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REGENERATIVE MEDICINE

Concise Review: MSC Adhesion Cascade—Insights into Homing and Transendothelial Migration

FRANZISKA NITZSCHE [a,b], CLAUDIA MÜLLER,[a] BARBARA LUKOMSKA,[c] JUKKA JOLKKONEN,[d] ALEXANDER DETEN,[e] JOHANNES BOLTZE [a,e,f]

Key Words. Cell adhesion molecules • Cell migration • Chemokine receptors • Integrins • Mesenchymal stem cells • Stem/progenitor cell • Leukocytes

ABSTRACT

Mesenchymal stem cells (MSCs) are promising candidates for adult cell therapies in regenerative medicine. To fully exert their potential, efficient homing and migration toward lesion sites play an important role. Local transplantation deposits MSC in spatial proximity to the lesion, but often requires invasive procedures. Systemic administration routes are favored, but require the targeted extravasation of the circulating MSC at the site of injury. Transplanted MSC can indeed leave the blood flow and transmigrate through the endothelial barrier, and reach the lesion site. However, the underlying processes are not completely dissolved yet. Recent in vitro and in vivo research identified some key molecules scattered light on the extravasation mechanism. This review provides a detailed overview over the current knowledge of MSC transendothelial migration. We use the leukocyte extravasation process as a role model to build a comprehensive concept of MSC egress mechanisms from the blood stream and identified relevant similarities as well as important differences between the extravasation mechanisms. STEM CELLS *2017;35:1446–1460*

SIGNIFICANCE STATEMENT

Our manuscript provides a concise overview on the current knowledge of the mesenchymal stem cell (MSC) extravasation and homing mechanisms. We highlight relevant similarities and important differences to leukocyte migration, which are important for research and potential downstream clinical implementation of MSC-based therapies.

INTRODUCTION

Mesenchymal stem cells (MSCs) are widely used for experimental therapies of neurodegenerative as well as immune disorders, diseases of the skeletomuscular system, and even tumors [1–4]. Moreover, first clinical applications are underway, attempting to capitalize on the cells' beneficial effects and immunocompatibility [5, 6]. A particularly interesting MSC ability is their homing capability, allowing the cells to navigate to sites of injury and inflammation [7]. MSCs are believed to exert migratory behavior resembling that of leukocytes [8–10] with respect to cytokine responsiveness and the ability for transendothelial migration (TEM, diapedesis). This distinct homing capability enables application of minimally invasive systemic delivery strategies in clinical practice, facilitating a widespread implementation of novel MSC-based treatment protocols. One the other hand, systemic MSC administration might lead to adverse events such as pulmonary [11] or (cerebral) microembolism [12] under certain circumstances. While MSC homing behavior has been well described in numerous scenarios, clarity about definite mechanisms and regulation of MSC migration is still lacking [13–15]. Hence, detailed knowledge on the MSC migration process is needed to avoid potential adverse events in the clinical translation of systemic treatment protocols.

This review summarizes current knowledge on navigation, homing, and TEM mechanisms of MSCs after systemic administration.

MSC HOMING DEFINITION: WHERE DO WE STAND AND WHAT DO WE NEED?

Most attempts to define "MSC homing" resulted in relatively vague, mechanistically oriented and descriptive definitions. Some approaches are simply equating it with migration, ultimately resulting in the delivery of cells to the site of injury [16, 17], and subsequent exertion of local effects [18]. Importantly, these definitions lack clarity about how and where homing starts as

[a]Department of Ischemia Research, Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany; [b]Department of Radiology, McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; [c]NeuroRepair Department, Mossakowski Medical Research Centre, Warsaw, Poland; [d]Department of Neurology, Institute of Clinical Medicine, University of Eastern, Kuopio, Finland; [e]Translational Centre for Regenerative Medicine, Leipzig University, Leipzig, Germany; [f]Department of Translational Medicine and Cell Technology, Fraunhofer Research Institution for Marine Biotechnology and Institute for Medical and Marine Biotechnology, University of Lübeck, Lübeck, Germany

Correspondence: Franziska Nitzsche, M.Sc., Fraunhofer Institute for Cell Therapy and Immunology, Department of Ischemia Research, Perlickstr. 1, D-04103 Leipzig, Germany; or Department of Radiology, McGowan Institute for Regenerative Medicine, University of Pittsburgh, 3025 East Carson Street, Pittsburgh, Pennsylvania 15203, USA. Telephone: 49-341-35536-3150; Fax: 49-341-35536; e-mail: frn5@pitt.edu

Received January 11, 2017; accepted for publication February 23, 2017; first published online in STEM CELLS *EXPRESS* March 18, 2017.

1066-5099/2017/$30.00/0

http://dx.doi.org/10.1002/stem.2614

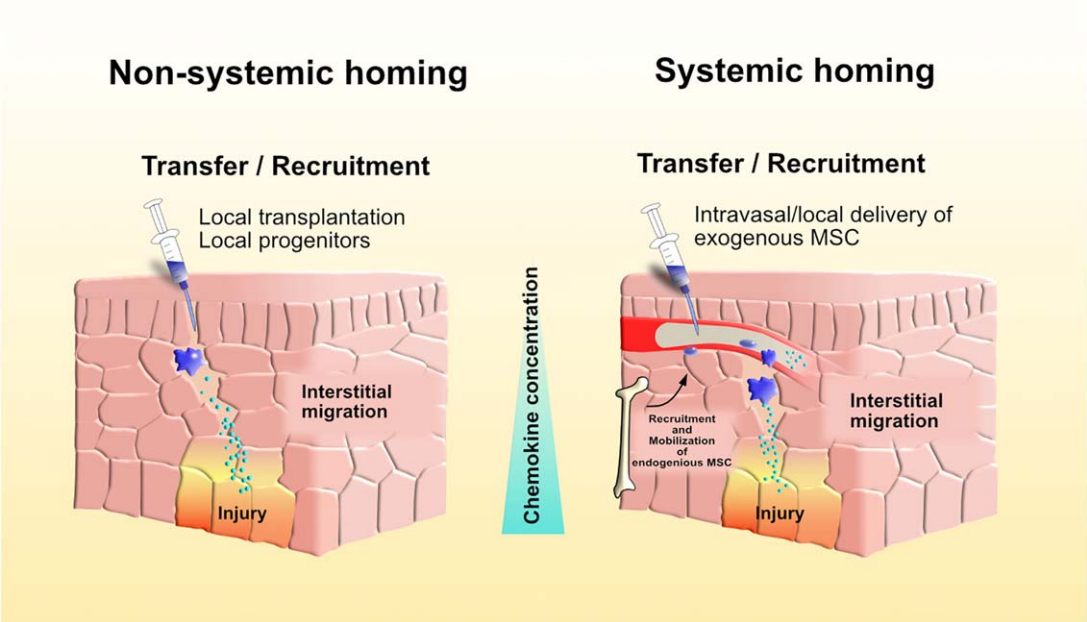

Figure 1. Systemic and nonsystemic homing. Recruitment of local progenitors or transplanted mesenchymal stem cell (MSC) is based on a chemokine gradient released from the injured site. Systemic homing is inevitable after intravasal transplantation and requires active or passive MSC extravasation followed by chemokine guided interstitial migration toward the injury. Occasionally, endogenous MSCs are recruited to the injured tissue via the vascular system. Local transplantation circumvents the journey through the vascular system, so the nonsystemic homing is based on migration through tissue in spatial proximity to the injury. Abbreviation: MSC, mesenchymal stem cell.

well as when it is terminated, or its characteristics [15]. The most comprehensive definition so far was given by Karp et al. who called for a more nonmechanistic definition and determined MSC homing as the active or passive arrest of a MSC within the vasculature followed by transmigration across the endothelium [13].

