



Cite this: *Biomater. Sci.*, 2014, **2**, 1548

Received 16th April 2014,  
Accepted 7th July 2014  
DOI: 10.1039/c4bm00128a  
[www.rsc.org/biomaterialsscience](http://www.rsc.org/biomaterialsscience)

## Biophysical regulation of hematopoietic stem cells

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Blood is renewed throughout the entire life. The stem cells of the blood, called hematopoietic stem cells (HSCs), are responsible for maintaining a supply of all types of fresh blood cells. In contrast to other stem cells, the clinical application of these cells is well established and HSC transplantation is an established life-saving therapy for patients suffering from haematological disorders. Despite their efficient functionality throughout life *in vivo*, controlling HSC behaviour *in vitro* (including their proliferation and differentiation) is still a major task that has not been resolved with standard cell culture systems. Targeted HSC multiplication *in vitro* could be beneficial for many patients, because HSC supply is limited. The biology of these cells and their natural microenvironment – their niche – remain a matter of ongoing research. In recent years, evidence has come to light that HSCs are susceptible to physical stimuli. This makes the regulation of HSCs by engineering physical parameters a promising approach for the targeted manipulation of these cells for clinical applications. Nevertheless, the biophysical regulation of these cells is still poorly understood. This review sheds light on the role of biophysical parameters in HSC biology and outlines which knowledge on biophysical regulation identified in other cell types could be applied to HSCs.

## Introduction

Stem cells have, by definition, the abilities to differentiate into specialized cell types and to self-renew. Among the adult stem cells (*i.e.* stem cells that are organ-specific, possess important roles in tissue maintenance and repair, and are present throughout the entire life) hematopoietic stem cells (HSCs) are the best-studied.<sup>1</sup> HSCs, the stem cells of the blood system, renew our blood with billions of fresh cells such as erythrocytes, T- and B-cells every day.<sup>2</sup> Since the 1960s, HSCs have been used in the clinical setting to treat patients that suffer

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from haematological disorders such as leukaemia or lymphoma.<sup>3</sup> However, the supply of HSCs for clinical applications is limited in terms of the availability of matching donors (= number of available transplants) and the number of HSCs per transplant. The latter is the case in particular when umbilical cord blood is used as an alternative source of transplantable HSCs.<sup>4</sup> Steps toward efficient HSC proliferation *in vitro* could severely contribute to an improved supply with HSCs for transplantation and thereby help tens of thousands of patients. However, this remains a challenge, as conventional cell culture systems thus far have failed to reproduce the efficiency of the natural HSC environment in terms of controlling cellular behaviour.

In 1978, Schofield proposed the niche hypothesis for HSCs.<sup>5</sup> Niches are highly specialized microenvironments in which the stem cells receive all the signals they need in order to maintain their stem cell character (*i.e.*, the ability to self-renew and to differentiate). In the case of HSCs these niches are located in the red bone marrow of trabecular bones. Up until now, several locations and entities have been described as HSC niches and new factors, parameters and cell-types that play a role in HSC niches are continuously being discovered. This continuously adds complexity to the system.<sup>6</sup> Despite the fact that for other stem cells, such as mesenchymal stem cells, the importance of physical parameters in stem cell biology has been widely accepted, surprisingly little is known on the impact of (bio)physical parameters on HSCs. The aim of this review is to give an overview on the current understanding of the biophysical regulation of HSCs and to discuss the possibilities of transferring existing concepts of biophysical regulation mechanisms to HSCs.

## The stem cell niche

Stem cell niches are specific three-dimensional microenvironments that regulate adult stem cell survival, maintenance, proliferation and differentiation.<sup>7</sup> The signals through which the niche controls stem cell function are (i) direct contact and communication between stem cells and their adjacent supporting niche cells, (ii) interactions with the extracellular matrix, and (iii) stimulation by soluble components such as growth factors, hormones, cytokines or chemokines.

Studies have located HSC niches either close to the endosteum ('endosteal niche'),<sup>8–10</sup> adjacent to bone marrow sinusoids<sup>11</sup> or next to arterioles<sup>12</sup> ('perivascular niches'). Current literature suggests that these are functionally distinct microenvironments that host distinct types of HSCs.<sup>6,13</sup> Each of these niches is created by multiple cell types that contribute to niche function and HSC regulation.<sup>14</sup> In other words, there is not a singular niche cell that regulates HSCs alone. Instead, the niches orchestrate the functions of multiple niche cell types in order to fulfil their task (Table 1).

A range of different cell populations have been used to study HSCs. They differ in terms of species (mainly mouse or human) as well as markers that were used for their

**Table 1** Cells, ECM molecules and physical parameters in the bone marrow/the HSC niche

Category	Components	References
Extracellular matrix	Collagens type I, II, III, IV, V, VI, X	25–27
	Laminins	19,28
	Fibronectin	25,27,29
	Nidogen	30
	Tenascin-C	27,30
	Thrombospondin	26,27
	Vitronectin	26
	Decorin	26
	Fibulin	31,32
	Hyaluronic acid	33,34
	Osteopontin	21,22,35
	Endothelial cells	11
	Mesenchymal stem/stromal cells	36
Cellular components	Perivascular cells	6
	CAR (CXCL-12 abundant reticular) cells	37,38
	Non-myelinating Schwann cells	39
	Macrophages	40–42
	Sympathetic nerves	43
	Osteoblasts	8–10
	Osteoclasts	44,45
	Osteocytes	46
	Three-dimensional architecture	29,47–51
	Mechanical properties	52–54
Physical parameters	Nanostructural features	47,52,55–57
	Shear stress/fluid flow	58–60

purification. As a result, different hematopoietic subpopulations as well as cell populations that are more or less enriched with HSC subtypes have been applied. Whereas suitable markers for the identification and purification of HSCs are available in mice, such markers are still missing for human HSCs and it appears that the murine and human hematopoietic system have remarkable differences.<sup>15</sup> Nevertheless, the term 'HSC' is used throughout this review for readability reasons. In order to acknowledge the potential impact of species and subpopulations on the biophysical regulation of HSCs, footnotes provide information on the cell populations used in the respective study wherever necessary.

