Chapter 1
A Review of Bioreactors and Mechanical Stimuli

The increased need to accelerate the healing process of critical size defects in the bone led to the study of optimal combination of cells, materials and external stimuli to obtain fully differentiated tissue to the injured site. Bioreactors play a crucial role in the control over the development of functional tissue allowing control over the surrounding chemical and mechanical environment. This chapter aims to review bioreactor systems currently available for monitoring mesenchymal stem cells (MSCs) behaviour under mechanical stimuli and to give an insight of their effect on cellular commitment. Shear stress, mechanical strain and pulsed electromagnetic field bioreactors are presented, and the effect of multiple conditions under varying parameters such as amplitude, frequency or duration of the stimuli on bone progenitor cells differentiation is considered and extensively discussed with particular focus on osteogenic and chondrogenic commitment.

1.1 Introduction to the Tissue Engineering Approach

1.1.1 Mesenchymal Stem Cells and External Environment

Long bone fracture gaps can be repaired through the use of natural and synthetic grafts seeded with cells to enhance tissue formation. For this purpose, the tissue engineering (TE) approach aims to use cells directly harvested from the donor and then expand them in cultures to reach the desired number. Osteoblasts are the most obvious choice for bone TE purposes as they are the main precursors of the bone. Despite this, their low proliferation rate and their fully differentiated state present issues. Moreover, there are problems related to the lack of tissue source and morbidity (Finkemeier 2002). As a consequence, mesenchymal stem cells (MSCs) currently are the next cellular target (Salgado et al. 2004) to satisfy the demand for an increased proliferation rate and a reduced amount of surgical intervention. Indeed, MSCs present high proliferation rates and can also be obtained from several sources such as bone marrow, adipose tissue or cord blood. Their undifferentiated state allows them to differentiate towards diverse lineages such as osteoblasts, chondrocytes, adipocytes or myocytes (Caplan 2007). After expansion and seeding

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onto the scaffolds, cells usually are stimulated through bioreactors to drive their differentiation towards a defined pathway and to obtain fully differentiated tissue to implant. Applying external stimuli, cells activate biochemical pathways defining the functional properties of the resulting engineered tissue (Hoffman et al. 2011). For example, chemical stimulation was found to be particularly promising. On this regard, fibroblast growth factors (FGFs) showed to increase self-renewal and to maintain cell multi-lineage differentiation potential, transforming growth factors (TGFs) and serum-free medium-induced chondrogenesis; bone morphogenic proteins (BMPs) and dexamethasone were instead the most relevant chemical factors inducing osteogenesis and have already been employed for clinical treatments such as spinal fusion and long bone fractures (Wilson et al. 2005).

Another stimuli having an impact on cells differentiation is the mechanical load. As a matter of fact, the bone is constantly under loading condition arising from the daily activities. Vigorous exercise induces up to 1000 microstrain in human bone, where 1000 microstrain equal to 0.01% change in length compared to the initial length, and are associated with bone mass increase (Klein-Nulend et al. 2012). As many evidences have shown the possibility to influence cell behaviour through mechanical stimulation (Ehrlich and Lanyon 2002; Kelly and Jacobs 2010), the use of external mechanical stimuli on cell differentiation has become an increasingly common practice nowadays.

1.1.2 Mechanical Stimuli and Cell Behaviour

Cell behaviour can be triggered by hydrostatic pressure, fluid shear stress, mechanical strain and electrical fields generated by interstitial flow passing on charged bone crystals. For example, continuous hydrostatic pressure decreases collagen production by osteoblasts, while intermittent compressive forces enhance osteoblast activity and decreased osteoclast resorption (Rubin et al. 2006). Hydrostatic pressure has also shown to play a role on chondrocyte behaviour as a constant stimulus was proved to lead to chondrogenesis, while intermittent strain led to hypertrophy (Rubin et al. 2006). When bone is loaded in tension, compression or torsion, the interstitial fluid is moved towards regions of low pressure to come back when the load is removed, inducing an oscillatory fluid flow of 0.8 Pa up to 3 Pa in vivo. This regime results in a dramatic amplification of local strains in proximity of the osteocyte processes (Klein-Nulend et al. 2012; Klein-Nulend et al. 2005). Osteocytes are able to sense this variation in the interstitial fluid as demonstrated by multiple studies where shear stress was found to trigger mechano-activated biochemical pathways regulating NO production in osteocytes (Vezeridis et al. 2006; Rubin et al. 2006). Osteocytes were found to be more responsive to mechanical stimuli than other cell types and are believed to play a role in regulating the activity of osteoblasts and osteoclasts (Klein-Nulend et al. 1995). Furthermore, mechanical stimuli were shown to regulate calcium deposition with osteoblast cells increasing mineralization as a result of cyclic loading (Sittichockechaiwut et al. 2009; Damaraju et al. 2014).

1.1.3 Cell Mechanotransduction

The effect of mechanical forces on bone cells is currently under investigation aiming to define a relationship between stimuli and differentiation. The key cues to better understand the effect of mechanical stimuli on cell commitment are (1) the forces applied by the cytoskeleton and the contractile components of cells on the surrounding environment, (2) how the stiffness of the surrounding environment influences cells through durotaxis and (3) how external mechanical stimuli generated by gravitational action, muscles and other cells are translated into biochemical processes. In skeletogenesis the differentiation of stem cells towards the osteogenic or chondrogenic pathway is regulated by many external factors (Kelly and Jacobs 2010; Mauck 2003) influencing cytoskeletal organization, shape, motility (Lim et al. 2010; McBeath et al. 2004) as well as the expression of transcriptor factors (Salazar and Ohneda 2012). For example, the Wnt/ß-catenin or Rho/ROCK signalling pathways are known to play a crucial role in controlling cell commitment towards the osteogenic or chondrogenic pathway through the expression of Sox9 and Runx2 at early stage of differentiation (Kelly and Jacobs 2010). Sox9 is put alongside with expression of collagen II, TGFβ and glycosaminoglycan (GAG) genes and identifies differentiation towards the chondrogenic lineage, while Runx2 identifies osteogenic differentiation and induces expression of collagen I and non-collagenous proteins such as alkaline phosphatase (ALP), osteocalcin (OC) and osteopontin (OP). OC and OP are markers for bone mineralization and help in regulating the size of mineral crystals deposited by mature osteoblasts (Clarke 2008).