As therapeutic MSC administration can be conducted either systemically or site-specific, it is necessary to extend the MSC homing definition to provide a global picture of enrolled processes and mechanisms. Homing from local clusters after site-specific transplantation follows chemotactic principles whereas systemic transplantation also involves travel in and egress from the vascular system. Hence, there is a need to distinguish between systemic and nonsystemic homing.

Nonsystemic homing (Fig. 1) requires either recruitment of local MSC or transplantation of exogenous cells close to the target area. Directed migration follows activation and polarization of MSC, during which a front pole is formed. The front pole guides interstitial locomotion by sensing chemokines released from injured or inflamed tissue. Migration is terminated after reaching the target site.

Systemic homing is a multistep process composed of three distinctive phases: (a) direct administration into or cell recruitment and ingress to the circulation, (b) extravasation at the lesion vicinity, and (c) interstitial migration toward the target site (Fig. 1). In the first phase, MSCs are recruited to the circulation from endogenous depots such as the bone marrow, or exogenous MSCs are systemically administered. Of note, egress of endogenous MSC into the circulatory system is an active process involving an additional migration step. In the circulation, MSCs are transferred to the site of injury via the vascular system, a process which is guided by chemokine gradients (see below). The subsequent extravasation phase comprises capture of flowing MSC, rolling along, and adhesion to the endothelium, followed by TEM to reach the perivascular space. Arrest at the endothelium as well as TEM are determined by MSC and endothelial surface proteins. The homing process is finally concluded by the trans-tissue and/or -interstitial MSC migration phase to the lesion site [19], which is also navigated by chemokine gradient.

MSC Extravasation

It is considered that MSCs should exit the circulation to be effective in tissue regeneration and repair after systemic delivery or recruitment from remote sites. Similar to leukocytes, this initially involves formation of contact between the endothelium and the circulating MSC, tethering, and endothelial rolling [20].

Pioneering work to reveal mechanisms behind leukocyte TEM was performed by the Butcher and Springer groups, suggesting a multistep model of leukocyte extravasation [21, 22] (Fig. 2). The conceptional ideas behind these processes have significantly improved and historical definitions of rolling and adhesion were dramatically reshaped with the discovery of adhesion surface molecules and their respective ligands [23, 24]. The model of "combinatory specificity" [23] was established in the context of selectin-mediated rolling, chemokine-triggered activation, and integrin-dependent arrest. Conventional knowledge suggests that MSC TEM is initiated similarly to that of leukocytes and hematopoetic stem cells [23]. Leukocyte homing and arrest can indeed serve as the conceptual role model for MSCs as both cell populations share many similarities. However, striking differences with respect to these processes have also been described (Table 1).

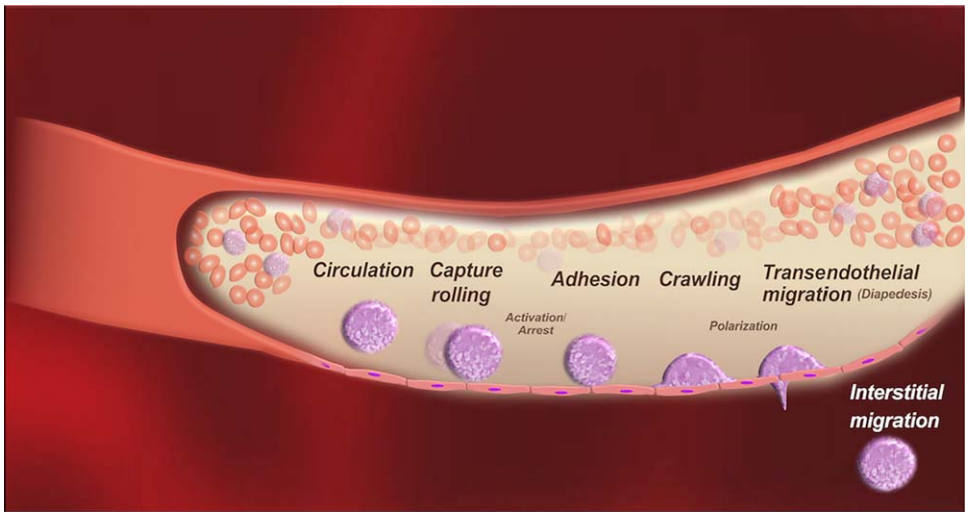

Figure 2. Multistep model of leukocyte and mesenchymal stem cell (MSC) extravasation. Pro-inflammatory mediators initiate a cascade of endothelial and leukocyte/MSC adhesion and motility responses. Initially, the flowing cell is captured through the interaction of surface molecules and a low affinity contact to the vascular wall is established. The cell then slows down during rolling until arrest. Firm attachment and intraluminal crawling are mediated by interaction of adhesion molecules. During crawling, the cell polarizes and scans the endothelium for exit cues. At the right spot, the cell overcomes the endothelial barrier, the endothelial basement membrane, and the pericyte sheath before continuing chemokine guided interstitial migration.

Table 1. Comparison of leukocyte and MSC extravasation

Parameter	Leukocyte	MSC
Intravasation	Active	Active (+ Passive by transplantation)
Extravasation	Active	Active + Passive
Duration	About 20 minutes	60–120 minutes
Routes	Transcellular, paracellular	Transcellular, paracellular, (integration)
Rolling	Mandatory	Possible, but not required
Firm adhesion	Mandatory	Mandatory
Crawling	Formation of invadosomes, protrusion, filo-, and lamellipodia; lateral migration	Formation of pseudo- and filopodia, membrane blebbing; no lateral migration
Crossing the endothelial barrier	Guided by endothelial cells, Rho dependent, PI3K signaling	MSC-endothelial cooperation, formation of transmigratory cups
Crossing the basement membrane	Interplay of several MMPs and TIMPs	MMP-2/TIMP-3 interplay, Urokinase-type plasminogen activator

Abbreviations: MMP, Matrix metalloproteases; MSC, mesenchymal stem cell; PI3K, Phosphoinositide 3-kinase; TIMP, Tissue inhibitor of metalloproteases.

As known from electron microscopy studies, leukocyte TEM is an active process, requiring structural modification of the transmigrating cell. In contrast, MSCs can also become passively arrested within capillaries, potentially representing an alternative to active adhesion. MSCs, being significantly larger than leukocytes, become entrapped in small-diameter vessels during passive arrest [13, 25]. It is unclear whether or not passive MSC arrest initiates TEM, and the fate of passively arrested MSCs is still undetermined. The only difference between active and passive arrest verified so far is the alteration of the local blood flow. During active stop, the cell flattens swiftly, preventing local blood flow disturbances. In contrast to selectin- and integrin-mediated capture, cell flattening is not observed during passive arrest, what can lead to local blood flow impairments. A particular risk is "nonspecific" passive arrest in pulmonary, hepatic, splenic, and cerebral capillaries, which can cause microembolism [26–29]. Understanding of passive arrest mechanisms is therefore essential for MSC therapy development and, potentially, optimization with respect to safety and efficacy.

Another remarkable difference between leukocytes and MSC was found in TEM duration. While leukocytes can complete diapedesis within 20 minutes, MSC diapedesis requires the three- to sixfold time [30, 31]. Although there seems to be a difference between duration of the migration itself, a long MSC presence in the circulation will not lead to an enhanced migration [30].

