Regulation of α2β1-mediated Fibroblast Migration on Type I Collagen by Shifts in the Concentrations of Extracellular Mg²⁺ and Ca²⁺

John J. Grzesiak, George E. Davis, Daniel Kirchhofer, and Michael D. Pierschbacher
La Jolla Cancer Research Foundation, 10901 North Torrey Pines Road, La Jolla, California 92037

Abstract. Extracellular Ca²⁺ can reverse the Mg²⁺-dependent, α2β1-mediated adhesion of WI38 human fibroblasts to type I collagen substrates. Affinity chromatography data also demonstrate that Ca²⁺ can specifically elute the fibroblast α2β1 integrin bound to type I collagen-Sepharose in Mg²⁺. In modified Boyden chamber migration assays, Mg²⁺ alone supports the α2β1-mediated migration of fibroblasts on type I collagen substrates, while Ca²⁺ does not. However, a twofold enhancement in migration was observed when combinations of the two cations were used, with optimal migration observed when the Mg²⁺/Ca²⁺ ratio was higher than one. Inhibitory mAbs directed against various integrin subunits demonstrate that these observed cation effects appear to be mediated primarily by α2β1. These data, together with reports that under certain physiological conditions significant fluctuations in the concentrations of extracellular Ca²⁺ and Mg²⁺ can take place in vivo, suggest that the ratio between these two cations is involved in the up- and downregulation of integrin function, and thus, may influence cell migratory behavior.
affibodies, exhibits the same Ca\(^{2+}\)-inhibitable binding characteristics as \(\alpha_\beta_1\), (Staatz et al., 1989), and that the Mg\(^{2+}\)-dependent, \(\alpha_\beta_1\)-mediated adhesion of platelets to collagen is also inhibited by Ca\(^{2+}\) (Santoro, 1986). One of the integrins mediating adhesion to collagen on fibroblasts is also an \(\alpha_\beta_1\), very similar to that found on platelets (Kunicki et al., 1988), though, depending on the cell type on which it is expressed, this receptor may display altered ligand specificity (Languino et al., 1989; Kirchhofer et al., 1990; Elices and Hemler, 1989; Lotz et al., 1990).

In this study we have examined the potential regulatory role of extracellular Ca\(^{2+}\) and Mg\(^{2+}\) on the \(\alpha_\beta_1\)-mediated migration of fibroblasts on type I collagen. The results presented here suggest that integrin-mediated fibroblast migration on type I collagen can be regulated, at least in part, by small changes in divalent cation concentration. We demonstrate that fibroblastic cells which express both \(\alpha_\beta_1\) and \(\alpha_\beta_3\) exhibit migration in the presence of Mg\(^{2+}\) on type I collagen but not on the control, vitronectin, while in the presence of Ca\(^{2+}\), the cells migrate on vitronectin but not on type I collagen. Furthermore, we demonstrate that migration on collagen is significantly enhanced when both cations are present, but is optimal when the ratio of Mg\(^{2+}\)/Ca\(^{2+}\) concentrations is slightly higher than one. mAbs directed against the \(\alpha_\beta_1\) and \(\beta_1\) integrin subunits inhibit this migration on type I collagen, suggesting that these observed cation effects are mediated through \(\alpha_\beta_1\). These data suggest a possible physiological role for the divalent cations, Mg\(^{2+}\) and Ca\(^{2+}\), in the modulation of integrin-mediated cell migration.

**Affinity Chromatography**

W138 cells were detached with 4 mM EDTA and washed with PBS. The final cell pellet (06 ml) was resuspended in PBS and labeled with 3 mCi of \(^{125}\)Iodo-iodide according to the lactoperoxidase method (Lehien et al., 1982). The reaction was stopped with DME containing 0.02% sodium azide, and the cells were washed with cold PBS. The cells were extracted at 4°C for 20 min in TBS containing 100 mM octyl glucopyranoside (Cal- biochem Corp., LaJolla, CA) and 2 mM PMSF (Sigma Chemical Co., St. Louis, MO). The lysate was centrifuged at 12,000 g for 20 min. The supernatant was supplemented with 3 mM Mg\(_\text{Cl}_2\) and applied onto bovine type I collagen-Sepharose prepared by coupling the protein to cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions (Sigma Chemical Co.). After 2 h of incubation, the column was washed with 20 bed volumes of TBS containing 30 mM octyl glucopyranoside and 3 mM Mg\(_\text{Cl}_2\). The column was eluted with 3 mM Ca\(_\text{Cl}_2\) and subsequently with 10 mM EDTA in TBS containing 50 mM octyl glucopyranoside and the fractions analyzed by 7.5% SDS-PAGE under nonreducing conditions and autoradiography.

