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# Sensing the Difference: The Influence of Anisotropic Cues on Cell Behavior

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## Abstract

From tissue morphogenesis to homeostasis, cells continuously experience and respond to physical, chemical and biological cues commonly presented in gradients. In this article we focus our discussion on the importance of nano/micro topographic cues on cell activity, and the role of anisotropic milieus play on cell behavior, mostly adhesion and migration. We present the need to study physiological gradients *in vitro*. To do this, we review different cell migration mechanisms and how adherent cells react to the presence of complex tissue-like environments and cell-surface stimulation in 2D and 3D (*e.g.* ventral/dorsal anisotropy).

# 1.- Introduction

When considering cell response to topographical cues, we initially think on classical contact guidance to grooves. This was first reported by 1911, when Harrison observed cells aligning to spider silk (Harrison, 1911). In the 1950s Weiss and Garber termed this phenomenon contact guidance (Weiss and Garber, 1952). The field was popularized in the 1980s through a collaboration between a cell biologist, Adam Curtis, and an electronic engineer interested in miniaturization, Chris Wilkinson (Curtis and Wilkinson, 1997), who observed that surface topography gradients can be sensed by adherent cells at the microscale and later, the nanoscale.

In this article we will focus on the design of novel anisotropic polymeric surfaces and how different parameters influence cell fate. Then we will review cell migration mechanisms in different environments and the influence that bi-phasic (ventral/dorsal) gradients play on cell activity in *in vivo*-like milieus.

# 2.- Anisotropic topography and cellular function

## Anisotropic biomaterials

The interplay between heterogeneity and anisotropy persists as the predominat strategy adapted by nature to optimize the function of biological materials. The former relates to the spatial variation (point to point) of the material properties, whereas the latter closely relates to the directional dependence (Ranganathan et al., 2011). Material topography, and in particular micro and nanoscale anisotropic structures, can affect cellular morphology as well as cellular behavior (**Table 1**) (Losert et al., 2013; Cassidy et al*.,* 2014; Londono et al*.,* 2014; Azeem et al*.,* 2015). It is thus necessary to develop a fundamental understanding on the mechanistic processes induced by anisotropic structures in cell biology, in order to gain further insights into the design of biological materials.

Novel techniques of nanofabrication and material processing, such as lithographic (Diehl et al., 2005; Zhu et al., 2005; Dalby et al., 2006; Smith et al., 2014) self-

assembly (Tsai et al., 2014), electrospinning (Gaharwar et al., 2014) and scratching techniques (Peng et al., 2010; Wang et al., 2013a), offer the ability to create *anisotropic substrates* with feature widths and depths ranging from the macroscale to the microscale down to features sizes as small as 5 nm facilitating the aquisition of information to help understand this field (Peng et al., 2010; Wang et al., 2013a) (**Figure 1**). These techniques are particularly suited for the generation of anisotropic micro-nanofeatures in polymeric formulations and in particular in thermoplastics. However, with specific reference to load-bearing biomaterials, the topographical modification of metals and their alloys has received much attention recently and several novel techniques including electrical anodization (Xie et al., 2011), acid etching (Att et al., 2009) and femtosecond laser processing (Tavangar et al., 2013) have recently been described.

Both micro (Itala et al., 2003; Germanier et al., 2006; Herrero-Climent et al., 2013), and nanoscale (Biggs et al., 2008; Cassidy et al., 2014; Azeem et al., 2015) groove/ridge topographies are well documented as powerful modulators of contact guidance, being relevant in tissue engineering and biomaterials manufacture. A principal design tenet of anisotropic grating substrates is that of biomimetic ECM design, attempting to mimic the topographical structures imparted by several fibrous components of the ECM. Collagen, being the most abundant protein in the body, encompasses individual fibril elements (*e.g.* with dimensions of 20–30 nm) to fibril bundles (*e.g.* from 15–400 μm in diameter). Key to this is that nanogroove surfaces may promote cellular polarization as well as promote aligned self-assembly of ECM components with motifs critical in cell adhesion such as fibronectin and vitronectin. Indeed, the elongated cellular morphology and alignment induced by grooved substrates may resemble the natural state of tissue resident cell populations *in vivo*, and studies indicate that most, if not all, cells, notably fibroblasts (Dalby et al., 2003; Garland et al., 2014), osteoblasts (Lenhert et al*.,* 2005; Wu and Wang, 2013), neurons (Yim et al*.,* 2007; Tonazzini et al*.,* 2014) and MSCs (Dalby et al*.,* 2006; McMurray et al*.,* 2013), undergo significant morphological and functional responses to anisotropic topographies.

The extent to which groove geometry and order can influence cell function is an often overlooked consideration in designing next generation biomaterials. Studies by Tonazzini et al. show that the loss of neurite guidance is not linear with noise on microgrooved topographies, but is a threshold effect, correlating with changes in focal adhesion (FA) maturation and spatial organization (Tonazzini et al., 2013). Here the authors found that nanogratings with a controlled amount of random nanotopographical noise - or defects - influenced neurite contact guidance at a threshold noise level of aproximately 40% to 50%. Interestingly, a further study by Gamboa *and col.* have identified that the groove orientation does not have to be linear; rather cell can gain spatial information from anisotropic features. Indeed, cells can cross over individual grooves when they arranged in a waveform pattern, residing both inside and outside of each wave pattern, yet aligning linearly along the long axis of the pattern (Gamboa et al., 2013).

