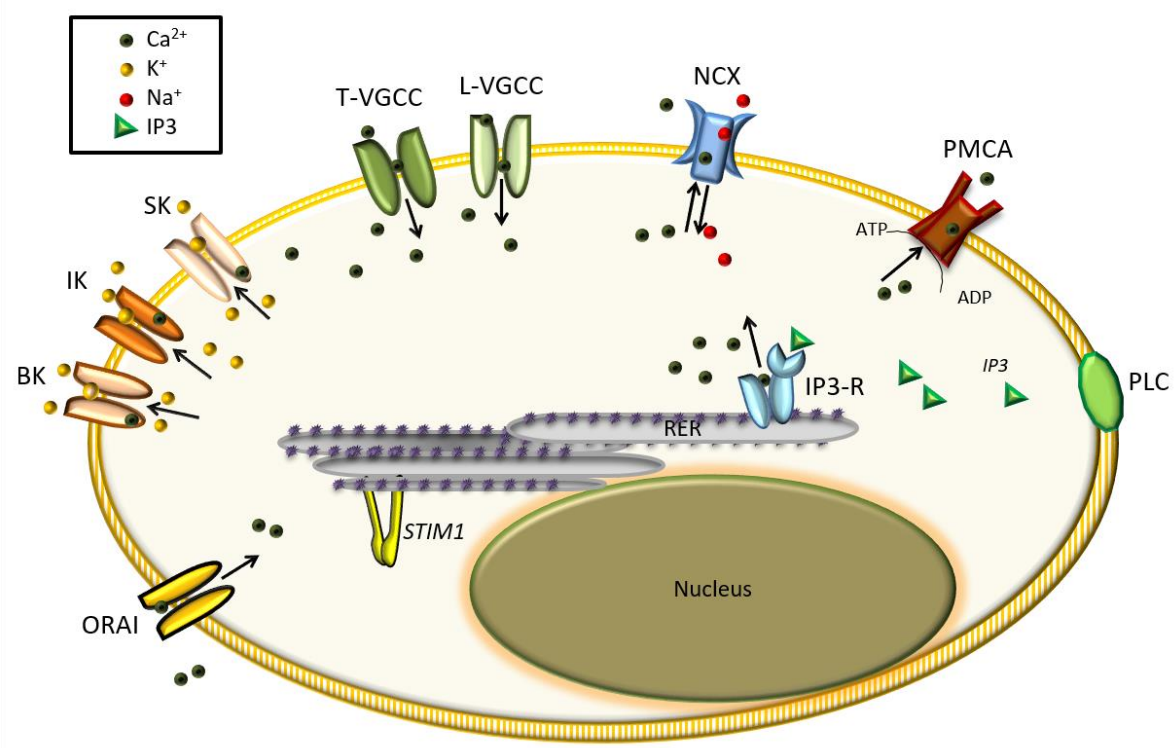

Fig.2 *Schematic cartoon of the molecular components expressed in hBM-MSC mediating transmembrane Ca^{2+} transport and the regulation of intracellular Ca^{2+} concentration and oscillations during osteogenesis. BK, IK, SK: Big, Intermediate, Small-conductance Ca^{2+}–activated K^+ channels transport potassium outside the cell in the presence of intracellular Calcium. L-VGCC, T-VGCC: L- and T-type voltage-gated Ca^{2+} channels mediate calcium influx in response to Vm depolarization. NCX: Na/Ca exchanger transports sodium inside and calcium outside the cell. PMCA: plasma membrane Ca^{2+} ATP-ase extrudes excessing calcium from cytoplasm to extracellular space. PLC: phospholipase C cleaves phospholipids producing several products including IP3. IP3, IP3-R: inositol 1,4,5 triphosphate, inositol 1,4,5 triphosphate receptor. IP3 binds its receptor promoting calcium release from RER to cytoplasm. STIM1: STromal Interaction Molecule, endoplasmic calcium sensor. ORAI: Calcium selective channel. STIM/ORAI coupling and uncoupling are part of the store operated calcium entry (CRAC) which mediates the store operated Calcium current (SOC). See text for further details.*

A quantitative analysis of Ca^{2+}-related events during MSC osteogenic differentiation has been attempted by adopting a panel of biophysical tools including electrophysiology and Calcium fluorimetry (Petecchia et al. 2015). Results showed that in undifferentiated MSCs, L-type VGCC are expressed and active only in a small population percentage, from 15% to 40% (Li et al. 2005; Petecchia et al. 2015) when growing in control proliferative medium. Instead, this proportion increases to about 50% after growing up to 34 days in the osteoinductive medium (ODM) containing a mixture of dexamethasone, ß-glycerophosphate and ascorbic acid. Similarly, the mean amplitude of the inward Calcium current for each cell was not affected by the addition of the osteoinductive compounds in the medium, even after thirty days of incubation. This suggests a substantial insensitivity of L-type VGCCs to the osteogenic mixture which in fact doesn't seem to be able by itself to commit stem cells toward osteogenesis to a large extent. L-type VGCC are normally expressed in all mature osteoblasts, but only in a few MSCs. The increase of the number of MSCs expressing L-type VGCCs is a good indicator of osteogenesis progression. Furthermore, cells exposed to a pulsed low frequency (75 Hz, 2 mT) electromagnetic field (PEMF) to attempt an improvement of the osteogenic process provide some insight on mechanism. The application of low energy magnetic fields to cells, tissues or organisms is suitable to obtain non-ionizing and non-thermal interactions between the magnetic field and the biological sample. A plethora of studies has shown that these interactions produce significant biological effects, for instance on the intracellular concentration of Ca^{2+} (Walleczec 1992). Over time, the responsiveness to weak EMF has been assessed in many cell types, including MSCs (Fassina et al. 2008; Schwartz et al. 2008; Ceccarelli et al. 2013), and it is now considered a general property widely, exploited also in research and for therapeutic purposes.

Petecchia and collaborators showed that when MSCs were exposed daily for 10 minutes to PEMF, both the percentage of cells expressing functional L-type VGCCs and the Calcium current amplitude per cell increased dramatically, independently of the incubation with ODM or not. It is noteworthy that neither Na^+ nor K^+ channels expressed in undifferentiated MSCs changed significantly in PEMF-stimulated sample (Petecchia et al. 2015) during the differentiation process, further confirming the central relevance of Ca^{2+} in MSC osteogenesis. A consistent trend was observed in the cytosolic basal Calcium concentration. By means of Fura-2 fluorescence imaging applied from the early to the late phase of osteogenesis, a gradual and persistent cytosolic free Calcium rise from 63 nM to a plateau value of 300 nM was observed in PEMF-stimulated MSCs. In addition, a synergistic effect between ODM and PEMF, especially in the early 10 days of differentiation was also noted. The observed discrepancy regarding ODM effectiveness towards VGCC expression and intracellular Calcium concentration variation strengthens the idea that different stimuli impact different Calcium-related pathways in osteogenesis. PEMF, for instance, has a big role in osteogenesis, favouring VGCC expression, but inducing a smaller increase of basal $[Ca^{2+}]_i$ with respect to ODM.

Like many other types of excitable and non-excitable cells, spontaneous Calcium oscillations are also generated in undifferentiated MSCs. According to Kawano and collaborators, Ca^{2+} is released from internal stores through InsP3 receptors and enters the cell mainly through SOCs, whereas the Na^+/Ca^{2+} exchangers and the PMCA pumps contribute to sustain oscillations (Kawano et al 2003). Oscillations are completely blocked by La^{3+} and by the absence of Ca^{2+} in the bath. Application of NCXs or PMCAs blockers inhibits the development

of oscillations as well (Kawano et al 2003). The interval between oscillation is around 3 min, the duration varies between 0.8 and 13.5 minutes. In addition, certain families of K^+ and Cl^- channels collaborate with Calcium transporters in the regulation of oscillations. Calcium oscillation frequency has been shown to be around 3.3 mHz and some studies show that their information is downstream decoded by MAPK and NFAT4 (Tao et al. 2011; Varga et al. 2011).