Previous studies to reveal the mechanisms of TEM were mainly based on MSCs from exogenous sources and involved in vitro cultivation and expansion [31–33]. Endogenous MSCs reside within the bone marrow or perivascular niche and migrate toward (lesion) sites after mobilization [34, 35]. Detection and studying of endogenous MSC migration is, however, challenging and resulted in controversial results, as their characteristics and immunophenotype might differ from culture-expanded MSCs [36]. Eggenhofer et al. presented two potential recruitment routes for endogenous MSCs. The first hypothesis describes the attraction and migration of MSCs from the bone marrow through the circulation. This process is guided by cytokines, chemokines, and growth factors such as

stem cell factor or stromal-derived factor 1 (SDF-1), all of which are released from the injury site. The second hypothesis assumes that local MSCs are recruited from within the injured tissue and migrate through the stroma or reach their destination via microcapillaries [36]. In contrast to migration of exogenous MSCs, however, reports on (transendothelial) migration mechanism of endogenous MSCs are scarce, not providing enough information to draw a comprehensive picture so far. One the other hand, there is no study indicating fundamental mechanistic differences between migration of endogenous and exogenous MSC toward a lesion site.

Molecular Signals Controlling MSC Extravasation

A multitude of molecular signals and messengers including growth factors and chemokines [37] plays an essential role for the MSC homing process. Most of these trafficking signals also control the well-orchestrated sequential process of leukocyte extravasation. They were first identified to control neutrophil diapedesis before being later found to navigate lymphocytes extravasation in general [22]. This inspired the concept of a ubiquitous cell migration control system. In fact, MSC extravasation is governed by the same surface receptors and migratory cues, although MSC extravasation displays some important differences to leukocyte egress as shown above (Table 1). The most important molecules and events for leukocyte and MSC homing and extravasation are given in Table 2.

Priming and Requirements for Extravasation

Endothelial cells contribute to extravasation by eliciting crucial signal responses upon contact with the extravasating cell [69]. This requires endothelial activation being characterized by an upregulation of receptors on the endothelium in response to mediators released by inflamed tissue. Those mediators are soluble factors such as tumor necrosis factor alpha (TNFα) or histamine, with the latter inducing activation of P-selectin on the endoluminal surface within a few minutes [49]. E-selectin expression is increased by TNFα and interleukin (IL)-1β and peaks after 3–4 hours [49]. Inflammatory mediators also stimulate vascular cell adhesion molecule 1 (VCAM-1) and Intercellular adhesion molcule 1 (ICAM-1) expression [31, 70]. The upregulation "primes" the endothelium for transmigration by activation of intracellular signaling pathways.

On the side of the extravasating cells, the expression of ligands is relevant for TEM and includes the upregulation of CD44 (HCAM) and CD49d (Very late antigen 4; VLA-4) [26]. Adhesion receptors permit the circulating cell to scan "the endothelium." After the matching, endothelial "signal expression pattern" was found [71], and receptor activation leads to firm adhesion.

Breakdown of the basal membranes and extracellular matrices as well as local chemokines favor locomotion and tissue reconstitution. The migrating cell literally "blazes" its trail through the surrounding tissue by using proteases being produced after cytokine or chemokine stimulation.

Taken together, a set of preconditions including expression, upregulation, and activation of adhesion molecules have to be fulfilled for extravasation of circulating cells. All relevant processes are described in more detail in the following paragraphs.

Cell Capture and Rolling

Selectin-Mediated Rolling. Selectins are evolutionary highly preserved cell surface receptors, which are expressed in the vascular system. They are composed of a single transmembrane chain and a unique N-terminal extracellular domain. The N-terminus contains a Ca^{2+}-dependent lectin domain, an epidermal growth factor (EGF)-like domain, and short consensus repeat units, which control ligand specificity, receptor stabilization and oligomerization [72]. Interaction of P-, L-, and E-selectins with P-selectin glyocoprotein ligand 1 (PSGL-1) mediates tethering and rolling of leukocytes (Fig. 3). Although PSGL-1 is expressed on all leukocytes, it is only functional when it is glycosylated correctly [23, 73]. The establishment of low-affinity contacts followed by perpetual formation and breakup of bonds between leukocytes and endothelium contributes to the reduction in the velocity of passing leukocytes [74]. Formation of selectin bonds is dependent from shear stress, what can be related to a "catch-bond" phenomenon of selectins [75]. Furthermore, it is known that selectin-mediated leukocyte rolling and deceleration is supported by formation of slings (= cell autonomous adhesive substrates) [76, 77] and microvilli flattening [47].

Contrasting leukocytes, selectin receptor expression is not observed on MSCs [26, 78]. While the coordinated rolling behavior of leukocytes is a prerequisite for adhesion, MSCs tethering and rolling cannot rely on selectin ligands. Selectin-mediated rolling may not be decisive for establishing firm adhesion as long as MSCs are slowed down or become passively arrested by other means, potentially including passive arrest. Most interestingly, MSC rolling depends on P-Selectin expression on activated endothelium in vitro and in vivo [9]. Although neither P-selectin glycoprotein ligand 1 (PSGL-1) nor CD24 expression was found on MSCs; blocking experiments revealed that P-selectin controls rolling. This strongly suggests the existence and relevance of other ligands (Fig. 3). Indeed, MSCs have been shown to express glycoproteins [79] and galectin-1 [80], which may represent alternative P-selectin ligands. Furthermore, interaction of MSCs with P-selectin might be mediated by a bridging mechanism involving platelets. A recent study showed that platelet depletion in a murine model of dermal inflammation decreased MSC trafficking [27]. Spatial proximity or direct contact of MSCs to platelets or neutrophil-platelet clusters may arbitrate adhesion to endothelium. Dependence of MSC rolling from shear stress was also demonstrated, suggesting a catch-bond mechanism.

Integrin-Mediated Rolling. Integrins are heterodimeric transmembrane receptors consisting of an α- and β-subunit. They participate in leukocyte rolling and mediate firm adhesion. In addition, integrin heterodimers are able to induce selectin-independent rolling and strengthen cell binding to the endoluminal layer [81]. Three different conformations result in low, intermediate, or high activity of integrins. This is crucial for ligand binding and affinity [82, 83]. Next, a distinct change in the integrin distribution on the cell (valency) induces cytoskeleton adaptation and formation of signalosomes [23, 84], and in turn influencing binding strength of captured cells to the endothelium. Finally, the interplay between VLA-4 ($\alpha4\beta1$) and VCAM-1 as well as the interaction of Lymphocyte function-associated antigen 1 (LFA-1) or Lymphocyte Peyer patch

