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Roles of mechanical force and CXCR1/CXCR2 in shear-stress-induced endothelial cell migration

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Abstract We previously demonstrated that CXCR1 and CXCR2 are novel mechanosensors mediating laminar shear-stress-induced endothelial cell (EC) migration (Zeng et al. in Cytokine 53:42–51, 2011). In the present study, an analytical model was proposed to further analyze the underlying mechanisms, assuming the mechanical force (MF) and mechanosensor-mediated biochemical reactions induce cell migration together. Shear stress can regulate both mechanosensor-mediated migration in the flow direction (Ms–M_{FD}) and mechanosensor-mediated migration toward a wound (Ms–M_W). Next, the migration distance, the roles of MF-induced cell migration (MF–M), and the mobilization mechanisms of mechanosensors were analyzed. The results demonstrated that MF–M plays an important role in 15.27 dyn/cm^2 shear-stress-induced EC migration but is far weaker than Ms–M_W at 5.56 dyn/cm^2. Our findings also indicated that CXCR2 played a primary role, in synergy with CXCR1. The Ms–M_{FD} was primarily mediated by the synergistic effect of CXCR1 and CXCR2. In Ms–M_W, when shear stress was beyond a certain threshold, the synergistic effect of CXCR1 and CXCR2 was enhanced, and the effect of CXCR1 was inhibited. Therefore, the retarding of EC migration and wound closure capacity under low shear flow was related to the low magnitude of shear stress, which may contribute to atherogenesis and many other vascular diseases.

Keywords HUVEC · Hemodynamic force · Mechanosensor · Synergistic effect · Wound progress · Angiogenesis

Abbreviations

MF	Mechanical force
MF–M	MF-induced cell migration
Ms–BR	Mechanosensor-mediated biochemical reactions
Ms–M_{FD}	Mechanosensor-mediated migration in the flow direction
Ms–M_W	Mechanosensor-mediated migration toward wound
D_n	The migration distance at the metering point n (h)
L_n	The cell position at the metering point n (h)
L_0	The original cell position
$D_{upstream}$	The net migration distance of cells from the upriver edge of wound
$D_{downstream}$	The net migration distance from the downriver edge of wound
D_{MF-M}	The migration distance induced by MF
$D_{Ms-M_{FD}}$	The migration distance of Ms–M_{FD}
D_{Ms-M_W}	The migration distance of Ms–M_W
RSI	Relative strength index
RSI_{MF-M}	The ratio of D_{MF-M} and D_{Ms-M_W}
$RSI_{Ms-M_{FD}}$	The ratio of $D_{Ms-M_{FD}}$ and D_{Ms-M_W}
D^*_{CXCR1}	The migration distance mediated by CXCR1 alone (in Ms–M_{FD})
D^*_{CXCR2}	The migration distance mediated by CXCR2 alone (in Ms–M_{FD})

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$D^*_{CXCR1/2}$	The migration distance mediated by the synergistic effect of CXCR1 and CXCR2 (in Ms–M_{FD})
D_{CXCR1}	The migration distance mediated by CXCR1 alone (in Ms–M_W)
D_{CXCR2}	The migration distance mediated by CXCR2 alone (in Ms–M_W)
$D_{CXCR1/2}$	The migration distance mediated by the synergistic effect of CXCR1 and CXCR2 (in Ms–M_W)
D_x	The migration distance mediated by other receptors (in Ms–M_W)
C^*_{CXCR1}	The distance ratio of CXCR1 to Ms–M_{FD}
C^*_{CXCR2}	The distance ratio of CXCR2 to Ms–M_{FD}
$C^*_{CXCR1/2}$	The distance ratio of the synergistic effect of CXCR1 and CXCR2 to Ms–M_{FD}
C_{CXCR1}	The distance ratio of CXCR1 to Ms–M_W
C_{CXCR2}	The distance ratio of CXCR2 to Ms–M_W
$C_{CXCR1/2}$	The distance ratio of the synergistic effect of CXCR1 and CXCR2 to Ms–M_W
C_x	The distance ratio of other receptors to Ms–M_W

Introduction

The migration of endothelial cells (ECs) is essential in a human's life. It is crucial for many physiological and pathological conditions, including embryonic development, immune surveillance, wound healing, and tumor formation and metastasis (Lauffenburger and Horwitz 1996). For example, tumor formation is accompanied by the construction of a new vascular network, which involves the migration of ECs from the pre-existing blood vessels into the tumor.

Cell migration has been regarded as a complex program of cellular behavior including sensing, polarization, cytoskeletal reorganization, and changes in adhesion and shape (Lamalice et al. 2007; Sheetz et al. 1999). The migration of ECs also can be thought of as a cyclical process that occurs in several stages (Petrie et al. 2009; Mofrad and Kamm 2006): perception of a motogenic signal from its surroundings, triggering the basic motility machinery; protrusion, the extension of the cell at the leading edge in the direction of movement; adhesion of the protrusion to the surrounding substrate or matrix; contraction of the cell that transmits a force from these protrusions at the leading edge to the cell body, pulling it forward; and release of the attachments at the rear, allowing net forward movement of the cell to occur. As long as a cell migrates, these single behaviors underlie a continuous regulation and feedback control to direct and coordinate oriented cell movements. In the absence of any external guiding factor, a cell will move in a directionally persistent manner while the protrusions and the subsequent new adhesions formed by a polarized cell are themselves directionally persistent (Petrie et al. 2009).

The 3D environment within the blood vessels is far more complex. ECs must integrate and coordinate with their surroundings and sense the motile stimuli. So far as is known, the directional migration of ECs is regulated by chemical and physical factors in the vascular system and involves three major mechanisms, namely chemotaxis, the directional migration toward a gradient of soluble chemoattractants; haptotaxis, the directional migration toward a gradient of immobilized ligands; and mechanotaxis, the directional migration generated by mechanical forces (Li et al. 2005). In vivo, ECs are in direct contact with blood flow and are constantly exposed to blood-flow-generated shear stress, which initiates the mechanotaxis and modulates the various steps of migration including extension at the leading edge, adhesion to the matrix, and release of adhesions at the rear (Lamalice et al. 2007). We and others have also shown that shear stress can modulate migration of ECs in blood vessels by affecting cell morphology (Levesque and Nerem 1985), cytoskeletal arrangement (Cheng et al. 2007b), and cell–cell junction (Liu et al. 1994; Melchior and Frangos 2010), and by activating mechanosensors, signaling pathways, and gene and protein expression (Chien et al. 1998; Zeng et al. 2011a). Different types of shear stress have different atherogenic properties, for instance, high shear flow with a net forward component is atheroprotective in the straight part of the aorta. In contrast, low shear flow does not have a net forward direction in the branch regions of the arterial trees, and hence these areas are prone to atherogenesis (Cheng et al. 2006; Chien 2007). Furthermore, an interesting phenomenon in our previous studies was discovered (Zeng et al. 2011a): under static conditions, cells migrated toward a wound to cover an equal area, but under conditions of laminar shear stress, they appeared to exhibit asymmetric migration. It is unclear how ECs integrate the directional signal with the basic motility machinery to modulate the directionally persistent migration.

Intrinsic cell directionality and external regulation are two sources of directional cell migration (Petrie et al. 2009). ECs sense a motogenic signal from their surroundings, such as a wound, triggering the basic motility machinery. The intrinsic migration includes many mechanosensor-mediated biochemical reactions. As an external guiding factor, shear stress affects the migration machinery.