***Anisotropic topography on cell adhesion***

Critically, nanogroove features seem to directly influence the formation and orientation of FA *in vitro* (Teixeira et al., 2004), probably due to the guiding of ECM proteins. A nice example has been described in the case of fibronectin, a protein which has been shown to selectively adsorb onto the ridge/groove boundaries (de Luca et al., 2015). At present no clear conclusions have been reached regarding the absolute dimensions required for cellular and FA alignment; rather this process is cell-specific and density dependent (Clark et al., 1990). It is probable however that an interplay between groove width and depth regulates cellular alignment, and a recent study by Crouch et al. indicated that a grating aspect ratio (depth to width) of 0.16 was required for 95% cellular alignment (Crouch et al., 2009). Additionally, anisotropic topographies can induce alignment of sub-cellular structures including filopodia (Fujita et al., 2009), nuclei (McKee et al., 2011) and neurite extensions (Tonazzini et al., 2013).

Recent studies indicate that FA alignment is generally more pronounced on patterns

with ridge widths between 1 and 5 μm than on grooves and ridged topographies with larger lateral dimensions (Biggs et al., 2008; Yang et al., 2010). Moreover, cells cultured on grooves with nanoscale widths produce FA that are non-polarized (Kim et al., 2013) or almost exclusively oriented obliquely to the topographic patterns (Teixeira et al., 2006). This occurs predominantly on topographical ridges as opposed to grooves, effectively limiting the length of FA formed perpendicular to the groove orientation. Thus, it arises that grooved nanoscale topographies can influence both the adhesion direction as well as adhesion reinforcement (Biggs et al., 2010).

***Anisotropic topography and cellular function***

The mechanisms by which anisotropic topography and ordered protein deposition influence cellular proliferation and differentiation require more research. Anisotropic arrays of topographical micro and nanoscale gratings have been shown to be potent tools in maintaining the phenotype of primary cells (Zhu et al., 2010) and in differentiating stem cells towards expressing osteogenic (Dalby et al., 2007; Biggs et al., 2009a; Watari et al., 2012), tenogenic (Wojciak et al., 1995), neurogenic (Jeon et al., 2014), adipogenic (Wang et al., 2012) and myogenic (Wang et al., 2013b) transcripts and proteins. The processes that mediate the cellular reaction to anisotropic surface structures, however, are not well understood and may be direct (Cassidy et al., 2014) or indirect (where the surface structure has affected the composition, orientation, or conformation of the adsorbed ECM components)(Perez-Garnes et al., 2011; Ballester-Beltran et al., 2012a).

Multiple studies with mesenchymal stem cell populations point towards the negative effects of micronscale grooves and pronounced cell polarization on osteospecific differentiation (Biggs et al., 2008; Watari et al., 2012), a process appearing to be more influenced by sub 500 nm topographies. Conversely more adipospecific (Wang et al., 2012) and myogenic (Wang et al., 2013b) differentiation is demonstrated to be enhanced by polarization through micronscale grooved topographies. Periodicity has also been shown to be important in modulating differential function in pluripotent cells. In particular if mesenchymal stem cells are cultured on grooves with a short

feature pitch and become highly aligned this reduces osteogenesis. However, if the pitch is increased so the grooves are more step cues (around 50 µm pitch) then osteogenesis is achieved with great efficiency (Biggs et al., 2009b).

The mechanism by which anisotropic grating surfaces influence cell function however are still being resolved; multiple studies suggest that cells cultured on topographical gratings modulate transcriptional events through adhesion-dependent phosphorylation of downstream signaling molecules (*e.g.* mediated focal adhesion kinases, FAK) (**Figure 2A**). FA pathway signaling has been shown to control essential cellular processes such as growth, survival, migration and differentiation. Interestingly, extensive evidence has also been generated on the involvement of extracellular signal-related kinase (ERK) 1/2 down-regulation following integrin-mediated FAK activation, in response to grooved substrates (Biggs et al., 2009b; Cassidy et al., 2014).

***A case of study: anisotropic surfaces and immune activation***

Besides analyzing the influence of anisotropic features in adherent cells, it seems that these surfaces can also induce a significant response in several *non-adherent* cell types (Kwon et al., 2012) (*e.g.* cells belonging to the immune system).

Several studies have reported on the effects of nanotopographical structures on immune cell motility (Song et al., 2012) and actin rich structures (*e.g.* lamella and filopodia) are reported to play a significant role in this process (Song et al., 2014). Emerging data suggest that the proteins involved in adhesive processes in cells of the immune system are analogous to those found in FA in adherent cells (Hocde et al., 2009), and that leukocyte binding to ECM components and adsorbed complement proteins can induce FAK-mediated immune cell activation (Bhattacharyya et al., 1999), phagocytosis (Kasorn et al., 2009) and chemokine mediated migration (Cohen-Hillel et al., 2009). Similarly, Bartneck and col. reported that a microstructured topography of regular grooves induced a pro-inflammatory

phenotype in macrophages which was not accompanied by release of pro-inflammatory mediators (Bartneck et al., 2010).

Although the immune response is tightly regulated by the complex interplay of events and interactions between its constituent cells, preliminary studies suggest that anisotropic topographies may be employed to induce cell activation and as in adherent cell types, this may be through FAK-mediated activation of critical signaling pathways.

## Perspectives on cell-material interactions

The interactions between cellular populations and engineered substrates are bidirectional; *e.g.* a mechanical cellular mediated deformation of the scaffold modulates the physical environmental properties. Consequently, in order to better understand the dynamic nature of topographical mediated direction of cellular function stimuli, responsive or "smart" materials are increasingly being employed in cell studies. New reports demonstrate that mechanically active anisotropic topographies can yield critical insight into the role of groove dimension on cellular function through dynamic modulation of the feature dimensions.