Table 2. Molecules involved in leukocyte and MSC extravasation

Molecule class	Molecule name	Expressed on			Ligand	Function	References
		Leukocytes	MSC	Endothelium			
Chemokine receptors (C–X–C chemokine receptor motif)	CXCR-1	Neutrophils, Monocytes	?	n.a.	IL-8 (CXCL-8)	Recruitment, attraction, activation	[37, 38]
	CXCR-2	+ Neutrophils, Monocytes	?	+	IL-8 (CXCL-8)	Recruitment, attraction, activation	[37, 38]
	CXCR-4	++ T-cells, Neutrophils, Monocytes	± (Varying degrees)	n.a.	SDF-1	Recruitment, attraction, activation	[36, 37, 39, 40]
Chemokine receptors (C–C chemokine receptor motif)	CCR-1	+ Monocyte, T-cells, Eosinophils, Basophils	± (<25% [38], Varying degrees)	n.a.	MIP-1, RANTES, MCP-3	Attractor; arrest	[37, 41]
	CCR-2	+ (Monocyte), T-cells	+	n.a.	MCP-1, MCP-2, MCP-3, MCP-4	Attractor; adhesion; transmigration	[37, 41–43]
	CCR-3, CCR-4	++ T-cells, Platelets, Basophils, Macrophages	+, Varying degrees	n.a.	MIP-1, MCP-3, (RANTES)	Attractor; spreading; arrest	[37, 41, 42, 44]
	CCR-5	+ Monocyte, T-cells	+	n.a.	MIP-1, RANTES	Arrest, transmigration	[37, 45]
	CCR-7	+ T-cells	± (<25% [38], Varying degrees)	n.a.	MCP-3, CCL-21	Attractor	[37, 41]
Selectines	E-selectin	–	–	++ (inflammatory site)	ESL-1, PSGL-1, CD44	Tethering; rolling	[46]
	P-selectin	++ Platelets	–	++ (Weibel Palade bodies)	PSGL-1	Tethering; rolling; arrest	[46]
	L-selectin	+	–	+	PSGL-1, CD34, MadCAM-1	Tethering; rolling; signaling	[24, 46]
Integrins	LFA-1	++ T-/B-cells, Monocytes, Neutrophils	–	–	ICAM-1, ICAM-2, ICAM-3	Arrest	[22, 24],[47]
	Mac-1	Monocytes, Neutrophiles	–	–	ICAM-1	Adhesion, transmigration	[22, 47]
	VLA-4	++ Monocytes, Eosinophils, T-/B-cells	±	–	VCAM-1, MadCAM-1	Rolling, arrest, adhesion, transmigration	[22, 24, 47]
	LPAM-1	+ T-/B-cells	–	–	MadCAM-1	Adhesion (ECM)	[22]
Immunoglobulin superfamily	ICAM-1, ICAM-2	+	–	++	LFA-1	Transmigration	[48, 49]
	JAM-A, JAM-B	+	–	+	JAM-A, JAM-B	Transmigration	[48]
	VCAM-1	–	–	++	VLA-4	Arrest, adhesion, transmigration	[48, 49]
	MadCAM-1	–	–	++	LPAM-1, L-selectin, VLA-4	Adhesion	[50]
	PECAM-1	+, ++ (Monocytes)	–	++	PECAM-1	Transmigration	[24, 48]
Proteases	MMP-1	++	+	n.a.	Cleavage of ECM components	Interstitial migration	[51, 52]
	MMP-2	++	++	n.a.	Cleavage of ECM components	Interstitial migration	[10, 51–53]
	MMP-9	++	–	n.a.	Cleavage of ECM components	Interstitial migration	[10, 32, 51]
	TIMP-1, TIMP-2	++	+	n.a.	Inhibition of MMPs	Interstitial migration	[51, 53]
	TIMP-3	++	+	n.a.	Inhibition of MMPs	Interstitial migration	[51]
	TIMP-4	++	–	n.a.	Inhibition of MMPs	Interstitial migration	[51, 53]
	TCF/LEF family	++	+	+	–		[54–56]

Table 2. *Continued*

Molecule class	Molecule name	Expressed on: Leukocytes	Expressed on: MSC	Expressed on: Endothelium	Ligand	Function	References
Transcription factors (TF)						(Negative) regulation of migration	
	KLF-2	+ T-cells	?	+	–	Regulation of trafficking	[57, 58]
	HIFs	++ T-cells	+	+	–	(Negative) regulation of migration, upregulation of chemokine receptors	[59–62]
Signaling molecules	FAK	+	+	+	Tyrosin kinase in focal adhesions	Signal transduction, chemotaxis, formation of lammelipodia, fiber polymerzation	[63],
	β-catenin	+	+	+	Wnt-signaling molecule	Coordination of cell adhesion, interaction with TF, transcription activation	[54, 55, 63]
	Rap-GTPases	+	+	+	Signaling proteins	Downstream T/B-cell reptor signaling, initiates adhesion	[63]
	Rho-GTPases	+	+	+	Signaling proteins	Cell polarization, formation of protrusions	[64, 65]
	MAPK	+	+	+	Serin/threonin kinase, phosphorylation of effectors	Mediates chemotaxis, actin polymerization, cytoskeletal changes, directing cell responses	[16, 63, 66]
	Src-Kinase	+	+	+	Kinase, phosphorylates FAK	LBRC-recycling, actin regulation	[63, 67]
	WASP	+	?	+	Downstream effector of Rho-GTPases	Initiates actin polymerization, regulates actin	[63, 68]

Abbreviations: CCR, C-C chemokine receptor; ECM, extracellular matrix; ESL-1, E-selectine ligand 1; FAK, focal adhesion kinase; HIF, Hypoxia inducible factor; ICAM, Intercellular adhesion molecule; IL, interleukin; JAM, junctional adhesion molecule; KLF, Krueppel-like factor; LBRC, lateral border recycling compartment; LEF, Lymphoid enhancer binding factor; LPAM, Lymphocyte Peyer patch adhesion molecule; MCP-3, Monocyte chemotactic protein; MIP-1, Macrophage inflammatory protein 1; MMP, Matrix metalloprotease; MSC, mesenchymal stem cell; n.a., not applicable; RANTES, Regulated on Activation, Normal T Cell Expressed and Secreted; SDF-1, stromal-derived factor 1; TCF, T-cell factor; VCAM, Vascular cell adhesion molecule 1; VLA-4, Very late antigen 4; PECAM, Platelet endothelial cell adhesion molecule; PSGL-1, P-selectin glycoprotein ligand 1; ++, high expression; +, moderate expression; ±, partial expression or varying expression level; –, no expression; ?, not known.

adhesion molecule (LPAM) ($\alpha4\beta7$) with MadCam-1, VCAM-1, or ICAM-1 induces rolling behavior of leukocytes [23]. β2-Integrins are crucial, as they reduce leukocyte velocity following selectin-mediated capture [23, 84]. Binding of L- or E-selectin triggers the initial change in integrin affinity from low to intermediate state and allows transient binding of ICAM-1.

MSCs express a broad spectrum of integrins including β1, β2, α1, α2, α3, α4, α5, α6, and αV [85, 86]. Particularly, expression of α4 subunit varies under different isolation and cultivation techniques as well as between individual donors and, importantly, species. This resulted in different observations regarding MSC adhesion behavior [9, 30, 85]. Noncovalent assembling of α4 and β1 is necessary to form VLA-4, an important mediator of endothelial rolling and arresting at sites of inflammation. A few studies pointed out the importance of VLA-4 for MSC adhesion on inflamed endothelium, but it remains open what role naturally expressed integrins play in MSC rolling. Nevertheless, induced ectopic overexpression of the α4 subunit has been found to enhance bone homing and engraftment of transplanted MSCs in mice [87]. As the β1 subunit is abundantly expressed by MSCs, the subsequent assembling of both subunits resulted in increased expression of functional VLA-4 on the cells [87]. Interaction of β1 subunit with VCAM-1 and extracellular matrix (ECM) components is of particular importance for rolling and firm adhesion of MSC. Hence, blocking of β1 substantially decreases homing capabilities [88].

Cell Activation and Firm Adhesion to the Endothelium

Chemokine receptor interaction enables chemo- or haptotaxis of mobile cells. Once bound to their ligand, chemoattractant receptors direct migration, promote integrin adhesiveness, and stimulate degranulation as well as rearrangement of the cytoskeleton [22]. After initial endothelial activation, chemokines can be locally expressed at the vascular endothelial surface or in the ECM where they are bound to glycosaminoglycanes [89]. During slow rolling, the leukocyte is exposed to immobilized chemokines at the apical (endoluminal) site of endothelial cells [20]. Binding of the chemokines to leukocytic G protein-coupled receptors (GPCRs) induces downstream signaling, resulting in straightening of inactive integrin receptors and exposure of a binding pocket [82] (Fig. 3). Monocyte GPCRs can cause Phospholipase C (PLC) activation, resulting in an increase in intracellular Ca^{2+}-levels and triggering an enhancement of integrin affinity. Ca^{2+}-ions activate guanine-exchange factors which in turn activate small GTPases such as Rap1 or Rho a [23]. One of the targets from this downstream signaling is talin, an intracellular adapter protein that moves to the cell membrane and interacts with the β-subunit of the integrin receptor [81]. Distance widening between cytoplasmic tails of both subunits leads to straightened position of the integrin receptor, and thus, exposure of a binding pocket. The resulting affinity increase as well as further integrin recruitment is particularly important for establishing strong and shear-resistant leukocyte adhesion to the endothelial cells [23, 47, 90]. In addition, actin polymerization is enhanced after leukocyte activation, and thus, promotes formation of lamellopodia necessary for probing the endoluminal surface [91].

