PLCβ3 mediates cortactin interaction with WAVE2 in MCP1-induced actin polymerization and cell migration

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ABSTRACT
Monocyte chemotactic protein 1 (MCP1) stimulates vascular smooth muscle cell (VSMC) migration in vascular wall remodeling. However, the mechanisms underlying MCP1-induced VSMC migration have not been understood. Here we identify the signaling pathway associated with MCP1-induced human aortic smooth muscle cell (HASMC) migration. MCP1, a G protein–coupled receptor agonist, activates phosphorylation of cortactin on S405 and S418 residues in a time-dependent manner, and inhibition of its phosphorylation attenuates MCP1-induced HASMC G-actin polymerization, F-actin stress fiber formation, and migration. Cortactin phosphorylation on S405/S418 is found to be critical for its interaction with WAVE2, a member of the WASP family of cytoskeletal regulatory proteins required for cell migration. In addition, the MCP1-induced cortactin phosphorylation is dependent on PLCβ3-mediated PKCδ activation, and siRNA-mediated down-regulation of either of these molecules prevents cortactin interaction with WAVE2, affecting G-actin polymerization, F-actin stress fiber formation, and HASMC migration. Upstream, MCP1 activates CCR2 and Gαq/11 in a time-dependent manner, and down-regulation of their levels attenuates MCP1-induced PLCβ3 and PKCδ activation, cortactin phosphorylation, cortactin–WAVE2 interaction, G-actin polymerization, F-actin stress fiber formation, and HASMC migration. Together these findings demonstrate that phosphorylation of cortactin on S405 and S418 residues is required for its interaction with WAVE2 in MCP1-induced cytoskeleton remodeling, facilitating HASMC migration.

INTRODUCTION
Cell migration plays an essential role in the development of organisms, repairing tissues, and defending against pathogens (Mitchison and Cramer, 1996; Stupack et al., 2000). However, cell migration contributes to several pathological processes as well. To cite a few examples, migration of tumor cells, synovial fibroblasts, leukocytes, and smooth muscle cells are involved in tumor metastasis, rheumatoid arthritis, atherosclerosis, and neointimal hyperplasia, respectively (Østerud and Bjørklid, 2003; Yamaguchi et al., 2005; Gerthoffer, 2007; Lefevre et al., 2009). Vascular smooth muscle cell (VSMC) migration is an abnormal phenomenon underlying atherogenesis and intimal hyperplasia after angioplasty, vascular stent implantation, and organ transplantation (Clowes et al., 1989; Gerthoffer, 2007). A plethora of molecules produced at the site of vascular injury by dysfunctional endothelium or inflammatory cells are involved in the modulation of VSMC migration (Berk, 2001). Monocyte chemotactic protein 1 (MCP1; also known as CCL2), in addition to its ability to attract monocytes, memory T-cells and dendritic cells in the mediation of inflammation (Carr et al., 1994; Xu et al., 1996), also exhibits potent chemotactic activity toward VSMCs (Singh et al., 2012; Kundumani-Sridharan et al., 2013). MCP1 binds to CCR2 and CCR4, which belong to the superfamily of G protein–coupled receptors (GPCRs), and transmits the cues from cell surface to inside the cell (Craig and Loberg, 2006; Deshmane et al., 2009). Vascular smooth muscle cell (VSMC) migration is an abnormal phenomenon underlying atherogenesis and intimal hyperplasia after angioplasty, vascular stent implantation, and organ transplantation (Clowes et al., 1989; Gerthoffer, 2007). A plethora of molecules produced at the site of vascular injury by dysfunctional endothelium or inflammatory cells are involved in the modulation of VSMC migration (Berk, 2001). Monocyte chemotactic protein 1 (MCP1; also known as CCL2), in addition to its ability to attract monocytes, memory T-cells and dendritic cells in the mediation of inflammation (Carr et al., 1994; Xu et al., 1996), also exhibits potent chemotactic activity toward VSMCs (Singh et al., 2012; Kundumani-Sridharan et al., 2013). MCP1 binds to CCR2 and CCR4, which belong to the superfamily of G protein–coupled receptors (GPCRs), and transmits the cues from cell surface to inside the cell (Craig and Loberg, 2006; Deshmane et al., 2009). MCP1 and its receptor CCR2 play a role in the pathogenesis of atherosclerosis (Boring et al., 1998; Gu et al., 1998) and intimal...
hyperplasia (Roque et al., 2002), although the downstream signaling events of the MCP1–CCR2 axis are not well studied.