1.1.4 Bioreactors for Tissue Engineering

To find a correlation between mechanical forces and cell differentiation, complex bioreactors providing a controlled micromechanical environment were developed combining advanced scaffold designs and mechanical conditioning systems (Zhang et al. 2010; Tanaka 1999; Thorpe et al. 2013). Bioreactors facilitate the monitoring and control of biological or biochemical processes undergoing within the scaffold during the bone-forming process. Bioreactors are generally adapted to fit within an incubator that controls the external environment guaranteeing physiological conditions: 37 °C temperature, 5% CO_2 concentration and 99% humidity. A requirement for cell culture bioreactors is inertia to the harsh chemistry of the biological environment preventing corrosion and toxic reactions. Moreover, the diffusion limit and uniform distribution of cells in the scaffolds are key factors to consider in the development of functional tissue. With this purpose, bioreactors aim to maximize the supply of nutrients and oxygen to cells seeded in internal areas exceeding the diffusion limit distance of 100–200 μm (Ratcliffe and Niklason 2002) in order to maintain their viability. Exchange of substances within the scaffold during the seeding can be also used to help increasing seeding efficiency and uniform

distribution of cells (Sobral et al. 2011). For this purpose, current techniques employ convection of medium by perfusion, centrifugation and spinner flasks (Zhang et al. 2010). Moreover, bioreactors can be designed to apply shear strain forces, mechanical strain or pulsed electromagnetic fields with a high control over the stimulation in order to reproduce the biological environment and clarify the relationship between mechanical stimulation and tissue formation.

1.2 Bioreactors for Fluid Flow-Induced Cell Differentiation

A homogeneous cellular distribution and a good exchange of nutrients and oxygen within the scaffolding material are the first step in the development of functional engineered tissue. Due to the three dimensional architecture of novel scaffolds, static seeding is no longer an optimal method as it leads to a low seeding efficiency, cellular inhomogeneous distribution and low diffusion of fluids or gases in the internal regions causing cell apoptosis. In order to overcome these limitations, different systems were considered which are spinner flask (SF), rotating wall vessel (RWV), biaxial rotating (BXR) and perfusion bioreactors. These systems are more efficient compared to the static methods where molecule exchange occurs by diffusion because those novel systems induce a convective flow, enhancing cell attachment, proliferation and differentiation.

1.2.1 Rotating Bioreactors

SF bioreactors consist in a vessel provided with side arms for gas exchange and a stirring mechanism able to create a flow though the culture media (Fig. 1.1a). In order to avoid scaffolds fluctuation, pins are connected to the top lid for allocating samples. SF bioreactors were shown to increase the seeding efficiency compared to static methods (Mauney et al. 2004) and to induce osteogenic differentiation though the expression of ALP and OC and increased calcium deposition (Meinel et al. 2005).

RWV bioreactors consist in a hollow cylinder provided with an external chamber for scaffolds allocation and working as medium reservoir, rotating along the radial axis (Fig. 1.1b). The laminar flow generated by the rotating motion results in low shear stress preventing cell detachment and partially overcome the diffusional limitations characteristic of static and SF seeding methods. Despite this, lower cell number and matrix production were observed compared to SF methods because scaffolds are free to float inside the chamber hitting against the walls of the rotating vessel. Solutions include (1) fixing scaffolds to the cylindrical structure as in rotating

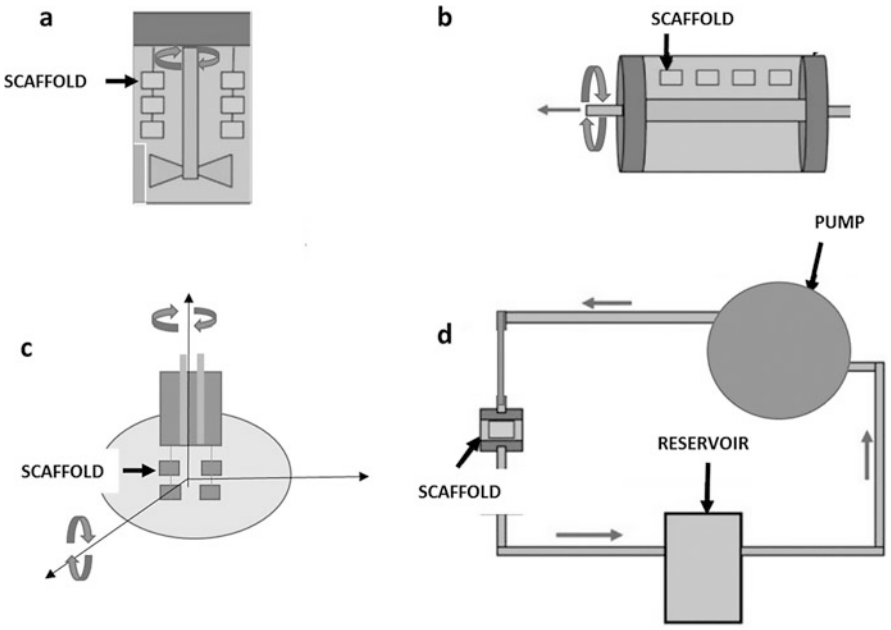

Fig. 1.1 Bioreactors for seeding and differentiation of MSCs due to effect of fluid flow. Spinner flask (**a**) and rotating wall vessel (**b**) bioreactors provide rotation towards an axis, while the biaxial rotating wall vessel (**c**) systems allow rotation in two directions providing homogeneous shear stress distribution in the culture chamber. Closed loop perfusion bioreactor (**d**) scheme employing a serial multichamber configuration. (Figures adjusted from Zhang et al. 2010)