Active, surface molecule-mediated MSCs adhesion to the endothelium is discussed as a core mechanism of the homing process [13]. Expression of numerous chemokine receptors including C-C chemokine receptors (CCR)2, CCR4, CCR7, CCR10, CXCR5, CXCR6, and CXCR4 was already shown for MSCs [89, 92, 93]. It is still unclear how these chemokine receptors contribute to extravasation. Although expressed, CXCR4 for instance does not contribute to TEM in MSCs [86]. In contrast, other researchers presented evidence that CXCR4 plays a major role for MSC homing [39, 93–96]. It is not unlikely that those conflicting results simply derive from different handling, isolation, and cultivation conditions. In vitro assays and screenings have identified the cytokine receptor CCR2 as being necessary for organ-specific homing [97, 98].

It was reported that enforced hematopoietic cell E- and L-selectin ligand (HCELL) expression on human MSCs leads to robust osteotropism [99]. Expression of HCELL induces binding to endothelial E-selectin. Interaction of HCELL with selectin also affects adhesion of MSC to VCAM-1 and triggers activation of G-protein signaling while bypassing chemokine receptor signaling [100]. Blocking experiments showed that firm adhesion is established by VLA-4/VCAM-1 interaction which in turn is mediated by Rac1/Rap1 GTPase signaling [99]. So far, the full range of downstream mediators involved in signaling for MSC TEM has not been elucidated, with only a few signaling pathways being identified so far (Fig. 3). Phosphokinase C (PKC), for example, is upregulated in an IL-8 dependent manner in human MSCs [101] and Phosphoinositide 3-kinase (PI3K)/Act plays a corresponding role [30]. As integrins lack enzymatic activity, an intracellular adapter is required to transduce signals from outside or from the inside [102]. One of those adapters is the integrin-linked kinase (ILK). It is conceivable that ILK also plays a role for MSC TEM and signaling. Song et al. have shown that overexpression of ILK in MSC leads to their increased adhesion to the infarcted myocardium and to improved cell survival after hypoxia [103].

Endothelial Scanning, Crawling, and Cell Polarization

Leukocytes search for exit cues after establishing a firm endothelial adhesion [91]. To this end, the leukocyte "scans" the endothelium along chemotactic gradients and moves along the inner vessel walls (=crawling). Endothelial crawling requires integration of inside-out integrin activation upon chemokine binding, outside-in integrin signaling, and chemokine receptor-induced cytoskeletal remodeling [104].

Lateral movement is accompanied by formation of protrusions, lamellipodia, filopodia, and invadosomes for motility and pathfinding and is observed as soon as leukocytes are starting to polarize [31, 32] (Fig. 4). Signaling via src family kinases and activation of syk are crucial for cellular polarization and cell shape changes [105]. Chemokine-GPCR downstream signaling triggers reorganization of the leukocyte actin cytoskeleton, generating a protrusive front pole and a contractile uropod. Furthermore, Rho/ROCK signaling is linked to formation of protrusions and lamellipodia [65] in response to chemokine signals [24]. Crawling behavior was observed with or against the blood stream as well as perpendicular to it [47]. It is assumed that crawling allows the leukocyte to sense the best location for TEM but is not essential to the process itself because inhibition of crawling only prolongs, but not prevents TEM [106].

MSCs also require polarization before TEM. The intracellular adapter molecule FROUNT, linked to CCR2, is necessary to polarize MSCs, which is followed by CCR2 clustering and leads to cytoskeletal reorganization [97]. MSCs show little lateral