Cell migration requires spatial and temporal coordination of cytoskeletal proteins leading to actin polymerization, and actin polymerization is mediated by a large number of actin-binding proteins (Vicente-Manzanares et al., 2005). Among them, actin-related protein 2/3 (Arp2/3), cortactin, Wiskott–Aldrich syndrome proteins (WASP and N-WASP), and verprolin homologous protein 2 (WAVE2) play important roles in actin polymerization, which, in turn, facilitates cell migration (Yamaguchi and Condeelis, 2007). Arp2/3 complex is the major component of actin nucleation and mediates actin polymerization by interactions with cortactin, N-WASP, and WAVE2 (Mullins and Pollard, 1999; Suetsugu et al., 2001; Urano et al., 2001). Whereas N-WASP plays a role in filopodium formation (Miki et al., 1998a), WAVE2 plays a role in lamellipodium formation required for cell migration (Miki et al., 1998b; Takahashi and Suzuki, 2009). Cortactin was initially identified as a Src substrate and later found to be a nucleation promoting factor (Wu et al., 1991; Urano et al., 2001). The basic structure of cortactin comprises an N-terminal acidic (NTA) region, actin-binding repeats, a proline-rich region, and an SH3 domain at the C-terminal end. The NTA domain of cortactin interacts directly with Arp2/3 to activate the Arp2/3 complex and facilitates cell migration by enhancing lamellipodium formation and adhesion assembly (Weed et al., 2000; Bryce et al., 2005). The fourth actin-binding repeat of cortactin interacts with F-actin to facilitate lamellipodium formation (Weed et al., 2000). In addition, cortactin reinforces the interaction of Arp2/3 complex with F-actin by maintaining F-actin networks from undergoing depolymerization (Weaver et al., 2001). The SH3 domain present in the C-terminus binds to proteins such as N-WASP or WASP-interacting protein, which promote actin nucleation and hence actin polymerization (Kinley et al., 2003; Martinez-Quiles et al., 2004). The potential of cortactin to interact with several cytoskeletal and signaling proteins through its SH3 domain also links this protein to various cellular processes, including invadopodium and lamellipodium formation (Bryce et al., 2005; Artyom et al., 2006). The capacity of cortactin to interact with many of these cytoskeletal and signaling proteins appears to be regulated by its posttranslational modifications. It was reported that phosphorylation at the Y421, Y470, or Y486 residue in the proline-rich domain is required for its role in the development of lamellipodial protrusions and cell migration (Huang et al., 1998; Wang et al., 2011). On the other hand, phosphorylation at S405 and S418 by Pak1 and Erks (Grassart et al., 2010; Kelley et al., 2010) is required for its interaction with N-WASP in activating the Arp2/3 complex, leading to actin polymerization and lamellipodium formation (Martinez-Quiles et al., 2004; Kelley et al., 2010). Cortactin can also be phosphorylated at S113 by Pak3 and S298/348 by protein kinase D (Webb et al., 2006; Eiseler et al., 2010). However, although S113 and S298 phosphorylations of cortactin have been shown to exert a negative effect on actin polymerization and cell migration, the role of S348 phosphorylation is unclear (Webb et al., 2006; Eiseler et al., 2010). In addition to phosphorylation, deacetylation of cortactin by HDACs is required for its interaction with F-actin and cell migration (Zhang et al., 2007, 2009). In previous studies, we reported that MCP1 mediates VSMC migration and proliferation (Potula et al., 2009; Singh et al., 2011). We also reported that MCP1 induces HASMC F-actin stress fiber formation, migration, and proliferation via activation of NFATc1-mediated cyclin D1-CDK6-PKN1-CDK4-PAK1 signaling (Kundumani-Sridharan et al., 2013; Singh et al., 2012). However, the upstream signaling events of MCP1 in the modulation of HASMC migration are not known. Therefore, in the present study, we examined the role of cortactin in the mechanisms by which MCP1 induces HASMC migration. Here we show that MCP1, via activation of CCR2, Gαq/11, phospholipase Cβ3 (PLCβ3), and protein kinase Cα (PKCα), stimulates phosphorylation of cortactin at S405 and S418, leading to its interaction with WAVE2 and promoting actin polymerization and HASMC migration.

RESULTS

Role of cortactin phosphorylation in MCP1-induced HASMC migration

Cortactin is an actin nucleation–promoting factor and a crucial molecular scaffold for actin assembly and organization, which are necessary for endocytosis, cell migration, and invasion (MacGrath and Koleske, 2012). Serine phosphorylation of cortactin is important for cancer cell migration (Kruchten et al., 2008). Therefore, to understand the mechanism of MCP1-induced HASMC migration, we tested the role of cortactin. MCP1 induces serine/threonine phosphorylation of cortactin in a time-dependent manner (Figure 1A). The S113, S298, S348, S405, and S418 residues are potential sites of phosphorylation in cortactin (MacGrath and Koleske, 2012). To determine which of these serine residues are phosphorylated, we mutated each of these serine residues to alanine by site-directed mutagenesis and tested their effects on MCP1-induced cortactin phosphorylation. Whereas S113A, S298A, and S348A mutants had no effect, the S405A and S418A mutants reduced MCP1-induced cortactin phosphorylation, suggesting that MCP1 induces phosphorylation of cortactin at S405 and S418 residues in HASMCs (Figure 1B). To determine whether cortactin mediates actin polymerization by MCP1, we immunoprecipitated cortactin from control and 2-h MCP1–treated cells and tested its effect on G-actin polymerization in vitro. The anti-cortactin immunoprecipitates of MCP1-treated cells exhibited increased G-actin polymerization compared with untreated cells (Figure 1C). In addition, overexpression of S405A and S418A mutants of cortactin prevented MCP1-induced G-actin polymerization (Figure 1D). Consistent with these observations, overexpression of S405A/S418A mutants of cortactin also attenuated HASMC F-actin stress fiber formation and migration (Figure 1, E and F), suggesting that phosphorylation of cortactin on S405 and S418 residues is required for MCP1-induced HASMC migration. To validate these findings, we also tested the effect of cortactin small interfering RNA (siRNA) on MCP1-induced HASMC F-actin stress fiber formation and migration and found that downregulation of cortactin levels prevents HASMC F-actin stress fiber formation and migration (Figure 1, G and H).