The space between cells is filled by the extracellular matrix (ECM), which provides structural stability as well as anchorage sites for cell adhesion and migration. Furthermore, the ECM regulates cell functions such as morphology and development.<sup>16</sup> The ECM is secreted by cells into the extracellular space. It is a complex composition of various molecules that can be categorized into two groups: (i) structural proteins (including glycoproteins) that provide structural integrity for tissues, and (ii) proteoglycans and glycosaminoglycans, which provide the capacity to hold substantial amounts of water and to bind growth factors.<sup>17</sup>

The structural proteins of the bone marrow ECM (including collagens, laminins and fibronectin) provide anchorage sites that are recognized by cells through specific cell adhesion molecules. In this way, the ECM plays a crucial role in the retention of stem cells in their niches as well as their mobilization from the niche.<sup>16</sup> Integrins – which directly link the ECM with the intracellular actin cytoskeleton – are an important



family of cell adhesion receptors in this context. Furthermore, laminins and fibronectin act not only as structural elements that provide anchorage sites for cells. They also influence HSC<sup>†,‡</sup> proliferation, differentiation, and the cell's ability to engraft.<sup>18–20</sup> Osteopontin – another member of the structural proteins of the bone marrow ECM – was shown to negatively regulate both the HSC pool size *in vivo* in mice as well as the *in vitro* proliferation of human HSCs.<sup>†,‡,21,22</sup>

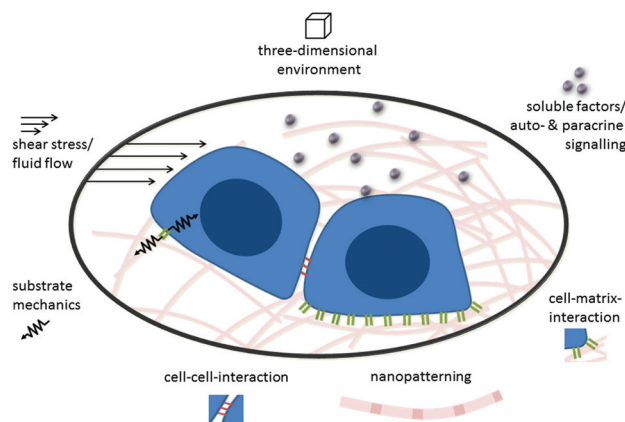
Proteoglycans consist of a protein core with covalently bound glycosaminoglycans. Their ability to bind and present growth factors to stem cells contributes to cell adhesion and migration as well as playing a role in regulating differentiation.<sup>23</sup> Among the glycosaminoglycans, hyaluronic acid is an important component of the bone marrow ECM that impacts HSC behaviour *via* interaction with its receptor CD44.<sup>24</sup> Table 1 contains a comprehensive list of known bone marrow ECM components.

Many ECM components are distributed throughout the bone marrow unevenly. They are more or less enriched in certain compartments (including compartments described to be potential HSC niches). While fibronectin appears to be expressed ubiquitously in the bone marrow<sup>27</sup> (endothelial<sup>61</sup> and endosteal<sup>25</sup> regions), osteopontin and collagen type I are restricted to endosteal regions.<sup>21,25</sup> Most laminin isoforms that are detected in the bone marrow are found in association with blood vessels, sinusoids and endothelial cells – in other words in vascular regions.<sup>19</sup> Variations in ECM composition may contribute to the different functions of the bone marrow regions (and potential niche microenvironments). These functions might be elicited not only by differences in the biological and/or (bio)chemical properties of the ECM molecules but also by differences in their biophysical properties. For example, the substrate stiffness – characterized by the Young's modulus  $E$  – varies broadly in the different potential niche microenvironments. They range from very soft in the marrow ( $E = 0.3$  kPa)<sup>62</sup> to intermediate in endothelia (0.5–2.0 kPa)<sup>63</sup> and vessel walls (5–8 kPa)<sup>64,65</sup> to relatively stiff in the osteoid matrix (35 kPa).<sup>66</sup>

In spite of the progress made so far, characterizing the composition of the niche is still a matter of ongoing research and engineering an artificial stem cell niche is considered to be one of the major challenges in hematopoietic research.<sup>26</sup> In the attempt to design and recreate stem cell niches, physical niche properties are emerging as important HSC influencing parameters that must be taken into account.

## Environmental sensing

Cells sense their environment through specific receptors that can recognize their matching counterpart molecule (in other words, their ligand) when it is nearby. In addition to recognizing the type of ligand that is bound by the receptor, cells can also “feel” other environmental and ligand cues, such as rigid-



**Fig. 1** Environmental stimuli that influence cells. Cells are naturally localized in three-dimensional environments. In their *in vivo* environments they are subjected to biochemical and biological signals elicited by cell–cell interactions including direct contacts and communication through soluble factors as well as cell–matrix interactions. At the same time, cells are stimulated by the physical parameters of the environment, such as substrate mechanics, shear forces and the nanopatterning of the surrounding matrix.

ity or elasticity. In other words, the cell can integrate both the chemical nature of its environment as well as physical cues generated by external and internal forces. In this way cells can sense the mechanical properties of the surrounding tissue, nanostructural features, and shear forces.<sup>67</sup> Fig. 1 gives a schematic overview of the different biological, chemical and physical stimuli in the environment of stem cells.

Cells in living tissues are closely connected to other cells and the ECM *via* cell adhesion molecules (CAMs). These molecules connect the cell's interior to its external environment and typically consist of an extracellular domain, a transmembrane region and an intracellular domain that elicits intracellular signalling or interacts with the cytoskeleton of the cell. Most CAMs can be grouped into distinct families: integrins, cadherins, selectins and the immunoglobulin superfamily.<sup>68</sup> Integrins are the most common cellular receptors for ECM molecules. They are heterodimeric molecules, each composed of one alpha and one beta chain.<sup>69,70</sup> The 18 different alpha and 8 beta chains identified so far dimerize non-covalently to form the 24 integrins known today.<sup>71</sup> The extracellular domain of integrins recognizes and binds to specific ECM molecules. The intracellular domain is linked to the actin cytoskeleton and also associates with signalling molecules such as kinases and phosphatases.<sup>72,73</sup>

Signalling through integrins is a bi-directional process that functions both outside-in and inside-out.<sup>70</sup> Upon binding of an ECM element to the extracellular part of the integrin receptor, the cell receives information on the biochemical and biophysical properties of its environment. Integrins, in their function as signal transmitters, then trigger an intracellular cascade of signalling events, including phosphorylation and signalling through small G-proteins (outside-in signalling). Local cytoskeleton dynamics are changed in response, which

<sup>†</sup> Human CD34<sup>+</sup> cells.