Intracellular Calcium oscillations produce spatial temporal patterns of $[Ca^{2+}]_i$ useful to improve Calcium signalling efficiency and specificity; indeed $[Ca^{2+}]_i$ regulates a plethora of vital functions and, due to the complexity of their physiology, in MSCs more than in other cells the role of oscillations is very challenging, being involved in gene expression, proliferation and differentiation. Likely Calcium oscillations in MSCs have to do with fluctuations of membrane potential and currents, thanks to the involvement of the big conductance Ca^{2+} activated-K^+ channels (BK, see below) which are finely modulated by $[Ca^{2+}]_i$ and in turn control Vm modulation (Kawano et al. 2003). Furthermore, autocrine/paracrine mechanisms are involved in Ca^{2+} oscillation. The localization of the intracellular release and the temporal pattern of Calcium oscillation produces further information for the commitment to a specific progeny lineage. Ca^{2+} oscillations can occur in response to external stimuli and in particular to electrical or magnetic stimulation. Sun and collaborators (2007), using Fluo-4 fluorescence imaging, demonstrated that during chemically induced osteogenesis Calcium oscillations within 30 minutes decreased from 8.6 in undifferentiated MSC to 3.5 in the early phase of differentiation, very close to the value observed in mature osteoblasts. Furthermore applying a noninvasive electrical stimulation ranging from 0.1 to 1 mV/cm they observed an acceleration in Calcium mineralization. Thus Calcium oscillations can be physically or chemically manipulated to facilitate osteodifferentiation of MSC.

All main subtypes of Calcium-activated K^+ channels (KCa), characterized by big (BK), intermediate (IK) or small (SK) conductance, are widely expressed in stem cells and play multiple roles in relation to their diversity, achieved by alternative splicing, heteromerizations and auxiliary subunits (Pchelintseva et al. 2018). Being K^+ channel, they do not directly contribute to $[Ca^{2+}]_i$, but rather are regulated by $[Ca^{2+}]_i$ and by its oscillations (Kawano 2003). Indeed, responding with different sensitivity to Ca^{2+} and membrane potential (V m) they control a wide range of cellular functions, including proliferation and differentiation. BK and IK are highly expressed in MSCs and influence all steps of their osteogenic maturation: initiation, proliferation and migration (Pchelintseva et al. 2018). Silencing BK through shRN, the expression of early osteo or adipogenesis marker, osteocalcin and PPAR-g respectively (Zhang et al. 2014) is reduced. Similarly in BK-KO osteoblasts a reduction in osteocalcin osteopontin and RUNX2 was observed (Hei et al. 2016) and blockage of KCa channels with the generic pore blocker TEA turned out in enhanced expression of the stem-related genes.

Mechanobiology of MSCs

Cells are exposed to a plethora of mechanical, electrical and biochemical cues as part of their microenvironment. Cells are able to sense these signals and accordingly tune the expression of mechanosensitive genes, ultimately leading to changes in cell growth, morphology, differentiation potential and proliferation (Jaalouk and Lammerding 2009;

Martino et al. 2018). This is especially relevant for cells that have the potential to develop into diverse lineages, namely stem cells.

Seminal work by Discher and colleagues (Discher et al. 2005; Engler et al. 2006) brought to light that mechanical properties play a key role in MSCs differentiation. In particular, MSCs commitment to phenotype is heavily dependent on substrate (and therefore tissue) stiffness**.** Tissues in the human body vary in terms of elastic modulus, from fatty and brain tissues that are soft (0.5-1 KPa) to much stiffer tissues such as bone (15000-20000 KPa). Indeed, Engler found that when cultured on soft polyacrylamide (PAA) hydrogels, MSCs display a filopodia rich morphology. When increasing hydrogel stiffness, cell morphology adapts, and MSCs differentiate into spindle-shaped muscle cells; or osteocytes in the stiffest substrate. This can be further observed in Fig.3a**,** where the stem cell phenotype changes as the substrate stiffness increases.

There is a set of coordinated efforts led by the ECM and neighbouring cells that provide biochemical and mechanical signals directing cell differentiation, proliferation, survival and migration. Cells probe their mechanical environment under the form of different cues: substrate stiffness, shear flow, gravity or hydrostatic pressure. This is rendered possible by the presence of several transmembrane receptors e.g., G protein coupled receptors, integrins and ion channels, which allow the transduction of the above mechanical cues into intracellular signals (Vining and Mooney 2017)**.**

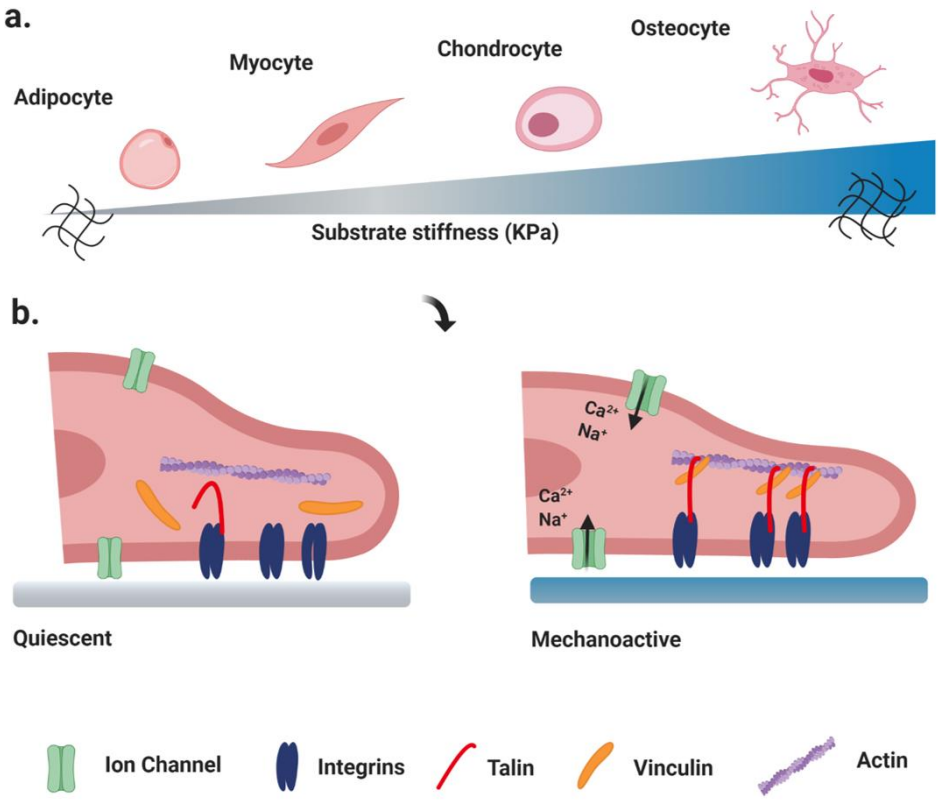

Fig.3 ***a)*** *Diagram highlighting the ideas described by Engler et al. (2006), where substrate stiffness is able to drive MSC differentiation. As substrate stiffens, MSCs differentiate into stiffer tissue cells, such as osteocytes.* ***b)*** *The cell's attachment to the substrate via integrins*

is a plausible bi-directional force transducer across the cell membrane. Integrins bind the ECM, allowing the interaction between the intracellular environment (cytoskeleton) and the ECM via focal adhesion proteins (present in the illustration are talin and vinculin, although more than 160 molecules form the integrin adhesome, see Geiger et al. 2009). The linkage of intracellular cytoskeletal molecules triggers intracellular signalling cascades that regulate cellular migration, proliferation and, eventually, differentiation (Lee and Liu 2014). Figure inspired by Mohammed et al. 2019. Created with BioRender.com

Stiffer substrates promote the formation of focal adhesions (FAs), that is, macromolecular protein clusters that connect and tether the cell to the extracellular matrix (Fig.3b)**.** Signalling molecules such as focal adhesion kinase (FAK) and paxillin are activated by the formation of this protein cluster via phosphorylation and thus, activate mechano-responsive signalling, such as RhoA and MAPK (Huveneers and Danen 2009; Vining and Mooney 2017). On the contrary, when unphosphorylated, FAK inhibits this pathway and thus, the polymerisation of actin. The phosphorylation of FAK also activates key biochemical pathways that are then transduced into the nucleus to expedite the process of mechanotransduction. The activation of these signalling pathways is important for tissue-specific transcription factor activation and the transduction of mechanical signals that impact differentiation (Childs et al. 2016).