Shape memory polymers have recently been employed for this purpose and can be designed with a physiological transition temperature to provide an alternate morphology or topography when subjected to physiological temperatures (*e.g.* between 32 and 37°C), providing dynamic mechanical cues or anti-adhesive surfaces (Ebara et al., 2014). A study by Gong et al. investigated microgroove surface patterns formed on a cross-linked poly(ε-caprolactone) substrate. Here, a dynamic response to cyclic temperature (*e.g.* between 32 and 41°C) resulted in the modulation of the microgroove dimensions, influencing cell shape and the cytoskeletal arrangement of adherent bone marrow stem cells, which upregulated myospecific genes and proteins (Gong et al., 2014).

Although a large number of studies have reported the effects of nanotopographical structures on modulations to cell function *in vitro* (Dalby et al., 2007; Biggs et al., 2009b), a limited number of studies *in vivo* have been conducted (Fernandez-Yague et al., 2014), particularly in osteointegration and de novo bone formation (Xia et al., 2012b; Svensson et al., 2013). A recent study by Yin et al demonstrated the significant effects of anisotropy in a rat Achilles tendon repair model. Here anisotropic scaffolds and enhanced alignment resulted in tendon regeneration, while randomly ordered isotropic scaffolds induced ectopic bone formation. Interestingly the influence of anisotropic micro and nanotopography on osteospecific function seems to be most effectively translated to an *in vivo* response when applied in combination with microscale grating features, indicating a possible synergy between the cellular (micro) and subcellular (nano) in directing regeneration (Kim et al., 2014a).

Considering these indications it appears logical to expect significant efforts to translate findings from model (*e.g.* polymer) surfaces into orthopaedic materials such as ceramics and metals. However, these materials are hard to manufacture due to the hardness of the materials and the brittle nature of ceramics. Also, while structural orthopaedic ceramics, such alumina/zirconia, tend to be highly bio-inert, metals such as titanium and its alloys are highly adhesive to cells due to their reactive oxide layers and both these low and high adhesion environments can mask topographical effects. However, recent advancements in pre-sintering embossing of green ceramics has illustrated that groove patterns in alumina with different widths can be used to influence fibrous tissue (narrow width, 20 µm pitch) and hard tissue (150 µm pitch with 100 µm groove/50 µm ridge) growth from human osteoprogenitor cells (Nadeem et al., 2013) similarly to results observed on polymers (Biggs et al., 2008).

For metals, there is plenty of literature showing potential effects on more random topographies from etching, blasting and anodising (Olivares-Navarrete et al., 2014; Sjöström et al., 2013; Sjöström et al., 2012; McNamara et al., 2011; Anselme et al.,

2000) due to the need for fast fabrication protocols. However, Anselme et al. have indicated potential positive effects for grooves in metals. We further note that other cell types have been shown to be responsive to topographies in metals – again though, roughened rather than grooved (Kim et al., 2015).

As we can observe, anisotropic surfaces can stimulate *in vitro* and *in vivo* cell activity. However, it is still unclear how cell adhesion can be affected by topographic cues. In the next section of this review, we will consider the best understood mechanisms for cell adhesion and migration, focusing further discussions on the influence of ventral and dorsal stimulation on adherent cells.

# 3.- Cell motility

During the last years it has been demonstrated that cell anchorage and motility play important roles in a wide spectrum of biological processes, such as mechano and chemotaxis (Charras and Sahai, 2014; Andreas et al., 2014), as well as wound healing and tissue repair (Gattazzo et al., 2014). Furthermore, similar to tissue repair, several diseases (*e.g.* cancer) have evolved to use adhesion and migration as part of their pathogenic strategy (Farahani et al., 2014), making research on cell migration an extremely relevant field and showing the necessity to study this process in detail.

Since anisotropic surfaces play a potent role on cell migration, in the next section we will briefly describe different mechanisms used by migrating cells to displace into complex environments, putting into context topographic cues detected on 2D surfaces or 3D milieus.

## Cell migration on 2D: Following the front

### *Cell migration mediated by the protrusion of lamellipodia*

One of the most researched migration mechanisms on flat surfaces, at the single-cell scale, corresponds to *mesenchymal migration.*

*Mesenchymal migration* is recognized by the presence, adhesion and protrusion of lamellipodium. From the molecular point of view, this type of migration requires cell-matrix adhesion structures (*e.g.* protein clusters) named *adhesomes*, which are composed by intra-, trans- and extra- cellular proteins (**Figure 2A**).

The most important mediator between cells and their milieu are heterodimer transmembrane proteins known as integrins. Integrins comprises α- and β- chains. β-subunits interconnect extracellular matrix proteins (*e.g.* collagen, fibronectin, elastin, etc.) with the cytoskeleton (*e.g.* actin stress fibers), allowing the sensing of elastic and topographical properties of the milieus where they are anchored through the exertion of traction forces (**Figure 2A**). Cell-matrix adhesion and mechanosensing is mediated by non-receptor tyrosine kinases, the most notable of which is FAK, a molecule constitutively associated with the β-integrin subunit. FAK is localized at adhesion structures. It promotes talin recruitment to modulate adhesion reinforcement, and further unraveling of these stimuli by intracellular kinases (*e.g.* FAK) bound to transmembrane integrins and linking proteins (*e.g.* vinculin and paxillin) (Leal-Egaña et al., 2013; Vicente-Manzanares and Horwitz, 2011; Ridley et al., 2003).

***Adhesion structures and cell polarization***

*Mesenchymal* migration is a cyclic process defined by cell polarization (Ridley et al., 2003). The first step consists in the protrusion of the membrane in the direction of migration. Two different types of protrusion have been identified: lamellipodia (large and broad) and filopodia (spike-like). In both cases, they are driven by actin polymerization and stabilized by their adherence to extracellular matrix (ECM) proteins.

Differences in function have been observed between these protrusions: while filopodia are constituted by parallel actin fibers and are well designed to serve as 'sensors' and explore the environment; lamellipodia are constituted by branched actin capable of supporting traction forces involved in cell displacement.