Cortactin phosphorylation at S405 and S418 residues is required for its interaction with WAVE2

Although cortactin plays an important role in actin nucleation and polymerization, it requires interactions with other cytoskeletal proteins in mediating these effects. Toward this end, we studied its interactions with WAVE2, which also plays an important role in cell migration (Yamazaki et al., 2003). Coinmunoprecipitation experiments showed that cortactin interacts with WAVE2 in a time-dependent manner (Figure 2A). To determine the role of cortactin phosphorylation in its interaction with WAVE2, we tested the effects of S405A and S418A mutants. Overexpression of S405A/S418A mutants of cortactin prevented its interaction with WAVE2 by MCP1 (Figure 2B). In addition, siRNA-mediated depletion of WAVE2 inhibited MCP1-induced G-actin polymerization, F-actin stress fiber formation, and HASMC migration (Figure 2, C–E). These observations suggest that cortactin plays an important role in MCP1-induced HASMC migration via its interaction with WAVE2, promoting G-actin polymerization and F-actin stress fiber formation.
PKCδ mediates cortactin phosphorylation in MCP1-induced HASMC migration
PKCs are key intracellular enzymes activated by calcium and diacylglycerol (DAG), alone or in combination, or independent of both of these molecules (Mellor and Parker, 1998). To determine the mechanisms underlying MCP1-induced cortactin phosphorylation, we tested the time-course effect of MCP1 on activation of conventional (α, β, γ) and novel (ε, η, θ) PKCs. MCP1 induced phosphorylation of PKCα, PKCε, and PKCθ in a time-dependent manner in HASMCs (Figure 3A). Because the time course of PKCθ and PKCε phosphorylation correlated with the time course of cortactin phosphorylation, we next focused on the role of these novel PKCs in MCP1-induced cortactin phosphorylation. Adenovirus-mediated expression of dnPKCθ blocked MCP1-induced cortactin phosphorylation and its interaction with WAVE2 (Figure 3, B and C). On the other hand, adenovirus-mediated expression of dnPKCε had no effect on MCP1-induced cortactin phosphorylation (Figure 3B). These findings suggest that PKCθ but not PKCε mediates MCP1-induced cortactin phosphorylation. Consistent with these observations, adenovirus-mediated expression of dnPKCθ also attenuated MCP1-induced G-actin polymerization, F-actin stress fiber formation, and HASMC migration (Figure 3, D–F). To confirm the role of PKCθ in MCP1-induced cortactin phosphorylation and its interaction with WAVE2, we used the siRNA approach as well. Down-regulation of PKCθ levels prevented cortactin phosphorylation and its interaction with WAVE2, which, in turn, hampered cortactin-mediated G-actin polymerization, F-actin stress fiber formation, and HASMC migration (Figure 4, A–E).

Role of PLCβ3 in PKCδ activation and cortactin phosphorylation in MCP1-induced HASMC migration
Many reports suggest that GPCR agonists activate PLCβ3 in mediating their cellular effects (Kelley et al., 2006; Lyon et al., 2014). To test the role of PLCβ3 in MCP1-induced cortactin phosphorylation and actin cytoskeleton remodeling, we first studied the time-course effect of MCP1 on the steady-state levels of PLCβ3 isoforms. MCP1 had no effect on PLCβ3-1 levels, and the presence of PLCβ4 was not detected in HASMCs (Figure 5A). Next, using a fluoregenic PLC substrate, WH-15 (Huang et al., 2011), we studied the effect of MCP1 on their activities. MCP1 induced PLCβ3 but not PLCβ1 or PLCβ2 activities in a time-dependent manner in HASMCs (Figure 5B). Furthermore, depleting PLCβ3 levels by its short hairpin RNA (shRNA) expression plasmid inhibited phosphorylation of PKCθ and cortactin (Figure 5C). Furthermore, down-regulation of PLCβ3 levels using its shRNA suppressed MCP1-induced cortactin–WAVE2 interactions, G-actin polymerization, F-actin stress fiber formation, and HASMC migration (Figure 5, D–G).

CCR2 but not CCR4 activation is required for PLCβ3- and PKCδ-dependent cortactin phosphorylation in MCP1-induced HASMC migration
MCP1 mediates its effects via its receptors CCR2 and CCR4 (Craig and Loberg, 2006). However, in HASMCs, it is not known which of these receptors mediates MCP1-induced cytoskeleton remodeling, leading to their migration. Both CCR2 and CCR4 are present in HASMCs as detected by Western blot analysis, and treatment with MCP1 did not affect their steady-state levels (Figure 6A). To study the role of these receptors in MCP1-induced HASMC migration, we used their antagonists, CCR2A and CCR4A, respectively. CCR2A significantly inhibited MCP1-induced PLCβ3 activity compared with CCR4A (Figure 6B). On the other hand, MCP1-induced PKCδ phosphorylation depended on activation of either CCR2 or CCR4, although preferentially CCR2 (Figure 6C). CCR2A but not CCR4A also prevented MCP1-induced cortactin phosphorylation, cortactin–WAVE2 interactions, G-actin polymerization, F-actin stress fiber formation, and HASMC migration (Figure 6, C–G).

Gaq/11 mediates MCP1-induced PLCβ3- and PKCδ-mediated cortactin phosphorylation and HASMC migration
To understand further how CCR2 mediates the effects of MCP1 on HASMC migration, we studied the role of G proteins. Treatment with MCP1 did not affect the steady-state levels of Gaq, Gα11, Gα12, or Gα13 (Figure 7A). However, MCP1 caused a time-dependent dissociation of Gaq/11 but not Gα12 or Gα13 from CCR2 (Figure 7B), suggesting that MCP1 activates Gaq/11-coupled CCR2 receptors in HASMCs. Silencing Gaq/11 using their siRNAs prevented MCP1-induced PLCβ3 activity (Figure 7C). Down-regulation of Gaq/11 also attenuated MCP1-induced PKCδ and cortactin phosphorylation (Figure 7D). In addition, depletion of Gaq/11 levels inhibited MCP1-induced cortactin–WAVE2 interactions, G-actin polymerization, F-actin stress fiber formation, and HASMC migration (Figure 7, E–H).