<sup>‡</sup> Murine mononuclear cells from bone marrow.



in turn leads to the generation of force. These processes lead to directly observable alterations, *e.g.*, in cell shape and motility, and later cause changes on the transcriptional level. This ultimately affects cell proliferation, differentiation and survival.<sup>67</sup> The control over the affinity of integrins to their extracellular ligands (as part of their adhesion molecule function) also occurs *via* signals which originate from the cell's interior and act on the cytoplasmic tail of the integrins. These signals trigger conformational changes in the extracellular ligand-binding domain of the receptor and thereby lead to changes in ligand affinity (inside-out signalling).<sup>74</sup>

When integrins establish contact with the ECM nascent adhesive structures, so-called focal complexes,<sup>75</sup> appear. In anchorage-dependent cells such as fibroblasts these focal complexes are transient. They can disappear with time or mature into larger, more stable adhesive structures – the focal adhesions. Mature focal adhesions have an elongated appearance and are found at the ends of actin stress fibres.<sup>67,76</sup> It appears that integrin-mediated contacts to the ECM formed by HSCs differ from those found in obligatory adherent cells, because the actin cytoskeleton of HSCs does not develop stress fibres.<sup>77</sup> Although HSCs† express zyxin<sup>78</sup> – a marker that is considered specific for mature focal adhesions and that is not detected in nascent focal complexes<sup>79</sup> – it still seems most likely that HSCs do not form mature focal adhesions. Nevertheless, many of the substrates that were shown to elicit mechanosensitive responses in HSCs do actually target integrins.<sup>52,54,62</sup> This suggests that, similar to their role in anchorage-dependent cells, integrins are likely to act as environmental sensors in HSCs, even though the composition and maturation of the associated multiprotein complexes might differ.

These differences are accompanied by some striking phenomenological differences between HSCs and anchorage-dependent cells. HSCs,† instead of showing spreading behaviour on 2D surfaces, polarize and form a uropod.<sup>80–82</sup> They exhibit an amoeboid-type migration behaviour, which leads to a fast migration speed of several  $\mu\text{m}$  per minute.<sup>54</sup> These phenomenological observations in 2D cell culture systems indicate a more transient nature of integrin-mediated contacts between ECM molecules and HSCs, compared to contacts with anchorage-dependent cells.

### Integrin-actin link

The formation of adhesive structures that link cells to the ECM first requires integrin activation. This involves a conformational re-organization of the dimer, resulting in an enhanced affinity to its ligand.<sup>83</sup> The connection of the cytoplasmic domain of the integrin beta-chain with the actin cytoskeleton *via* kindlins and talins is a crucial step during this activation process.<sup>84–88</sup> As described for anchorage-dependent cells, it has also been shown for HSCs§ that adhesion to the ECM is talin-dependent.<sup>89</sup> HSCs are assumed to express kindlin-3

(synonym: FERMT3), because this member of the kindlin family is specifically found in hematopoietic cells and platelets, including hematopoietic organs during murine fetal development and bone marrow mononuclear cells in adult mice.<sup>90,91</sup>

Following the engagement of talin and kindlin, vinculin binds to talin and fosters the clustering of multiple activated integrins. Vinculin also strengthens the integrin-actin cytoskeleton linkage by binding to actin through its tail domain.<sup>92,93</sup> However, experiments in which vinculin expression was silenced have provided evidence that vinculin is actually not necessary for integrin-dependent adhesion in HSCs,§ but is indispensable for integrin-independent repopulation of the bone marrow by HSCs.§<sup>89</sup> These results indicate that vinculin plays a different role in HSCs than in anchorage-dependent cells.

Actin assembly and flow lead to cell locomotion. In non-muscle cells actin movement depends on non-muscle myosin II activity. Non-muscle myosin II exists in two isoforms (IIA and IIB) both of which are expressed by HSCs. Both isoforms show a cortical localization in freshly isolated, uncultured HSCs.† Myosin IIB accumulates in the cortical regions of the uropod during the process of adhesion-induced polarization, whereas myosin IIA localization is diffuse in the cytoplasm. Isoform switching seems to regulate HSC differentiation and motility.<sup>62,77</sup>

Motility and migration are essential to HSC function in embryogenesis and adulthood.<sup>94</sup> However, the cytoskeletal motility apparatus of HSCs has not been investigated in great detail, as of yet. Gene expression analysis of HSCs† using cDNA arrays, *e.g.*, revealed the expression of several members of this apparatus, namely alpha-actinin, dynein, dynamin 2, dynactin 1 and drebrin 1.<sup>95</sup> Among these, alpha-actinin is of particular interest in the context of biophysical regulation of cells, because it is involved in the integrin-mediated force transmission.<sup>96</sup> Its role in adhesion and force transduction in HSCs is yet to be elucidated.

### Integrin-mediated signalling

Besides connecting the receptor to the actin cytoskeleton, the intracellular multiprotein complex around ligand-bound integrin clusters is also involved in transmitting signals into the cell. Many different components of the integrin ‘adhesome’ have been described and their interplay is highly complex.<sup>97</sup> Here, we will focus on some key elements that are known to play a role in HSCs and/or the mechanotransduction of stem cells.

The most prominent integrin-linked signalling molecule is the focal adhesion kinase (FAK), which is a non-receptor tyrosine kinase. It is a key element of many signalling pathways that lie downstream of integrins. FAK plays a central role in adhesion turnover, the activation of small GTPases of the Rho family, and crosstalk between integrin- and growth factor receptor mediated signalling.<sup>98</sup> FAK interacts with talin and paxillin.<sup>99–101</sup> It binds and is involved in the phosphorylation and activation of its substrates including paxillin and guanine

§ Murine Lin<sup>−</sup> Sca1<sup>+</sup> cKit<sup>+</sup> cells (LSK cells).





nucleotide exchange factors (which are important for signalling *via* small G-proteins).<sup>102</sup> The FAK homologue Pyk2 (synonym RAFTK) has been described as the principal 'focal adhesion kinase' in HSCs.<sup>†103</sup> Pyk2 shares many properties with FAK. However, these kinases are not redundant and do possess individual capacities.<sup>69</sup> Both, Pyk2 and FAK are necessary for proper HSC function.