After generation of mature adhesions at the lamellipodia in the cell edge, traction forces generated by the actomyosin network pull the cell body in the direction of the lamellipodia, producing the unidirectional displacement of the cell. During this process, the Arp 2/3 complex plays a preponderant role, inducing actin polymerization at the lamellipodia and generating dendritic actin networks (branches) with pre-existing filaments at the cell front. This polarization defines the final direction of the migration. Simultaneously, during the translocation of the cell body, the Arp 2/3 complex is inhibited at the trailing edge of the cells, inducing detachment of adhesomes, and release of the rear part of the cells from the substrate (Hanein and Horwitz, 2012; Vicente-Manzanares and Horwitz, 2011).

Adhesomes attachment/detachment and cell polarization is regulated by two families of GTPases with antagonistic activity and localization in the cell: Rho and Rac. While Rho proteins activate actin polymerization, promote cell adhesion and generate a leading edge due to their interaction with the Arp 2/3 complex; Rac GTPases produce the disassembly of the actin fibers and the detaching of integrins from ECM proteins, as observed at the trailing edge (Petrie and Yamada, 2012; Vicente-Manzanares and Horwitz, 2011).

Finally, it is important to indicate that, even though it seems that the biological machinery involved in cell migration could also be related to the sense of topographic cues, there is not clear information about the mechanisms by which cells can sense differences in structure and geometrical organization of 2D milieus during their displacement.

## Cell motility within complex 3D milieus

During recent years, several biophysical mechanisms used for cells during migration have been studied, which are described in the next section of this review. These migration mechanisms, mostly observed in migrating cells within complex

environments (*e.g.* micro-channels, filamentous 3D milieus), can give us clues about the way cells could interact with topographic cues found in 3D milieu.

***Cell migration mediated by the protrusion of lobopodia***

When cells migrate within 3D elastic environments, a special type of protrusion, named lobopodia, can be observed. Lobopodia are mostly represented as non-sharp cylindrical protrusions that might be driven by intracellular pressure rather than actin polymerization (Petrie and Yamada, 2012). Moreover, and although it has been shown that lobopodia contraction is mediated by RhoA-ROCK-myosin II signaling, this type of migration still remains less understood and poorly characterized (Petrie et al., 2014).

With respect to lobopodia, there is little information about structure and composition, although it has been proposed that the myosin IIa acts through vimentin filaments (polarized to the anterior of lobopodial cells and anchored to the nucleus, likely by nesprin-3) easing cell migration (DeSimone and Horwitz, 2014). Particularly, Petri *and col.* (2014) suggests that the nucleus physically divides the cell in 2 compartments, maintaining differences in hydrostatic pressure between the leading and the rear parts of the cell.

***Cell migration mediated by generation of blebs***

Displacement through plasma membrane blebbing is one of simplest biological mechanisms used by migrating cells (Fackler and Grosse, 2008; Charras and Paluch, 2008). Traditionally named *amoeboid* migration, this process is characterized by the formation of spherical membrane protrusions (*e.g.* blebs) (**Figure 2B**), produced by contraction of the actomyosin cortex. This phenomena is dependant on cortical tension and the integrity of actin cortex (Charras and Paluch, 2008).

From the molecular point of view, *amoeboid* migration is dependent on the activity of Rho GTPase and actomyosin contractility. However, it is noteworthy to remark that, differently to *mesenchymal* migration, the blebbing mechanism generates low

traction forces since the Arp 2/3 complex is not required (Bergert et al*.,* 2012). As bleb motility requires less energy than *mesenchymal* cell translocation, this mechanism has been mostly studied on metastatic cells, due to their capacity to escape from anti-tumor treatments based on protease inhibitors, or during their speedy migration onto low adherent surfaces and/or confined environments.

It is important to indicate that, although several cancers exhibit only one migration style, several cancer lines can easily switch from *mesenchymal* to *amoeboid* displacement when they are embedded within three-dimensional (3D) matrices (Bergert et al., 2012; Paluch and Raz, 2013). Moreover, Bergert and col. demonstrated that when the Arp 2/3 complex is inhibited (*e.g.* by using the compound CK-666 in Walker cells cultured on 2D surfaces), lamellipodia are replaced by bleb formation, probably by controlling the actin cortex and/or cortical tension (Bergert et al*.,* 2012).

However, it is important to indicate that even though current research has demonstrated the use of blebs as a mechanism for cell migration, there is a lack of information concerning the mechanisms involved in translocation and forces implied in cell movement (Paluch and Raz, 2013; Charras and Paluch, 2008). Then, and although it is still unclear, the mechanisms by which cells translocate themselves using blebs appear related to periodicity in the formation of protrusions and the regular generation of small pseudopodia on the leading edge allowing the translocation of the cytosol and intracellular organelles through them (Paluch and Raz, 2013; Charras and Paluch, 2008).

It is important to indicate that, although migration mediated by blebs have been mostly studied on scaffolds exhibiting topographic cues (*e.g.* micro channels, fibrous matrices), there is no information regarding the influence that these topographic cues play on cell migration. However, and as was previously noted, first attempts to solve this question have been focused on immune-cells exhibiting an amoeboid migration fashion.

***Cell migration mediated by flux of ions and water***

Recently, Stroka *and col.* demonstrated *in vitro* that neoplastic cells migrating in confined 3D matrices use an actin/myosin-independent mechanism based on the permeation of ions and water through trans-membrane channels (**Figure 2C**). This new translocation strategy, named the *osmotic engine model* (Papadopoulos and Saadoun, 2014; Stroka et al. 2014a), can explain cell displacement within 3D environments (*e.g.* 3µm-wide channels) in the presence of myosin, Rho/Rock kinase and/or β1 integrin inhibitors (Stroka et al. 2014b; Balzer et al., 2012).