**Immunoprecipitation**

To perform immunoprecipitations, mAbs were absorbed overnight at 4°C onto anti-mouse IgG-agarose (Sigma Chemical Co.). The beads were washed with TBS containing 50 mM octyl glucopyranoside, 1 mM Mg\(_\text{Cl}_2\), 1 mM Ca\(_\text{Cl}_2\), and 1 mM PMSF (wash buffer) and added to receptor fractions. After incubation for 6 h at 4°C the beads were washed five times with the wash buffer described above, added to SDS-PAGE sample buffer, boiled, and the eluted material was analyzed by SDS-PAGE on 7.5% gels under nonreducing conditions. The gels were dried and exposed to x-ray film (X-OMAT AR; Eastman Kodak Co., Rochester, NY).

**Migration Assays**

Migration assays were conducted using the modified Boyden chamber as previously described (Banai et al., 1990). Briefly, the chamber consists of two compartments separated by a filter, and migration is measured by counting the number of cells crossing the membrane through pores of defined size. Lower chambers were filled with modified, serum-free DME without Ca\(^{2+}\), Mg\(^{2+}\), or PO\(_4^{3-}\) (Gibco Laboratories, Grand Island, NY). Various Ca\(_\text{Cl}_2\), Mg\(_\text{Cl}_2\), and/or EGTA concentrations were then added along with 20 ng/ml PDGF (Gibco Laboratories). 10-μm-pore polycarbonate membrane filters (Poretics Corp., Livermore, CA) that had been previously washed with water, the attached cells on the upper side of the filter were removed for 3 h at 37°C. After the incubation period, the upper chamber was removed and the filter was fixed in 3% paraformaldehyde and stained with 0.5% toluidine blue in 3.7% formaldehyde. Excess stain was washed away with water, the attached cells on the upper side of the filter were removed and the migrated cells on the underside were quantitated by counting two high-powered fields (at a magnification of 200) per well.

**Results**

**Ca\(^{2+}\) Reverses the Mg\(^{2+}\)-dependent, \(\alpha_\beta_1\)-mediated Attachment of Fibroblasts to Type I Collagen Substrates**

The integrin profile of W138 human fibroblasts has been shown by immunoprecipitation to include \(\alpha_\beta_1\) (Vogel et al., 1990). When W138 cells were tested for their attachment activity to bovine type I collagen in the presence of increasing concentrations of either Mg\(^{2+}\) or Ca\(^{2+}\), we found that they adhered well in the presence of Mg\(^{2+}\) alone but not at all in the presence of Ca\(^{2+}\) alone (not shown). mAb P1E6, directed against the \(\alpha_2\) integrin subunit, inhibited this Mg\(^{2+}\)-dependent attachment in a concentration-dependent manner.
Figure 1. The effects of Ca\(^{2+}\) and Mg\(^{2+}\) on the cell detachment (A) or attachment (B) of cells to surfaces coated with bovine type I collagen after initial incubation with the alternate cation. 3.0 x 10\(^4\) WI38 cells were added to each well of microtiter plates coated with 10 \(\mu\)g/ml bovine type I collagen and incubated for 45 min at 37°C in the presence of either 2.5 mM Mg\(^{2+}\) or 2.5 mM Ca\(^{2+}\). After the initial incubation a titration of the alternate cation was added to the wells and reincubated for another 45 min. Attached cells were fixed with 3% paraformaldehyde and stained with 0.5% toluidine blue in 37% formaldehyde. The cells were then solubilized with 2% SDS and quantitated by measuring the absorbance at 600 nm. The results are the mean ± SD of three experiments done in triplicate.