In point of fact, focal adhesions formation and the subsequent signalling cascades that occur inside the cell are of interest for driving the differentiation of MSCs. Following the work of Engler (Engler et al. 2006), it was clear that the cell cytoskeleton plays an important role in MSCs fate determination; where morphological changes correlate with the expression of lineage-specific markers. FAs increase in number and size during osteogenic differentiation. Conversely, the opposite happens for adipogenic regulation. The number of FAs can be tuned via substrate stiffness or patterning. This relationship can be seen in past literature, where MSCs were differentiated by stereo-topographical cues given by precisely controlled silicon nanowires. Here, when exposed to silicon nanowires of different physical properties, osteogenic differentiation could be induced via integrin signalling in the absence of differentiation medium (Kuo et al. 2012). Another example of how integrin signalling has been exploited for stimulating osteogenic differentiation is the work by Lavenus and colleagues. Here, stiff titanium plaques with small non-patterned nanostructures were shown to promote the differentiation of MSCs into osteocytes more efficiently when compared to planar substrates with or without added osteogenic supplements (Lavenus et al. 2011).

It is now generally accepted that substrate patterning as well as the addition of polymers or proteins to the substrate can be used to stimulate cell adhesion, which, in turn, increases cytoskeleton contraction and subsequent osteogenic phenotype stimulation (Anderson et al. 2016; Mobasseri et al. 2017; Tsimbouri et al. 2014). Other active methods have also been exploited to induce MSCs differentiation such as cyclic compressive loading and shear flow which have been shown to play a part in lineage choice for these cells (Huang et al. 2004; Marie 2008; Shyy and Chien 2002).

More recently, researchers have exploited nano vibrational stimulation to influence differentiation of MSCs (Tsimbouri 2017). This method attempts to mimic the naturally occurring nano vibrations of the cell's microenvironment. Nano vibration stimulation increased the contractility of the cytoskeleton and actin reorganization, similar to previously

mentioned mechanical differentiation methods (Anderson et al. 2016; Kuo et al. 2012; Lavenus et al. 2011).

Mechanosensing via ion channels in MSCs

Mechanotransduction is a long term process of adaptation, in which cells exploit a plethora of sensory machineries to probe the physical properties of the environment and respond to external mechanical stimuli. The sustained activation of this mechanism is an important driver of cell adaptation, that can lead to profound cytoskeletal rearrangements and - for non specialized cells - fate commitment (Paluch et al. 2015). On a shorter time scale, transmembrane adaptor proteins, such as integrins, are responsible for relaying mechanical stimuli across the cellular membrane, and mechanosensitive (MS) proteins provide the cell with a sort of “sense of touch”. Among all the MS molecular players directly involved in the differentiation of MSCs, particular attention has been raised by mechanosensitive ion channels.

MS ion channels are responsible for rapid mechanotransduction processes. These channels are expressed in the cell plasma membrane of many different cell types, including MSCs (Coste et al. 2010). They were first associated with mechanotransduction in the late 1970s, when the research of Hudspeth and Corey linked hearing with the activation of MS ion channels (Corey and Hudspeth 1979).

Most of these channels, when activated by force, allow transmembrane ion flux, as well as forming molecular complexes with stress fibres in the cell. These channels are potently affected by substrate rigidity, that tunes their ability to induce rapid electrical and Calcium signals that in turn, have the potential to activate several biochemical signalling cascades (Kobayashi and Sokabe 2010). Mechanical stress, for example, is sensed by MS ion channels in stem cells that then impact their differentiation via articulated intracellular signalling cascades. Ca^{2+} is the primary downstream effector upon MS ion channel activation in non-excitatory cells, such as MSCs (He et al. 2018).

In 2010, a specific MS ion channel family received attention for its role in transducing mechanical cues in vivo. The Piezo channels are non-selective cation channels that have been found to be essential for relaying mechanical processes. This family of channels is composed of two subfamilies: Piezo1 and Piezo2 (Ridone et al. 2019). Piezo1 was first identified as a mechanically activated transmembrane protein in a neuroblastoma cell line, N2A (Coste et al. 2010; Ranade et al. 2015) and it is now widely recognized as a key player in mechanotransduction associated to directly applied forces forces, such as shear stresses (Li et al. 2014), mechanical properties of the local microenvironment (Pathak et al. 2014) or cytoskeleton (Cox et al. 2016) and membrane properties (Ridone et al. 2020). The Piezo1 channel, in particular, has been associated with human neural stem cell (hNSC) and MSC lineage choice in recent literature (Pathak et al. 2014). Pathak and colleagues found that Piezo1 activity was an important determinant in lineage choice of hNSCs via Ca^{2+} influx, nuclear co-localisation of Yes associated protein (Yap) and altered neural-glial specification of the stem cells. Similarly, Sugimoto and colleagues (Sugimoto et al. 2017) investigated the role of Piezo1 in perceiving changes in hydrostatic pressure (HP) in MSCs. They found that this MS ion channel was activated by an increase in HP. Furthermore, they showed that a

known Piezo 1 agonist, Yoda1, promoted BMP-2 expression and osteoblast differentiation. The opposite was true when Piezo1 was inhibited with the use of GsMTx4, a channel peptide antagonist isolated from the venom of *Grammostola Spatulata,* a Chilean tarantula spider (Suchyna et al. 2000) or by inhibiting Piezo1 expression by siRNA. Most recently, (Mousawi et al. 2020) linked Piezo1 channel expression in MSCs with increased migration via the induction of the extracellular signalling molecule, ATP and ensuing P2 receptor signalling. ATP is a key signalling molecule in MSCs that holds an important role in regulating cell proliferation, migration and differentiation via activation of the P2 receptor purinergic signalling and downstream proline tyrosine kinase 2 (PYK2) and mitogen activated protein kinase/ extracellular signal-regulated kinase (MEK/ERK) signalling. In this instance, it is implied by the authors that ATP induction is dependent on $[Ca^{2+}]_i$, which fluctuates upon Piezo1 channel opening. Thus, in this paper, the channel's permeability to Ca^{2+} ions has been exploited as a tool to monitor channel activation, and subsequently downstream biochemical signalling linked to mechanotransduction events (Fig.4).