Subsequent to integrin–ECM interaction, a further family of non-receptor kinases – the Src family kinases (SFK) – is quickly activated. These can either bind to the cytoplasmic tail of the integrin  $\beta$ -chain directly or to FAK. They can phosphorylate FAK and FAK-associated proteins.<sup>69</sup> The SFKs Lyn, Hck and Fgr are specific to the hematopoietic compartment and play a role in cytokine-induced mobilization of HSCs§ from the bone marrow to the circulation as well as in the engraftment of transplanted HSCs¶ to the bone marrow.<sup>104,105</sup>

The active interaction of SFKs with FAK is essential for the integrin-mediated regulation of Rho GTPases.<sup>106</sup> The small Rho GTPases Rho and Rac play major roles in regulating the actin cytoskeleton. Their activation is regulated by guanine nucleotide exchange factors that promote GDP release from the GTPases and subsequent GTP loading. Some of these nucleotide exchange factors are also substrates of FAK (as mentioned above).<sup>67,107</sup> Rho GTPases play an important role in regulating HSC§ migration, proliferation and self-renewal. The ubiquitously expressed Rho GTPase Rac1, in particular, is essential in HSC§ homing and engraftment.<sup>108</sup> The hematopoietic cell-specific Rho family member Rac2 is critical for HSC¶ migration and adhesion. It also modulates Rac1 and Cdc42 activities.<sup>109,110</sup> Cdc42 plays a role in HSC§ migration and retention in the niche.<sup>111</sup> RhoA negatively regulates human HSC\*\* migration. And, correspondingly, active RhoA supports the retention of HSCs\*\* in their niche.<sup>112</sup>

Paxillin is recruited to integrin adhesion sites early on during integrin-mediated signalling.<sup>113</sup> It is able to bind directly to the cytoplasmic tail of the integrin  $\alpha$ 4 chain<sup>114</sup> and has several protein-interaction modules and phosphorylation sites. Phosphorylation regulates the interaction of paxillin with other proteins. Paxillin's main function is to mediate and orchestrate the binding of signalling molecules and cytoskeleton-linking molecules to the cytoplasmic domain of integrins, thereby contributing to the regulation of adhesion turnover and cell migration.<sup>69</sup>

Detailed analyses of the signalling pathways downstream of integrins in HSCs† are available for the process of chemokine-stimulated cell migration. During this process, phosphorylation of FAK, Pyk2, and paxillin as well as the adaptor molecules P130CAS (synonym: BCAR1), Crk and Crk-L is induced.<sup>115,116</sup> Fig. 2 summarizes the molecules that are known to be involved in integrin–actin-linkage and integrin-mediated signalling in HSCs.

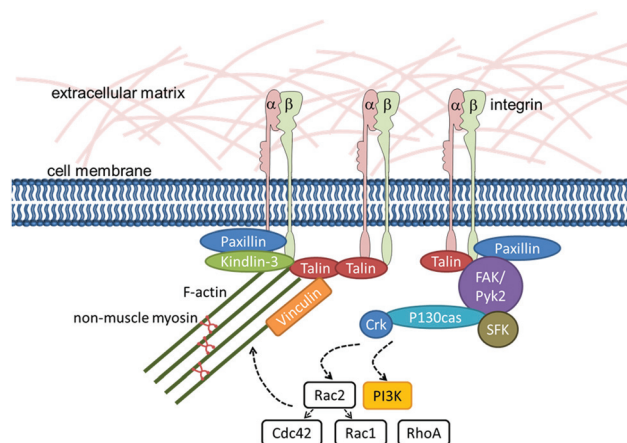


Fig. 2 Integrin–actin interaction and the integrin-signalling complex. The illustration highlights the molecules downstream of integrins that are expressed by HSPCs and/or play a role in HSPC biology.

## Mechanical properties

### Sensitivity to matrix stiffness

In their niches, stem cells experience microenvironments with specific mechanical properties. Research using polymeric materials with an adjustable E-modulus has revealed that both embryonic and adult stem cells are sensitive to substrate stiffness.<sup>117–120</sup> Engler *et al.* were the first to report on the ability to control and direct the differentiation of mesenchymal stem cells by changing the E-modulus of the substrate. Interestingly, cellular differentiation reflected the matrix's stiffness. For example, on soft matrices (resembling the brain's mechanical properties) mesenchymal stem cells differentiated into cells of the neural lineage.<sup>117</sup>

In 2010, Holst and co-workers reported that the proliferation of HSCs§,†† was increased when they cultured murine bone marrow cells or human mononuclear cells from umbilical cord on tropoelastin-coated substrates. Tropoelastin is the most flexible known biomolecule and its stretchability proved to be the pro-proliferative parameter in this experimental setup.<sup>53</sup> Both HSC† adhesion and migration depend on the mechanical properties of the substrate, as we could show employing fibronectin-coated poly(ethylene glycol) (PEG) hydrogels. Our research showed that HSCs† adhered better and were more motile on stiffer hydrogels than on softer ones.<sup>54</sup> Similarly, it has been reported that the elasticity of collagen hydrogels influences HSC morphology.<sup>52</sup>

The mechanical properties that stem cells experience in their stem cell niche are determined by the ECM and the adjacent niche cells. Osteoblasts, which represent a major cell type in the endosteal HSC niche, respond to adrenergic stimulation (as it occurs during mobilization of HSCs from their niche to the blood circulation) by changing their morphology – they flatten.<sup>43</sup> And, as our research shows, they simultaneously change their mechanical properties by getting stiffer.<sup>54</sup> This is

¶ Murine Lin<sup>−</sup> Sca1<sup>+</sup> cells.

¶ Murine Lin<sup>−</sup> cKit<sup>+</sup> cells.

\*\* Human Lin<sup>−</sup> mononuclear cells from umbilical cord blood.

†† Human Lin<sup>−</sup>, CD34<sup>+</sup>, CD38<sup>+</sup> cells.



a first hint suggesting that niche stiffness is not a static parameter, but rather a property that is dynamically regulated during physiological processes.

One example of changing tissue stiffness caused by physiological processes is the impact of ageing. In tissues like skin both the composition of the ECM and molecular cross-linking in the ECM change during ageing, resulting in matrix stiffening.<sup>121,122</sup> These changes are considered cell-extrinsic as they are a result of the aging cellular environment. In addition, HSCs also undergo cell-intrinsic age-related modifications that lead to a decline in their functionality.<sup>123–125</sup> These may lead to age-related changes in the mechanosensitivity of HSCs, as the small Rho GTPase Cdc42 was shown to play a central role in HSC ageing. Cdc42 activity is increased in aged HSCs and is associated with a loss of polarity in these cells.<sup>123,124</sup> As Cdc42 acts downstream of integrins and is involved in the regulation of the actin cytoskeleton, it may also be involved in the mechanosensing processes downstream of integrins. Whether ageing impacts biophysical cues (including matrix stiffness and mechanosensing processes) in the hematopoietic compartment, however, is yet to be determined.