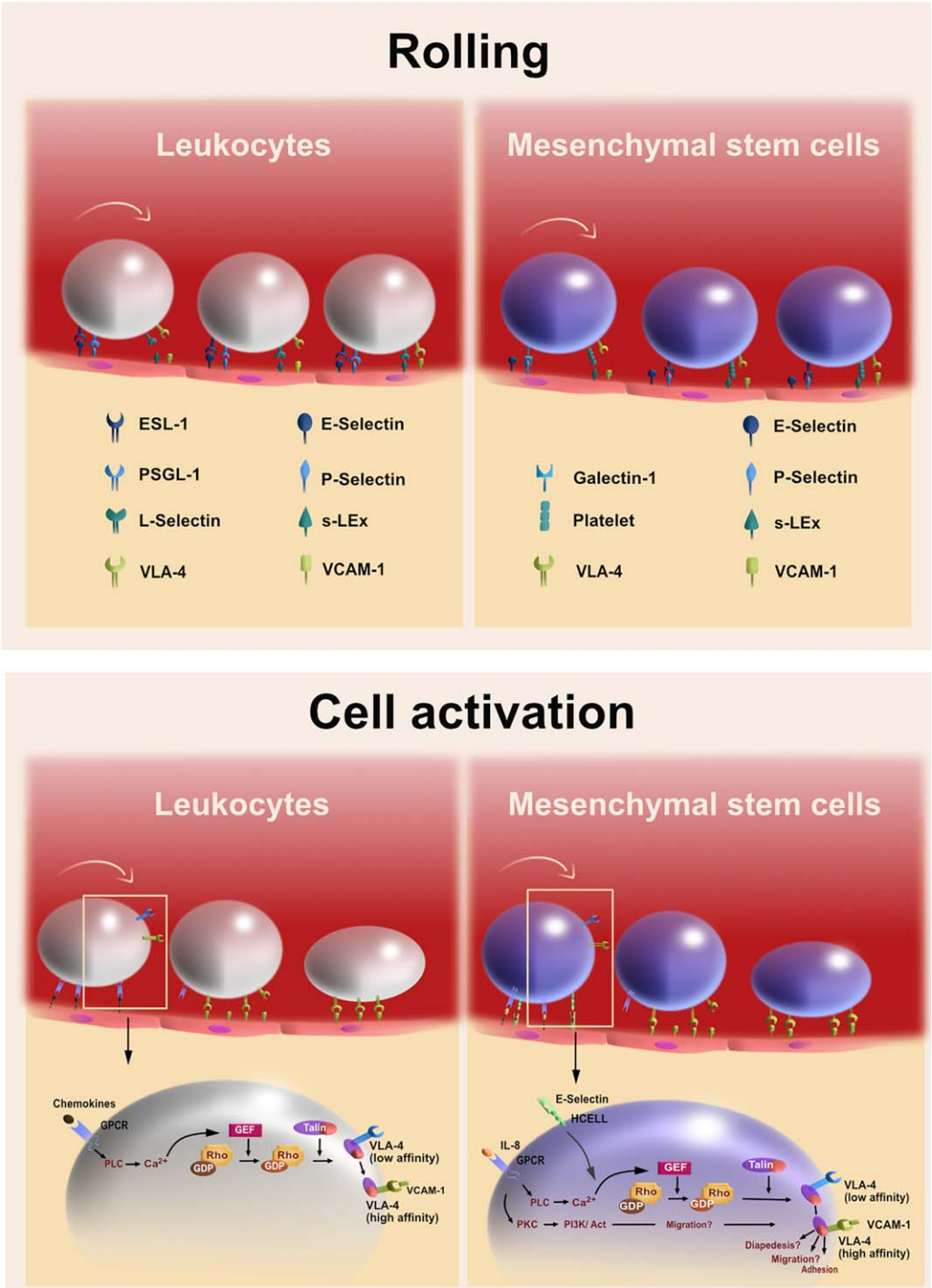

Figure 3. Rolling, cell activation, and firm adhesion of leukocytes and mesenchymal stem cell (MSC). Rolling along the inner vascular wall is mediated by selectins and integrins. Transitory bonds are established and dissolved between selectin receptors and their respective ligands. Tethering activates the integrin receptors. E-selectin and LFA1 play an important role in leukocyte rolling. MSC rolling is obligatory and is facilitated by galectin1 or platelet bridging that act as alternatives for selectin-mediated adhesion. Cell activation and firm adhesion to activated endothelium: A chemokine binds to its cognate G protein-coupled receptor. Small GTPases get activated and target the cytoplasmic domain of the VLA-4 receptor via the adaptor protein talin. This interaction causes an allosteric switch exposing the extracellular binding site. The erected form of the integrin receptor further facilitates receptor clustering, lateral movement, and triggers migration, adhesion, or diapedesis. While the contributing molecules and signaling pathways are relatively well investigated, their role in MSC activation is primarily derived from the leukocyte model. Abbreviations: ESL-1, E-selectin ligand 1; GEF, guanine-exchange factor; GPCR, G protein-coupled receptor; HCELL, hematopoietic cell E- and L-selectin ligand; IL-8, interleukin-8; PLC, Phospholipase C; PSGL-1, P-selectin glycoprotein ligand 1; s-LEx, sialyl Lewis x; VCAM, Vascular cell adhesion molecule 1; VLA-4, Very late antigen 4.

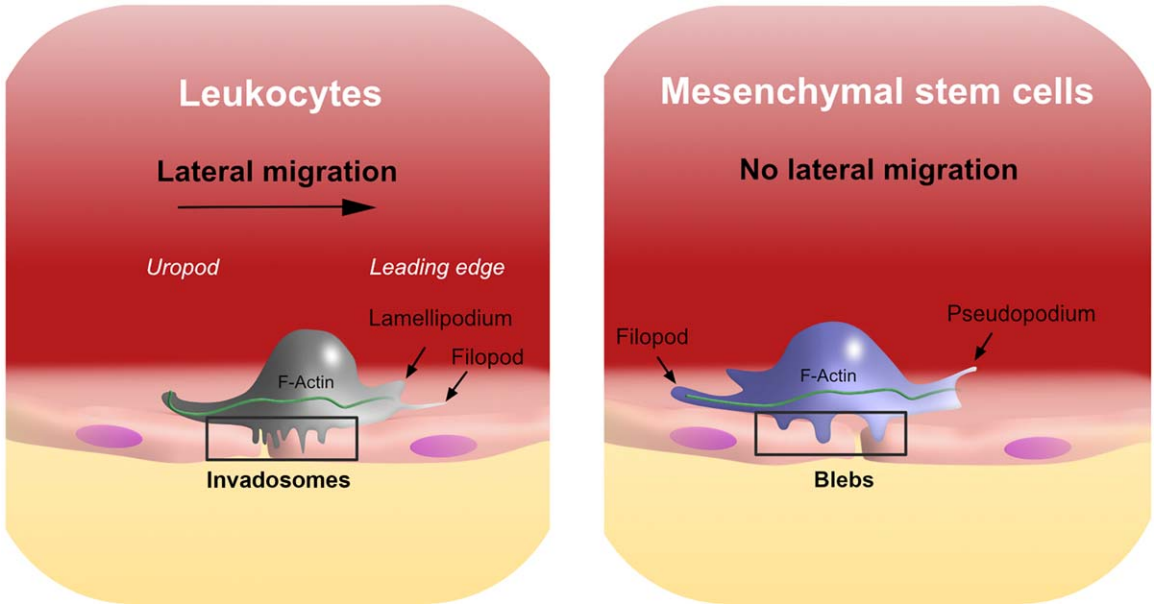

Figure 4. Leukocyte crawling versus mesenchymal stem cell (MSC) crawling. During crawling onto the endothelial layer, the leukocyte moves into various directions to find the optimal spot for transendothelial migration. The leukocyte polarizes and forms cytoplasmic extensions (filopodes and lamellipodes). Additional leukocytic membrane protrusions (invadosomes) are advancing into the endothelial cell body and are mediated by ICAM-1. MSC crawling does not occur in an extensive manner and lateral movement is reduced. The MSC advances against the endothelial barrier by the formation of blebs.

migration on the endothelium during polarization, which is in contrast to leukocytes (Fig. 4). However, MSCs are able to form filopodia after stimulation with CXCL-9 and crawl on endothelial cells. This is followed by formation of pseudopodia and spreading in vitro [26]. In distinction from leukocytes TEM, MSC TEM does not involve invadosomes or lamellipodia. Instead, MSCs share a number of interesting characteristics with the extravasation of germ cells or metastatic tumor cells including non-apoptotic blebbing [31, 107]. MSCs are supposed to form bleb-like protusions (cup-like structures) on the cell surface, particularly at sites of close contact with the endothelium [31].

Crossing the Endothelial Barrier

Overcoming the Endothelial Monolayer. Any transmigrating cell has to overcome three barriers: endothelial cells, the basement membrane, and the pericyte sheat [23]. The guidance of the leukocyte through endothelial junction (or pores) requires a strict coordination of the interaction between endothelial monolayer and the transmigrating leukocyte [47]. When the leukocyte has found an optimal spot for TEM, integrin binding to ICAM-1 or VCAM-1 activates signaling pathways that lead to high receptor trafficking in the endothelial cell (Fig. 5). Furthermore, ICAM-1 and VCAM-1 clustering facilitates the formation of transmigratory cups, which are finger-like protrusion surrounding the adherent leukocyte and providing directional guidance [90, 91]. The inter-endothelial cell–cell connections formed by adherens (Vascular endothelial cadherin, VE-cadherin), gap (connexins), and tight junctions (occludin, claudin, and junctional adhesion molecules) are disassembled upon activation and clustering of ICAM-1 or VCAM-1. This eases the leukocyte passage [24, 91]. Importantly, endothelial connections are subsequently recycled in the so-called lateral border recycling compartment providing new membranous components surrounding the passing leukocyte. The leukocyte itself has to relocate integrin adhesion molecules when passing the endothelium, what comprises inactivation of GTPases, antagonizing Rho/Rap activities, as well as regulation by PI3K signaling and PKC [47]. In particular, PI3K signaling is associated with disruption of endothelial tight junctions [108].

Also MSCs and endothelium actively cooperate to enable guidance by transmigratory cups, a form of endothelial protrusions [31]. It can be assumed that MSC-endothelial cooperation may induce similar endothelial signaling and reorganization as seen after leukocyte adhesion. However, in vitro life cell imaging studies on transmigrating MSCs suggested an important role of blebs that might mediate intercellular forces against endothelium [31, 109] (Fig. 5). Moreover, bone marrow-derived MSC TEM was shown to depend from PI3K and ROCK in knock down experiments [110]. In accordance to leukocytes, Rho was identified as a regulator of cytoskeletal activation and a modulator of MSC transmigration. Rho inhibition led to resolution of actin stress fibers and increased the amount of cytoplasmatic protrusions. Rho inhibition also enhanced chemotactic migration toward PDGF, HG, LPA, and S1P [111].