As far as is known, shear stress can activate a number of mechanosensors of ECs, including membrane proteins such

as receptor tyrosine kinase, integrins, G proteins and G protein-coupled receptors, Ca^{2+} channels, intercellular junction proteins, membrane lipids, and membrane glycocalyx (Chien 2007). The CXCR1 and CXCR2 receptors, known as G protein-coupled receptors, are novel mechanosensors mediating laminar shear-stress-induced EC migration, a finding which was validated in our previous study (Zeng et al. 2011a). It is well known that the CXCL8 receptors CXCR1 and CXCR2 exist on HUVECs in vitro (Murdoch et al. 1999; Salcedo et al. 2000; Schraufstatter et al. 2001; Zeng et al. 2011a); the expression of CXC chemokine receptors may be affected by culture conditions and various stimuli (Feil and Augustin 1998). Several studies have shown that HUVECs express low levels of CXCR1 and CXCR2 under static conditions (Murdoch et al. 1999; Salcedo et al. 2000; Schraufstatter et al. 2001; Zeng et al. 2011a). Our previous study has shown that both mRNA and protein expression of CXCR1 and CXCR2 in EA.hy926 cells is upregulated by 5.56 dyn/cm^2 laminar shear stress but downregulated by 15.27 dyn/cm^2 (Zeng et al. 2011a). Our previous study also confirmed that the mRNA expression of CXCR1 and CXCR2 in HUVECs showed the same change trend as that in EA.hy926 cells (Zeng et al. 2011a). Furthermore, the migratory response of EA.hy926 cells induced by shear stress can be significantly suppressed by anti-CXCR1 alone, anti-CXCR2 alone, or anti-CXCR1 in combination with anti-CXCR2. In addition to shear stress, CXCL8 can induce cell migration. It is well known that the affinity of CXCR1 and CXCR2 to CXCL8 is different with different cell types. For example, migration is primarily mediated by CXCR1 in leukocytes (Chuntharapai and Kim 1995), and by CXCR2 in NIH3T3 cells (Schraufstatter et al. 2003). CXCR2 is the receptor responsible for ELR^+ CXC chemokine-mediating microvascular EC migration under static conditions (Addison et al. 2000; Mestas et al. 2005). Both CXCR1 and CXCR2 are involved in melanoma cell migration (Singh et al. 2010). However, whether there are differences between CXCR1 and CXCR2 in mediated shear-stress-induced EC migration is as yet unclear.

Furthermore, it is well known that shear stress, transmitted either directly to the cell membrane or via the mechanosensors to the cell interior, affects cell shape, viscoelasticity, cell–cell junction, expression of specific genes, and protein synthesis, ultimately leading to cell migration. Studies have shown that the effects of shear stress are mixed and complicated (Petrie et al. 2009; Stroka and Aranda-Espinoza 2010). However, cell deformation can be ignored because cell–cell junction, cell shape, and structural stiffness under the same conditions (in terms of shear stress magnitude as well as duration) are consistent. Accordingly, the motile cell could be treated as a rigid body, thus turning the complicated cell motion into a simple rigid body motion. The motion of cells induced by shear stress could be expressed in terms of two symmetric tensors according to Newton's third law. One could accelerate cell migration in the direction of flow while the other prevents cell motion in the opposite direction. The net migration due to these tensors and the intrinsic migration together cause the directional EC migration. As a result, those factors that have the potential for migration can be divided into two parts: mechanical force (MF) and mechanosensor-mediated biochemical reactions (Ms–BR). We further assume that mechanosensors have different roles in cell migration; some mechanosensors sense the directional information to mediate cell migration in the direction of flow, and others mediate migration toward the wound. Therefore, the cell migration induced by them can be understood in terms of mechanical force-induced migration (MF–M), mechanosensor-mediated migration in the flow direction (Ms–M_{FD}), and mechanosensor-mediated migration toward the wound (Ms–M_W). Based on this hypothesis and our experimental data, a mathematical model of shear stress that initiates mechanotaxis and modulates cell migration was proposed to further analyze the underlying mechanisms, such as the roles of MF and the mobilization mechanisms of mechanosensors (CXCR1 and CXCR2) by using the theories of mathematics and physics.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord vein with 0.1% Collagenase II (Sigma, St. Louis, MO) and maintained in M-199 medium supplemented with 20% fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 20 μg/ml endothelial cell growth supplement (ECGS, Millipore, Billerica, MA). Cells were seeded onto a glass slide that had been precoated with fibronectin (50 μg/ml), and all cell cultures were grown in a humidified 5%/95% CO_2/air incubator at 37°C. Confluent cultures of HUVECs exhibited the typical cobblestone morphology, and most of those cells contained factor VIII-related antigen and Weibel-Palade body.

Scratch wound migration assay

Cell migration was measured using a monolayer scratch injury assay, as described previously (Fan et al. 2000; Liu et al. 2007; Zeng et al. 2011a). Briefly, the ECs were seeded on glass chamber slides and cultured until confluence. To further isolate the phenomenon of cell migration,

2 mM hydroxyurea, which can completely inhibit HUVEC proliferation, was used to eliminate any confounding effect of shear stress on proliferation. Then, a uniform straight scratch was created in the cell monolayer by using a plastic Cell Scraper (Corning, Corning, NY). Monolayers were washed gently, marked (for reference) and photographed using an inverted microscope (Model CKX41SF, Olympus Optical, Tokyo, Japan) before shear-stress experiments.

ECs exposed to shear stress

The shear-stress experiments were performed as previously described (Cheng et al. 2007a; Gojova and Barakat 2005; Zeng et al. 2011a). In brief, laminar shear flows were applied in the direction orthogonal to the wound. The circulation fluid was serum-free M-199 medium with 50 ng ml^{-1} anti-CXCR1 and/or anti-CXCR2 (Santa Cruz Biotechnology, Santa Cruz, CA). The flow system was kept at 37°C and ventilated with 95% humidified air with 5% CO_2. Three levels of shear stress were imposed in this study: 5.56, 10.02, and 15.27 dyn/cm^2. Images of the wounds were acquired after exposure to shear stress for 1, 2, 4, and 8 h. Shear controls were concurrently performed by using the circulation fluid M-199 medium without antibodies. Static controls were also concurrently performed.

Migration distance

EC migration during the wound closure was analyzed using the Image Pro Plus 6.0 image analysis software (Media Cybernetics, Bethesda, MD). The acquired images were converted from pixels to micrometers with the use of a calibration image (Albuquerque et al. 2000; Savla and Waters 1998). For each experiment, 10 cells were randomly chosen along each edge of the wound, and the migration distances of those cells were respectively computed by

$$D_n = L_n - L_o \tag{1}$$

where D_n is the migration distance, L_n the cell position at the metering point n (h), and L_o the original position.

Modeling of shear-stress-induced directional migration

It was assumed the MF and Ms–BR induced the directional cell migration together. The motion of MF–M obeys Newton's third law. The Ms–BR includes both Ms–M_{FD} and Ms–M_W. Then,

$$D_{\text{upstream}} = D_{\text{MF–M}} + D_{\text{Ms–M}_{\text{FD}}} + D_{\text{Ms–M}_W} \tag{2}$$

$$D_{\text{downstream}} = D_{\text{Ms–M}_W} - D_{\text{Ms–M}_{\text{FD}}} - D_{\text{MF–M}} \tag{3}$$

where D_{upstream} is the net migration distance of cells from the upriver edge of the wound, $D_{\text{MF–M}}$ is the migration distance induced by MF, $D_{\text{MS–M}_{\text{FD}}}$ that induced by Ms–M_{FD}, $D_{\text{Ms–MW}}$ that induced by Ms–M_W, and $D_{\text{downstream}}$ the net migration distance from the downriver edge of the wound.