Although this process is currently under study, mathematical and biochemical analysis have revealed that this type of migration requires the coordinated activity of polarized ion channels and aquaporins, which modulate fluxes of ions and water from the leading (flux in) to the trailing edge (flux out), where the sodium hydrogen exchanger-1 (NHE-1) and aquaporin 5 (AQP5) - recruited by cortical actin polymerization- seem to play a preponderant role on cell motility and regulation of intracellular pH (Stroka et al. 2014a; Stroka et al. 2014b).

***Role of the nucleus on cell migration within 3D milieus***

The nucleus is the largest and stiffest organelle in cells. Since cell migration within 3D matrices is restricted by the pore cut-off/size distribution, cells need to squeeze the nucleus through via myosin-II mediated contraction (Friedl et al., 2011).

During the lobopodia-based migration, via the actomyosin contractility, the nucleus acts as a piston pressurizing the leading compartment. This results in bleb formation, lobopodia formation and protrusion, and finally cell migration. Recently, Petri and col. compared the intracellular hydrostatic pressure in front of the nucleus, for cells cultured on/in 2D and 3D environments, finding differences of approximately 10 fold between these 2 systems (from ≈ 300 to 700 Pa in lamellipodia compared to ≈ 2200 Pa in lobopodia). According to the authors, when cells are confined within defined 3D micro channels (approximately 20 µm diameter), the nucleus compartmentalize

the intracellular volume, this then acts as a piston, which enhances the hydrostatic pressures at the frontal edge of the cell with respect to the rear edge (≈ 2400 v/s 900 Pa). This results in bleb formation, lobopodia formation and protrusion, and finally cell migration. Molecularly, the actomyosin-vimentin-nesprin-3 complex (*e.g.* intermediate filaments) seems to be responsible for pulling the nuclei to the front of the cell, and its influence on the cell displacement within 3D channels (Petrie et al., 2014).

***Cell migration on nano/micro topographic designed environments***

It is known that cells can switch spontaneously between different migration mechanisms after remodeling cytoskeleton arrangement depending on their environment. Thus, *in vitro* and *in vivo* gradients may lead to changes in cell migration among other important cellular processes. In almost all cases, the migration fashion will be dependent on the balance between actin polymerization, rear contractility and adhesion. First attempts to explain this behavior have been done in 3D micro-channels after analysis of actin organization at the leading edge. According to the authors, there are two different F-actin networks involved in cell sensing and migration: one polymerizing at the cell-matrix interface (Wilson et al., 2013) and a "free" fragment growing on the leading edge. Both structures can communicate and interact mechanically (Wilson et al., 2013), suggesting that cytoskeleton, compartmentalization and hydrostatic pressures produced by the nucleus as well as sensing of topographic characteristics of the cell milieu by migrating cells are intimately related. However, as was previously discussed, the biological mechanism by which cells recognize topographic cues, and their influence on cell migration, is still far away for being explained.

# 4.- Physiological gradients and bi-phasic biophysical stimulation

Traditionally, cell culture has been performed on 2D substrates where cells sense and respond to ventral stimulation (*e.g.* topographical cues). However, standard flat

surfaces differ from *in vivo* milieus, where cells are subjected to complex 3D gradients, such as differential ventral/dorsal exposure to liquid-gas phases (*e.g.* lungs), shear stress produced by fluid flow (*e.g.* endothelial tissues) or surrounded by an extracellular matrix (*e.g.* parenchymal tissues) (Figure 3).

These complex environmental stimuli have been reconstituted *in vitro*, with the purpose to study cell behavior in physiological-like conditions. Here we will focus on the simultaneous ventral/dorsal stimulation of cells, seeking to recapitulate the physiological cell behavior.

## Fluid Dynamic Stimulation

This type of stimulation can be divided in 2 groups, according to the physical sub-phase involved in cellular stimulation:

***Liquid-gas phase stimulation***

The epithelium is a polarized tissue where cells are ventrally anchored to the ECM and dorsally contacting a gas phase. Thus, fully immersed epithelial cells cultures (*e.g.* traditional culture strategy on 2D substrates) differ from the physiological environment. Consequently, alternative methods allowing dorsal contact to a gas phase have been developed (*e.g.* roller bottles, rocking platforms and inverted cultures) (Voisin et al., 1977).

Nowadays, the use of the air-liquid Interface culture (ALi, **Figure 3A**) has become a traditional system used to cultivate epithelial cells because resembles the physiological condition (de Borja Callejas et al., 2014; Zamora et al., 1983; Zamora et al., 1986; Voisin et al., 1977) and thus makes possible to perform drug testing, among other analysis, in physiologically relevant environments (Ashraf et al., 2015; Kooter et al., 2013; Kim et al., 2014).

***Dynamic fluid flow stimulation***

Blood vessels are internally covered by endothelial cells, exposing their ventral surfaces to parenquimal tissues (*e.g.* ECM), and dorsally to the lumen of the vessels: being stimulated by forces produced by fluid displacement (*e.g.* shear stress). Therefore, new *in vitro* culture systems providing constant flow or even pulsatile stress have been performed (**Table 2**) (**Figure 3B**).

It is now well characterized that the excitation of the dorsal receptors stimulates protein Kinase C (PKC) and the mitogen-activated protein (MAP) kinase pathways, resulting in gene transcription modulation and control of *e.g.* endothelial growth and vaso-active substances secretion such as endothelin-1 and endothelial nitric oxide synthase-3 (Vozzi et al., 2014).