Discussion
Many studies have demonstrated that MCP1 is involved in vascular wall remodeling (Schober et al., 2004; Singh et al., 2012; Kundumani-Sridharan et al., 2013). VSMC migration requires cytoskeletal remodeling (Gerthoffer, 2007). In characterizing the role of cytoskeletal remodeling in MCP1-induced VSMC migration, we found that MCP1 induces serine/threonine phosphorylation of cortactin in HASMCs. It was reported that cortactin binds to Arp2/3 and acts as a nucleation-promoting factor to enhance actin polymerization and F-actin formation, facilitating cell migration (Weed et al., 2000; Urino et al., 2001; Weaver et al., 2001). Previous studies showed that phosphorylation of cortactin on S405/S418 is needed for its role in actin polymerization and invadopodium and lamellipodium formation, which are required for cell migration (Ayala et al., 2008; Kelley et al., 2010). In the present study, we found that MCP1 induces phosphorylation of cortactin on S405 and S418 but not on S113, S298, and S348 residues, and overexpression of S405/S418A mutants prevents MCP1-induced G-actin polymerization, F-actin stress fiber formation, and HASMC migration. These observations indicate that cortactin phosphorylation at S405/S418 residues is required for its role in MCP1-induced cell migration. The finding that down-regulation of cortactin levels by its siRNA inhibits G-actin polymerization, F-actin stress fiber formation, and HASMC migration also supports the role of cortactin in HASMC migration. To understand the potential mechanisms underlying the role of cortactin in the regulation of MCP1-induced HASMC migration, we found that cortactin interacts with WAVE2 and promotes actin polymerization. In addition, our results showed that phosphorylation of cortactin at S405/S418 residues is essential for its interaction with WAVE2 in mediating G-actin polymerization, F-actin stress fiber formation, and HASMC migration by MCP1. Furthermore, depletion of WAVE2 levels prevented MCP1-induced G-actin polymerization, F-actin stress fiber formation, and HASMC migration. In addition, other studies showed that the C-terminal verprolin homology, cofilin homology, and acidic region domain of WAVE2 enhances the branching efficiency of actin filaments (Suettsugu et al., 2001). It was also reported that cortactin in concert with N-WASP activates the Arp2/3 complex and enhances G-actin polymerization (Helgeson and Nolen, 2013). Furthermore, phosphorylation of cortactin on S405/S418 is critical for its interaction with N-WASP and to activate the Arp2/3 complex (Martinez-Quiles et al., 2004). On the basis of these findings, along with our observations, we suggest that the
FIGURE 1: Cortactin phosphorylation at S405/S418 is required for MCP1-induced HASMC migration. (A) Quiescent HASMCs were treated with vehicle or MCP1 (50 ng/ml) for the indicated time periods, and cell extracts were prepared. Equal amounts of protein from control and each treatment were analyzed for cortactin phosphorylation by reciprocal immunoprecipitation using anti-cortactin and anti-pSer/Thr antibodies, followed immunoblotting with the indicated antibodies. Nonimmune immunoglobulin G (IgG) was used as a negative control for immunoprecipitation. (B) HASMCs were transfected with green fluorescent protein (GFP)–tagged cortactin expression vector with or without S113A, S298A, S348A, S405A, or S418A mutations, quiesced, and treated with vehicle or MCP1 for 2 h. Equal amounts of protein from control and each treatment were immunoprecipitated with anti-pSer/Thr antibodies, and the immunocomplexes were analyzed by Western blotting using anti-GFP antibodies. (C) Equal amounts of protein from control and various time periods of MCP1-treated cells were immunoprecipitated with anti-cortactin antibodies. After eluting from the immunocomplexes, cortactin was incubated with pyrene-actin monomers, and the rate of actin polymerization was measured as described in Materials and Methods. (D) Cells were transfected with GFP-tagged cortactin expression vector with or without S405A or S418A mutations, quiesced, and treated with vehicle or MCP1 for 2 h. Equal amounts of protein from control and each treatment were immunoprecipitated with anti-GFP antibodies. After release from the immunocomplexes, the GFP-tagged cortactin was analyzed for actin polymerization as described for C. (E) Cells were seeded onto glass coverslips in a six-well
plate, transfected with GFP-tagged cortactin expression vector with or without S405A or S418A mutations, quiesced, treated with vehicle or MCP1 for 6 h, and stained for F-actin stress fiber formation with rhodamine-conjugated phalloidin. The images were captured using an inverted Zeiss fluorescence microscope (AxioObserver Z1) via a 40×/NA 0.6 objective and AxioCam MRm camera without any enhancements, and F-actin levels were quantified using ImageJ software. Scale bar, 50 μm. (F) Cells were seeded into ibidi chambers, transfected with GFP-tagged cortactin expression vector with or without S405A or S418A mutations, quiesced, and subjected to wound-healing cell migration assay in response to vehicle or MCP1 as described in Materials and Methods. (G) Cells were transfected with siControl or siCortactin, quiesced, treated with vehicle or MCP1 for 6 h, and stained for F-actin as described for E. (H) All the conditions were the same as for G, except that cells were subjected to wound-healing cell migration assay in response to vehicle or MCP1. The bar graphs in A, B and E–H represent mean ± SD values of three independent experiments. *p < 0.05 vs. vehicle control or CTTN (WT) or siControl; **p < 0.05 vs. MCP1, CTTN (WT) + MCP1, or siControl + MCP1.
FIGURE 2: Cortactin phosphorylation at S405/S418 is essential for its interaction with WAVE2 in HASMC migration.