### Mechanotransduction

As described in the previous chapter, HSCs are sensitive to the mechanical properties of their environment. But how do they transmit the mechanical signals provided by the environment to the cell's interior? A prerequisite for the cell's ability to sense the mechanical properties of substrates is the existence of forces acting between the cell and the substrate. In order to react to the physical stimulus that is elicited by the mechanical properties of the environment, the stimulus must be translated into a biochemical signal in the cell's interior which is (i) readable by the cell, and (ii) results in a direct response (e.g., change of morphology or, on a longer timescale, gene expression). When a cell interacts with an ECM with distinct mechanical properties, the cell will bind to its specific ligand through an integrin that, on the one hand, connects the ECM to the cytoskeleton and, on the other hand, elicits intracellular signalling. As described above, the resulting outside-in and inside-out signalling leads to local changes in the cytoskeleton. This results in the generation of internal forces that counteract the externally applied forces. This dynamic interplay of reciprocally pulling forces leads to a condition in which the cytoskeleton is under isometric tension, thereby stabilizing the cell morphology and enabling the cell to sense external forces and react appropriately.<sup>126–128</sup> The direct mechanical cross-talk between integrins and the actin cytoskeleton (i) provides pulling forces that are a prerequisite for the successful development of adhesive structures and (ii) appears to be a key element in the mechanosensing of cells. Many molecules are involved in linking integrins to the actin cytoskeleton, regulating their dynamics and eliciting signalling cascades. At the same time, the assembly of this multiprotein complex is dependent on the generation of force at many different levels and steps. Therefore, it appears that it is not a single protein which acts as 'the one and only' mechanosensor. Instead, the

entire multiprotein complex – including its molecular network that acts in response to applied and generated forces – has to be regarded as a mechanosensory unit.<sup>67</sup> In the following, some molecules of this network that appear to be of particular importance in the mechanotransduction process of HSCs will be highlighted (Fig. 3).

Non-muscle myosin II is a central player in the generation of force in cells. Since Engler *et al.* first reported on the mechanosensitivity of mesenchymal stem cells, it has become generally accepted that non-muscle myosin II is involved in the mechanotransduction process of these cells. Pharmacological inhibition of non-muscle myosin II using blebbistatin has revealed its role in mechanosensing of mesenchymal stem cells<sup>117</sup> as well as HSCs.<sup>53</sup> Non-muscle myosin II is composed of two heavy chains, two essential light chains, and two regulatory light chains. The heavy chains interact with the actin cytoskeleton and host the motor unit. The essential light chains act as stabilizing elements. The regulatory light chains contain several phosphorylation sites. Phosphorylation of these sites, e.g., by myosin light chain kinase (MLCK) or Rho-associated protein kinase (ROCK), contributes to the regulation of myosin II activity. The heavy chains carry additional phosphorylation sites, whose phosphorylation may influence myosin II activity even more directly.<sup>77</sup> The expression level as well as phosphorylation pattern of non-muscle myosin II appear to depend on the stiffness of the underlying matrix.<sup>129</sup> As mentioned earlier, HSCs† express both non-muscle myosin II isoforms A and B. While non-muscle myosin IIA is expressed throughout the entire hematopoietic differentiation, myosin IIB vanishes with differentiation. When cultured on stiff substrates (34 kPa, resembling the osteoid matrix of the bone endosteum) myosin IIB is polarized in the uropod of adhering, polarized HSCs. In contrast, on soft matrices that resemble bone marrow in their stiffness (0.3 kPa) myosin IIB polarization is not observed. Myosin IIB polarization contributes to the asymmetric cell division of HSCs. During this process, myosin IIB accumulates in the daughter cell which expresses higher levels of the HSPC marker CD34. Myosin IIA activity is enhanced on stiff matrices (more myosin IIA is dephosphorylated at S1943), whereas on soft matrices it is decreased (phosphorylation at S1943 increased).<sup>62</sup> This suggests that non-muscle myosin II is a central element in mechanotransduction processes and biophysical regulation in anchorage-dependent cells and HSCs likewise.

Like myosin II, the signalling pathways downstream of integrins are also subjected to mechanosensitive signalling. We could show that phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) plays a role in those mechanotransduction processes downstream of integrins that regulate HSC† adherence and motility. When PI3K is specifically inhibited, adhesion and motility on stiff matrices are reduced down to a level similar to that observed on soft matrices.<sup>54</sup> Which signals act up- and downstream of PI3K remains to be elucidated.

Another particularly interesting potential mechanosensor located downstream of integrins is the adaptor molecule P130CAS, which undergoes conformation changes under



mechanical stretching. As it is being stretched phosphorylation sites are exposed and become available as substrates for interacting kinases of the Src family.<sup>130</sup> Whether P130CAS in HSCs acts in a similar manner is the subject of further studies.

All in all, as HSC sensitivity to matrix stiffness has been a topic of investigation only during the last 4 years, very little is known on mechanotransduction in these cells. Most studies describe mechanosensitivity phenomenologically rather than elucidate the underlying molecular mechanisms. Therefore, exploring mechanotransduction in HSCs remains a hot topic for future investigation.

### Transducing mechanical signals into the nucleus

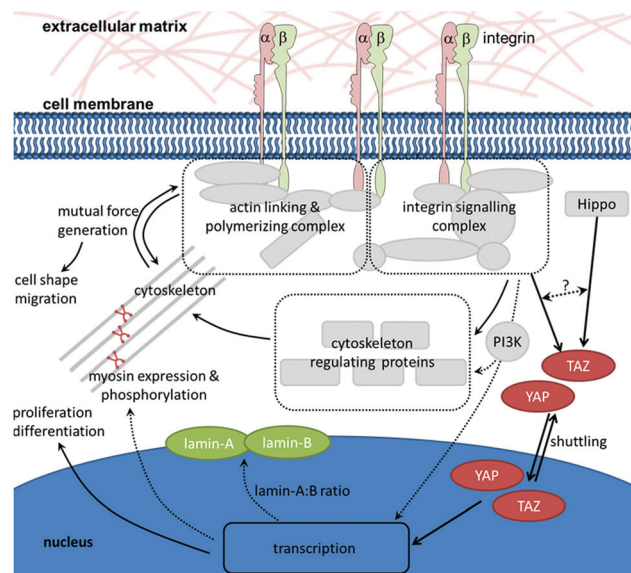
Short-term effects of mechanical signals on HSCs include altered adhesion and migration<sup>54</sup> whilst, on a longer time-scale, HSCs react with changes in their proliferation and differentiation.<sup>53</sup> Changes in protein conformation, complex composition and posttranslational modifications (such as phosphorylation) are sufficient for immediate responses to intracellular forces that are elicited by the mechanical properties of the environment. Long-term reactions, in contrast, necessitate modifications in gene expression. This requires that the mechanical signal is first translated into the cell's interior and then, in a second step, into the nucleus. Here, the arriving signal can lead to changes in the activity of transcription factors as well as the nuclear or cytoplasmic localization of molecules that ultimately affect gene expression.