bed bioreactors (Rauh et al. 2011), (2) employing scaffolds with lower density than water (Yeatts and Fisher 2011) or (3) preventing contact with the walls by optimization of the rotation rate (Zhang et al. 2010).

According to a study by Zhang (Zhang et al. 2010), the gold standard seeding performances are given by biaxial rotating bioreactor (BXR). It consists in a spherical chamber equipped with pins for scaffolds allocation, a reservoir for culture media and a perfusion system (Fig. 1.1c). The spherical chamber is able to rotate simultaneously in two perpendicular axes overcoming diffusion problems observed with SF. Moreover, it prevents cell detachment phenomena observed in RWV, thanks to the spaces for scaffold allocation. In summary, BXRs provide all the advantages of the perfusion systems while overcoming the "cell washout" phenomena observed in perfusion bioreactors. Indeed by not allocating the scaffold directly in the flow stream, cell detachment from the side of the scaffold facing the oncoming flow is prevented, resulting in higher homogeneous distribution of cells. BXRs increase considerably cell attachment, proliferation, molecule diffusion and osteogenic differentiation compared to SF, RWV and even perfusion bioreactors working in optimal conditions (Zhang et al. 2010).

1.2.2 *Perfusion Bioreactors*

In the last decade, the attention turned towards perfusion bioreactors (Fig. 1.1d) composed by a chamber fitting the geometry of the scaffold, a medium reservoir for supply of nutrients and a waste reservoir. Some perfusion bioreactors are closed loop and do not use a waste reservoir but nutrients are continuously pumped into the system (Kausar and Kishore 2013). Perfusion bioreactors force the fluid through the entire scaffold allowing cells to reach the interior of the structure and enhancing homogeneous distribution and optimal supply of gases and nutrients. The first challenge developing perfusion systems is related to prevent air bubbles formation as the presence of air is the main cause of local stress variation as it blocks the passage of fluid increasing the local flow rate and inhomogeneous condition inside the culture chamber, which might compromise the seeding process. A similar effect is observed when scaffolds are not completely anchored to the walls of the bioreactor chamber. In this case, void areas arise and become the preferred pathway for fluid to flow. The shear stresses generated by the fluid flowing through the scaffold are not only dependent on the inlet flow rate but also on the scaffold pore size and interconnectivity (Melchels et al. 2011; Porter et al. 2005; Chen et al. 2011). Despite the difficulties in developing efficient perfusion systems, a number of studies have studied the effect of perfusion flow on cell attachment, proliferation, matrix production and differentiation. While turbulent flow caused mainly cell detachment or programmed cell death due to the high shear stress (Cherry 1993), laminar regimes such as continuous, oscillating and pulsating flow led to satisfactory results and increased performances compared to static conditions. The effect of velocity and number of cycles on cell attachment was elucidated by Koch et al. who applied an oscillatory perfusion flow showing that velocities up to 5 mm/s were necessary in order to obtain uniform cell distribution in the interior of the scaffold (Fig. 1.2). He also demonstrated that the main effects on seeding efficiency were elicited by the number of cycles applied rather than the velocity used. Indeed, a lower number of cycles led to higher seeding efficiency. This suggests a dual role of shear stress which promotes cell attachment at the early stages of the seeding process but causes cell detachment if applied for long periods of time. The velocity of fluid flow was also found to significantly affect the viability of cells on the exterior of the scaffold as increased cell apoptosis was found associated to increasing shear stress regime (Fig. 1.3). These outcomes underline the need to define the optimal conditions enhancing uniform cell distribution, high seeding efficiency and cell viability.

Continuous unidirectional flow of cell suspension was also demonstrated to increase cell attachment and distribution (Vunjak-Novakovic et al. 1999; Wendt et al. 2003), ECM production and osteogenic differentiation (Scaglione et al. 2006; Bjerre et al. 2011; Papadimitropoulos et al. 2013; Koch et al. 2010; Sikavitsas et al. 2005). Moreover, a laminar flow oscillating in nature mimics the in vivo conditions applied to bone cells and stimulates calcium production in osteoblast-like cells (Koch et al. 2010) and human bone marrow stromal cells (Li et al. 2004). However, pulsating flow was found to be the most efficient in enhancing mineralization