Overcoming the Basement Barrier and the Pericyte Sheat. Finally, migrating leukocytes enter the subendothelial space via a lamellopodial leading edge (Fig. 5). This movement is guided by platelet endothelial cell adhesion molecule (PECAM-1, CD31), CD99, and junctional adhesions. The endothelial basal membrane consists of laminins and collagen type IV. Proteolytic cleavage by matrix-metalloproteinases or elastases facilitates leukocytic passing of this barrier. Beside degradation mechanisms, existence of leukocyte-permissive regions is discussed aligning with gaps between adjacent pericytes. Those gates show low matrix protein deposition and are enlarging in response to different inflammatory stimuli [112]. The leukocyte

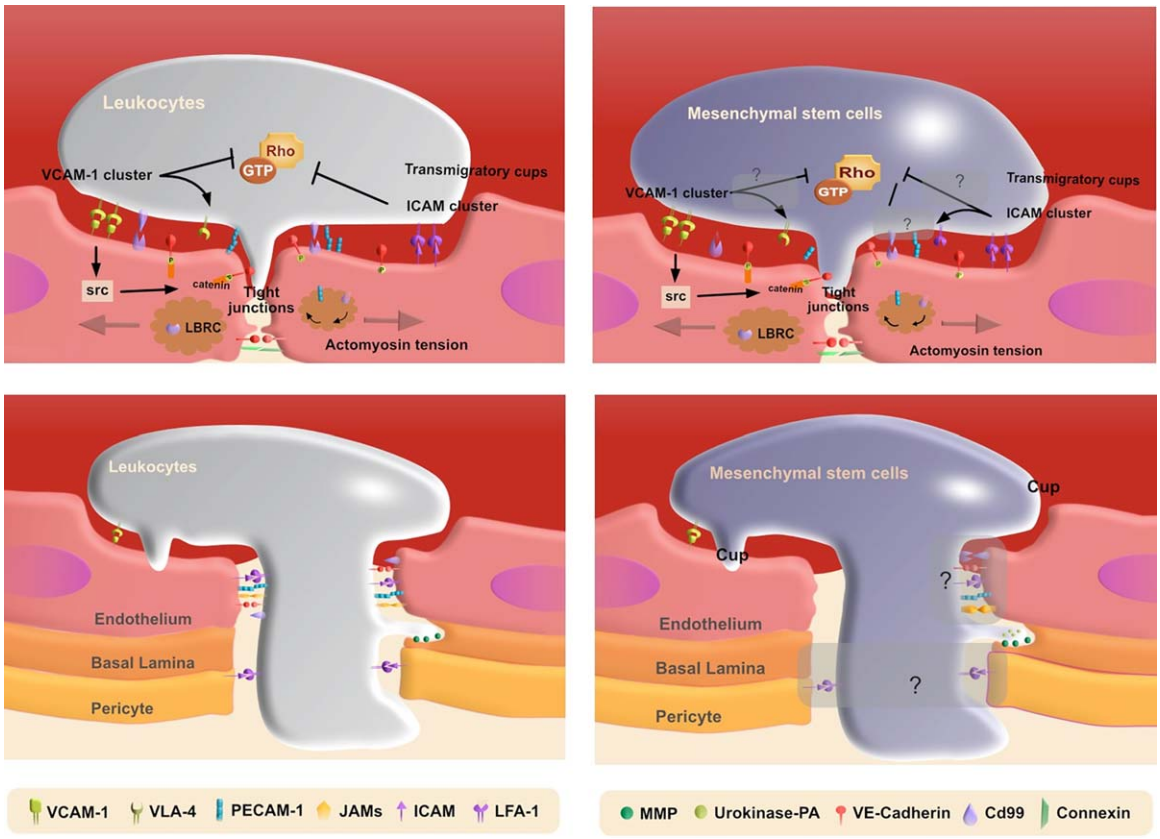

Figure 5. Leukocyte and mesenchymal stem cell (MSC) crossing of endothelial barrier. Transendothelial migration (TEM) involves coordinated assembly and disassembly of adhesion molecules (VE-cadherin, integrins, connexins, and tight junctions). Endothelial adhesion molecules are temporarily occupied by the transmigrating cell addressing complex signaling pathways. Receptor internalization and recycling within the lateral border recycling compartment are mediated by phosphorylation and dephosphorylation, and can further involve proteolytic cleavage and alteration of cytoskeletal anchorage. Cytoskeletal remodeling facilitates opening of endothelial junctions and eases the passage of the transmigrating cell. As soon as the leading edge reaches the basal lamina, proteolytic enzymes are released for basal lamina breakthrough. An ICAM-1 interplay regulates the passage through the pericyte sheath. Of note, molecular signaling during TEM of MSC is not completely elucidated yet, leaving several ambiguities. Abbreviations: ICAM, Intercellular adhesion molecule 1; JAM, junctional adhesion molecule; LBRC, lateral border recycling compartment; LFA-1, Lymphocyte function-associated antigen 1; MMP, Matrix metalloprotease; PA, Plasminogen activator; PECAM, Platelet endothelial cell adhesion molecule 1; VCAM, Vascular cell adhesion molecule 1; VLA-4, Very late antigen 4; VE, Vacular endothelial cadherin.

is then attracted by pericytes expressing ICAM-1 and is further guided by pericyte-borne chemoattractants [113].

Urokinase-type plasminogen activator (PA) has been found in MSC protrusions (Fig. 5) and can support migration of MSCs under ERK 1,2 MAPK signaling [114]. Overcoming the endothelial basement barrier by MSCs involves proteases including collagenases and metalloproteinases such as Matrix metalloprotease 2 (MMP-2). MMPs are able to degrade the basal membrane collagen type IV, and Tissue inhibitor of metalloprotease 3 (TIMP-3). MMP-9 seems to play a minor role [10, 32, 52], while knock down of MMP-2 severely impairs the migration of MSC over endothelial basement membrane [10].

Different Ways Trough the Endothelial Barrier

Two main routes for leukocyte TEM have been described [74, 115]. Paracellular migration between neighboring endothelial cells is used to pass endothelial cell junctions and accounts for the majority of extravasating leukocytes [23]. Besides that, a transcellular route directly through an individual endothelial cell was observed for 5%–20% of leukocytes and occurs especially after contact to ICAM-1 [90, 91].

Paracellular migration is also the main route observed for MSC TEM. Importantly, it does not induce morphological changes to the endothelial barrier. However, it decreases transendothelial electrical resistance and alters localization of endothelial tight junctions [116]. Formation of a gap between endothelial cells by dissolving of inter-endothelial cell contacts allows MSC to overcome the endothelial barrier [30, 110, 116]. Another mechanism called active vascular expulsion depends on endothelial pocketing. This extravasation process requires activity from endothelial cells to guide migrating cells though the endothelial barrier [117]. Integrins are required for pocketing while vascular breakdown necessitates MMPs. Of note, endothelial pocketing is so far described only for MSC forming multicellular spheres or cardiospheres after cultivation on ultralow attachment culture dishes [117] .

Transcellular diapedesis occurs by the formation of an intracellular window for cell passage in a tunnel-like manner, and was recently described for leukocytes [115]. It is unclear though whether this also plays a major role for MSC TEM as only a single publication [31] reported a route directly through the endothelial cell so far.

Furthermore, an integration process, defined by endothelial retraction as well as MSCs spreading and integration was described [30, 32, 118]. That mechanism is usually not considered a TEM process, but may involve similar induction mechanisms as "true" TEM.