The D_{upstream} and $D_{\text{downstream}}$ can be calculated according to Eq. 1. Then, $D_{\text{MF–M}} + D_{\text{MS–M}_{\text{FD}}}$ and $D_{\text{Ms–MW}}$ can be obtained from Eqs. 2 and 3:

$$D_{\text{MF–M}} + D_{\text{MS–M}_{\text{FD}}} = (D_{\text{upstream}} - D_{\text{downstream}})/2 \tag{4}$$

$$D_{\text{MS–M}_W} = (D_{\text{upstream}} + D_{\text{downstream}})/2 \tag{5}$$

Determination of the $D_{\text{MF–M}}$ and $D_{\text{Ms–M}_{\text{FD}}}$

The receptors that mediate EC migration include CXCR1, CXCR2, and other mechanosensors (Zeng et al. 2011a). The effect of other mechanosensors on Ms–M_{FD} is neglected in the present study. The MF–M was independent of CXCR1 and CXCR2, but Ms–M_{FD} can be inhibited by those antibodies. Considering the synergistic effect of CXCR1 and CXCR2, then,

$$D_{\text{MF–M}} + D_{\text{Ms–M}_{\text{FD}}} = D_{\text{MF–M}} + D^*_{\text{CXCR1}} + D^*_{\text{CXCR2}} + D^*_{\text{CXCR1/2}} \tag{6}$$

where D^*_{CXCR1}, D^*_{CXCR2} and $D^*_{\text{CXCR1/2}}$ respectively represent the migration distance (in Ms–M_{FD}) mediated by CXCR1 alone, CXCR2 alone, and the synergistic effect of CXCR1 and CXCR2.

If the CXCL8 receptors are functionally blocked by anti-CXCR1 alone, D^*_{CXCR1} and $D^*_{\text{CXCR1/2}}$ are each equal to zero. Then,

$$D_{\text{MF–M}} + D_{\text{Ms–M}_{\text{FD}}} = D_{\text{MF–M}} + D^*_{\text{CXCR2}} \tag{7}$$

Similarly, if the CXCL8 receptors are functionally blocked by anti-CXCR2 alone, D^*_{CXCR2} and $D^*_{\text{CXCR1/2}}$ are each equal to zero. Then,

$$D_{\text{MF–M}} + D_{\text{Ms–M}_{\text{FD}}} = D_{\text{MF–M}} + D^*_{\text{CXCR1}} \tag{8}$$

If the CXCL8 receptors are functionally blocked by anti-CXCR1 in combination with anti-CXCR2, D^*_{CXCR1}, D^*_{CXCR2} and $D^*_{\text{CXCR1/2}}$ are each equal to zero. Then,

$$D_{\text{MF–M}} + D_{\text{Ms–M}_{\text{FD}}} = D_{\text{MF–M}} \tag{9}$$

By combining Eqs. 6–9, $D_{\text{MF–M}}$, $D_{\text{MS–M}_{\text{FD}}}$, D^*_{CXCR1}, D^*_{CXCR2} and $D^*_{\text{CXCR1/2}}$ can be obtained, respectively.

Roles of MF in shear-stress-induced cell migration

The roles of MF in shear-stress-induced cell migration can be analyzed by a relative strength index (RSI), which is defined as the ratio of $D_{\text{MF–M}}$ and $D_{\text{Ms–MW}}$:

$$\mathrm{RSI}_{\mathrm{MF-M}} = \frac{D_{\mathrm{MF-M}}}{D_{\mathrm{MS-M_W}}} \times 100\% \quad (10)$$

where $\mathrm{RSI}_{\mathrm{MF-M}}$ is the RSI of MF–M.

Roles of Ms–M_{FD} in shear-stress-induced cell migration

We can also obtain the RSI of the Ms–M_{FD}:

$$\mathrm{RSI}_{\mathrm{Ms-M_{FD}}} = \frac{D_{\mathrm{Ms-M_{FD}}}}{D_{\mathrm{Ms-M_W}}} \times 100\% \quad (11)$$

where $\mathrm{RSI}_{\mathrm{MS-M_{FD}}}$ is the RSI of Ms–M_{FD}.

The mobilization mechanisms of mechanosensors in Ms–M_{FD}

The distance ratio of each receptor to Ms–M_{FD}, such as C^*_{CXCR1}, C^*_{CXCR2} and $C^*_{\mathrm{CXCR1/2}}$ can be computed, respectively, by D^*_{CXCR1}, D^*_{CXCR2} and $D^*_{\mathrm{CXCR1/2}}$ divided by $D_{\mathrm{Ms-M_{FD}}}$. Based on those ratios, we can obtain the mobilization mechanisms of mechanosensors in Ms–M_{FD}.

The mobilization mechanisms of mechanosensors in Ms–M_W

Considering the synergistic effects of CXCR1 and CXCR2, and the other mechanosensors, then,

$$D_{\mathrm{Ms-M_W}} = D_{\mathrm{CXCR1}} + D_{\mathrm{CXCR2}} + D_{\mathrm{CXCR1/2}} + D_{\mathrm{x}} \quad (12)$$

where $D_{\mathrm{CXCR1}} + D_{\mathrm{CXCR2}} + D_{\mathrm{CXCR1/2}} + D_{\mathrm{x}}$ represent the migration distance (in Ms–M_W) mediated by CXCR1 alone, CXCR2 alone, the synergistic effect of CXCR1 and CXCR2, and other receptors, respectively.

If the CXCL8 receptors are functionally blocked by anti-CXCR1 alone, D_{CXCR1} and $D_{\mathrm{CXCR1/2}}$ are each equal to zero. Then,

$$D_{\mathrm{Ms-M_W}} = D_{\mathrm{CXCR2}} + D_{\mathrm{x}} \quad (13)$$

Similarly, if the CXCL8 receptors are functionally blocked by anti-CXCR2 alone, D_{CXCR2} and $D_{\mathrm{CXCR1/2}}$ are each equal to zero. Then,

$$D_{\mathrm{Ms-M_W}} = D_{\mathrm{CXCR1}} + D_{\mathrm{x}} \quad (14)$$

If the CXCL8 receptors are functionally blocked by anti-CXCR1 in combination with anti-CXCR2, then D_{CXCR1}, D_{CXCR2}, and $D_{\mathrm{CXCR1/2}}$ are each equal to zero. Then,

$$D_{\mathrm{Ms-M_W}} = D_{\mathrm{x}} \quad (15)$$

From Eqs. 12–15, D_{CXCR1}, D_{CXCR2}, $D_{\mathrm{CXCR1/2}}$, and D_{x} can be obtained, respectively. Then, the distance ratio of each receptor to Ms–M_W, including C_{CXCR1}, C_{CXCR2}, $C_{\mathrm{CXCR1/2}}$, and C_{x} can be computed, respectively, as D_{CXCR1}, D_{CXCR2}, $D_{\mathrm{CXCR1/2}}$, and D_{x} divided by $D_{\mathrm{Ms-M_W}}$. Based on those ratios, we can obtain the mobilization mechanisms of mechanosensors in Ms–M_W.