The influence of shear stress has also been studied on additional cell lineages, such as vascular smooth muscle cells, since these can be encounter in endothelium-desquamated injuries and are therefore, involved in the response to vascular wounds. Furthermore, the effect on osteocytes has been studied, since the fluid flow over the osteocyte network has been suggested to be the main cellular signal-generating factor (*e.g.* as a result of mechanical loading of bone) (Piekarski and Munro, 1977; Cowin and Weinbaum, 1998; Knothe Tate et al., 1998; Knothe Tate et al., 2000) (**Table 2**).

## Solid phase stimulation

Cells embed within 3D milieus are subjected to physical (*e.g.* stiffness and pores), chemical (*e.g.* cytokines and growth factors) and biological (*e.g.* cell-cell contact and cell-ECM adhesion) gradients during tissue morphogenesis and homeostasis. Among the strategies developed to recreate this environment, hydrogels represent a convenient and well-studied system due to their structural and compositional similarities to natural ECM and the advances in material chemistry that allow tunable physical, chemical and functional properties (Sant et al., 2010; Pedron et al., 2015).

Here, we will focus on cell culture systems that trigger bi-phasic adhesion signaling such as, sandwich culture, cell sheet engineering and dorsal stimulation (*e.g.* by atomic force microscopy, AFM), (**Figure 3C**, **D** and **E** respectively)(Takahashi et al., 2013). In these systems, even if cells are not completely embedded within a 3D matrix, the stimulation of dorsal cell receptors triggers cell adhesion and specific signaling pathways that determine cell fate as a balance between ventral and dorsal stimulation.

Sandwich cultures involve confining cells within two substrates – dorsally and ventrally - with the same or different properties. One of the best known examples are collagen constructs, used preferentially in culture of hepatocytes since these cells lose their metabolic functions when attached to 2D substrates. However the use of sandwich cultures made of ECM proteins (*e.g.* collagen on the top and the bottom), allow hepatocytes to maintain their polarization and metabolic activity (*e.g.* albumin, urea, transferrin, fibrinogen, and bile salt secretion, as well as cytochrome activity (P450 activity) restored (Swift et al., 2010 and Dunn et al*.,* 1992 respectively). Furthermore, this type of culture allows hepatocytes to generate bile canalicular networks (Bi et al., 2006). Thus, this system is a very straightforward tool to study *in vitro* hepatocellular function, such as protein synthesis and/or drug metabolism (Xia et al*.,* 2009; Xia et al., 2012a).

Cell-sheet engineering techniques are based in stacking 2D cell layers, including the underlying extracellular matrix, to generate a 3D tissue that consists of alternate layers of cells and ECM. The technique enables the study of more complex environments, mimicking the multidimensional organization of some tissues and organs. This system stresses the key role of dorsal stimulation for hepatocyte cultures and shows that the 3D co-culture of hepatocyte and endothelial cells results in a powerful tool to gain more comprehensive knowledge of liver metabolism, detoxification and signaling pathways *in vitro* (Harimoto et al., 2002; Kim and Rajagopalan, 2010; Kim et al., 2012).

We, among others, have been involved in the research of new alternatives for sandwich cultures, such as the use of ventral and dorsal surfaces with different properties/characteristics (*e.g.* stiffness and/or topological patterns) (Ballester-Beltrán et al., 2015c). This system has shown cell morphology to be related to substrate rigidity and the ability of cells to reorganize ECM proteins adsorbed to these surfaces (Beningo et al., 2004; Ballester-Beltran et al., 2012; Ballester-Beltrán et al., 2014). Furthermore, it has been shown that sandwich-like cultures can modify cell spreading/elongation and migration rates depending on the substrates properties, resulting in closer 3D environment behaviors (Beningo et al., 2004; Ballester-Beltrán et al., 2015a; Ballester-Beltrán et al., 2015b).

Cells cultured within these bi-phasic systems adhere to the ventral and dorsal substrates, exerting tensional forces on each surface (*e.g.* spatially tunable mechanotransduction). This was first observed when osteosarcoma U2OS cells were ventrally attached to 2D fibronectin-coated glass. In this configuration, cells were dorsally stimulated by atomic force microscopy (AFM) using a fibronectin-coated cantilever (**Figure 3E**). In response to this stimulus, cells adhered and spread on the cantilever forming new fibrillar actin fibers connecting both surfaces, which enabled the cells to contract and pull the cantilever (Chaudhuri et al., 2009). Hence, cell interaction mediated by dorsal stimulation triggers changes in the cytoskeleton arrangement and generates new adhesion structures. This might link ventral and dorsal sides, allowing a complex response to bi-phasic stimuli, such as the fact that dorsal stimulation induces modification of ventral adhesions (Fuentes et al., 2011; Ballester-Beltrán et al., 2015a), indicating the existence of a clear balance between ventral and dorsal stimulation.

When aligned electrospun fibers are used to stimulate dorsal cell adhesion, cells elongate and align in the direction of the fibers. Interestingly, when sandwich-like cultures consist of aligned fibers contacting cells ventrally, and a flat surface stimulating cells dorsally, cells remain aligned to ventral fibers. These results suggest that the topographical cues play a major role irrespective of which surface they are

presented to (Ballester-Beltran et al., 2013). As expected, this alignment is depending on the cell adhesion to fibers, since cells were not able to align when they were coated with non-adhesive proteins (*e.g.* albumin).

# Outlook and conclusions

In this review we have shown the important role gradients have *in vivo* and different methods to recapitulate them *in vitro*. We had special attention to the study of topographical cues since its ability to mimic the fibrillar structure of the ECM is key to unravel cell biology *in vivo*.

Novel methodologies would allow studying these surface gradients in closer 3D physiological environments. For example, here we have shown those offering a bi-phasic stimulation (adhesion-gas; adhesion-fluid flow and adhesion-adhesion). These technical strategies could be combined with 2D gradients on both dorsal and ventral sides, in order to investigate 3D environments-like (*e.g.* topographical gradients on a 2D ventral substrate and fluid flow through the z axis). These studies would set up the basis to answer complex biological questions as well as new methods to mimic the micro/nano pathological surroundings and drug assessment.