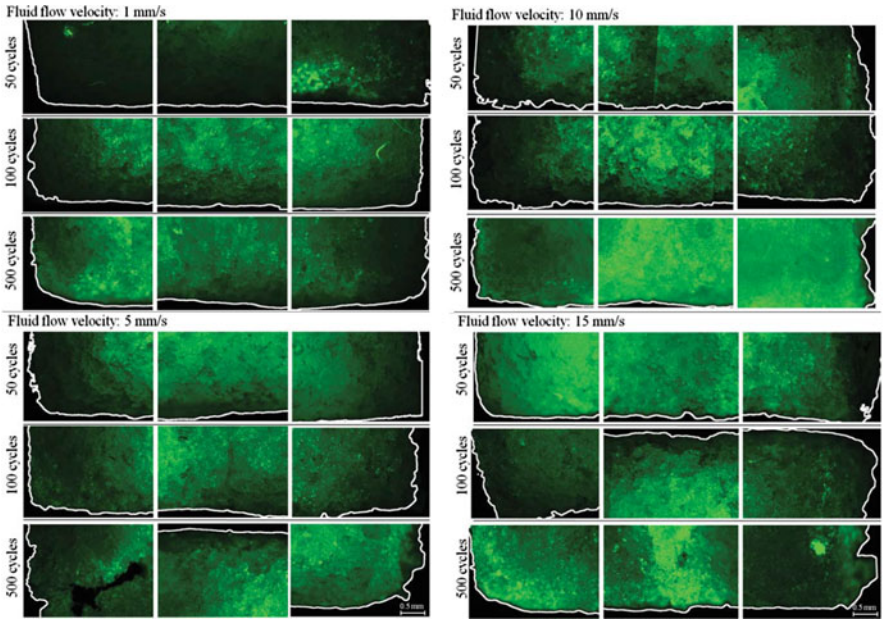

Fig. 1.2 Effect of velocity and number of cycles on cell attachment in the interior of the scaffold. (Koch et al. 2010)

(Jacobs et al. 1998; Bancroft et al. 2002), inhibiting cell apoptosis (Tan et al. 2008) and regulating matrix deposition (Vezeridis et al. 2006; Tan et al. 2007). The main drawback of perfusion bioreactors is the high amount of reagents needed, which has led to the development of perfusion microfluidic systems.

Microfluidic systems are easy to develop, require a low amount of reagents and above all allow to perform parallel experiments (Beebe et al. 2002). The new generation "lab on a chip" microfluidic devices permit repeatability of experimental conditions, testing simultaneously multiple samples. Due to their high versatility, they have already found application in the development of in vitro vascular implants (Khan et al. 2012). Polydimethylsiloxane (PDMS) is the most commonly used material for microfluidic perfusion culture systems since it is non-cytotoxic, autoclavable, gas permeable, flexible and easy to mold. Moreover, PDMS has low autofluorescence, and it is light transparent finding application for fluorescence and optical imaging (Kim et al. 2007). For cellular culture purposes, a glass-PDMS configuration is the preferred choice (Plecis and Chen 2007) as PDMS can be easily covalently bonded to glass substrates by surface activation through gas plasma treatments (Bhattacharya et al. 2005; Millare et al. 2008). Microfluidics systems made of glass-PDMS are currently used as support for 2D and 3D culture studies on the differentiation towards muscular tissue (Tourovskaia et al. 2005); the effect of different flow rates on cell morphology and proliferation (Kim et al. 2006), liver toxicology (Kane et al. 2006), cell seeding and monitoring (Toh et al. 2007); and

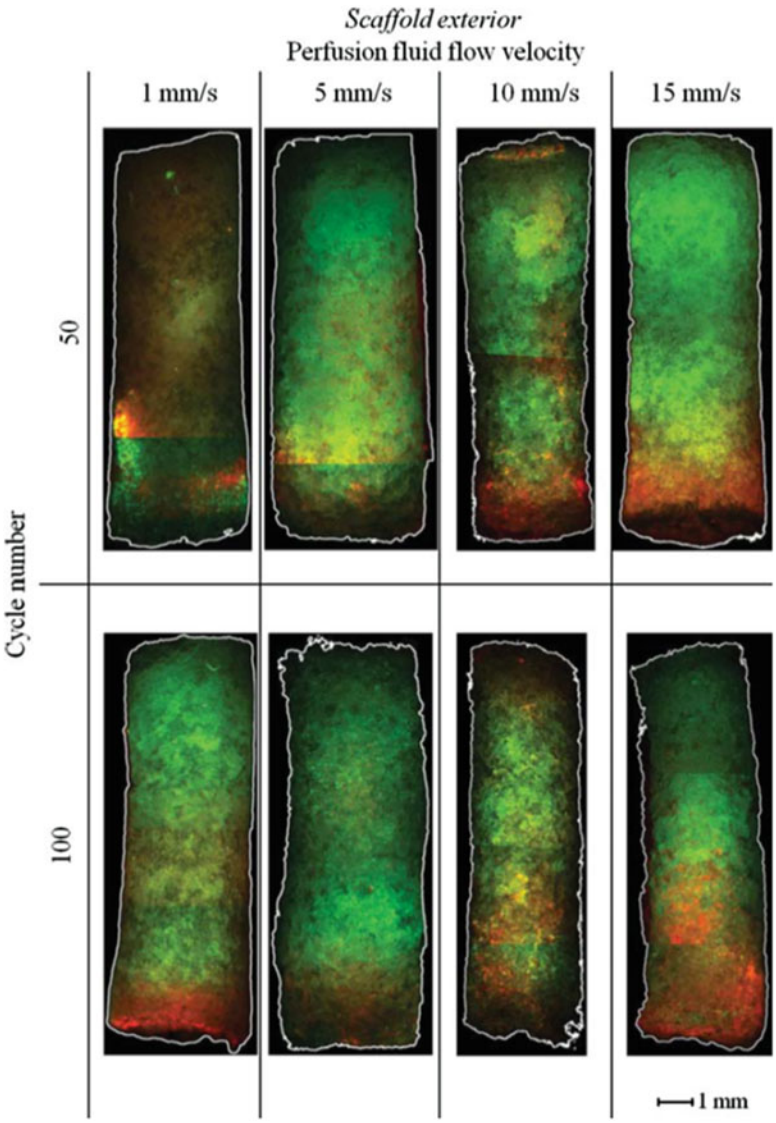

Fig. 1.3 Cell distribution on the exterior of the scaffold employing different velocities and number of cycles. Alive cells are shown in green, while apoptotic/dead cells are shown in red. (Koch et al. 2010)