Grzesiak et al. Divalent Cation Regulation of \(\alpha_\beta_1\)-mediated Migration

Ca\(^{2+}\) Specifically Elutes the \(\alpha_\beta_1\) Integrin from Bovine Type I Collagen-Sepharose

To study this apparent reversibility in cation-dependent adhesion further, surface \(^{125}\)I-labeled WI38 membrane extracts supplemented with 3 mM Mg\(^{2+}\) were chromatographed over bovine type I collagen-Sepharose and the column was subsequently eluted with 3 mM Ca\(^{2+}\). As shown in Fig. 2 A, all of the bound integrin was eluted from the column with Ca\(^{2+}\), and subsequent elution with 10 mM EDTA released no additional receptor. Fig. 2 B shows by immunoprecipitation that the integrin eluted was indeed \(\alpha_\beta_1\). After extensive dialysis back into 3 mM Mg\(^{2+}\), this purified receptor rebound to the column and was again eluted with Ca\(^{2+}\) (not shown). In a reverse experiment, where WI38 extracts supplemented with 3 mM Ca\(^{2+}\) were chromatographed over GRGDSPK-Sepharose, cry/\(\beta_3\) bound to the column and could not be eluted with 3 mM Mg\(^{2+}\). Elution could only be achieved with EDTA (not shown).

To confirm that the elution of \(\alpha_\beta_1\) was due to the addition of Ca\(^{2+}\) and not the removal of Mn\(^{2+}\), we conducted two additional experiments. Mg\(^{2+}\)-supplemented WI38 extracts were loaded onto a type I collagen-Sepharose column and eluted first with Mn\(^{2+}\), then Ca\(^{2+}\). Again, all of the integrin was released with Ca\(^{2+}\) while none was eluted with Mn\(^{2+}\) (data not shown). We also loaded WI38 extracts supplemented with 1 mM Mg\(^{2+}\) onto a type I collagen-Sepharose column and eluted with 4 mM Mg\(^{2+}\), 3 mM Mg\(^{2+}\) plus 1 mM Ca\(^{2+}\), and finally EDTA. The change to 4 mM Mg\(^{2+}\) caused no integrin to be released. However, when 3 mM Mg\(^{2+}\) plus 1 mM Ca\(^{2+}\) was introduced, the integrin was eluted, clearly indicating that Ca\(^{2+}\) reverses the Mg\(^{2+}\)-dependent binding of \(\alpha_\beta_1\) to type I collagen (not shown). It is noteworthy that the detachment by Ca\(^{2+}\) of cells previously bound in Mg\(^{2+}\) (Fig. 1 A) correlates well with this affinity chromatography result in that Ca\(^{2+}\) appears to weaken the Mg\(^{2+}\)-dependent integrin-ligand interactions. In other exper-
Figure 2. Affinity chromatography of extracts of I25-I-surface-labeled WI38 cells on bovine type I collagen-Sepharose. Extracts of surface-iodinated WI38 cells were supplemented with 3 mM MgCl2 and applied onto a 1.0 ml collagen-Sepharose column. After washing with wash buffer (see Materials and Methods) containing 3 mM Mg2+, the column was eluted with 3 mM Ca2+ followed by 10 mM EDTA (A). The eluted fractions were analyzed on a 7.5 % SDS-polyacrylamide gel under nonreducing conditions followed by autoradiography. Eluted material from Lanes 2-4 was used in immunoprecipitation experiments using mAbs P1H5 against α2, P1B5 against α3, 147 against α1, and 8E6 against vitronectin (B). The immunoprecipitates were separated by SDS-PAGE (7.5 %) under nonreducing conditions, and protein bands were visualized by autoradiography. The molecular mass markers were myosin (200 kD), phosphorylase b (97 kD), and BSA (67 kD). The positions of the subunits α2 and β1 are indicated.

The attachment/detachment data together with the affinity chromatography results suggested that Ca2+ and Mg2+ could be involved in the modulation of integrin function, such as in mediating a cellular process like migration. In modified Boyden chamber migration assays, we found that while WI38 fibroblasts were migratory on type I collagen in Mg2+ and not in Ca2+ (Fig. 3 A), a twofold enhancement of migration was observed when these cations were used in combination, with maximum migration observed when Mg2+/Ca2+ ratios were higher than one (Fig. 4, A and B). Specifically, Fig. 4 B shows that in the presence of 1.5 mM Mg2+ without Ca2+, migration is about half that observed when optimal combinations of the two cations are used. As a control, WI38 cells migrated on vitronectin substrates in the presence of Ca2+ alone but, surprisingly, not in Mg2+ (Fig. 3 B), indicating that the β1 integrin has different cation requirements for the promotion of migration versus adhesion (Kirchhofer et al., 1991). It should be noted that maximal migratory activity is achieved at significantly lower combined divalent cation concentrations (<4 mM) than maximal adhesion (5–10 mM) in a single cation. Fig. 5 shows a relative comparison between the maximum fibroblast migration observed on type I collagen substrates in Ca2+ alone (Fig. 5 A), Mg2+ alone (Fig. 5 B), and in combination with slight excess of Mg2+ (Fig. 5 C).