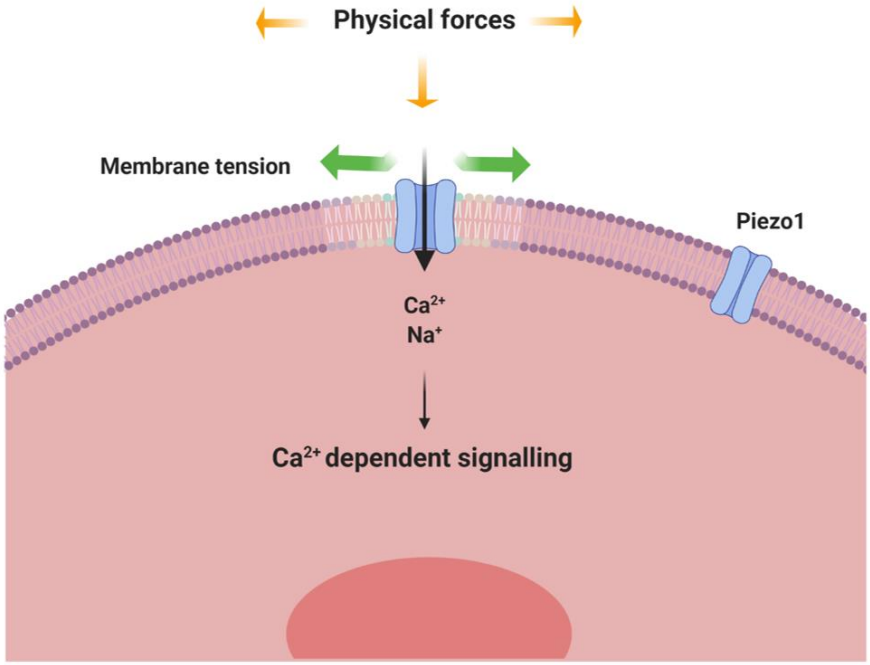

Fig.4 *Proposed mechanism of action of the Piezo1 channel. Mechanical forces, shear flow, membrane tension and composition, all these factors are able to trigger a conformational change in the channel piezo1. The gating of the channel is associated with a local flattening of the membrane and the opening of the central pore, with corresponding flux of cations, including Ca^{2+} which activates intracellular Calcium signalling pathways. As discussed in the text, the exact role of Ca^{2+} signalling in stem cells differentiation is unclear. However, increased intracellular Ca^{2+} signalling induces the upregulation of transcription factors that cause the expression of osteoblast-specific genes, thus stimulating osteogenesis (Viti et al. 2016). Figure inspired by Parpaite and Coste 2017. Created with BioRender.com*

MSC mechanics and differentiation

MSC lineage commitment is highly affected by surrounding or internal cell mechanics. Many key players involved in perceiving and transducing that response into a biological outcome have been described here; can these players be used to describe and characterise cell phenotype along osteogenesis? From a morphological perspective, cell shape towards maturation has been explored for this purpose, as described previously in this work. However, mechanical markers have also been highlighted for describing the MSCs differentiation process. In 2007, Titushkin and Cho (2007) characterised the biomechanical properties of MSCs by AFM and average membrane tether length. Both methods showed increased stiffness in fully differentiated osteoblasts when compared to undifferentiated MSCs (assessed by Young's Modulus and membrane tether length). However, when MSCs were cultured for 10 days in osteogenic media, their biomechanical properties matched those of differentiated osteoblasts. Accordingly, this work determined that actin cytoskeleton remodelling, from thick stress fibres to thin filamentous networks, plays a pivotal role in determining the modulation of MSCs cellular mechanics at the early stage of osteogenesis. In the work of Maloney et al. (2010), AFM and optical stretching (OS), were used to describe the mechanical properties of single primary MSCs in attached and suspended states. Mechanical changes over population doublings (PDs) were compared with molecular markers (assessed by flow cytometry) and with differentiation potential (assessed via established cell differentiation protocols). The results pointed towards the existence of mechanical markers that were consistent with MSCs phenotype; as passage number increased, so did stiffness and stress fiber radius in the attached state. However, the fact that suspended cells do not match these findings restricts the possibility of using mechanical measurements to sort through MSC populations in a high throughput fashion. More recently, Bartolozzi et al. (2020) discussed a biophysical approach for quantifying the mechanical phenotype of single cells along their differentiation pathway using biomechanical testing of a human foetal osteoblast (hFOB) cell line. Single cell nanoindentation measurements along hFOB maturation again showed that cell stiffness (described by Young's Modulus) increased along the maturation process, with a marked initial stiffening (~5-10 days) that saturates over time (~15 days); a trend that can be fitted on a sigmoidal curve. This work also investigated morphological features along the maturation process of hFOB cells, which intuitively corroborated biomechanical characterisation: osteoblasts changes in stiffness highly matched measurements of cell area, perimeter, eccentricity, topography, thickness, and volume. Together, cell morphology and mechanics can concomitantly describe and quantify osteoblast maturation.

Morpho-mechanics are thus intrinsically linked with the mechano-transduction processes associated with bone formation. The plasma membrane mechanosensory channel Piezo1, has recently been found to have a central role in bone formation and anabolism (Li et al. 2019; Sun et al. 2019). In the work of Sun et al. (2019) activation of Piezo1 was shown to be critical in the formation and mechanical-load dependent remodelling of bone in mouse models. It is therefore reasonable to connect cell stiffening in bone formation, with force sensing processes, mediated via channels such as Piezo1 that permeate the cell to Ca^{2+} ions. Together, these cues provide biophysical phenotypic markers along osteogenesis that describe this process in a non-invasive manner.

Conclusions

MSCs have emerged as a powerful biomedical tool in cell and tissue engineering and regenerative medicine (Fitzsimmons 2018). The possibility to monitor and finely control the differentiation process towards a specific lineage is the key to unleash their therapeutic potential. In this review, we present a range of biophysical methods intended to address this challenge, providing label-free biomarkers to identify the biological stage along the osteogenic differentiation pathway. The shape of the cell, the organization of the cytoskeleton, the calcium homoeostasis, the mechanical properties, all these characteristics progressively change, and correlate with the phenotype of the population. Some connections between these indicators are explicit, as the structure of the cytoskeleton is expected to impact the shape and mechanical properties of the cell. In other conditions, the link is more subtle, as the one connecting mechanics and calcium through the functionality and expression of mechanosensitive ion channels. The proposed biophysical methods are powerful tools to monitor the differentiation stage of MSCs, but they can also extend the current view of the process, and suggest future directions to better understand the complex and multi-faceted process of differentiation.

Declarations

Conflicts of interest/Competing interests
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Consent to participate
All authors agreed with the content of the manuscript and all gave explicit consent to submit.

Consent for publication
All authors made substantial contributions to the work; drafted the work or revised it critically for important intellectual content; approved the version to be published; and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

References

- Alford AI, Kozloff KM, Hankenson KD. Extracellular matrix networks in bone remodeling. Int. J. Biochem. Cell Biol. 2015;65:20–31.
- Anderson HJ, Sahoo JK, Ulijn RV, Dalby MJ. Mesenchymal stem cell fate: Applying biomaterials for control of stem cell behavior. Front. Bioeng. Biotechnol. 2016;4:38.
- Aquino-Martínez R, Artigas N, Gámez B, Rosa JL, Ventura F. Extracellular calcium promotes bone formation from bone marrow mesenchymal stem cells by amplifying the effects of BMP-2 on SMAD signalling. PLoS One. 2017;12(5):e0178158.
- Banik BL, Riley TR, Platt CJ, Brown JL. Human Mesenchymal Stem Cell Morphology and Migration on Microtextured Titanium. Front Bioeng Biotechnol. 2016;4:41.