comparison between cell lineages response to hydrostatic pressure (Park et al. 2012). Creating a robust sealed channel and avoiding bubble formation (Kim et al. 2007) are among the main challenges to currently face in the design of an efficient microfluidic system. In general, the fluid flow in a microfluidic perfusion system defines cell seeding efficiency and nutrients and gases delivery and can be used to transport

molecules probing cells to perform cellular assays to test for cellular activities or viability (Jeon et al. 2000). Normally fluid infusion processes are controlled by external pumps and valves and can employ multiple inputs. Among the applications mentioned above which can be controlled by fluid flow, cell seeding represent a particularly delicate issue as it needs optimization to avoid cells settling due to low shear stresses as well as cell viability and detrimental effects caused instead by high shears. On this matter, cell settling is normally overcome by minimizing the distance between cell reservoir and culture chamber or employing a viscous carrier to decrease the settling rate or by rotating the reservoir (Kim et al. 2007).

1.3 Bioreactors for Mechanically Loaded-Induced Cell Differentiation

1.3.1 Common Bioreactor Types

Common bioreactor systems for mechanical-induced differentiation include a vessel containing the culture media, space to allocate scaffolds and clamping parts aiming to apply tension or compression stimuli through an external computer control. In simple stretching devices, the extremities of the scaffold are anchored to grips connected to external automatic controls and able to move on a plane and transmit the displacement to the structure (Fig. 1.4a). Four-point bending devices (Fig. 1.4b) are another widely used and fairly simple configuration. The working principle consists in placing the structure on two vertical pillars and applying a force perpendicular to the plane of the structure (Mauney et al. 2004). Both setups allow high computed-control over the mechanical stimuli employing load and displacement sensors, actuators and an external control interface. Four-point bending systems equipped with micromanipulators and cameras were extensively used also to transmit tension as well as compression stimuli though the use of a piezoelectric actuators bending when voltage is applied (Tanaka 1999). Applying voltage, a piezoelectric layer shrinks while the other stretches, bending the actuator and transmitting the resulting displacement to the sample (Fig. 1.4c). When the polarity is inverted, the actuator bends on the other direction, allowing to test cell behaviour under both stimuli in 2D collagen layers. The addition of multiple chamber configurations allows high-throughput studies and increases repeatability and reproducibility of the tested conditions (MacQueen et al. 2012). Recently, novel bioreactors are developed to fit in incubators and maximize sterile conditions such as the BOSE ElectroForce® Systems (Fig. 1.4d) already employed in studies on scaffold mechanical characterization (Abrahamsson et al. 2010; Brunelli et al. 2017a), hMSCs differentiation (Thorpe et al. 2013; Brunelli et al. 2017b) and vascularization of bone grafts (Kong et al. 2012). The biodynamic chamber works as a bioreactor providing (1) sterile and isolated environment, (2) samples immersed state preventing drying phenomena, (3) high controlled tension or compression stimuli

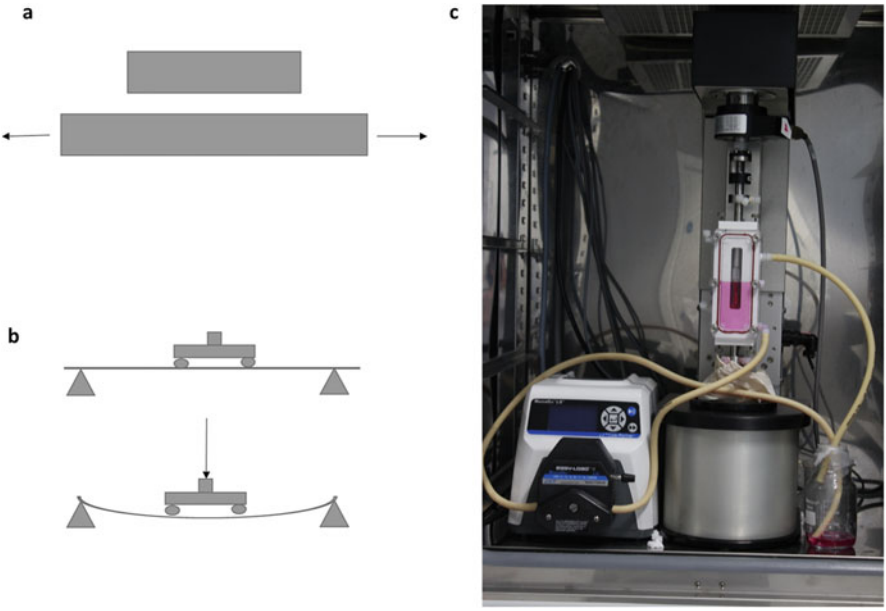

Fig. 1.4 Stretching principle to apply tension stimuli (**a**); four-point bending system (**b**) while applying deformation on 2D-seeded substrates; (**c**) BOSE ElectroForce® equipped with culture chamber forms mechanical stimulation and simultaneous perfusion of media

and simultaneously fluid shear stress by an external pumping system and (4) multiple motor configuration for parallel experiments.