Because migration was enhanced when the two cations were present together, we questioned whether the migration observed in the presence of Mg2+ alone is dependent on the efflux of Ca2+ from the cell. This seems unlikely because no migration was observed without the addition of cations, even after a 3 h incubation on either type I collagen or the control ligand, vitronectin, which exhibits migratory activity in Ca2+ alone (Fig. 3 B). Moreover, the addition of 5 mM EGTA to wells containing 3 mM Mg2+ reduced fibroblast migration on type I collagen substrates by only ~25% of that observed in the presence of 3 mM Mg2+ alone, but completely abolished migration on vitronectin substrates in the presence of 3 mM Ca2+ (data not shown). Some nonspecific chelation of Mg2+ by EGTA is the likely explanation for the observed reduction in migration on collagen.

Antibodies Directed Against the Integrin α2 and β1 Subunits Inhibit Fibroblast Migration on Type I Collagen

mAbs directed against various integrin subunits were tested for inhibitory activity of migration under the optimal conditions of 2.5 mM Mg2+/1.5 mM Ca2+ (Fig. 4 A). As shown in Fig. 6, P1H5 and P1E6, directed against the α2 subunit, and A1B2, directed against the β1 subunit, essentially completely inhibited WI38 fibroblast migration on type I collagen. No inhibition was observed in the presence of equivalent concentrations of PIB5 directed against the α3 subunit, BIIG2 directed against the α2 subunit or a polyclonal antiserum directed against α2β3. In control migration studies on vitronectin, laminin, and fibronectin, we determined that the antibodies showing no inhibition on type I collagen were indeed functional blockers on their respective ligand (not shown). Thus, it appears that the integrin α2β1 is the target of the divalent cation effects described in this study.
Figure 3. Effects of Ca\(^{2+}\) and Mg\(^{2+}\) on fibroblast migration through ligand coated filters. Using modified Boyden chambers, 3.0 \(\times\) 10\(^4\) WI38 cells were added to each upper chamber and allowed to migrate, through filters of defined pore size (10 \(\mu\)m), towards PDGF in the lower chamber for 3 h at 37°C in the presence of various concentrations of Ca\(^{2+}\) (○) or Mg\(^{2+}\) (●). The filters were coated with 10 \(\mu\)g/ml bovine type I collagen (A) or vitronectin (B). After incubation, filters were fixed in methanol and stained with Diff-Quik Solution II (Scientific Products, McGaw Park, IL). After rinsing, adherent cells were removed from the upper side of the filter and migrated cells on the underside were counted by taking the mean of two high-powered fields (magnification of 400x) per well using an inverted, light microscope (model CK2; Olympus Corp., Lake Success, NY). The results represent the mean ± SD of three experiments done in triplicate. Migration on type I collagen and the control, vitronectin, are compared with each other and all values are expressed as a percentage of maximum migration (68 cells ± SD/high-powered field), which was observed on type I collagen. No migration was observed in the absence of divalent cations.