Statistical analysis

Data of migration distance are presented as means ± SD obtained from three different experiments, unless otherwise indicated. By using the theories of data analysis (Lyons 1991), the combined errors in individual indirect measurements were calculated, and then the combined results of different experiments are presented. Statistical analysis was performed by one-way ANOVA test using SPSS 17.0 software package. Differences in means were considered significant if $P < 0.05$.

Results and analysis

Distance of cell migration

The migration distances of ECs were obtained as shown in Fig. 1. As in EA.hy926 cells (Zeng et al. 2011a), EC migration was asymmetric. Specifically, the cell migration distance increased with the magnitude of shear stress and exposure time upriver of the wound but these differences were not observed in downriver. The asymmetry increased with the exposure time. Interestingly, the migration distance of ECs on both sides of the wound decreased when the cells were treated with functional CXCL8 receptor antibodies, but the asymmetry remained (Fig. 1b–d). Compared to the results under static control, it was found that there was a greater distance upriver and a smaller distance downriver. It was indicated that CXCR1 and CXCR2 are involved in the mechanosensor-mediated EC migration toward the wound (Ms–M_W) and that laminar shear flow provided a mechanical force for cell migration in the direction of flow.

Additionally, the smaller migration distance of cells upriver of the wound under 5.56 dyn/cm^2 also suggested that 5.56 dyn/cm^2 led to retarding wound closure in comparison with 15.27 dyn/cm^2 (Fig. 1a).

Roles of MF in shear-stress-induced cell migration

Based on our mathematical model, the migration distance $D_{\mathrm{MF-M}}$ and $D_{\mathrm{Ms-M_W}}$ were obtained. Then, the ratio of $D_{\mathrm{MF-M}}$ and $D_{\mathrm{Ms-M_W}}(\mathrm{RSI}_{\mathrm{MF-M}})$ was further analyzed to determine the roles of MF in EC migration (Fig. 2). The results have shown that $\mathrm{RSI}_{\mathrm{MF-M}}$ was maintained at about 106% under 15.27 dyn/cm^2 conditions, 37% under 10.02 dyn/cm^2, and 27% under 5.56 dyn/cm^2. These results indicated that MF plays an important role in

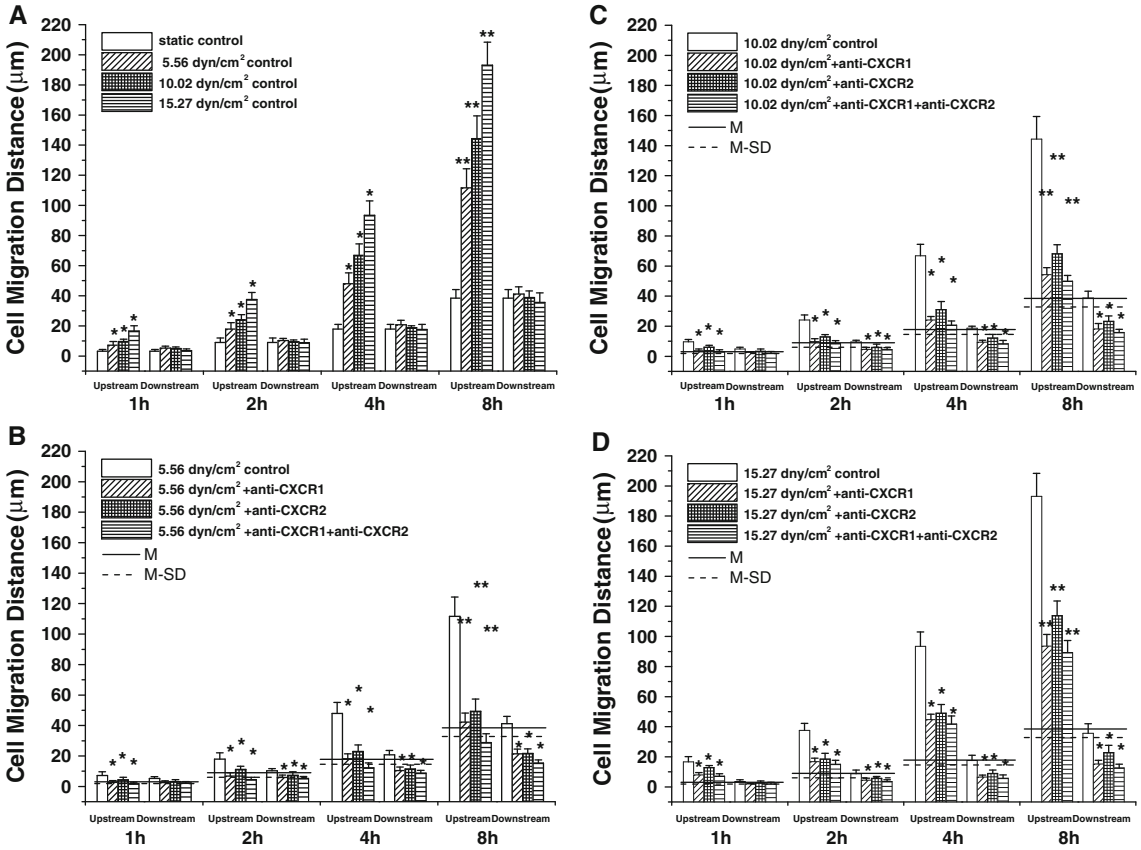

Fig. 1 The cell migration distance of EC during exposure to laminar shear stress. **a** Static conditions. Laminar shear-stress-induced asymmetric EC migration. Cell migration distance induced by **b** 5.56 dyn/cm^2; **c** 10.02 dyn/cm^2; **d** 15.27 dyn/cm^2 conditions. The asymmetry increased with exposure time. The cell migration distance decreased when cells were treated with functional CXCL8 receptor antibodies, but the asymmetry remained. This suggests that laminar shear flow provides a mechanical force (MF) for cell migration in the direction of flow. Mean ± SD is shown. *Control* indicates cells that were treated without CXCL8 receptor antibodies under various conditions. *M* Mean of migration distance in static control, *M–SD* mean − SD of migration distance in static control. Differences in means were considered significant if $P < 0.05$. $^*P < 0.05$, $^{**}P < 0.01$ versus control at the same time

15.27 dyn/cm^2-induced EC migration but is far weaker than Ms–M_W in 5.56 dyn/cm^2-induced EC migration.

Roles of Ms–M_{FD} in shear-stress-induced cell migration

The $RSI_{Ms-M_{FD}}$ was also obtained as shown in Fig. 3. Under 5.56 dyn/cm^2 conditions, the $RSI_{Ms-M_{FD}}$, which was kept at a relatively high level (about 98%) under 15.27 dyn/cm^2 conditions, increased with exposure time and reached statistical significance at 8 h (about 64% at 8 h vs. 21% at 1 h, $P < 0.05$). It was also increased with exposure time under 10.02 dyn/cm^2 conditions, which was near the level under 15.27 dyn/cm^2 at 8 h. These results suggested that the response of those mechanosensors to the flow directional information was dependent on the magnitude of shear stress.

Overall, the reduced roles of MF–M and Ms–M_{FD} under 5.56 dyn/cm^2 conditions suggest that the 5.56 dyn/cm^2-retarded cell migration and wound closure capacity are related to the lower magnitude of shear stress.