- Barradas AM, Fernandes HA, Groen N, Chai YC, Schrooten J, van de Peppel J, van Leeuwen JP, van Blitterswijk CA, de Boer J. A calcium-induced signaling cascade leading to osteogenic differentiation of human bone marrow-derived mesenchymal stromal cells. Biomaterials. 2012 (11):3205-15.
- Bartolozzi A, Viti F, De Stefano S, Sbrana F, Petecchia L, Gavazzo P, Vassalli M. Development of label-free biophysical markers in osteogenic maturation. J Mech Behav Biomed Mater. 2020;103:103581.
- Barty A, Nugent K, Paganin D, Roberts A. Quantitative optical phase microscopy. Opt Lett. 1998;23:817–9.
- Bianco P, Cao X, Frenette PS, Mao JJ, Robey PG, Simmons PJ, Wang CY. The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nat Med. 2013;19:35-42.
- Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology and potential applications. Stem Cells. 2001;19(3):180–92.
- Biggs MJP, Richards RG, Gadegaard N, Wilkinson CDW, Oreffo ROC, Dalby MJ. The use of nanoscale topography to modulate the dynamics of adhesion formation in primary osteoblasts and ERK/MAPK signalling in STRO-1+ enriched skeletal stem cells. Biomaterials. 2009;30(28):5094–103.
- Bonnans C, Chou J, Werb Z. Remodelling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol. 2014;15:786–801.
- Briggs T, Treiser MD, Holmes PF, Kohn J, Moghe PV, Arinzeh TL. Osteogenic differentiation of human mesenchymal stem cells on poly(ethylene glycol)-variant biomaterials. J Biomed Mater Res A. 2009;91(4):975-84.
- Caplan AI. Mesenchymal stem cells. J Orthop Res. 1991;9:641–50.
- Ceccarelli G, Bloise N, Mantelli M, Gastaldi G, Fassina L, De Angelis MG, Ferrari D, Imbriani M, Visai L. A comparative analysis of the in vitro effects of pulsed electromagnetic field treatment on osteogenic differentiation of two different mesenchymal cell lineages. Biores. Open Access. 2013;2:283-94.
- Childs PG, Boyle CA, Pemberton GD, Nikukar H, Curtis ASG, Henriquez FL, Dalby MJ, Reid S. Use of nanoscale mechanical stimulation for control and manipulation of cell behaviour. Acta Biomater. 2016;34:159-68.
- Corey DP, Hudspeth AJ. Response latency of vertebrate hair cells. Biophys. J. 1979;26, 499–506.
- Coste B, Mathur J, Schmidt M, Earley TJ, Ranade S, Petrus MJ, Dubin AE, Patapoutian A. Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. Science. 2010;330(6000):55-60.
- Cox CD, Bae C, Ziegler L, Hartley S, Nikolova-Krstevski V, Rohde PR, Ng C-A, Sachs F, Gottlieb PA, Martinac B. Removal of the mechanoprotective influence of the cytoskeleton reveals PIEZO1 is gated by bilayer tension. Nat. Comm. 2016;7:10366.
- Curry AS, Pensa NW, Barlow AM, Bellis SL. Taking cues from the extracellular matrix to design bone-mimetic regenerative scaffolds. Matrix Biol. 2016;52-54:397-412.
- Cutiongco MFA, Jensen BS, Reynolds PM, Gadegaard N. Predicting gene expression using morphological cell responses to nanotopography. Nat Commun. 2020;11:1384.
- Dalby MJ, Biggs MJP, Gadegaard N, Kalna G, Wilkinson CDW, Curtis ASG. Nanotopographical stimulation of mechanotransduction and changes in interphase centromere positioning. J Cell Biochem. 2007a;100(2):326–38.

- Dalby MJ, Gadegaard N, Herzyk P, Sutherland D, Agheli H, Wilkinson CDW, Curtis ASG. Nanomechanotransduction and interphase nuclear organization influence on genomic control', J Cell Biochem. 2007b;102(5):1234–44.
- Dalby MJ, Gadegaard N, Tare R, Andar A, Riehle MO, Herzyk P, Wilkinson CD, Oreffo RO. The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder. Nat Mater. 2007c;6:997–1003.
- Dalby M, García A, Salmeron-Sanchez M. Receptor control in mesenchymal stem cell engineering. Nat Rev Mater. 2018;3:17091.
- Dimitriou R, Mataliotakis GI, Angoules AG, Kanakaris NK, Giannoudis PV. Complications following autologous bone graft harvesting from the iliac crest and using the RIA: A systematic review. Injury. 2011;42(2):S3–S15.
- Discher DE, Janmey P, Wang YL. Tissue cells feel and respond to the stiffness of their substrate. Science. 2005;310(5751):1139-43.
- Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315–7.
- Domura R, Sasaki R, Ishikawa Y, Okamoto M. Cellular Morphology-Mediated Proliferation and Drug Sensitivity of Breast Cancer Cells. J Funct Biomater. 2017;8(2):pii: E18.
- Donnelly H, Salmerón-Sánchez M, Dalby MJ. Designing stem cell niches for differentiation and self-renewal. J. R. Soc. Interface. 2018;15(145):20180388.
- Elefteriou F, Benson M, Sowa H, Starbuck M, Liu X, Ron D, Parada L, Karsenty G. ATF4 mediation of NF1 functions in osteoblast reveals a nutritional basis for congenital skeletal dysplasiae. Cell Metab. 2006;4(6):441–51.
- Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix Elasticity Directs Stem Cell Lineage Specification. Cell. 2006;126:677–89.
- Fassina L, Saino E, Visai L, Silvani G, Cusella De Angelis MG, Mazzini G, Benazzo F, Magenes G. Electromagnetic enhancement of a culture of human SAOS-2 osteoblasts seeded onto titanium fiber-mesh scaffolds. J Biomed Mater Res A. 2008;87(3):750-9.
- Ferraro P, Wax A, Zeev Zalevsky. Coherent Light Microscopy, Imaging and Quantitative Phase Analysis. Springer-Verlag; 2011.
- Fitzsimmons REB, Mazurek MS, Soos A, Simmons CA. Mesenchymal Stromal/Stem Cells in Regenerative Medicine and Tissue Engineering. Stem Cells Int. 2018;2018:8031718.
- Ford J. Red blood cell morphology. Int J Lab Hematol. 2013;35(3):351-7.
- Friedenstein AJ. Precursor Cells of Mechanocytes. Int Rev Cytol. 1976;47:327–59.
- Gasser JA, Kneissel M. Bone Physiology and Biology. In: Smith SY, Varela A, Samadfam R, editors. Bone Toxicology. Cham: Springer International Publishing; 2017. pp. 27–94.
- Ge C, Cawthron WP, Li Y, Zhao G, Macdougald OA, Franceschi RT. Reciprocal Control of Osteogenic and Adipogenic Differentiation by ERK/MAP Kinase Phosphorylation of Runx2 and PPARγ Transcription Factors. J Cell Physiol. 2016; 231(3):69–81.
- Ge C, Xiao G, Jiang D, Franceschi RT. Critical role of the extracellular signal–regulated kinase–MAPK pathway in osteoblast differentiation and skeletal development. J Cell Biol. 2007;176(5):709–18.
- Geiger B, Spatz JP, Bershadsky AD. Environmental sensing through focal adhesions. Nat Rev Mol Cell Biol. 2009;10:21–33.