Such effects have been described for transcriptional cofactors YAP (yes-associated protein) and TAZ (transcriptional coactivator with PDZ binding motif; synonym: WWTR1).<sup>131,132</sup> YAP and TAZ are closely related proteins that shuttle between the nucleus and the cytoplasm. Inside the nucleus they interact with transcription factors such as TEA domain family members. YAP and TAZ regulate organ size as well as adult stem cells.<sup>133–137</sup> Both are downstream effectors of the Hippo pathway, the pathway that regulates YAP and TAZ localization and degradation.<sup>138</sup> YAP and TAZ have been implicated as major effectors in mechanotransduction, because they communicate mechanically elicited signals into the nucleus, thereby affecting gene transcription. On stiff substrates YAP and TAZ are located inside the nucleus and are active, whereas on soft matrices YAP and TAZ are positioned in the cytoplasm and are functionally inactive.<sup>131</sup> As force transduction is closely associated with the actomyosin cytoskeleton, YAP and TAZ activity depend on the integrity of the actin cytoskeleton and Rho activity (which regulates cytoskeletal dynamics).<sup>131,139–141</sup> Crosstalk between the two known parallel-acting YAP and TAZ regulating cues – the Hippo pathway and cytoskeletal dynamics – has been proposed.<sup>132</sup> Concerning its occurrence, YAP is detectable at low levels only in murine long-term HSCs (Lin<sup>−</sup> Sca1<sup>+</sup> cKit<sup>+</sup> Flt3<sup>−</sup> CD34<sup>−</sup>), but not in murine short-term HSCs (Lin<sup>−</sup> Sca1<sup>+</sup> cKit<sup>+</sup> Flt3<sup>−</sup> CD34<sup>+</sup>) or Lin<sup>+</sup> hematopoietic lineages. Furthermore, YAP expression seems to not impact HSC function, which is in contrast to the role ascribed to YAP in other tissue-specific adult stem cells.<sup>142</sup> Therefore, it appears questionable whether the results

obtained for YAP concerning mechanotransduction in, *e.g.*, mesenchymal stem cells also hold true for HSCs.

The nuclear lamina has been proposed as an additional element that regulates YAP translocation. Research on mesenchymal stem cells has shown the nucleoskeleton protein lamin-A to be more abundant in cells cultured on stiffer substrates than in those grown on softer substrates. Additionally, lamin-A conformation proved to be mechanosensitive.<sup>143</sup> Lamin-A also coregulates key factors such as YAP and serum response protein (SRP).<sup>143</sup> As lamins determine the mechanical properties of the nucleus, the relative abundance of the two isoforms lamin-A and -B plays an important role in transmigration processes of hematopoietic cells, including HSCs.<sup>†144</sup> Transmigration can occur both during homeostasis and disease as well as after pharmacological induction. A small number of HSCs constantly leave their niche in the bone marrow, enter circulation by migrating across the endothelium of bone marrow sinusoids, circulate in the body, and return into the niche.<sup>145</sup> The percentage of circulating HSCs can dramatically increase during alarm situations, such as infections or blood loss. In clinical settings (*e.g.*, transplantation), mobilization of HSCs into the blood circulation can be induced in the donor through pharmacological treatment with cytokines such as G-CSF.<sup>146</sup>

Although some progress has been made in studying mechanotransduction, a detailed picture is lacking of how



**Fig. 3** Mechanosensing pathways in HSCs. The drawing schematically depicts the mechanosensory units and molecules known to play a role in HSCs. The integrin adhesion and signalling complexes that act in response to externally applied and internally generated forces can be regarded as one mechanosensing unit that acts as a whole. Cellular signalling and structural factors like PI3K, YAP and TAZ, non-muscle myosin II as well as lamin-A and -B are involved in or regulated by mechanotransduction. Pathways that remain to be elucidated are indicated by dashed arrows, the question mark indicates that crosstalk between the Hippo and mechanotransduction signalling pathways has been proposed.





mechanical signals are transduced into the nucleus of HSCs (Fig. 3). Along the series of events that comprise the mechano-transduction signalling pathway in HSCs less and less is known about the components and events involved in passing along the signal the further downstream you look. Nevertheless, lessons learned from other cell types such as mesenchymal stem cells might provide useful information.

In addition to mechanical signals that create direct effects in HSCs (like matrix stiffness), indirect effects through neighboring niche cells that influence HSC behaviour and that are mechanosensitive (*e.g.*, mesenchymal stem cells, osteoblasts, osteocytes and endothelial cells<sup>117,147–150</sup>) also seem likely. For example, stiffer matrices can induce differentiation of mesenchymal stem cells into the osteoblast lineage.<sup>117</sup> This might, in turn, influence HSCs, as osteoblasts are known to provide signals to HSCs that are important in HSC maintenance *in vivo* in the niche<sup>8–10</sup> and *in vitro*.<sup>†151</sup> Osteocytes are subject to mechanical load in bone. In response to mechanical signals, they release signals that regulate osteoblast and osteoclast activity.<sup>148</sup> Both osteoblasts and osteoclasts are involved in HSC niche formation in mice.<sup>44</sup> Furthermore, osteocytes themselves are involved in the regulation of HSC mobilization in mice.<sup>46</sup> Endothelial cells stiffen and contract in response to stiffer substrates, which facilitates transmigration of neutrophils through endothelial cell layers.<sup>150</sup> Transmigration through vascular endothelia takes place during mobilization, homing and trafficking of HSCs. It would be interesting to investigate whether endothelial cells in the HSC micro-environment are also mechanosensitive and if the effects observed for neutrophil transmigration also hold true for HSC transmigration.

In conclusion, the direct as well as the indirect effects of matrix stiffness should be considered when trying to manipulate HSC behaviour through biophysical cues.