Mobilization mechanisms of mechanosensors in Ms–M_{FD}

The migration distance ratios of each receptor to Ms–M_{FD} have shown that the Ms–M_{FD} is primarily mediated by the synergistic effects of CXCR1 and CXCR2, while the roles of CXCR1 and CXCR2 under all those conditions could be negligible (Fig. 4).

Mobilization mechanisms of mechanosensors in Ms–M_W

The mobilization mechanism of mechanosensors was assessed by the migration distance ratio of each receptor to Ms–M_W. The receptors that mediated EC migration included CXCR1, CXCR2, and other mechanosensors. The

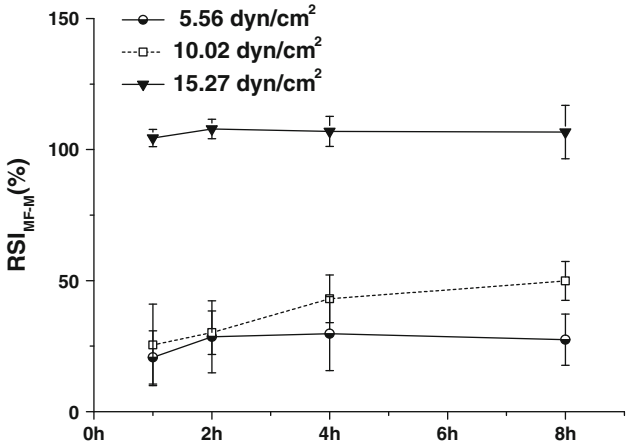

Fig. 2 The relative strength index (RSI) of mechanical force-induced migration (MF–M). RSI_{MF-M} was maintained at about 106% under 15.27 dyn/cm^2 conditions, 37% under 10.02 dyn/cm^2, and 27% under 5.56 dyn/cm^2. Mean ± SD is shown

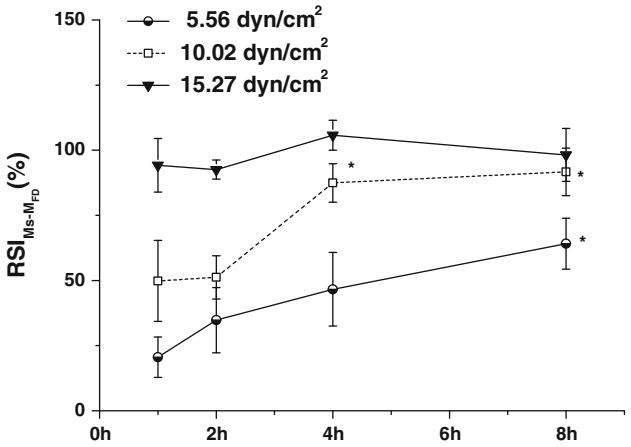

Fig. 3 The relative strength index (RSI) of mechanosensor-mediated migration in the flow direction (Ms–M_{FD}). Under 5.56 dyn/cm^2 conditions, the RSI of Ms–M_{FD}, which was kept at a relatively high level (about 98%) under 15.27 dyn/cm^2 conditions, increased with exposure time and reached statistical significance at 8 h (about 64% at 8 h vs. 21% at 1 h, $P < 0.05$). Mean ± SD is shown. Differences in means were considered significant if $P < 0.05$. *$P < 0.05$ versus the RSI of Ms–M_{FD} at 1 h

synergistic effect of CXCR1 and CXCR2 was also considered in the present study. The results were obtained as shown in Fig. 5. Under 5.56 dyn/cm^2 conditions, the migration distance ratio of each receptor to Ms–M_W did not change with exposure time (Fig. 5a). In particular, the ratios of CXCR1, CXCR2, and the synergistic effect of CXCR1 and CXCR2 to Ms–M_W were about 30.9, 10.9, and 26.6%, respectively.

Under 10.02 dyn/cm^2 conditions, the migration distance ratios of CXCR1, CXCR2, and the synergistic effect of CXCR1 and CXCR2 to Ms–M_W were, respectively, about

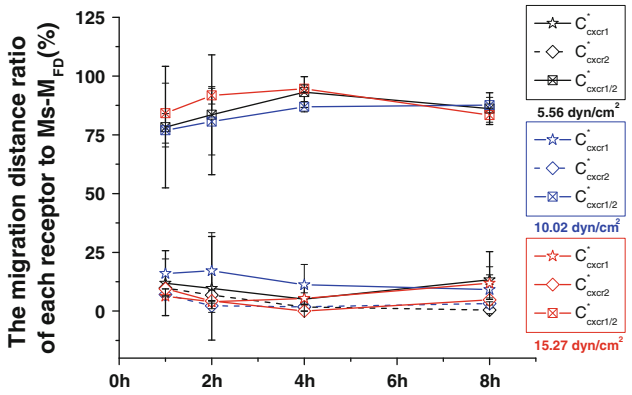

Fig. 4 The mobilization mechanisms of mechanosensors in Ms–M_{FD}. They were assessed in terms of the migration distance ratio of each receptor to Ms–M_{FD} during exposure to different laminar shear flows. Those receptors that mediated EC migration in the flow direction only focused on CXCR1 and CXCR2. The synergistic effect of CXCR1 and CXCR2 was considered in the present study. The roles of CXCR1 and CXCR2 alone under all those conditions could be negligible. The Ms–M_{FD} was primarily mediated by the synergistic effect of CXCR1 and CXCR2, and can be defined in terms of the migration distance ratio of CXCR1, CXCR2, the synergistic effect of CXCR1 and CXCR2, and other mechanosensors to Ms–M_{FD}, respectively. Mean ± SD is shown

30.5, 8.7, and 25.4% at 1 h, and about 16.2, 4.4, and 43.2% at 2–8 h (Fig. 5b).

Under 15.27 dyn/cm^2 conditions, the migration distance ratios of CXCR1 and of the synergistic effect of CXCR1 and CXCR2 to Ms–M_W were, respectively, about 32.1 and 18.5% at 1 h, and about 12.3 and 41.2% at 2–8 h. Of note, the ratio of CXCR2 to Ms–M_W was maintained at a level of 3.0–5.4% (Fig. 5c).

However, the migration distance ratios of other mechanosensors to Ms–M_W did not change with exposure time (Fig. 5d).

In particular, the migration distance ratio of CXCR1 to Ms–M_W was much larger than that of CXCR2 (Fig. 5a–c). But it was smaller than the synergistic effect of CXCR1 and CXCR2 under 10.02 and 15.27 dyn/cm^2 conditions for 2–8 h ($P < 0.01$). Therefore, the ratio of CXCR2 to Ms–M_W was maintained at a particularly low level. Both CXCR1 and the synergistic effect of CXCR1 and CXCR2 played predominant roles in 5.56 dyn/cm^2 shear-stress-induced Ms–M_W and the early phase (1 h) of 10.02 and 15.27 dyn/cm^2 shear-stress-induced Ms–M_W. However, the synergistic effect of CXCR1 and CXCR2 was more important in the later phase (2–8 h) of 10.02 and 15.27 dyn/cm^2. This suggests that CXCR2 plays a primary role in synergy with CXCR1. When shear stress was beyond a certain threshold, the synergistic effect of CXCR1 and CXCR2 was enhanced, and the effect of CXCR1 was subsequently inhibited.