1.3.2 Mechanical Load and Cell Commitment

By employing stretching devices or four-point bending systems, mesenchymal stem cells osteogenic commitment was studied on 2D surfaces or 3D structures (Table 1.1). 2D silicone membranes coated with collagen were considered to study MSCs behaviour under tension, showing increased osteogenic differentiation through synthesis of BMP2 and collagen 1 in multiple studies (Kearney et al. 2010; Rui et al. 2011; Friedl et al. 2007). Haudenshild et al. demonstrated the dual effect of tension and compression on hMSCs seeded in 3D alginate phosphate scaffolds (Haudenschild et al. 2009). Volume, surface area, skeletal length and diameter of cells were quantified by confocal images and revealed variation in cell morphology depending on the stimuli received. Compression stimuli led to round and shorter cells while tension led to more elongated and spread cells compared to controls. Moreover, gene microarray screening and RT-PCR analysis showed upregulation of a wide range of osteogenic genes and downregulation of chondrogenic genes in samples undergoing tension stimuli. The opposite expression

Table 1.1 Comparison between studies on the effect of mechanical forces on skeletogenesis

Cells	Scaffolds	Differentiation media	Parameters	Differentiation	References
Stretching devices					
MSCs	Silicon membrane	–	0.5% strain	Osteogenesis↑	Kearney et al. (2010)
			0.17 Hz		
			4 h/day		
			3 days/week		
		–	4 and 8% strain	Osteogenesis ↑	Rui et al. (2011)
			0.5 Hz		
			4 h/day		
		Osteogenic	0.3% strain	Osteogenesis↑	Friedl et al. (2007)
			1 Hz		
			15 min/day		
			2 or 8% strain	Strain 8%:	Jagodzinski et al. (2004)
			1 Hz	Osteogenesis↑	
			2 h/day		
			3 days/week		
Four-point bending					
BMSCs	Partially demineralized bone	Osteogenic	0.2 mm	Depending on dexamethasone concentration:	Mauney et al. (2004)
			0.2 Hz		
			250 cycles/day	Osteogenesis↑	
Osteoblasts	Collagen gels	–	0.3% strain	Low amplitude,	Tanaka et al. (2003)
			Low VS broad frequencies	High frequencies:	
				Osteogenesis ↑	
MSCs	Collagen gels	–	0.8, 0.9% strain	Chondrogenesis↓	Au-Yeung et al. (2010)
			1, 0.5, 0.1 Hz	Osteogenesis↓	
			1 h/day		

(continued)

Table 1.1 (continued)

Cells	Scaffolds	Differentiation media	Parameters	Differentiation	References
			7 days/week		
Porcine MSCs	Agarose hydrogels	Chondrogenic	10% + 1% strain	Chondrogenesis ↓	Thorpe et al. (2008)
			1 Hz		
			1 h/day		
			5 days/week		
		Chondrogenic VS untreated	10% strain	Supplementing chondrogenic media: Chondrogenesis ↑	Thorpe et al. (2010)
			1 Hz		
			1 h/day		
			5 days/week		
Other systems					
Porcine MSCs	Agarose hydrogels	Chondrogenic	10% strain	Confined compression: Chondrogenesis ↑	Thorpe et al. (2013)
			1 Hz		
			10 sec/day		
			5 days/week		
MSCs	Poly ethylene hydrogels	Chondrogenic VS untreated	10% strain	Chondrogenesis ↑	Terraciano et al. (2007)
			1 Hz		
			1, 2, 2.5, 4 h/day		
	Fibrin polyurethan 3D scaffolds	Chondrogenic	15, 20, 30% strain	High strain, high frequency: chondrogenesis ↑	Li et al. (2010)
			0.1, 1 Hz		
	Poly ethylene hydrogels	–	15% strain	Chondrogenesis↓	Steinmetz and Bryant (2011)
			0.3 Hz	Osteogenesis↓	
			4 h/day		
			14 days		
	Agarose hydrogels	–	9% strain	54, 120 mins: Chondrogenesis ↑	Elder et al. (2001)
			0.03, 0.15,0.33 Hz		
			12, 54, 120 min/day		
			3 days/week		

profile was characteristic in samples undergoing compression. Due to their remarkable properties in terms of biocompatibility, biomimetic, easy molding and transmission of uniform distribution of stresses through the structure, hydrogels were mainly considered as scaffolds for compression studies. Moreover, hydrogels allow accessibility to the core of the structure through fluorescence and optical light, enabling to monitor the conditions of cells placed in the interior volume of seeded scaffolds (Thevenot et al. 2008). For example, collagen gel scaffolds allowed real-time monitoring of cells and fibre alignment in multiple studies. Both static and cyclic loading conditions were shown to affect cell alignment inducing cells to orient parallel to the direction of the applied stress (Au-Yeung et al. 2010). However, collagen orientation, GAG and cellular metabolism variations were absent, suggesting that mechanical loading alone has no effect on the collagen remodelling action performed by hMSCs.

1.3.3 Loading Parameters Affecting Cells Response

Chemical cues have a high impact on the modulation of cellular response to mechanical forces. For example, the osteogenic commitment of bone marrow stromal cells (BMSCs) cultured in demineralized bone scaffolds and undergoing cyclic tension was found to be strictly related to the concentration of dexamethasone, varying ALP and OP expression. Absence as well as high amounts of dexamethasone (100 nM) led to suppression of osteogenic markers. Similar results were obtained investigating compression stimuli. When coupled with chondrogenic media, mechanical compression increased chondrogenesis gene expression (Thorpe et al. 2008), but compression forces alone showed to induce no significant differences in cell phenotype compared to free swelling samples in multiple studies (Terraciano et al. 2007; Thorpe et al. 2010).