## Nanopattern

One of the characteristic features of collagen type I fibrils is their banded structure with a periodicity of 67 nm, which can be observed using transmission electron and atomic force microscopy. The first three-dimensional model of collagen fibrils that accounted for the gap and overlap regions of monomers in the fibril that elicit the 67 nm banding was proposed in 1963.<sup>152,153</sup> Since then, it has become evident that the ECM is highly organized and structured from the microscopic down to the nanometer scale. This means that a cell that interacts with the ECM receives – in addition to the chemical information provided by the molecule's amino acid sequence and composition – mechanical as well as (nano)structural and geometrical information. Cells are able to integrate these different signals and to respond specifically to nanostructural features of their environment.<sup>47,67</sup> In order to investigate the impact of nanometer scale distances between specific adhesive ligands on cellular behaviour, nanopatterned substrates were developed that allow the positioning of ligands with precision in the

nanometer range. Using block-copolymer micellar nanolithography, gold nanoparticles with a typical diameter of less than 10 nm were deposited on glass or silicon substrates in a quasi-hexagonal order. The lateral distance between individual gold nanoparticles can be adjusted between ~20 and 300 nm, simply by altering the production parameters.<sup>154–156</sup> Specific biofunctionalization of the gold nanoparticles is enforced by either passivating the area in between the gold nanoparticles by covalent attachment of PEG or by transferring the nanoparticle arrays to PEG hydrogels. Next, the gold nanoparticles are specifically functionalized *via* thiol chemistry with a biomolecule of choice.<sup>157,158</sup>

RGD is a minimal integrin recognition motif that can be found in ECM molecules such as fibronectin. The RGD sequence in fibronectin is located in a loop structure.<sup>159</sup> On artificial surfaces, a cyclic RGD peptide (cRGD) is usually employed, as it best mimics the amino acid sequence and the secondary structure of the naturally occurring integrin recognition motif. In our experiments with cRGD functionalized gold nanoparticle arrays, we could show that fully differentiated tissue cells such as fibroblasts and osteoblasts are highly sensitive to the nanostructured presentation of their ligands. Their adhesive and migratory behaviour as well as the assembly of focal adhesions are influenced by the ligand presentation on the nanometer scale.<sup>160–165</sup> At lateral distances of 58 nm (and less) between the cRGD functionalized nanoparticles the cells adhered, spread out and developed mature focal adhesions. At distances of 73 nm and more, adhesion, spreading and focal adhesion development were impaired.<sup>160–162,164</sup> The cells were also more motile on these substrates. Using substrates with lateral spacing gradients, it was shown that MC3T3 osteoblasts polarized and migrated towards smaller distances.<sup>163,165</sup>

Similarly, mesenchymal stem cell adhesion and differentiation are also influenced by nanoscale substrate features such as nanotopography and lateral spacing.<sup>166–170</sup> For example, increasing the lateral distance between RGD ligands was shown to lead to diminished cell spreading and lower numbers of mature focal adhesions. At the same time, differentiation into the osteogenic lineage was impaired, while differentiation into adipocytes was increased.<sup>168</sup> On nanotube arrays, small tube diameters promoted adhesion without differentiation, whereas cells on larger nanotubes were strongly elongated and differentiation into osteoblast-like cells was enhanced.<sup>169</sup> On nanoscale gratings, mesenchymal stem cells aligned along the gratings and neuronal markers in these cells were up-regulated compared to neuronal marker expression in cells on flat substrates.<sup>170</sup>

The first report pointing at an influence of nanometer-sized features on HSCs described the enhancing effect of nanofibre scaffolds on the adhesion and proliferation of HSCs.<sup>†56</sup> In the following years it became increasingly clear that, like anchorage-dependent cells, HSCs are influenced by the nanopatterning of their environment. We could show that the lateral distance between several ECM-derived ligands severely influenced the adhesive behaviour of HSCs.<sup>†</sup> Our findings show





that the critical lateral distance between functionalized nanoparticles (*i.e.* a threshold value: at distances smaller than this value HSPCs are able to adhere, whereas at larger distances no adhesion occurs) is dependent on (i) cell type, (ii) the receptor that is targeted in the cell membrane and (iii) the presented ligand.<sup>57</sup> For example, for the small ligand cRGD that is recognized by the integrin  $\alpha 5 \beta 1$  receptor of HSPCs the critical distance is  $\sim 40$  nm, whereas for a fibronectin protein domain (that also contains the RGD sequence and targets the same receptor in the membrane) the value lies between 85 nm and 110 nm. Explanations for the observed discrepancy between HSC adhesion to cRGD and the fibronectin domain are two-fold: (i) the fibronectin protein domain is larger and more flexible than the small cRGD peptide. This enables it to bridge larger distances (however, this cannot completely account for the substantial difference of  $\sim 60$  nm). (ii) The fibronectin domain carries not only the adhesive RGD sequence but also synergy sequences that stabilize binding to the integrin receptor. The increased binding stability should facilitate cell adhesion at lower ligand concentrations (in other words, binding at greater lateral distances between functionalized nanoparticles is possible).

In addition to influencing HSC† adhesion, nanopatterning also affects other cell functions such as gene expression,<sup>57</sup> lipid raft clustering (which is assumed to be a prerequisite for successful signal transduction *via* transmembrane receptors), and receptor distribution in the cell.<sup>55</sup>

Little is known about the impact of nanotopography – meaning all sorts of nanoscale topological surface features – on HSCs. Despite the attention that the influence of nanotopography on mesenchymal stem cells has gained,<sup>166,167</sup> such studies have not been performed with HSCs, probably because it was assumed that HSCs do not adhere strongly enough to materials surfaces to be able to sense nanotopographical features.<sup>171</sup> However, studies using nanofiber scaffolds have shown that, in addition to biochemical surface functionalization, topographical surface texture influences HSC† adhesion and expansion. HSCs† adhered better and multiplied to higher cell numbers on nanofiber scaffolds than on flat surfaces.<sup>56,172</sup> These findings support the idea that nanotopography, like nanopatterning, can indeed influence HSC behaviour.

## Shear stress

HSCs develop in the aorta-gonad-mesonephros region during embryogenesis. During this process, shear stress elicited by blood flow is an important factor that impacts arising HSC precursors. Blood flow and the resulting shear stress even appear to be a prerequisite for successful HSC development.<sup>58,60</sup> Studies have revealed that the influence of biomechanical forces on hematopoiesis commences during the embryonic stages. They also indicate that nitric oxide is a major mediator of shear stress-induced signalling in HSC precursors in both zebrafish and mice.<sup>58,60</sup>

Adult HSCs in mammals are localized in the bone marrow and only small numbers circulate in the body.<sup>146</sup> As they are circulating in the blood flow of mice, HSCs experience shear stresses at the vessel walls that in some regions exceed  $600 \text{ dyne cm}^{-2}$ . In humans these shear stresses are lower by one order of magnitude.<sup>173</sup> It has been shown in human HSCs† that shear stress impacts HSC adhesion and rolling behaviour on hyaluronan.<sup>174,175</sup> However, most adult HSCs are found in bone marrow, where they might not be subjected to fluid flow directly. Nevertheless, other cells that are implicated in HSC niche functions do experience shear stress and could influence HSCs through paracrine signalling. Endothelial cells and also pericytes *e.g.*, might be influenced by the relatively slow fluid flow in the vasculature that feeds the medullary cavities of bone. Furthermore, the fluid flow in the lacunar-canalicular network in compact bone ( $6\text{--}50 \text{ dyne cm}^{-2}$ ) may, in addition to transporting nutrients and signalling molecules to osteocytes, also lead to their mechanical stimulation.<sup>59</sup>