(A) Quiescent cells were treated with vehicle or MCP1 for the indicated time periods, and cell extracts were prepared. Equal amounts of protein from control and each treatment were immunoprecipitated with anti-cortactin or anti-WAVE2 antibodies, and the immunocomplexes were analyzed by immunoblotting with the indicated antibodies. Nonimmune IgG was used as a negative control for immunoprecipitation. (B) HASMCs were transfected with GFP-tagged cortactin expression vector with or without S405A or S418A mutations, quiesced, and treated with vehicle or MCP1 for 2 h. Equal amounts of protein from control and each treatment were immunoprecipitated with anti-GFP antibodies, followed by immunoblotting with the indicated antibodies. (C) Cells were transfected with siControl or siWAVE2, quiesced, and treated with vehicle or MCP1 for 2 h, and equal amounts of protein from control and each treatment were immunoprecipitated with anti-cortactin antibodies. After elution from the immunocomplexes, the cortactin was
cortactin–WAVE2 interactions probably intensify the actin branching that is required for MCP1-induced HASMC migration.

In elucidating the upstream mechanisms of cortactin phosphorylation by MCP1, we observed that MCP1 activates PKCδ and PKCε with a time course that overlaps the time course of cortactin phosphorylation. However, adenovirus-mediated expression of dnPKCδ but not dnPKCε inhibited MCP1-induced cortactin phosphorylation at S405/418. Down-regulation of PKCδ levels by its siRNA also inhibited MCP1-induced cortactin phosphorylation. These results indicate that PKCδ but not PKCε mediates MCP1-induced cortactin phosphorylation. Previous reports showed that Pak1, MeK, or Erk5 can phosphorylate cortactin on S405 and S418 residues (Martinez-Quiles et al., 2004; Grassart et al., 2010; Kelley et al., 2010). It was also reported that PKCδ interacts with cortactin and organizes actin dynamics in early endosomes (Liado et al., 2008). Other reports have shown that cortactin is phosphorylated at S113 by Pak3 and at S298/348 by protein kinase D (Webb et al., 2006; Eiseler et al., 2010). However, in our study, MCP1 does not stimulate cortactin phosphorylation at S113, S298, or S348. In addition, our findings reveal that MCP1-induced activation of PKCδ is critical for actin polymerization, F-actin stress fiber formation, and HASMC migration. Other reports have shown that PKCδ by myosin light chain phosphorylation mediates epidermal growth factor–induced fibroblast contractility and motility (Iwabu et al., 2004). PKCδ also promotes smooth muscle cell migration by platelet-derived growth factor–BB (PDGF-BB) or mechanical stress (Li et al., 2003; Kamiya et al., 2007). Another study demonstrated that lysophosphatidic acid induces fibroblast migration via phosphorylation and activation of PKCδ (Gan et al., 2012). In view of all of these findings, we suggest that PKCδ via phosphorylation of cytoskeletal or contractile proteins mediates the migration of various cell types in response to different agonists.

PLCs are widely expressed enzymes that hydrolyze phosphatidylinositol 4,5-bisphosphate and generate inositol 1,4,5-trisphosphate and DAG (Berridge and Irvine, 1989). Because novel PKCs require DAG for their activation, we examined the role of PLCδs in MCP1-induced PKCδ activation. We observed that MCP1 stimulates PLCβ3 activity with a time course that overlaps the time course of PKCδ phosphorylation. We further discovered that PLCβ3, via PKCδ, mediates MCP1-induced cortactin phosphorylation, cortactin/ WAVE2 interactions, G-actin polymerization, F-actin stress fiber formation, and HASMC migration. Previous studies showed that activation of PLCβ2/3 is essential for chemooattractant-mediated regulation of PKCs, and loss of both genes prevented PKC activation and T-cell migration (Li et al., 2000; Bach et al., 2007). On the basis of these observations and present findings, we suggest that various PLCδs, by activating different PKCs, play an important role in the modulation of cell migration.

Many studies suggest that Gαq or Gβγ subunits of Gq/11 heterotrimeric G proteins regulate PLCδs (Smrcka and Sternweis, 1993; Biber et al., 1997). We investigated the role of MCP1 receptors and G proteins in the regulation of PLCβ3 activity. We found that CCR2 but not CCR4 mediates PLCβ3 activity. Although CCR2 couples to various G proteins, including Gq/11, G14/16 and Gi (Araki and Charo, 1996; Kuang et al., 1996), in response to MCP1, only Gαq/11 dissociates from CCR2, indicating that MCP1 activates Gq/11 down-stream of CCR2. Furthermore, like CCR2, down-regulation of Gq/11 completely inhibited MCP1-induced PLCβ3 and PKCδ activation and cortactin phosphorylation, resulting in diminished F-actin stress fiber formation and HASMC migration. Our findings are also consistent with previous reports that Gαq/11 directly activates PLCβ3 (Lyon et al., 2013). A role for Gαq/11 in endothelial and neuronal cell migration has also been demonstrated (Zeng et al., 2002; Ando et al., 2010).

It is important to note that despite the presence of both the receptors, only CCR2 and not CCR4 mediates MCP1-induced cortactin phosphorylation and its interaction with WAVE2 in facilitating G-actin polymerization, F-actin stress fiber formation, and HASMC migration via activation of Gαq/11-PLCβ3-PKCδ signaling. However, CCR4 was partially involved in PKCδ activation by MCP1. Because CCR4 had no major role in HASMC migration, this observation may indicate that the CCR4-dependent PKCδ activation might be linked to other responses of HASMCs to MCP1. It was demonstrated that CCR2 mediates MCP1-induced VSMC migration and proliferation in neointimal development (Roque et al., 2002). In addition, other reports showed that CCR2 mediates MCP1-induced human adventitial and eosinophilic leukemia cell migration (Lee et al., 2009; Si et al., 2012). MCP1 and CCR2 have also been shown to enhance macrophage accumulation and immune responses in the pathogenesis of atherosclerosis (Boring et al., 1998; Charo, 1999; Gu et al., 1999). On the basis of all of these observations and as shown in Figure 8, we speculate that CCR2, upon activation by MCP1, triggers Gαq/11-PLCβ3-PKCδ–dependent cortactin phosphorylation and its interaction with WAVE2 in the modulation of HASMC migration and thereby restenosis.