Discussion

Evidence derived from studies of integrin-ligand interactions using integrin-mediated cell adhesion, affinity chromatography, and modified Boyden chamber migration assays demonstrate that Ca\(^{2+}\) can reverse the Mg\(^{2+}\)-dependent, \(\alpha_5\beta_1\)-mediated adhesion and dramatically affect the migration of fibroblasts to type I collagen substrates. That WI38 cells were still capable of Mg\(^{2+}\)-dependent adhesion after preincubation with Ca\(^{2+}\) alone suggests that exposure to Ca\(^{2+}\) is not detrimental to the cell or receptor. We also demonstrate that Ca\(^{2+}\) can specifically elute the fibroblast \(\alpha_5\beta_1\) integrin from a type I collagen-Sepharose column when it is bound in the presence of Mg\(^{2+}\). Interestingly, while WI38 cells migrate on type I collagen in the presence...
participate with the proposed cation binding sequences on wards et al., 1988; Loftus et al., 1990; Dransfield et al., environments (Kirchhofer et al., 1991). We and others (Ed-
two receptors function in different extracellular cation en-
vironments. It has also been proposed that CT3fl~ can be
of Mg 2+ alone and not in Ca 2+ alone, a combination of Mg 2+
and Ca 2+ with Mg 2+ in a slight excess caused a twofold en-
hancement of migration. Taken together, these results sug-
gest that the relative concentrations of extracellular Mg 2+
and Ca 2+ could be involved in the regulation of the function
of this integrin and could influence the migratory behavior
of fibroblasts. It has also been proposed that α3β1, can be
regulated by a shift in divalent cation concentration (Elices
et al., 1991). These data, together with our results demon-
strating that Ca 2+ can also elute α3β1 and α3β1 bound to
ligand in Mg 2+ (unpublished observations), may reflect a
general inhibitory effect of Ca 2+ on the function of β1 inte-
grins and indicate that Mg 2+/Ca 2+ ratios may critically
affect the binding function of β1 integrins.

In recent studies using α3β1 and α3β1, two integrins shar-
ing a common α subunit, we were surprised to find that the
two receptors function in different extracellular cation en-
vvironments (Kirchhofer et al., 1991). We and others (Ed-
wards et al., 1988; Loftus et al., 1990; Dransfield et al.,
1990) have proposed that residues on the β subunit might
participate with the proposed cation binding sequences on
the α subunit to provide the sixth coordination site (-Z) for
divalent cation binding. Recent crystallographic studies of
the cation-binding loops (EF loops or hands) in parvalbum-
min, demonstrate that cations with an ionic radius closest to
1 Å are favored thermodynamically. Those with smaller
ionic radii are more constraining, and thus require more
energy. Of all the physiological cations, Ca 2+ comes closest
with an ionic radius of 0.94 Å, and this accounts for its
demonstrated higher affinity binding in these cation-binding
loops (Lehky et al., 1977; Pechère, 1977; Wnuk et al.,
1982). Mg 2+, however, with an ionic radius of 0.65 Å, also
binds in these loops and crystallographic data show that the
coordination spheres of parvalbumin that contain these
cation-binding domains contract and become more con-
strained in the presence of Mg 2+ when compared with Ca 2+
(Declercq et al., 1991). If one assumes that the same cation-
dependent conformational changes occur in the cation-
binding domains of integrins, and involvement of the β
subunit in at least one of these domains is critical for ligand
binding, a mechanism based on fluctuations in the relative
concentrations of Mg 2+ and Ca 2+, resulting in changes in
the affinity of integrins, could explain how their function is
up- and downregulated during a cellular process such as
migration.

Under normal physiological conditions, the extracellular
environment has about a 1.5 mM higher concentration of
Ca 2+ than Mg 2+ (Olinger, 1989). Our in vitro results suggest
that fibroblasts are capable of a certain level of α3β1-
mediated adhesion (Fig. 1, A and B) and migration (Fig. 4,
A and B) on type I collagen under these conditions. However,
our data also suggest that the potential exists for a twofold
increase in this activity. It is not obvious whether sufficient
Mg 2+ could be recruited to affect the extracellular Mg 2+
concentration. Interestingly, the concentration of intracellular
Mg 2+ in the typical mammalian cell is reported to be be-
tween 15 and 30 mM while intracellular Ca 2+ is only about
1–2 mM (Polimeni and Page, 1973; Henrotte, 1988; Caddell
and Reed, 1989; Alberts et al., 1989). In the case of tissue
injury, for example, it is possible that a local increase in the
extracellular Mg 2+ levels might occur as the damaged tissue
releases its cellular contents. One could speculate that such
an increase in extracellular Mg 2+ might stimulate strong
platelet adhesion to collagen through α3β1 (Santoro, 1986).
A Mg 2+ gradient, set up locally from the site of injury,
along with growth factors released from the platelet, may
then provide the stimulus and directional signaling necessary
to mobilize fibroblasts and other cells required for a success-
ful wound healing response. This mechanism appears plausi-
ble in light of the results showing that even subtle changes
in this cation ratio can dramatically affect a cell's ability to
migrate on type I collagen.