- Ghali O, Broux O, Falgayrac G, Haren N, Van Leeuwen JPTM, Penel G, Hardouin P, Chauveau C. Dexamethasone in osteogenic medium strongly induces adipocyte differentiation of mouse bone marrow stromal cells and increases osteoblast differentiation. BMC Cell Biol. 2015;16(1):1–15.
- Giannoudis PV, Chris Arts JJ, Schmidmaier G, Larsson S. What should be the characteristics of the ideal bone graft substitute?. *Injury*. 2011;42(2):S1.
- Hei H, Gao J, Dong J, Tao J, Tian L, Pan W, Wang H, Zhang X. BK Knockout by TALEN-Mediated Gene Targeting in Osteoblasts: KCNMA1 Determines the Proliferation and Differentiation of Osteoblasts. Mol Cells. 2016;39(7):530-5.
- He L, Ahmad M, Perrimon N. Mechanosensitive channels and their functions in stem cell differentiation. Exp. Cell Res. 2018;374:259–65.
- Heubach JF, Graf EM, Leutheuser J, Bock M, Balana B, Zahanich I, Christ T, Boxberger S, Wettwer E, Ravens U. Electrophysiological properties of human mesenchymal stem cells. J Physiol. 2004;554:659-72.
- Huang C-YC, Hagar KL, Frost LE, Sun Y, Cheung HS. Effects of Cyclic Compressive Loading on Chondrogenesis of Rabbit Bone-Marrow Derived Mesenchymal Stem Cells. Stem Cells. 2004;22:313–23.
- Huveneers S, Danen EHJ. Adhesion signaling - Crosstalk between integrins, Src and Rho. J. Cell Sci. 2009;122:1059-69.
- Ingber DE. Tensegrity I. Cell structure and hierarchical systems biology. J Cell Sci. 2003a;116(7):1157–73.
- Ingber DE. Tensegrity II. How structural networks influence cellular information processing networks. J Cell Sci. 2003b;116(8):1397–1408.
- Ito T, Itakura S, Todorov I, Rawson J, Asari S, Shintaku J, Nair I, Ferreri K, Kandeel F, Mullen Y. Mesenchymal stem cell and islet co-transplantation promotes graft revascularization and function. Transplantation. 2010;89(12):1438–45.
- Jaalouk DE, Lammerding J. Mechanotransduction gone awry. Nat Rev Mol Cell Biol. 2009;10:63–73.
- Kawaguchi N, Sundberg C, Kveiborg M, Moghadaszadeh B, Asmar M, Dietrich N, Thodeti CK, Nielsen FC, Moller P, Mercurio AM, Albrechtsen R, Wewer UM. ADAM12 induces actin cytoskeleton and extracellular matrix reorganization during early adipocyte differentiation by regulating beta1 integrin function. J Cell Sci. 2003;116(Pt 19):3893-904.
- Kawano S, Otsu K, Shoji S, Yamagata K, Hiraoka M. Ca(2+) oscillations regulated by Na(+)-Ca(2+) exchanger and plasma membrane Ca(2+) pump induce fluctuations of membrane currents and potentials in human mesenchymal stem cells. Cell Calcium. 2003;34(2):145-56.
- Kawano S, Shoji S, Ichinose S, Yamagata K, Tagami M, Hiraoka M. Characterization of Ca(2+) signaling pathways in human mesenchymal stem cells. Cell Calcium. 2002;32(4):165-74.
- Kobayashi T, Sokabe M. Sensing substrate rigidity by mechanosensitive ion channels with stress fibers and focal adhesions. Curr Opin Cell Biol. 2010;22(5):669-76.
- Kou SS, Waller L, Barbastathis G, Sheppard CJ. Transport-of-intensity approach to differential interference contrast (TI-DIC) microscopy for quantitative phase imaging. Opt Lett. 2010;35:447–9.
- Kronenberg HM. Developmental regulation of the growth plate. Nature. 2003;423(6937):332-6.

- Kuo SW, Lin HI, Hui-Chun Ho J, Shih YRV, Chen HF, Yen TJ, Lee OK. Regulation of the fate of human mesenchymal stem cells by mechanical and stereo-topographical cues provided by silicon nanowires. Biomaterials. 2012;33:5013–22.
- Lane S, Williams D, Watt F. Modulating the stem cell niche for tissue regeneration. Nat Biotechnol. 2014;32:795–803.
- Langelier E, Suetterlin R, Hoemann CD, Aebi U, Buschmann MD. The chondrocyte cytoskeleton in mature articular cartilage: structure and distribution of actin, tubulin, and vimentin filaments. J Histochem Cytochem. 2000;48(10):1307-20.
- Lavenus S, Berreur M, Trichet V, Pilet P, Louarn G, Layrolle P. Adhesion and osteogenic differentiation of human mesenchymal stem cells on titanium nanopores. Eur Cells Mater. , 2011;22:84–96.
- Lee K, Kim K, Jung J, Heo J, Cho S, Lee S, Chang G, Jo Y, Park H, Park Y. Quantitative Phase Imaging Techniques for the Study of Cell Pathophysiology: From Principles to Applications. Sensors. 2013;13(4):4170-91.
- Lee OK, Liu Y-S. In Search of the Pivot Point of Mechanotransduction: Mechanosensing of Stem Cells. Cell Transplant. 2014;23:1–11.
- Li GR, Sun H, Deng X, Lau CP. Characterization of ionic currents in human mesenchymal stem cells from bone marrow. Stem Cells. 2005;23:371-82.
- Li J, Hou B, Tumova S, Muraki K, Bruns A, Ludlow MJ, Sedo A, Hyman AJ, McKeown L, Young RS, Yuldasheva NY, Majeed Y, Wilson LA, Rode B, Bailey MA, Kim HR, Fu Z, Carter DA, Bilton J, Imrie H, Ajuh P, Dear TN, Cubbon RM, Kearney MT, Prasad RK, Evans PC, Ainscough JF, Beech DJ. Piezo1 integration of vascular architecture with physiological force. Nature. 2014;515(7526):279-82.
- Li X, Han L, Nookaew I, Mannen E, Silva MJ, Almeida M, Xiong J. Stimulation of piezo1 by mechanical signals promotes bone anabolism. Elife. 2019;8:1–22.
- Loewke NO, Pai S, Cordeiro C, Black D, King BL, Contag CH, Chen B, Baer TM, Solgaard O. Automated Cell Segmentation for Quantitative Phase Microscopy. IEEE Trans Med Imaging. 2018;37(4):929–40.
- Long EG, Buluk M, Gallagher MB, Schneider JM, Brown JL. Human mesenchymal stem cell morphology, migration, and differentiation on micro and nano-textured titanium. Bioact Mater. 2019;4:249-55.
- Long F. Building strong bones: Molecular regulation of the osteoblast lineage. Nat Rev Mol Cell Biol. 2012;13(1):27–38.
- Loye AM, Kinser ER, Bensouda S, Shayan M, Davis R, Wang R, Chen Z, Schwarz UD, Schroers J, Kyriakides TR. Regulation of Mesenchymal Stem Cell Differentiation by Nanopatterning of Bulk Metallic Glass. Sci Rep. 2018;8:8758.
- Maloney JM, Nikova D, Lautenschläger F, Clarke E, Langer R, Guck J, Van Vliet KJ. Mesenchymal stem cell mechanics from the attached to the suspended state. Biophys J. 2010;99:2479–87.
- Mao AS, Shin JW, Mooney DJ. Effects of substrate stiffness and cell-cell contact on mesenchymal stem cell differentiation. Biomaterials. 2016;98:184-91.
- Marie PJ. Transcription factors controlling osteoblastogenesis. Arch Biochem Biophys. 2008;473(2):98-105.
- Martino F, Perestrelo AR, Vinarský V, Pagliari S, Forte G. Cellular mechanotransduction: From tension to function. Front Physiol. 2018;9:824.