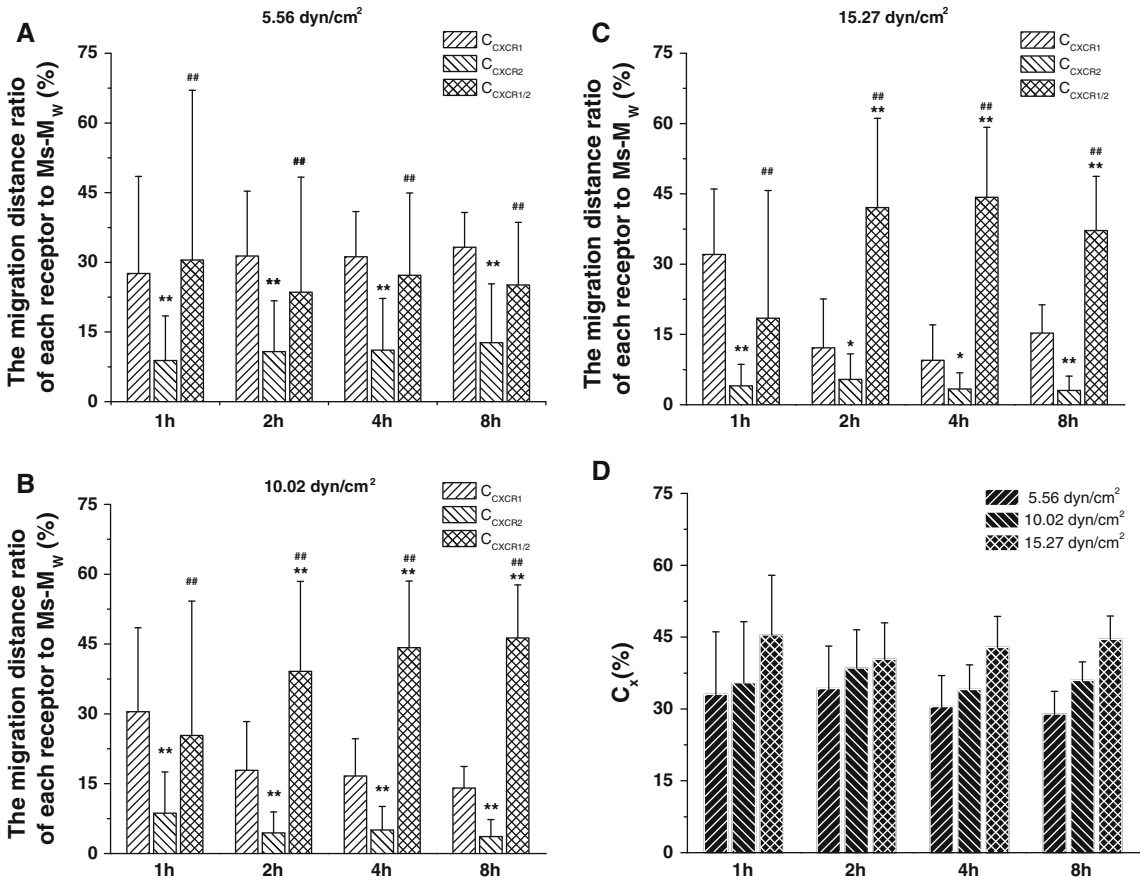

Fig. 5 The mobilization mechanisms of mechanosensors in Ms–M_W. They were assessed in terms of the migration distance ratio of each receptor to Ms–M_W. The receptors that mediated EC migration included CXCR1, CXCR2, and other mechanosensors. The synergistic effect of CXCR1 and CXCR2 was considered in the present study, and under 5.56 dyn/cm^2 (**a**), 10.02 dyn/cm^2 (**b**), and 15.27 dyn/cm^2 (**c**) conditions, C_x (**d**), C_{CXCR1}, C_{CXCR2}, $C_{CXCR1/2}$, and C_x can be defined, respectively, as the migration distance ratios of CXCR1, CXCR2, the synergistic effect of CXCR1 and CXCR2, and other mechanosensors to Ms–M_W. The ratio of CXCR2 to Ms–M_W was maintained at a certain low level. Both CXCR1 and the synergistic effect of CXCR1 and CXCR2 played predominant roles at 5.56 dyn/cm^2 and in the early phase of 10.02 and 15.27 dyn/cm^2. However, the synergistic effect of CXCR1 and CXCR2 was more important in the later phase of 10.02 and 15.27 dyn/cm^2. The ratio of other mechanosensors to Ms–M did not change with exposure time. Differences in means were considered significant if $P < 0.05$. $^*P < 0.05$, $^{**}P < 0.01$ versus C_{CXCR1} at the same time; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus C_{CXCR2} at the same time

Discussion

Our present investigation demonstrates the roles of mechanical force and the underlying mobilization mechanisms of mechanosensors, especially the roles of CXCR1 and CXCR2.

In the present study, both cell spreading and cell area were assumed to be invariable. Hydroxyurea at 2 mM can completely inhibit the proliferation of HUVECs (Cai et al. 2000). We have found that shear stress still enhances EC migration in the presence of hydroxyurea, and that 2 mM hydroxyurea is able to inhibit proliferation through its cytostatic effect without damaging the viability of HUVECs (Zeng et al. 2011b). To further isolate the phenomenon of cell migration, 2 mM hydroxyurea was used to eliminate any confounding effect of shear stress on proliferation. Thus, the differences in wound closure were considered primarily due to cell migration.

External regulation and intrinsic cell directionality induce directional cell migration together. A wound is a motogenic signal from the ECs' surroundings. An EC can sense it, triggering the basic motility machinery. The same migration distance towards the wound from both sides under static conditions indicated that the intrinsic migration was symmetric. In our previous studies, we found that the cell migration under static conditions was blocked by CXCR1/2 antibody (data not shown), indicating that CXCR1/2 contributes to migration toward the wound. The external guiding factors that regulate directional migration in vitro include chemical stimuli, either soluble in the medium [chemotaxis (Chung et al. 2001; Janetopoulos and Firtel 2008; Kolsch et al. 2008; Van Haastert and

Devreotes 2004)] or immobilized on the substrate surface [haptotaxis (Chen et al. 1997; Hsu et al. 2005; Li et al. 2005)], as well as mechanical stimuli [mechanotaxis (Chien 2008; Petrie et al. 2009; Uttayarat et al. 2008)]. The recent studies into the crosstalk between haptotaxis and mechanotaxis induced by shear stress during EC migration on collagen showed that 2 dyn/cm^2 did not affect haptotaxis compared to static control (Li et al. 2005); shear stress at 3 dyn/cm^2 or higher was sufficient to drive EC migration in the flow direction against the collagen gradient (Li et al. 2005); and the increase in shear stress from 2 to 10 dyn/cm^2 effectively switched the direction of $\sim$50% of ECs to migrate with the flow across the haptotactically derived collagen tracks over a 16 h period (Hsu et al. 2005). These results suggest that haptotactic and mechanotactic stimuli competitively direct EC migration, and above a certain threshold, shear stress can predominately regulate EC migration. Therefore, the direction of cell migration under flow depends on the magnitude and direction of fluid shear stress with respect to chemical stimuli. In the present study, the substrate surface was 50 μg/ml fibronectin, and the stable influence of fibronectin on mechanotactic stimuli-induced cell migration was ignored.