Another type of regulation which could influence an
integrin-mediated wound migratory response might be fac-
tors that deplete extracellular Ca 2+ levels. Ca 2+-binding pro-
tins released from platelets or other cells might serve lo-
cally as Ca 2+ chelators. It is well known that transmembrane
Ca 2+ fluxes via voltage-gated Ca 2+ channels in the plasma
membrane are associated with amoeboid cell movement
(Cooper and Schliwa, 1988). A recent report (Fujimoto et
al., 1991) suggests that a GPllb-llla complex-associated
Ca 2+ channel facilitates extracellular Ca 2+ influx across the
plasma membrane of platelets after thrombin stimulation.
Whether resulting from increases in extracellular Mg 2+, de-
Figure 6. Effects of inhibitory anti-integrin monoclonal/polyclonal antibodies on WI38 fibroblast migration on type I collagen. Migration was determined and quantitated as described in Fig. 3 in the presence of 2.5 mM Mg\(^{2+}\)/1.5 mM Ca\(^{2+}\) and the indicated purified antibody concentrations. 100% (mean = 150 cells ± SD/high-powered field) is defined as that observed with 2.5 mM Mg\(^{2+}\)/1.5 mM Ca\(^{2+}\) without any antibody. The results represent the mean ± SD of two experiments done in duplicate. No migration was observed in the absence of divalent cations.

Increases in extracellular Ca\(^{2+}\), or a combination of both, it seems possible that alterations of the extracellular cation environment can occur, and that these changes can influence the behavior of cells during an integrin-mediated migratory response.

Indirect support for such speculation has been documented where, in cases of trauma, such as severe burns, spinal cord injury, and myocardial ischemia, there is an increased requirement for extracellular Mg\(^{2+}\) during recovery from these injuries, and internal mechanisms are established in an attempt to maintain elevated extracellular Mg\(^{2+}\) levels even at the expense of healthy adjacent tissue (Hearse et al., 1978; Cunningham et al., 1987; Demediuk et al., 1990). A report illustrating that capillary endothelial cell proliferation and migration, both important for revascularization after trauma, are enhanced in the presence of elevated extracellular Mg\(^{2+}\) (Banai et al., 1990) suggests that these extracellular Mg\(^{2+}\) increases may provide a physiological stimulus for endothelial cell migration. Our data demonstrate that the same appears to be true for fibroblasts which are a predominant cell type responsible for re-establishing the extracellular matrix in wound areas (Clark, 1990). Support for this hypothesis is also suggested in a report demonstrating that increased extracellular Ca\(^{2+}\) significantly inhibits both keratinocyte chemotaxis and adhesion on type I collagen. Using an in vivo rat model, they demonstrated further that topical Ca\(^{2+}\) significantly delays wound contraction characteristic of a chronic or impaired wound, though the correlation to integrin function was not made (Sank et al., 1989). However, recent inhibitory mAb studies suggest that α\(5β1\) appears to be the integrin responsible for migration of the human keratinocyte cell line HaCaT on type I collagen (Scharffetter-Kochanek et al., 1992), and this integrin may account for the cation effects observed in the system described above. Finally, the leukocyte β\(3\) integrins appear to undergo conformational changes which stimulate their ligand binding functions (Springer, 1990). In one of these studies, a mAb was defined which reacted with all three β\(3\) integrins only after leukocyte activation and only in the presence of Mg\(^{2+}\) (Dransfield and Hogg, 1989). This work raised the possibility that activation may result in integrin conformational changes which allow Mg\(^{2+}\) binding or that Mg\(^{2+}\) binding is required for the conformational changes to occur.

Together, these findings suggest that during injury a resulting shift in the relative concentrations of extracellular Ca\(^{2+}\) and Mg\(^{2+}\) could facilitate and/or enhance a potential integrin-mediated wound migratory response. As the injury is repaired and the extracellular environment is normalized, the entire mechanism could downregulate, and the Mg\(^{2+}\)-dependent integrins would return to their normal physiological state. Such sensitivity to even subtle changes in the ratio between these two extracellular divalent cations may act to control the migration of cells such as fibroblasts, keratinocytes, leukocytes, and endothelial cells, all of which play crucial roles in a successful wound healing response.

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Grzesiak et al. Divalent Cation Regulation of αβ1-mediated Migration 1117