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# Figures and Tables

## ***Figures***

**Figure 1:** Schematization of the types of migration fashions described in this review. A) Cell migration mediated by the protrusion of lamellipodia (*Mesenchymal migration*). The circle shows a detailed structure of a focal adhesion and adhesome. B) Cell migration mediated by generation of blebs (*Amoeboid migration*), and C) Cell migration mediated by flux of ions and water (*Osmotic engine model*).

**Figure 2:** Traditional surfaces used to culture cells on 2D without/with nano/micro topographic cues. A) Flat surface, B) Contact guidance lines, C) Grooves, D) Aligned fibers, E) 2D confined environments, F) Nano/Micro pillars G) Nano/Micro pits.

**Figure 3:** Cell culture systems developed to mimic the physiological environment *in vitro*: A) Air-liquid interface culture, B) Cell culture under fluid flow conditions. C) Cell-sheet engineering, D) Sandwich culture and E) Localized dorsal stimulation using an AFM cantilever.

## ***Tables***

**Table 1:** Topographic characteristic of several of anisotropic polymer scaffolds and their influence on cellular differentiation.

**Table 2:** Effect of fluid flow on different cell lineages.

# Figures

**Figure 1**

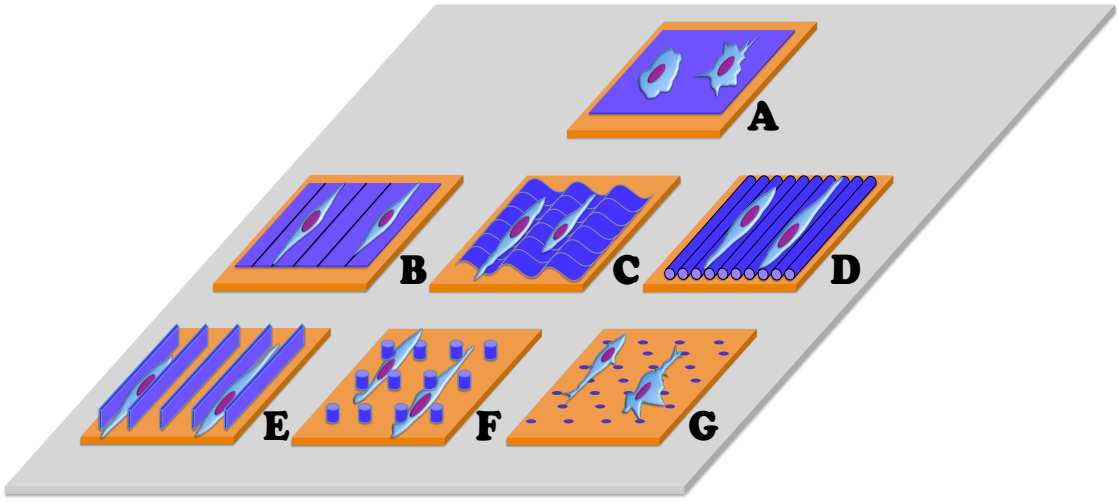

**Figure 2**

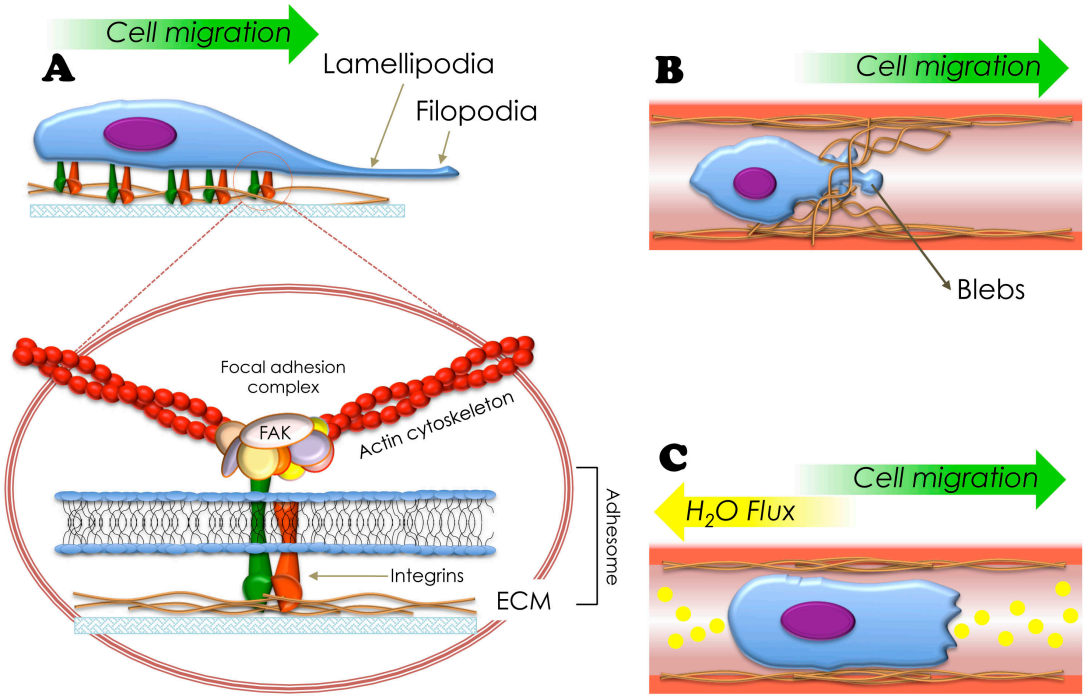
A
Cell migration
Lamellipodia
Filopodia
Focal adhesion complex
FAK
Actin cytoskeleton
Integrins
ECM
Adhesome
B
Cell migration
Blebs
C
Cell migration
$H_2O$ Flux