MATERIALS AND METHODS

Reagents

Anti-CCR4 antibody (AF5207), donkey anti-sheep horseradish peroxidase (HRP) antibody (HA016), and recombinant human MCP1 (279-MC/CF) were from R&D Systems (Minneapolis, MN). CCR2 antagonist (SC-202525), CCR4 antagonist (SC-221406), and anti-actin (SC-11408), anti-Gαq (SC-393), anti-Gα11 (SC-394; SC-390382), anti-Gα12 (SC-409), anti-Gα13 (SC-410), anti-GFP (SC-9996), bovine anti-goat HRP (SC-2350), anti-PKCε (SC-208), anti-PKCγ (SC-211), anti-PKCδ (SC-937), anti-PKCβ (SC-212), anti-PKCε (SC-1681), and anti–β-tubulin (SC-9104) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cortactin (3502), anti-pPKCa/β (9375), anti-pPKCa1 (9379), anti-PKCδ (9374), anti-PKCδ (9377), and anti-WAVE2 (3659) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti–phosphoserine/threonine antibody (ab17464) was bought from Abcam (Cambridge, MA). Goat anti-rabbit HRP (31460) and goat anti-mouse HRP (31437) antibodies were obtained from Thermo Scientific (Waltham, MA). Protein A Sepharose CL-4B (170780-01), Protein G Sepharose Fast flow (17061801), and ECL Western blotting detection reagents (RP2106) were from GE Healthcare (Pittsburgh, PA). Human cortactin siRNA (ON-TARGETplus SMARTpool l-010508-00-0005), human Gαq siRNA (ON-TARGETplus SMARTpool J-008562), human Gαq11...
FIGURE 3: PKCδ mediates MCP1-induced cortactin phosphorylation and its interaction with WAVE2 in HASMC migration. (A) Extracts of control and the indicated time periods of MCP1-treated HASMCs were analyzed for phosphorylation of the indicated PKC isomorph either by Western blotting using their phosphospecific antibodies or immunoprecipitation with anti-pSer/Thr antibody followed by immunoblotting with the indicated anti-PKC isomorph antibody. (B, C) HASMCs that were infected with ad-GFP, ad-dnPKCδ, or ad-dnPKCε and quiesced were treated with vehicle or MCP1 for 2 h, and cell extracts were prepared. Equal amounts of protein from control and each treatment were immunoprecipitated with anti-cortactin antibody, and the immunocomplexes were analyzed by Western blotting for the indicated proteins. Equal amounts of protein from the same cell extracts were also analyzed by Western blotting for GFP, PKCδ, and PKCε levels to show the overexpression of GFP, dnPKCδ, and dnPKCε. (D) All of the conditions were the same as for B, except that equal amounts of protein from control and each treatment were immunoprecipitated with anti-cortactin antibody, and eluted cortactin was assayed for actin polymerization as described in Figure 1C. (E) All of the conditions were the same as for B, except that after quiescence, the cells were subjected to wound-healing migration assay in response to vehicle or MCP1. The bar graphs in A–C, E, and F represent mean ± SD values of three experiments. *p < 0.05 vs. vehicle control or ad-GFP; **p < 0.05 vs. control + MCP1 or ad-GFP + MCP1.
FIGURE 4: Depletion of PKCδ levels inhibited MCP1-induced cortactin phosphorylation and its interaction with WAVE2 in HASMC migration. (A, B) HASMCs that were transfected with control or PKCδ siRNA and quiesced were treated with vehicle or MCP1 for 2 h, and cell extracts were prepared. Equal amounts of protein from control and each treatment were immunoprecipitated with anti-pSer/Thr or anti-cortactin antibodies, and the immunocomplexes were analyzed by Western blotting for the indicated proteins. Equal amounts of protein from the same cell extracts from each condition were also analyzed by Western blotting for PKCδ levels to show the efficacy of the siRNA on its target molecule level. (C) An equal amount of protein from cell extracts prepared as for A was immunoprecipitated with anti-cortactin antibodies, and the cortactin was eluted from the immunocomplexes and assayed for actin polymerization as described in Figure 1C. (D) All of the conditions were the same as for A, except that after quiescence, the cells were treated with vehicle or MCP1 for 6 h and stained for F-actin. (E) All of the conditions were the same as for A, except that after quiescence, the cells were subjected to wound-healing migration assay in response to vehicle or MCP1. The bar graphs in A, B, D, and E represent mean ± SD values of three experiments. *p < 0.05 vs. vehicle control or siControl; **p < 0.05 vs. siControl + MCP1.
FIGURE 5: PLCβ3 acts upstream of PKCδ in MCP1-induced cortactin phosphorylation and its interaction with WAVE2 in HASMC migration. (A) Cell extracts of control and the indicated time periods of MCP1-treated HASMCs were analyzed for PLCβ1-3 levels by Western blotting using their specific antibodies and normalized for β-tubulin levels. (B) The same cell extracts were analyzed for PLCβ activity. (C) G-actin polymerization was measured by F-actin levels. (D) Cortactin and WAVE2 levels were analyzed by Western blotting. (E) Wound closure was measured. (F) Representative images of IB and IP of cortactin and WAVE2 were shown. (G) Wound closure was measured.