In the absence of any external guiding factor, cells will move in a directionally persistent manner. In the present study, shear stress is an important external guiding factor. Urbich et al. (2002) reported that shear stress dose-dependently enhances migration of HUVECs over a laminar shear stress range of 5 to 45 dyn/cm^2. Our results showed that the wound closure was enhanced with the magnitude and exposure time of shear stress, and that 15.27 dyn/cm^2 induced greater migration than 5.56 and 10.02 dyn/cm^2. Albuquerque et al. (2000) reported that shear stress of 3 dyn/cm^2 promotes the largest acceleration in wound closure in both HUVECs and human coronary artery endothelial cells (HCAECs). It is possible that the different direction of flow resulted in the difference between Albuquerque et al.'s results and ours. The direction of flow in Albuquerque et al.'s study was oriented parallel to the wound, whereas in the present study it was orthogonal. In fact, EC migration during wound closure involves displacement both parallel and orthogonal to the direction of flow. In order to confirm how flow affects the directionality of cell migration, the migration distances of cells from the upriver edge and downriver edge of wound were calculated. The cells upriver of the wound migrated faster than those downriver (Fig. 1), suggesting that the direction of flow is very important for EC migration, that the upriver cells are the primary contributors to wound closure, and that the direction of cell migration parallel to flow is the principal one for wound closure.

Interestingly, the asymmetry increased with the magnitude and duration of shear stress. It also can't be completely inhibited by functional CXCR1 and CXCR2 antibody. The migration distances of cells on both sides of the wound decreased but, in comparison to under static control, the distances were larger upriver and smaller downriver. It was indicated that CXCR1/2 was involved in the shear-induced wound closure, and that shear-induced migration in the flow direction is present in these conditions. It might be induced by a directly mechanical force. Whether CXCR1/2 influences the EC migration toward flow direction needs to be explored in depth. However, this can't be directly made clear through comparison with the static control because the migration level of CXCL8 receptor antibody-treated cells upriver and downriver under these conditions was also decreased, while the cell migration under static conditions was blocked by CXCR1/2 antibody.

These results, which serve as the basis for our hypothesis and the purpose of our current study, are shown in the mind mapping in Fig. 6. In the following analysis, we assumed that shear stress can induce asymmetric EC migration either directly by MF or via Ms–BR, that Ms–BR includes both Ms–M_{FD} and Ms–M_W, and that CXCR1 and CXCR2 are involved in the Ms–M_{FD}. Our results showed that the Ms–M_{FD} was primarily mediated by the synergistic effects of CXCR1 and CXCR2. Therefore, the MF and Ms–M_{FD} were two important factors that induced cell migration in the flow direction. The direction of cell migration was regulated by MF and Ms–M_{FD}, especially by the MF and the synergistic effect of CXCR1 and CXCR2. Our results also showed the synergistic effect of CXCR1 and CXCR2 played a prominent role in Ms–M_W when shear stress was beyond a certain threshold, and subsequently the effect of CXCR1 was inhibited. Our previous study has shown that the CXCR1 and CXCR2 receptors are novel mechanosensors mediating laminar shear-stress-induced EC migration (Zeng et al. 2011a). In particular, it was documented that CXCR2 is responsible for mediating EC chemotaxis and angiogenesis by the ELR$^+$ CXC chemokine under non-flow conditions (Addison et al. 2000; Mestas et al. 2005). Our results showed that CXCR2 contributed little to the Ms–M_{FD} and Ms–M_W, but it played important roles in synergy with CXCR1. We speculate that CXCR1 and CXCR2 have a synergistic effect, but the specific mechanism is very complex and much is still to be learned.

Additionally, different magnitudes of shear stress were applied to the ECs in our study. A lower magnitude of MF under 5.56 dyn/cm^2 conditions may have been the cause of the reduced power, which wasn't able to drive the molecular machinery completely; it was assumed to be an important risk factor for retarding wound closure. Specifically, the migration distance of cells upriver of the wound under 5.56 dyn/cm^2 conditions was much smaller than that under 15.27 dyn/cm^2. This is due to the weak roles of

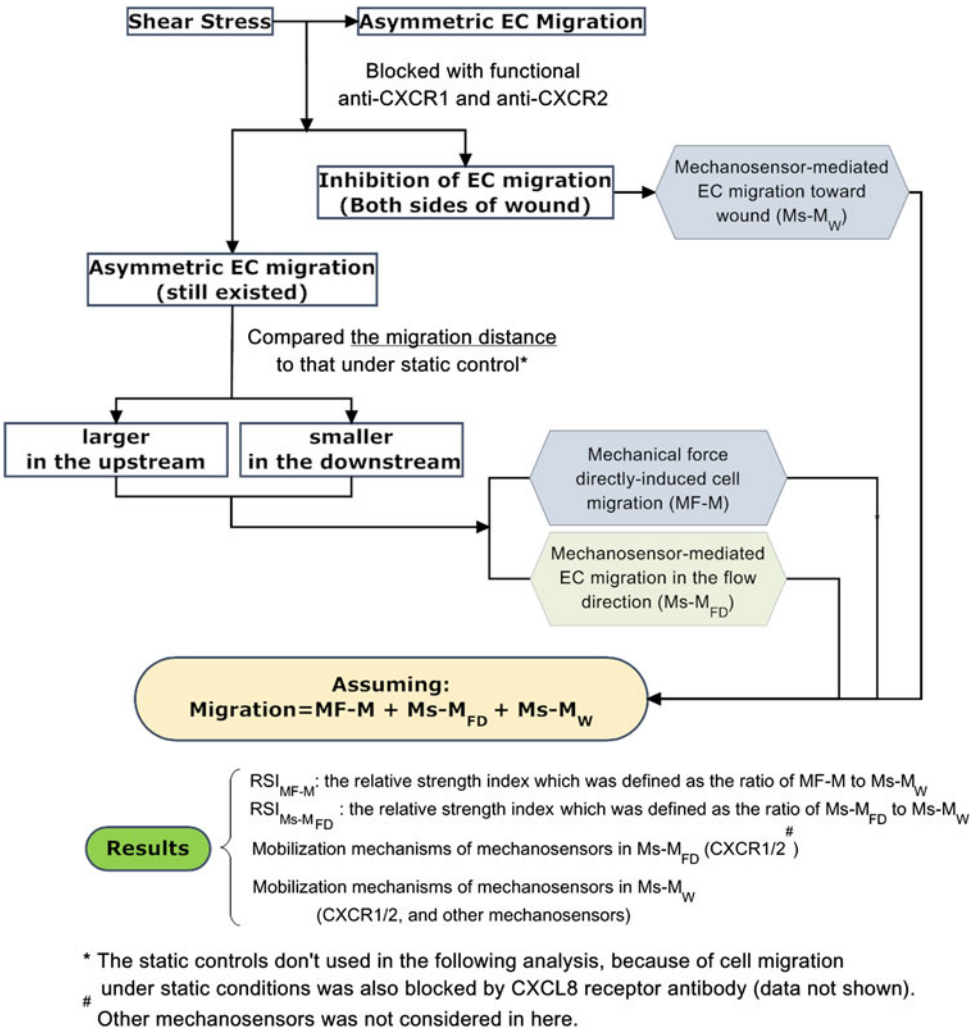

Fig. 6 The mind map represents the basis of our hypothesis and the purpose of our current study

MF–M and Ms–M_{FD}, and the magnitude of the synergistic effect of CXCR1 and CXCR2 in Ms–M_W being lower than a certain threshold. Our results also showed that the RSI_{MF-M} was maintained at a very low level under 5.56 dyn/cm^2 conditions, but the RSI_{Ms-MFD} increased with exposure time. It was suggested that the response of those mechanosensors to the flow directional information was dependent on the magnitude of shear stress. Furthermore, it has been documented that flow with low magnitude (Davies 1995), none in a net forward direction (Chien 2007) in the branch regions of the arterial trees, may result in EC dysfunction, leaving one prone to atherogenesis and many other vascular diseases (Barakat and Lieu 2003; Resnick et al. 2003). Therefore, applying an appropriate mechanical environment may be a promising strategy for prevention and treatment of cardiovascular diseases.