Amplitude, frequency and duration of the stimuli can play a role in the activation of mechanotransduction pathways (Mack et al. 2004) and in modulating osteogenic or chondrogenic protein expression. Applying 2% and 8% cyclic tensile strain on MSCs, ALP activity and OC expression were upregulated when 8% strain was applied regardless of the presence of dexamethasone (Jagodzinski et al. 2004). The effect of sinusoidal frequencies (S), broad frequencies (V) and a combination of both (S + V) stimuli was investigated on osteoblasts keeping constant amplitude and varying frequencies of the stimulus. OC was 2.6-fold higher when S+V was applied; other osteogenic markers were upregulated after 4 days from V exposure, but no significant differences were noticed by applying S alone (Tanaka et al. 2003). Varying amplitude and frequency, Li et al. observed increased chondrogenic marker expression of the TGF family as result of high strain and high frequency stimulations (Li et al. 2010). Low-amplitude high-frequency stimuli were shown to produce the same effect as high-amplitude low-frequency stimuli to activate bone formation (Ozcivici et al. 2010). Similar results were obtained in other studies where the duration of the stimuli and its frequency were varied. Long periods of stimulations

have no significant difference in driving cell commitment compared to results obtained by continuous loading with downregulation of both osteogenic and chondrogenic markers (Steinmetz and Bryant 2011). hMSCs are sensitive to accumulation of stress eliciting a stronger chondrogenic commitment to higher frequencies as well as to strain history which enhances chondrogenic differentiation over longer periods of stimulation (54 or 120 min versus 12 min) (Elder et al. 2001). Despite the high amount of studies claiming chondrogenesis commitment as a result of compression, short boosts of compression were found to activate the same response as dexamethasone elicits on matrix mineralization by hMSCs cultured in polyurethane (Sittichokechaiwut et al. 2010) and 3D PCL (Brunelli et al. 2017b) scaffolds. This suggests the possibility to induce osteogenic differentiation by compression forces within polymeric scaffolds.

1.4 Electromagnetic Field Bioreactors and Differentiation

Electromagnetic field (EMF) and pulsed EMF (PEMF) in vivo arise from the piezoelectric effect induced by bone deformation as a consequence of muscular action (Kramarenko and Tan 2003). EMF stimuli arise in vivo in two ways: (1) as a consequence of postural or walking activities causing displacement in the bone and resulting in EMF frequencies ranging between 5 and 30 Hz and (2) when bone fracture occurs giving rise to a negative potential due to accumulation of negative charges at the injured site (Antonsson and Mann 1985). In the recovery process, EMFs and PEMFs have a beneficial effect on patients affected by osteoporosis or non-union fractures, decreasing bone resorption action performed by osteoblasts or accelerating the bone-forming process by osteoblasts (Bassett et al. 1977; Aaron et al. 2004). In order to observe the effect of EMFs and PEMFs on cellular conformational changes, proliferation and differentiation, EMF-based bioreactors were developed. These systems consist of two Helmholtz coins hosting a chamber for scaffold allocation and connected to an external EMF generator (Fig. 1.5). Applying continuous stimuli of PEMF was found to have no effect on osteoblasts or BMSCs proliferation, ALP or calcium content up to day 14 where an increase in calcium deposition occurs in BMSCs at the expense of proliferation (Jansen et al. 2010). In other studies employing short resting periods between consecutive stimulations (8 h), EMF increased hMSCs proliferation, viability and multi-lineage differentiation (Sun et al. 2009). MEF was found to affect bone progenitor cell proliferation rate depending on their bone differentiation stage (BMSCs versus osteoblasts) and the presence of osteogenic media (Sun et al. 2010). BMSCs have a higher proliferation rate compared to untreated controls in presence of osteogenic media whereas previously differentiated osteoblasts decreased in cell number compared to untreated controls. Increased ALP and BMP2 were observed at early stages culturing BMSCs in osteogenic media. Following these findings, studies were performed using mainly BMSCs culture in osteogenic media in order to maximize the osteogenic performance (osteogenic BMSCs). Increased osteogenic markers

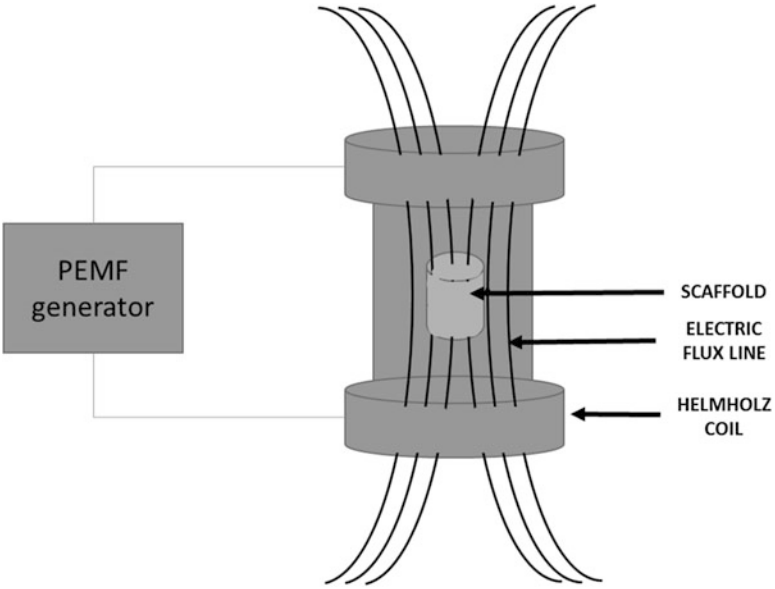

Fig. 1.5 Common design for PEMF bioreactors. (Figure adjusted from Zhang et al. 2010)