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siRNA (ON-TARGETplus SMARTpool J-010860), human PKCδ siRNA (ON-TARGETplus SMARTpool L-000324-00-0005), human WAVE2 siRNA (ON-TARGETplus SMARTpool L-012141-00-0010), and control nontargeting siRNA (D-001810-10) were purchased from Dharmacon RNAi Technologies (Chicago, IL). Lipofectamine 3000 transfection reagent (L3000-015), Hoechst 33342 (H3570), Prolong gold antifade mounting medium (P36930), Medium 231 (M231-500), smooth muscle growth supplements (S-007-25), and gentamicin/amphotericin solution (R-015-10) were purchased from Life Technologies (Carlsbad, CA), anti-CCR2 antibody (NB110-55674) from Novus Biologicals (Littleton, CO), and rhodamine-phaloidin (00027) from Biotium (Hayward, CA).

Cell culture
HASMCs were purchased from Invitrogen (Carlsbad, CA) and subcultured in Medium 231 containing smooth muscle cell growth supplements and 1× gentamicin/amphotericin. The cells were used between four and 10 passages for all of the experiments.

Adenoviral vectors and DNA constructs
Construction of Ad-GFP, Ax-PKC Δ/N (Ad-dnPKCΔ), and Ax-PKC Δ/N (Ad-dnPKCΔ) was described previously (Matsumura et al., 2003; Baijai et al., 2007). pGFp cortactin was a gift from Kenneth Yamada (National Institute of Dental and Cranial Research, National Institutes of Health, Bethesda, MD; Addgene plasmid #50728). The serine (S) residues at 113, 298, 348, 405, and 418 of cortactin were mutated to alanine (A) in pGFp-cortactin mammalian expression vector using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) and the following primers. S113A mutant: forward, 5′-TTTCCAAGCAGTCCGGCGGAAGTTGACTG-3′; reverse, 5′-CGAGCACCACCGCCGACTGTTGAAAAA-3′. S298A mutant: forward, 5′-TGGCCAAAGCCAGGCGCACAGACACAC-3′; reverse, 5′-TGAACGCTCTGGGCTGCCTGGTCTGCTGAC-3′. S348A mutant: forward, 5′-GAAGCTGTTGACCAGCAAAACAGTCATACAGACTAATGCTTTG-3′; reverse, 5′-TCAAGATGATGCTGACATGCTGACAGCTC-3′. S405A mutant: forward, 5′-GCCGCCCTGCTGGCCGGCGACCCT-3′; reverse, 5′-AGTGGCAGCCAGGGCGCCAGG-3′. S418A mutant: forward, 5′-GAGCAGCTCTGGGCTGCTTGAATGAG-3′; reverse, 5′-CTCATAGACGGGGGGCAGGGAGCC-3′. The mutant nucleotides are shown as bold letters. DNA sequence analysis using vector-specific primers verified the mutations. PLCβ3 shRNA expression plasmid was constructed using BLOCK-IT U6 RNA Entry Vector (Life Technologies) following the manufacturer’s instructions. Briefly, single-stranded DNA oligos encoding PLCβ3 shRNA (top strand, 5′-CACCAGAATTCTCCGAAACGTGTCACGGTCAAGAGACGTGACACGTCCTTCGAGGAAATT-3′; bottom strand, 5′-AAAAATTTCCGAAACGTGTCACGGTCAAGAGACGTGACACGTCCTTCGAGGAAATT-3′) were annealed and cloned into pENTR/U6 Δ/N (top strand, 5′-CACCAGAATTCTCCGAAACGTGTCACGGTCAAGAGACGTGACACGTCCTTCGAGGAAATT-3′; bottom strand, 5′-AAAAATTTCCGAAACGTGTCACGGTCAAGAGACGTGACACGTCCTTCGAGGAAATT-3′) were annealed and cloned into pENTR/U6 linear vector with overhangs. The complementary nucleotide overhangs in the primer sequences necessary for directional cloning are shown in bold letters. The positive constructs were confirmed by DNA sequencing. The EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA) was used to purify plasmids for all transfection purposes.

Transfections
HASMCs were transfected with nontargeted control or ON-TARGETplus SMARTpool siRNA at a final concentration of 100 nM using Lipofectamine 3000 transfection reagent according to the manufacturer’s instructions. Wherever appropriate, cells were transfected with plasmid DNAs at a final concentration of 2.5 μg/60-mm culture dish or 5 μg/100-mm culture dish using Lipofectamine 3000 transfection reagent according to the manufacturer’s instructions. After transfections, cells were recovered in complete medium overnight, grown for 36 h in serum-free medium, and used as required.

Cell migration
Cell migration was measured by wound-healing assay using culture inserts (bidi USA, Madison, WI) as described previously (Singh et al., 2015) with minor modifications. Briefly, HASMCs were seeded onto the culture inserts, allowed to grow for 24–48 h and quiesced. Transfections, wherever appropriate, were performed 1 d after seeding and quiesced for 24 h. Cell growth was arrested by the addition of 5 mM hydroxyurea at the start of the experiment. Images were captured at the indicated time points using an inverted microscope (Eclipse TS100; Nikon, Melville, NY) with 4×/numerical aperture (NA) 0.13 objective. Wound closure was measured by subtracting wound area at 24 h from that at 0 h by using ImageJ software (National Institutes of Health, Bethesda, MD). Cell migration was presented as percentage of wound closure.