All in all, the impact of shear stress on adult HSCs is still an open field. Even though fluid flow-induced shear stress may not play a major role inside the *in vivo* niche, it does play a role in the targeted *in vitro/ex vivo* proliferation and differentiation of HSCs.<sup>176,177</sup> Successful HSC proliferation and differentiation *ex vivo* is an important goal of current research in the field of hematopoiesis and would have tremendous impact on possible clinical applications. Any kind of cell expansion on a sufficiently large scale for clinical applications inevitably requires the use of large bioreactors in order to ensure an adequate supply with medium containing essential nutrients and growth factors for a growing cell population. Fluid flow is an intrinsic parameter of such bioreactors. In order to be able to control and predict HSC behaviour in bioreactors, investigations on how fluid flow-induced shear stress influences HSC behaviour, including proliferation and differentiation, are needed.

## 3D versus 2D

*In vivo* HSCs are located in a three-dimensional environment. Therefore, culturing HSCs on flat tissue-culture dishes or in suspension is a highly artificial situation for these cells and is insufficient for realistically simulating the natural *in vivo* situation.<sup>29</sup> One example of what may be missing in 2D cell culture systems is autocrine and paracrine signalling. These signals are important regulating mechanisms during hematopoiesis that act locally on short distances – either by acting back on the signalling cell itself (autocrine) or between adjacent cells/cells in close proximity (paracrine).<sup>178</sup> In standard cell culture systems with relatively large amounts of medium these signals are rapidly diluted upon release and, therefore, cannot act locally. In contrast, in three-dimensional systems the effects of local accumulation (by release) or depletion (by consumption) of diffusible factors can be reproduced.

Similarly, the diffusion of other soluble factors like nutrients or oxygen in the natural ECM leads to gradients that play



an important role in many cellular processes such as migration or homing.<sup>179</sup> These gradients can be mimicked more realistically by diffusion-limited 3D cell culture systems than standard 2D cell cultures.

Differences between the 2D and 3D culture systems include the distribution of soluble and diffusible factors as well as the way the ECM embeds the cells from all sides, rather than just providing one-sided contact. Providing a surrounding matrix leads to changes in the cells' adhesive behaviour, morphology and migration. It has been shown that the induction of certain morphologies is an important determinant towards driving the differentiation of mesenchymal stem cells.<sup>180</sup> This suggests that morphological changes induced by 2D cultures may also influence stem cell function. However, HSCs and their direct progenies (*i.e.* early hematopoietic progenitors) all have a similar round shape. Therefore, it seems unlikely that the concept of morphology that impacts differentiation can be transferred directly from mesenchymal to hematopoietic cells.

Nevertheless, it has become clear that cellular answers to substrate-bound as well as soluble signals differ in 2D and 3D systems. Therefore, an important future task will be to study a wide range of parameters in 3D systems that resemble the natural stem cell niches, including the influence of ECM-molecules, cell-cell interaction (contact and communication) and growth factors as well as biophysical signals (such as spatial distribution of signals on the micro- and nanoscale) and mechanical parameters.

Several different approaches to culture and investigate HSCs *in vitro* using 3D systems have been tested up until now: HSCs<sup>†,§</sup> have been encapsulated in hydrogels made of natural (*e.g.*, collagen, hyaluronic acid) or synthetic (*e.g.*, polyacrylates) monomers;<sup>48,50,52</sup> Other approaches included seeding of HSCs<sup>†</sup> onto scaffolds made of fibres or fibre meshworks,<sup>51</sup> as well as culturing HSCs<sup>†,‡,¶</sup> in macroporous scaffolds that resemble the spongy architecture of trabecular bones<sup>49,181–183</sup> (these host the red, blood-forming bone marrow); Furthermore, microwell-arrays have been used as quasi- or pseudo-3D environments for HSCs<sup>§,¶,¶¶</sup> in order to delineate signals and parameters that are present in the HSC niche.<sup>184–187</sup> All of these studies showed that HSCs respond and act differently in 3D materials than in standard 2D culture, backing the assumption that conventional 2D culture is a highly artificial situation for HSCs, which may even induce 'unnatural' cell behaviour. Using RGD-functionalized macroporous PEG scaffolds we determined that the support of HSCs<sup>†</sup> by feeder cells is more pronounced in 3D than on 2D scaffolds. Furthermore, only when combining 3D scaffolds and feeder cell support did more than 90% of the cells preserve CD34 marker expression throughout culture. Neither stromal support in 2D nor 3D scaffolds alone was able to support HSC marker maintenance to this degree.<sup>49</sup>

<sup>†‡</sup> Human mononuclear cells from umbilical cord blood.

<sup>§§</sup> Murine Lin<sup>−</sup> Sca1<sup>+</sup> cKit<sup>+</sup> CD150<sup>+</sup> cells.

<sup>¶¶</sup> Human CD133<sup>+</sup> cells.

Each of the afore-mentioned approaches for HSC 3D culture has its advantages and disadvantages – and each system can best imitate a different aspect of the natural 3D situation. A combination of the different approaches and techniques might be a promising future approach to mimic the natural niche as realistically as possible. Such artificial niches may be useful as model systems for fundamental research on HSC niches both in health and disease. Furthermore, they could be applied in drug screening systems, minimizing the need for animal testing. Last but not least, they are promising targets in the field of biomaterial-based HSC expansion for clinical use.

## Conclusions

Although after more than 50 years of research studying blood-forming stem cells HSCs are clearly the best studied adult stem cells today, surprisingly little is known about the biophysical regulation of these cells. More and more data suggest that HSCs are sensitive to physical signals. This allows the conclusion that to be able to direct HSC behaviour all types of environmental stimuli that act on the cell – biological, chemical as well as physical – must be taken into account. Guiding the behaviour and functions of HSCs by engineered materials is a difficult but promising future challenge. However, before being able to design such tailor-made materials, fundamental questions on the biophysical regulation of HSCs remain to be answered.

## Acknowledgements

The authors thank Nina Grunze for careful editing of the manuscript. C.L.-T. acknowledges support by the BMBF Nano-MatFutur programme (FKZ: 13N12968), the BioInterfaces programme of the Helmholtz-Association and the 'Käthe und Josef Klinz-Stiftung'. This work is also part of the excellence cluster CellNetworks at the University of Heidelberg. J.P.S. has a Weston Visiting Professorship at the Weizmann Institute of Science. The Max Planck Society is highly acknowledged for its support.

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