- Matsuoka F, Takeuchi I, Agata H, Kagami H, Shiono H, Kiyota Y, Honda H, Kato R. Morphology-based prediction of osteogenic differentiation potential of human mesenchymal stem cells. PLoS One. 2013;8(2):e55082.
- McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell. 2004;6(4):483-95.
- McNamara LE, Burchmore R, Riehle MO, Herzyk P, Biggs MJP, Wilkinson CDW, Curtis ASG, Dalby MJ. The role of microtopography in cellular mechanotransduction. Biomaterials. 2012;33(10):2835–47.
- McNamara LE, McMurray RJ, Biggs MJP, Kantawong F, Oreffo ROC, Dalby MJ, Salih V. Nanotopographical Control of Stem Cell Differentiation. J Tissue Eng. 2010; https://doi.org/10.4061/2010/120623
- McQuin C, Goodman A, Chernyshev V, Kamentsky L, Cimini BA, Karhohs KW, Doan M, Ding L, Rafelski SM, Thirstrup D, Wiegraebe W, Singh S, Becker T, Caicedo JC, Carpenter AE (2018). CellProfiler 3.0: Next-generation image processing for biology. PLoS Biol. 16(7):e2005970.
- Mehler VJ, Burns CJ, Moore ML. Concise Review: Exploring immunomodulatory features of Mesenchymal Stromal Cells in humanized mouse models. Stem Cells. 2018;37(3):298-305.
- Merola F, Miccio L, Memmolo P, Di Caprio G, Galli A, Puglisi R, Balduzzi D, Coppola G, Netti P, Ferraro P. Digital holography as a method for 3D imaging and estimating the biovolume of motile cells. Lab Chip. 2013;13(23):4512–6.
- Mobasseri R, Tian L, Soleimani M, Ramakrishna S, Naderi-Manesh H. Bio-active molecules modified surfaces enhanced mesenchymal stem cell adhesion and proliferation. Biochem Biophys Res Commun. 2017;483:312–7.
- Mohammed D, Versaevel M, Bruyère C, Alaimo L, Luciano M, Vercruysse E, Procès A, Gabriele S. Innovative Tools for Mechanobiology: Unraveling Outside-In and Inside-Out Mechanotransduction. Front Bioeng Biotechnol. 2019;1:162.
- Mousawi F, Peng H, Li J, Ponnambalam S, Roger S, Zhao H, Yang X, Jiang L-H. Chemical activation of the Piezo1 channel drives mesenchymal stem cell migration via inducing ATP release and activation of P2 receptor purinergic signaling. Stem Cells. 2020;38:410–21.
- Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, De Crombrugghe B. The Novel Zinc Finger-Containing Transcription Factor Osterix Is Required for Osteoblast Differentiation and Bone Formation. Cell. 2002;108:17–29.
- Nečas D, Klapetek P. Gwyddion: an open-source software for SPM data analysis. Cent Eur J Phys. 2012;10:181–8.
- Paino F, La Noce M, Giuliani A, De Rosa A, Mazzoni S, Laino L, Amler E, Papaccio G, Desiderio V, Tirino V. Human DPSCs fabricate vascularized woven bone tissue: a new tool in bone tissue engineering. Clin Sci (Lond). 2017;131(8):699–713.
- Paluch EK, Nelson CM, Biais N, Fabry B, Moeller J, Pruitt BL, Wollnik C, Kudryasheva G, Rehfeldt F, Federle W. Mechanotransduction: Use the force(s). BMC Biol. 2015;13:47.
- Panaroni C, Tzeng Y-S, Saeed H, Wu JY. Mesenchymal Progenitors and the Osteoblast Lineage in Bone Marrow Hematopoietic Niches. Curr Osteoporos Rep. 2014;12: 22–32.
- Park YK, Popescu G, Ferraro P, Kemper B. Quantitative Phase Imaging and Its Applications to Biophysics, Biology, and Medicine. Front Phys. 2020;7:226.
- Parpaite T, Coste B. Piezo channels. Curr Biol. 2017;27:243–58.

- Pathak MM, Nourse JL, Tran T, Hwe J, Arulmoli J, Le DTT, Bernardis E, Flanagan LA, Tombola F. Stretch-activated ion channel Piezo1 directs lineage choice in human neural stem cells. PNAS USA. 2014;111:16148–53.
- Paturzo M, Pagliarulo V, Bianco V, Memmolo P, Miccio L, Merola F, Ferraro P. Digital Holography, a metrological tool for quantitative analysis: Trends and future applications. Opt Lasers Eng. 2018;104:32-47.
- Pchelintsev E, Djamgoz MBA. Mesenchymal stem cell differentiation: Control by calcium-activated potassium channels. J Cell Physiol. 2018;233:3755-68.
- Petecchia L, Usai C, Vassalli M, Gavazzo P. Biophysical characterization of nanostructured TiO 2 as a good substrate for hBM-MSC adhesion, growth and differentiation. Exp Cell Res. 2017;S0014:4827.
- Petecchia L, Sbrana F, Utzeri R, Vercellino M, Usai C, Visai L, Vassalli M, Gavazzo P. Electro-magnetic field promotes osteogenic differentiation of BM-hMSCs through a selective action on Ca2+-related mechanisms. Sci Rep. 2015;5:13856.
- Pileggi A, Xu X, Tan J, Ricordi C. Mesenchymal Stromal (Stem) Cells to Improve Solid Organ Transplant Outcome: Lessons from the Initial Clinical Trials. Curr Opin Organ Transplant. 2013;18(6):672–81.
- Pinho S, Frenette S. Haematopoietic stem cell activity and interactions with the niche. Nat Rev Mol Cell Biol. 2019;20(5):303-320.
- Popescu G, Park Y. Quantitative phase imaging in biomedicine. J Biomed Opt. 2015;20(11):111201.
- Ranade SS, Syeda R, Patapoutian A. Mechanically Activated Ion Channels. Neuron. 2015;87(6):1162-79.
- Rangamani P, Lipshtat A, Azeloglu EU, Calizo RC, Hu M, Ghassemi S, Hone J, Scarlata S, Neves SR, Iyengar R. Decoding information in cell shape. Cell. 2013;154(6):1356-69.
- Rezakhaniha R, Agianniotis A, Schrauwen JTC, Griffa A, Sage D, Bouten CVC, van de Vosse FN, Unser M, Stergiopulos N. Experimental Investigation of Collagen Waviness and Orientation in the Arterial Adventitia. Biomech Model Mechanobiol. 2012;11:461–73.
- Ridone P, Vassalli M, Martinac B. Piezo1 mechanosensitive channels: what are they and why are they important. Biophys Rev. 2019;11:795–805.
- Ridone P, Pandzic E, Vassalli M, Cox C, Macmillan A, Gottlieb P, Martinac B. Disruption of membrane cholesterol organization impairs the activity of PIEZO1 channel clusters. J Gen Physiol. 2020; https://doi.org/10.1085/jgp.201912515
- Robertson SN, Campsie P, Childs PG, Madsen F, Donnelly H, Henriquez FL, Mackay WG, Salmerón-Sánchez M, Tsimbouri MP, Williams C, Dalby MJ, Reid S. Control of cell behaviour through nanovibrational stimulation: Nanokicking. Phil Trans R Soc A. 2018;376:20170290.
- Rodriguez JP, Gonzalez M, Rios S, Cambiazo V. Cytoskeletal organization of human mesenchymal stem cells (MSC) changes during their osteogenic differentiation. J Cell Biochem. 2004;93(4):721-31.
- Rueden CT, Schindelin J, Hiner MC, DeZonia BE, Walter AE, Arena ET, Eliceiri KW. ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinformatics. 2017;18:529.
- Sbrana F, Landini E, Gjeci N, Viti F, Ottaviani E, Vassalli M. OvMeter: an automated 3D-integrated opto-electronic system for Ostreopsis cf. ovata bloom monitoring. J Appl Phycol. 2017;29(3):1363-75.