**Figure 3**

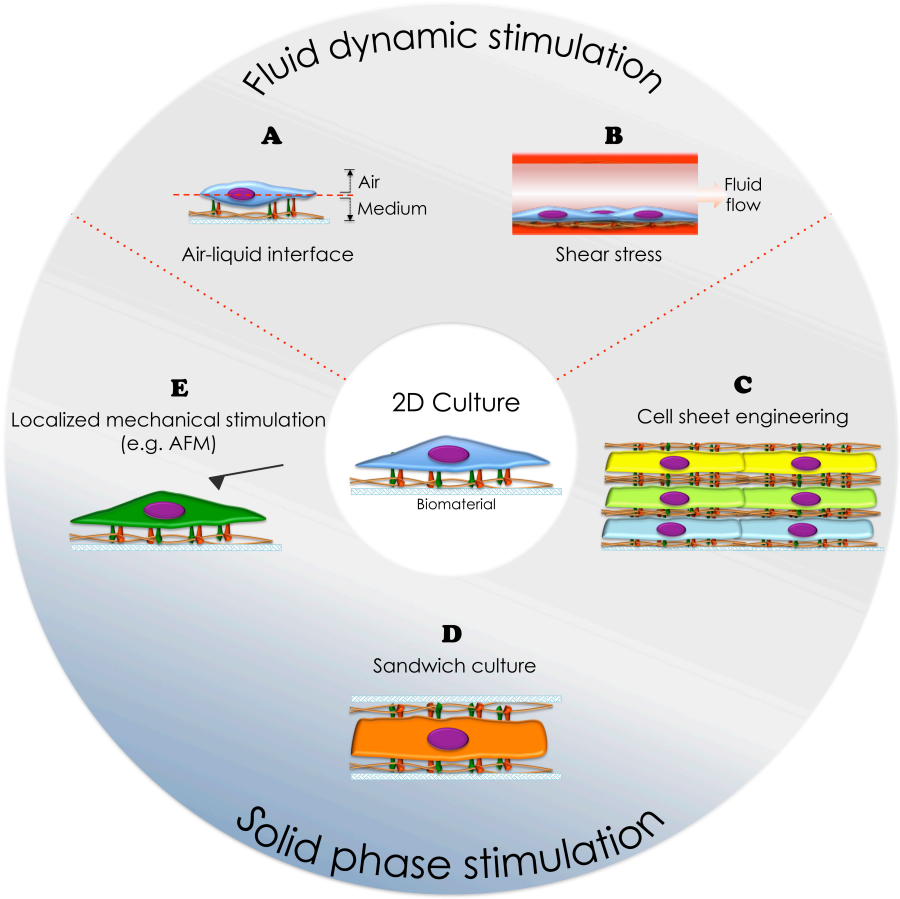
Fluid dynamic stimulation
A
Air
Medium
Air-liquid interface
B
Fluid flow
Shear stress
E
Localized mechanical stimulation (e.g. AFM)
2D Culture
Biomaterial
C
Cell sheet engineering
D
Sandwich culture
Solid phase stimulation

**Table 1**

| Height/ Depth | Width | Pitch | Cell type | Differential function | Ref |
|---|---|---|---|---|---|
| 650 nm | 500 nm | 3.0 µm | Upregulation of neuronal markers β-Tubulin III and NeuN | Upregulation of neuronal markers $\beta$ -Tubulin III | Marino et al., 2013 |
| 4.0 µm | 2.0 µm | 4.0 µm | Primary murine neural progenitor cells | Upregulation of neuronal marker TUJ1. | Wang et al., 2012; Chua et al., 2014 |
| 250 nm | 250 nm | 500 nm | Human embryonic stem cell line, H1 | Upregulation of neuronal markers MAP2 | Ankam et al., 2015 |
| 2.0 µm | 2.0 µm | 4.0 µm | Murine neural progenitor cells | Upregulation of neuronal markers TH, MAP2 and PITX3 | Tan et al., 2015 |
| 625 nm | 1500 nm | 3 µm | Human neural stem cells | Upregulation of neuronal Tuj1 and MAP2 and glial markers GFAP, O4 and Olig2 | Yang et al., 2014 |
| 150 nm | 250 nm | 500 nm | Human MSC | Upregulation of fibro/superficial zone cartilage formation markers PRG4 and COL1 | Wu et al., 2014 |
| 200 nm | 415 nm | 830 nm | Human MSC | No upregulation in the osteospecific marker ALP | Janson et al., 2014 |

**Table 2**

| Cell type | Effect of fluid flow | References |
|---|---|---|
| Endothelial cells | Cell alignment<br>Release of vaso-active substances (*e.g.* endothelin-1) and nitric oxide synthase. | Ishibazawa et al., 2011;<br>Vozzi et al., 2014. |
| Vascular smooth muscle cells | Decrease cell proliferation.<br>Increase NO production.<br>Increase of fibroblast growth factor (FGF)-2 production. | Papadaki et al., 1998;<br>Lindner et al., 1991. |
| Osteoblast cells | Induction of NO and prostaglandin E2 production.<br>Activation of growth factor signaling pathways.<br>Alteration in the Wnt/β-catenin and bone formation signaling pathway. | Smalt et al., 1997;<br>Jia et al., 2014. |
| Stem cells | Cell alignment.<br>Increase in the angiogenic and vasculogenic potential of endothelial cells derived from human embryonic stem cells (hESC).<br>Positive modulation of the stem cell proliferation and differentiation. | Metallo et al., 2008;<br>Huang et al., 2005;<br>Lim et al., 2013;<br>Potter et al., 2014. |

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