Strategies to Enhance MSC Homing, Migration, and Trafficking

Recent advances in molecular biology have augmented the opportunities for adjustment and manipulation of stem cell functionality and are mainly exerted by means of genetic engineering [119]. Genetic engineering of MSCs can be realized by numerous methods including the use of integrating and non-integrating viral vectors as well as delivery of plasmid-DNA or mRNA [119]. The element of modification is of similar importance. Mobility and homing via the SDF-1 axis is enhanced by transfection of CXCR4 [39, 94]. Integrating vectors have been used to insert chemokine receptors to MSCs using lentiviral vectors to transfer CXCR4 to rat MSC [120]. Although lentiviral modifications rise safety concerns due to the nonspecific integration sites, the efficiency particularly in resting or slowly proliferating cells is high [119]. Furthermore, it has been described that lentiviral transfection does not relevantly affect the differentiation potential [121]. Moreover, ectopic expression of alpha4/beta1 integrin in MSCs mediated by adenoviral transfection enhances homing to the bone in mice [87]. Adenoviruses are suitable to infect a broad variety of stem cells while having a low immunogenicity. Modification with integrating viral vectors generates long-term expression of the transgene, what makes them a practical tool for proof of principle research, but not for clinical use.

Another approach is transgene delivery via plasmids. Translocation of plasmid DNA to the nucleus is required to produce mRNA for protein translation, but the DNA does not integrate into the host genome and is only transiently translated, increasing the methods safety. This is believed to facilitate clinical use, but currently comes at the cost of low efficacy. To address this problem, a broad variety of transfection kits is available, but need to be optimized for MSCs. For instance, Marquez-Curtis et al. achieved a maximum transfection efficiency of 40% for cord blood derived MSCs using IBAfect while maintaining differentiation capabilities but affecting proliferation rate [39] .

Transient overexpression of relevant cell surface receptors can be also achieved by mRNA. For example, human MSCs were transfected with mRNA carrying the CXCR4 gene for CXCR4 tagged with green fluorescent protein [122]. The greatest advantage of mRNA transfection is the bypass of the nucleus since cytoplasmic mRNA is directly accessible for translation [119]. Electroporation [122] and cationic lipids [123] have been shown to improve mRNA transfection efficacy. Transfection is transient due to cytoplasmic instability of mRNA [124], allowing the transfected cell to home within a certain time window.

Besides the cell surface modification by genetically induced receptor expression, enzymatic manipulation of endogenously expressed surface proteins can be used [125]. An example for enzymatic conversion was given by Kerkela et al., who showed that cleavage of cell surface structures can increase MSC targeting and decreases pulmonary entrapment [126]. In particular, use of pronase instead of trypsin for detachment of cultured MSC resulted in faster lung clearance due to cleavage of fibronectin-binding receptors. Instead of cleavage, enzymatic modifications can be used to create molecules on the cells' surfaces. Generation of HCELL as an E-selectin ligand is achieved by glycosyltransferase (d)-programmed stereosubstitution, converting naturally expressed CD44 into its sialofucosylated glycoform [127].

Direct MSC capturing from blood flow by interaction of endothelial adhesion molecules with selective ligands is of limited effectiveness [128]. It can be improved though by noncovalent coupling of a PSGL-1-IgG1 fusion construct to the MSC surface using palmitated protein G (PPG) [129]. PPG-based modification is also suitable to bind antibodies against adhesion receptors on the inflamed endothelium as shown for ICAM-1 [130]. Tethering and rolling to P- and E-selectin carrying endothelial cells can further be enhanced by MSC surface modification using conjugation of sialyl Lewis X (sLeX) moiety via Biotin–Streptavidin under optimized conditions [5]. Hydrophobic tails can serve as an anchor for ligands with lipid or alkyl chains [131], but have been shown to offer little resistance to mechanical forces occurring during rolling and adhesion. Firm adhesion under shear stress can be achieved by coupling peptides to membrane proteins with N-hydroxy-succinimide polyethylen glycol 2 (NHS-PEG2). This construct serves as a linker and does not affect cell viability as well as proliferation and differentiation potentials [132].

Improving endothelial adhesion properties is a valid strategy to improve MSC homing, but migration can also be addressed through the manipulation of other regulators. For instance, the phosphoprotein focal adhesion kinase (FAK) serves as a central downstream cytoskeletal regulator of several receptors and proteins such as aquaporin 1 (AQP-1) [55], platelet-derived growth factor receptor [133], or EGF [134]. Enhanced migration of MSCs was shown after AQP-1 overexpression in a femoral fracture model. Accordingly, FAK and β-catenin were upregulated [55, 135]. Transcription factors, such as T-cell factor/Lymphoid enhancer-binding factor (TCF/LEF) [136] or Sox11 [137] control central stem cell characteristics including migratory behavior. Sox11 upregulation activates BMP/smad signaling pathway and initiates MSC migration [137]. Nuclear receptor nurr77 and nurr11 genes are enrolled in the migration of active cell populations [138]. Migration depends on quantitative relationships among the constituents of several pathways [134], and can therefore be affected and potentially controlled by factor regulation on various levels of the respective intracellular signaling cascades.

The aforementioned methods for enhanced homing are based on artificial modifications and thus generally harbor the risk of undesired side effects. Culture conditions, cell source, and manipulation can induce endogenous expression of surface and adhesion receptors [139, 140]. Expression profiles for surface receptors and thereby homing capabilities change considerably with culturing over several passages, and are further influenced by medium composition and oxygen concentration [141] or pretreatment [142]. For example, protein C kinase inhibitor Ro-31-8425 increases adhesion to ICAM binding domains of MSCs in vitro and in vivo [143]. Furthermore, cytokine pretreatment/preconditioning can augment MSC

adhesion and homing to ischemic tissue as shown for TNFα [144] and IGF-1 [145].

In living organisms, MSCs are physiologically situated in hypoxic environments with an oxygen concentration between 2% and 9% [61]. Therefore, maintenance of low oxygen during MSC cultivation supports their physiological characteristics [139], including surface receptor expression and stemness [146]. Hypoxic preconditioning also induces the expression of prosurvival genes, decreases ischemic cell death and increases trophic activity [147] particularly favorable properties for therapeutic transplantation.

Another approach involves the application of stimulating agents or pharmaceuticals to increase proliferation and mobilization of endogenous MSCs from the bone marrow upon injury. G-CSF and VEGF were considered to have a stimulating effect on MSCs, increasing the number of circulating MSCs in the peripheral blood [13, 148, 149]. It is, however, unclear whether mobilized MSCs also home more efficiently toward injury, or if just their number increases.

Clinical Relevance of (Improved) Systemic MSC Transplantation

The benefits of using MSCs for regenerative medicine are numerous and have been proven in many studies [1, 2, 150]. A particular advantage of MSCs compared with the application of certain pharmaceuticals is their capability to secrete a cocktail of bioactive factors in response to their environment [151]. This flexibility circumvents several difficulties as seen, for example, with drug dosing [151] or monodrug treatments. However, the therapeutic impact of this flexibility relies on the presence of MSCs in close spatial proximity to the injury. The invasive character of local transplantation might be not feasible for widespread clinical application. This particularly applies to sites of injury that are hard to reach or to organs such as the heart and the brain, to which a local injection would provide a considerable risk. In this context, a successful systemic transplantation including efficient homing toward injury sites is of significant relevance. Long-term treatment without a stressful intervention further allows repeated transplantation through a remote artery and vein and would, thus, ease the application. Furthermore, conditions have identified that even allow systemic MSC transplantation with high viability and maintenance of a defined secretome [152]. Understanding the process of TEM and factors contributing to it may provide the tools for enhancing MSC homing after systemic transplantation.

Author Contributions

F.N.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, conception of figures, final approval of manuscript; C.M.: conception of figures and graphical design of Figures 1–5, critical discussion of manuscript, final approval of manuscript; B.L. and J.J.: critical discussion of manuscript, final approval of manuscript; A.D.: conception and design, critical discussion of the manuscript, final approval of manuscript; J.B.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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