- Schakenraad K, Ernst J, Pomp W, Danen EHJ, Merks RMH, Schmidt T, Giomi L. Mechanical interplay between cell shape and actin cytoskeleton organization. 2019. arXiv:1905.09805 [physics.bio-ph]
- Schiller HB, Fässler R. Mechanosensitivity and compositional dynamics of cell-matrix adhesions. EMBO Rep. 2013;14(6):509–19.
- Schwartz Z, Simon BJ, Duran MA, Barabino G, Chaudhri R, Boyan BD. Pulsed electromagnetic fields enhance BMP-2 dependent osteoblastic differentiation of human mesenchymal stem cells. J Orthop Res. 2008;26(9):1250-5.
- Shih YR, Hwang Y, Phadke A, Kang H, Hwang NS, Caro EJ, Nguyen S, Siu M, Theodorakis EA, Gianneschi NC, Vecchio KS, Chien S, Lee OK, Varghese S. Calcium Phosphate bearing matrices induce osteogenic differentiation of stem cells through adenosine signaling. PNAS USA. 2014;111(3):990-5.
- Shyy JYJ, Chien S. Role of integrins in endothelial mechanosensing of shear stress. Circ Res. 2002;91:769–75.
- Silver IA, Murrills RJ, Etherington DJ. Microelectrode studies on the acid microenvironments behind the environments beneath adherent macrophage and osteoclast. Exp Cell Res. 1988;175(2):266-272.
- Sonowal H, Kumar A, Bhattacharyya J, Gogoi PK, Jaganathanet BG. Inhibition of actin polymerization decreases osteogeneic differentiation of mesenchymal stem cells through p38 MAPK pathway. J Biomed Sci. 2013;20:71.
- Suchyna TM, Johnson JH, Hamer K, Leykam JF, Gage DA, Clemo HF, Baumgarten CM, Sachs F. Identification of a peptide toxin from Grammostola spatulata spider venom that blocks cation-selective stretch-activated channels. J Gen Physiol. 2000;115:583–98.
- Sugimoto A, Miyazaki A, Kawarabayashi K, Shono M, Akazawa Y, Hasegawa T, Ueda-Yamaguchi K, Kitamura T, Yoshizaki K, Fukumoto S, Iwamoto T. Piezo type mechanosensitive ion channel component 1 functions as a regulator of the cell fate determination of mesenchymal stem cells. Sci Rep. 2017;7:1–14.
- Su H, Yin Z, Huh S, Kanade T. Cell segmentation in phase contrast microscopy images via semi-supervised classification over optics-related features. Medical Image Analysis. 2013;17:746-65.
- Sun S, Liu Y, Lipsky S, Cho M. Physical manipulation of calcium oscillations facilitates osteodifferentiation of human mesenchymal stem cells. FASEB J. 2007;21(7):1472-80.
- Sun W, Chi S, Li Y, Ling S, Tan Y, Xu Y, Jiang F, Li J, Liu C, Zhong G, Cao D, Jin X, Zhao D, Gao X, Liu Z, Xiao B, Li Yingxian. The mechanosensitive Piezo1 channel is required for bone formation. Elife. 2019;8:e47454.
- Szpalski C, Wetterau M, Barr J, Warren SM. Bone tissue engineering: current strategies and techniques part I: Scaffolds. Tissue Eng Part B Rev. 2012;18(4):246-57.
- Tanikake Y, Akahane M, Furukawa A, Tohma Y, Inagaki Y, Kira T, Tanaka Y . Calcium concentration in culture medium as a nondestructive and rapid marker of osteogenesis. Cell Transplant. 2017;26:1067-76.
- Tan YZ, Fei DD, He XN, Dai JM, Xu RC, Xu XY, Wu JJ, Li B. L-type voltage-gated calcium channels in stem cells and tissue engineering. Cell Prolif. 2019;52(4):e12623.
- Tao R, Sun HY, Lau CP, Tse HF, Lee HC, Li GR. Cyclic ADP ribose is a novel regulator of intracellular Ca2+ oscillations in human bone marrow mesenchymal stem cells. J Cell Mol Med. 2011;15:2684-96.

- Theveneau E, Mayor R. Cadherins in collective cell migration of mesenchymal cells. Curr Opin Cell Biol. 2012;24:677-84.
- Titushkin I, Cho M. Modulation of cellular mechanics during osteogenic differentiation of human mesenchymal stem cells. Biophys J. 2007;93:3693–702.
- Treiser MD, Yang EH, Gordonov S, Cohen DM, Androulakis IP, Kohn J, Chen CS, Moghe PV. Cytoskeleton-based forecasting of stem cell lineage fates. PNAS USA. 2010;107(2):610-5.
- Tsimbouri P, Gadegaard N, Burgess K, White K, Reynolds P, Herzyk P, Oreffo R, Dalby MJ. Nanotopographical Effects on Mesenchymal Stem Cell Morphology and Phenotype. J Cell Biochem. 2014;115:380–90.
- Tsimbouri PM, Childs PG, Pemberton GD, Yang J, Jayawara V, Oripiriyakul W, Burgess K, González-García C, Blackburn G, Thomas D, Vallejo-Giraldo C, Biggs MJP, Curtis ASG, Salmerón-Sánchez M, Reid S, Dalby MJ. Stimulation of 3D osteogenesis by mesenchymal stem cells using a nanovibrational bioreactor. Nat Biomed Eng. 2017;1:758-70.
- Tsimbouri PM, Murawski K, Hamilton G, Herzyk P, Oreffo ROC, Gadegaard N, Dalby MJ. A genomics approach in determining nanotopographical effects on MSC phenotype. Biomaterials. 2013;34(9):2177–84.
- Varga Z, Juhász T, Matta C, Fodor J, Katona É, Bartok A, Oláh T, Sebe A, Csernoch L, Panyi G, Zákány R. Switch of voltage-gated K+ channel expression in the plasma membrane of chondrogenic cells affects cytosolic Ca2+-oscillations and cartilage formation. PLoS One. 2011;6(11):e27957.
- Vining KH, Mooney DJ. Mechanical forces direct stem cell behaviour in development and regeneration. Nat Rev Mol Cell Biol. 2017;18:728–42.
- Viti F, Landini M, Mezzelani A, Petecchia L, Milanesi L, Scaglione S. Osteogenic differentiation of MSC through calcium signaling activation: Transcriptomics and functional analysis. PLoS One. 2016;11(2):e0148173.
- Walleczek J. Electromagnetic field effects on cells of the immune system: the role of calcium signaling. FASEB J. 1992;6:3177- 85.
- Wang C, Li Y, Yang M, Zou Y, Liu H, Liang Z, Yin Y, Niu G, Yan Z, Zhang B. Efficient Differentiation of Bone Marrow Mesenchymal Stem Cells into Endothelial Cells in Vitro. Eur J Vasc Endovasc Surg. 2018;55(2):257-65.
- Wang N, Tytell JD, Ingber DE. Mechanotransduction at a distance: Mechanically coupling the extracellular matrix with the nucleus. Nat Rev Mol Cell Biol. 2009;10(1):75–82.
- World Health Organization. World report on ageing and health. World Health Organization; 2015. https://apps.who.int/iris/handle/10665/186463
- Wu PH, Phillip JM, Khatau SB, Chen WC, Stirman J, Rosseel S, Tschudi K, Van Patten J, Wong M, Gupta S, Baras AS, Leek JT, Maitra A, Wirtz D. Evolution of cellular morpho-phenotypes in cancer metastasis. Sci Rep. 2015;5:18437.
- Yang J, McNamara LE, Gadegaard N, Alakpa EV, Burgess KV, Meek RMD, Dalby MJ. Nanotopographical Induction of Osteogenesis through Adhesion, Bone Morphogenic Protein Cosignaling, and Regulation of MicroRNAs. ACS Nano. 2014;8(10):9941–53.
- Yang L, Tsang KY, Tang HC, Chan D, Cheah KSE. Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. PNAS USA. 2014;111(33):12097–102.
- Yourek G, Hussain MA, Mao JJ. Cytoskeletal changes of mesenchymal stem cells during differentiation. ASAIO J. 2007;53(2):219-28.

- Yu VWC, Ambartsoumian G, Verlinden L, Moir JM, Prud'homme J, Gauthier C, Roughley PJ, St-Arnaud R. FIAT represses ATF4-mediated transcription to regulate bone mass in transgenic mice. J Cell Biol. 2005;169(4):591–601.
- Zahanich I, Graf EM, Heubach JF, Hempel U, Boxberger S, Ravens U. Molecular and functional expression of voltage-operated calcium channels during osteogenic differentiation of human mesenchymal stem cells. J Bone Miner Res. 2005;20(9):1637-46.
- Zhang S, Zhao C, Liu S, Wang Y, Zhao Y, Guan W, Zhu Z. Characteristics and multi-lineage differentiation of bone marrow mesenchymal stem cells derived from the Tibetan mastiff. Mol Med Rep. 2018;18(2):2097-109.
- Zhang YY, Yue J, Che H, Sun HY, Tse HF, Li GR. BKCa and hEag1 channels regulate cell proliferation and differentiation in human bone marrow-derived mesenchymal stem cells. J Cell Physiol. 2014;229(2):202-12.
- Zuo C, Chen Q, Asundi A. Comparison of Digital Holography and Transport of Intensity for Quantitative Phase Contrast Imaging. In: Osten W. (eds) Fringe 2013. Springer, Berlin, Heidelberg; 2014. pp. 137-42.