In particular, our present investigation focused on the roles of CXCR1 and CXCR2. The roles of other mechanosensors were considered in the Ms–M_W, but not in the Ms–M_{FD}. The roles of other mechanosensors in Ms–M_W didn't change with duration of shear stress. If those mechanosensors were not associated with CXCR1 and CXCR2 in Ms–M_{FD}, the roles of MF may be exaggerated in our results. However, it is difficult to separate the roles of other mechanosensors from the Ms–M_{FD}. Whether other mechanosensors can influence mechanosensor-mediated directional EC migration is a new subject and needs to be explored in depth.

Finally, our present study describes an analytical model for interpretation of EC migration under shear conditions to distinguish the source of migration between MF and Ms–BR. Based on our results and those of previous studies (del Álamo et al. 2008; Chiu et al. 2009; Lauffenburger and Horwitz 1996; Li et al. 2005; Zeng et al. 2011a), the description of a complex network depicts the molecular machinery that is involved in the shear-stress-induced directional EC migration, as shown in Fig. 7. If a cell is a machine, the motogenic

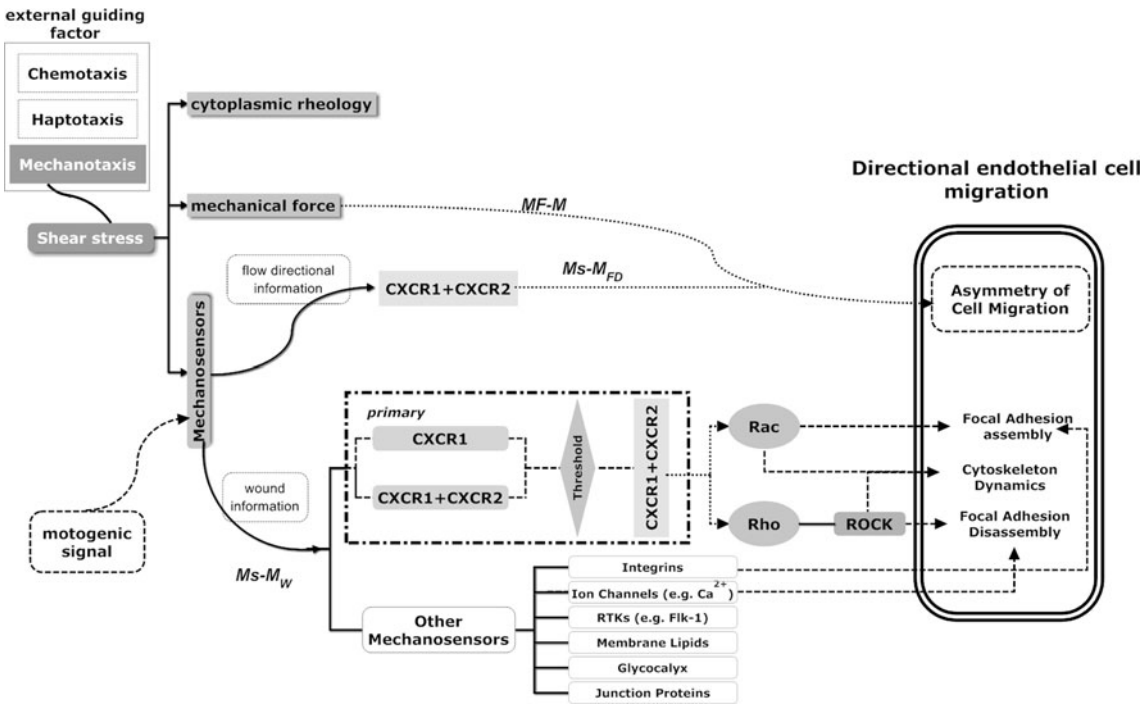

Fig. 7 Illustration of the molecular machinery involved in the shear-stress-induced directional EC migration. Cells sense the motogenic signal from their surroundings, triggering the basic motility machinery, and then regulate the persistent directional migration. Directional EC migration in vitro can be induced by chemotaxis, haptotaxis, and mechanotaxis. Haptotactic and mechanotactic stimuli are competitive. When shear stress is above a certain threshold, it is the predominant regulator of EC migration. In our study, it was assumed that the laminar shear-stress-induced directional EC migration could be divided into two parts: the externally regulated cell migration and the intrinsic cell directionality, or mechanical force-induced migration (MF–M) and mechanosensor-mediated biochemical reactions (Ms–BR). Shear stress induced asymmetric EC migration either directly by MF effects on cell membrane or via some mechanosensor-mediated migration in the flow direction ($Ms–M_{FD}$). The mechanical force that is subject to Newton's third law of motion provides a push tensor to the cells upriver, and a retard tensor downriver. The $Ms–M_{FD}$ was primarily mediated by the synergistic effect of CXCR1 and CXCR2. Additionally, CXCR2 played a primary role in synergy with CXCR1 in the mechanosensor-mediated migration toward the wound ($Ms–M_W$). When shear stress was beyond a certain threshold, the synergistic effect of CXCR1 and CXCR2 was enhanced, and the effect of CXCR1 was inhibited. A number of other mechanosensors of ECs can also be activated by shear stress, including the membrane proteins such as receptor tyrosine kinase (RTK), integrins, G proteins and other G protein-coupled receptors, Ca^{2+} channels, intercellular junction proteins, membrane lipids, and membrane glycocalyx. These mechanosensors and their sequential mechanotransduction pathways are very important to modulate the expression of different genes as well as the structure and function of ECs. CXCR1 and CXCR2 act through the adaptor molecules to activate upstream signaling molecules such as GTPase Rac and GTPase Rho, which then activate the downstream molecular kinase (ROCK), and then stimulate actin. Finally, the cytoplasmic rheology may also play an important role in mechanotransduction in adherent cells by providing a means to sense the direction of mechanical stimuli.

signal is the ignition system, and the mechanosensors are emerging as a breed of power converter for internal effects, while shear stress is an external power source. However, we ignored the cell deformation with exposure time. Recently, the mechanism of cell alignment was clarified (del Álamo et al. 2008). After continuous laminar flow shear stress, vascular endothelial cells gradually elongate and the directions of maximum and minimum cytoplasmic creep compliance become, respectively, parallel and perpendicular to flow direction. This mechanical alignment is accompanied by a transition of the cytoplasm to be more fluid-like in the flow direction and more solid-like in the perpendicular direction, while the cytoplasmic creep compliance increases at the downriver part of the cells. It may be that the cytoplasmic creep compliance increasing perpendicular to flow direction also contributes to the directional migration. Although the processes of cell migration were simplified here, it could be helpful in extending our understanding of cell motion, while providing some valuable reference information for further study.

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