expression and proliferation rate were achieved by applying PEMF over shorter periods at low amplitude. Osteogenic BMSCs undergoing 0.13 mT quasi-rectangular pulses at 7.5 Hz for 2 h a day showed higher production of ALP at day 7 and enhanced mineralization at day 28 compared to untreated controls (Tsai et al. 2009). The effect of frequency on BMSCs osteogenic marker expression was further investigated at 1mT of EMF by varying frequencies at 10, 30, 50 and 70 Hz. Enhanced proliferation was observed in samples stimulated at 10 Hz as well as expression of ALP and OC after a week of treatment. Despite this, enhanced cell viability was observed at 50 Hz together with maturation of osteoblasts after 2 weeks of exposure and extensive matrix mineralization (Liu et al. 2013). Similar studies were performed supplementing hMSCs with chondrogenic media and applying 5mT sinusoidal EMF at 15 Hz four times a day (45 min every 8 h) over 21 days demonstrating that sinusoidal low-frequency EMF stimulates and maintains differentiation towards a lineage when supplemented with specific growth factors (Mayer-Wagner et al. 2011).

1.5 Discussion

As reviewed above, mechanical stimuli affect MSCs shape, proliferation, matrix production and gene expression. Perfusion and biaxial rotating systems assist the seeding process at early stage of culture as well as the differentiation at late stage of

culture, by providing either homogeneous distribution of cell or stresses through the scaffold. Microfluidic systems are able to provide the required amount of nutrients preventing waste issues and uniform shear stress stimuli through the seeded structure. Moreover, they are also a support for real-time monitoring of cell activities by building perfusion chambers made of light transparent materials and by setting a system of pumps regulating the flow of different solutions reacting with cells cultured in the bioreactor. Furthermore, the microfluidic approach minimizes the handling of samples and consequently the risk of infections and also provides multichamber configurations allowing to perform parallel experiments and to increase the repeatability of the tested conditions. Microsystems known as "lab on a chip" are also employed for applying tension and compression in a controlled biological environment.

In terms of differentiation following mechanical stimuli, the most relevant effects are observed by fluid flow which demonstrates to induce osteogenic differentiation without the need for osteogenic supplements. A dynamic flow enhances not only cellular attachment but also proliferation and osteogenesis. Pulsing fluid flow is the best condition eliciting osteogenic differentiation as it closely mimics the regime characterizing the interstitial fluid flow caused by physiological movements in vivo. Compression and tension stimuli do not define a clear differentiation pathway as the addition of osteogenic or chondrogenic media was often required in order to investigate their effect on cell commitment. Both stimuli led to enhanced cell differentiation when coupled with specific media formulations: compression led to chondrogenesis differentiation, while tension evoked osteogenesis as largely demonstrated in hydrogels. However, different regimes in terms of amplitude, frequency and duration of the stimuli have a strong impact on the final outcomes, leading to contrasting results. Osteogenesis is enhanced by high-amplitude low frequency as well as by low-amplitude high-frequency regimes, while high-amplitude high-frequency stimuli induce chondrogenesis. The duration of the stimuli as well was found to play a role in the differentiation process. Long period of stimulation seems to prevent differentiation of cells into the bone or cartilage, while short burst of stimulation is more effective in eliciting differentiation. EMF and PEMF can be considered to obtain fully differentiated bone tissue decreasing the amount of time required for the healing process as already demonstrated in vivo on osteoporotic patients. Short bursts of low-amplitude high-frequency stimuli put alongside osteogenic media supplements demonstrate once more to induce proliferation and mineralization in hMSCs.

Mechanical loading stimuli were mainly investigated on hydrogels due to their biocompatibility, accessibility to common imaging methods and their ability in transmitting to cells uniform stress through the whole structure. However, more recently the focus moved to the investigation of cells commitment in 3D porous structures due to the demand for scaffolds able to transmit higher strain amplitudes to cultured cells. Despite this, porous polymeric 3D scaffolds prevent the use of common imaging techniques, and their deformation is not easily predictable as for hydrogels due to their complex geometry and mechanics, hence losing control over the forces sensed by cells. While the distribution of stress in hydrogels is uniform

guaranteeing the same level of stress through all the volume of the scaffold, porous polymeric scaffolds present a complicate architecture whose deformation can lead to local tensile forces in the inner areas of the scaffold as a result, for example, of external compression. In addition, shear stress caused by the fluid flowing in and out of the structure elicits a still unclear synergic action with the external mechanical strain. Microcomputed tomography imaging and computer modelling are recently employed in an increasing number of studies (Van Lenthe et al. 2007; Tuan and Hutmacher 2005; Lacroix et al. 2009) aiming to help defining locally the mechanical environment and correlate it to cells commitment.

1.6 Conclusions

The effect of shear stress, mechanical stimuli and EMF was extensively investigated in the last two decades helping defining the role of different environmental conditions on gene upregulation and proteins expression. Osteogenesis is enhanced or inhibited depending on the regimes applied, the scaffold employed as support for mechanical stimulation and the differentiation stage of cells. Pulsing shear stress is the most promising stimulus to drive cell commitment towards the osteogenic pathway as it closely mimics flow regimes observed in vivo during common activities. The optimal combination of parameters to apply to stem cells in terms of tension, compression and shear stress to obtain fully differentiate tissue is nowadays still ongoing work and needs further optimization, but the need of chemical supplements as support in eliciting cell response as a consequence of mechanical stimuli is becoming more and more prevalent. Multiple chamber bioreactors having small dimensions are increasingly used to provide control over the mechanical environment and experimental conditions and to reduce experimental variability. Studies employing bioreactors put alongside with computer modelling give a good chance to obtain the control needed over external and internal conditions to ultimately obtain well-defined protocols able not only to clarify cell transduction pathways but also to drive differentiation towards functional tissue for TE purposes.

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