Immunofluorescence staining
Immunofluorescence staining for F-actin was performed as described previously (Kundumani-Sridharan et al., 2013) with slight modifications. Briefly, HASMCs were seeded onto glass coverslips, allowed to grow for 24–48 h, and quiesced. After appropriate treatments, cells were fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, permeabilized in 0.3% Triton X-100 for 15 min, and blocked with 3% bovine serum albumin (BSA) in PBS. Cells were stained with rhodamine-labeled phalloidin for 1 h at room temperature. After washing with PBS, cells were counterstained with Hoechst 33342 for 5 min at room temperature and mounted onto glass slides with Prolong Gold antifade mounting medium. Fluorescence images were captured using a Zeiss Axioskop system (Carl Zeiss, Jena, Germany) with the appropriate excitation and emission filters.

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cell extracts prepared as for A were also analyzed for PLCβ1-3 activities as described in Materials and Methods. (C, D) HASMCs that were transfected with control or PLCβ3 shRNA plasmids and quiesced were treated with vehicle or MCPI for 2 h, and cell extracts were prepared. Equal amounts of protein from control and each treatment were immunoprecipitated with anti-pSer/Thr or anti-cortactin antibodies, and the immunocomplexes were analyzed by Western blotting for the indicated proteins. Equal amounts of protein from cell extracts prepared as for C were immunoprecipitated with anti-cortactin antibodies, and the cortactin was eluted from the immunocomplexes and assayed for actin polymerization as described in Figure 1C. (F) All of the conditions were the same as for C, except that after quiescence, the cells were treated with vehicle or MCPI for 6 h and stained for F-actin. (G) All of the conditions were the same as for C, except that after quiescence, the cells were subjected to wound-healing migration assay in response to vehicle or MCPI. The bar graphs in B, D, F, and G represent mean ± SD values of three experiments. *p < 0.05 vs. vehicle control or control shRNA; **p < 0.05 vs. control shRNA + MCPI.
FIGURE 6: CCR2 mediates cortactin phosphorylation and its interaction with WAVE2 in HASMC migration.

(A) Quiescent HASMCs were treated with vehicle or MCP1 for the indicated time periods, and cell extracts were prepared. Equal amounts of protein from control and each treatment were analyzed by Western blotting for CCR2 and CCR4 levels using their specific antibodies and normalized to β-tubulin. (B–D) Quiescent cells were treated with vehicle or MCP1 in the presence and absence of CCR2 antagonist (CCR2A) or CCR4 antagonist (CCR4A) for 30 min, and cell extracts were prepared. Equal amounts of protein from control and each treatment were analyzed for PLCβ3 activity, pPKCδ and pCortactin levels, and cortactin–WAVE2 interactions as described for Figures 5B, 3A, 1A, and 2A.
of cells were captured via an inverted Zeiss fluorescence microscope (AxioObserver Z1) with 40×/NA 0.6 objective and AxioCam MRm camera without any enhancements using the microscope operating and image analysis software AxioVision, version 4.7.2 (Carl Zeiss Imaging Solutions, Jena, Germany). The intensity of F-actin staining was measured using ImageJ software.

**Immunoprecipitation**

Cells with and without the indicated treatments were rinsed with cold PBS and lysed in 400 μl of lysis buffer (1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mm sodium orthovanadate in PBS) for 20 min on ice. The cell extracts were cleared by centrifugation at 14,000 rpm for 20 min at 4°C. The cleared cell extracts containing an equal amount of protein from control and each treatment were incubated with appropriate antibody overnight at 4°C, followed by incubation with protein A/G–Sepharose CL4B beads for 3 h with gentle rocking. The beads were collected by centrifugation at 1000 rpm for 2 min at 4°C and washed four times with lysis buffer and once with PBS.

| F | Control | MCP1 |
|---|---------|------|
| **F-actin levels** (fold change) |
| Vehicle | **+** | **+** |
| CCR2A | **+** | **+** |
| CCR4A | **+** | **+** |
| CCR2A + CCR4A | **+** | **+** |

respectively. (E) Cell extracts prepared as for B were analyzed for actin polymerization as describe in Figure 2C. (F) All of the conditions were the same as for B, except that after quiescence, the cells were treated with vehicle or MCP1 for 6 h and stained for F-actin. (G) All of the conditions were the same as for B, except that after quiescence, the cells were subjected to wound-healing migration assay in response to vehicle or MCP1. The bar graphs in A–D, F, and G represent mean ± SD values of three experiments. *p < 0.05 vs. vehicle control; **p < 0.05 vs. MCP1.
**FIGURE 7:** 
Gαq/11 mediates cortactin phosphorylation and its interaction with WAVE2 in HASMC migration.

(A) Quiescent cells treated with vehicle or MCP1 for the indicated time periods, and cell extracts were prepared. Equal amounts of protein from control and each treatment were analyzed by Western blotting for Gαq, Gα11, Gα12, and Gα13 levels using their specific antibodies and normalized to β-tubulin levels. (B) All of the conditions were the same as for A, except that equal amounts of protein from control and each treatment were immunoprecipitated with anti-CCR2 antibodies, and the immunocomplexes were analyzed by Western blotting for the indicated proteins. Nonimmune IgG was used as a negative control for immunoprecipitation. (C–E) HASMCs that were transfected with siControl, siGαq, or siGα11 and quiesced were treated with vehicle or MCP1 for 30 min, and cell extracts were prepared. Equal amounts of protein from control and each treatment were analyzed for PLCβ3 activity, pPKCδ and

[Further details and image annotations continued]
E

| siControl | + | + | – | – | – | – | – | – |
| siGαq | – | + | + | – | – | + | + | + |
| siGα11 | – | – | + | + | + | + | + | + |
| MCP1 | + | – | + | – | – | – | – | – |

IB:

- WAVE2
- Cortactin
- Gαq
- Gα11

F

G-actin polymerization (RFU)

- G-actin + anti-CTTN IP (siControl + Vehicle)
- G-actin + anti-CTTN IP (siControl + MCP1)
- G-actin + anti-CTTN IP (siGαq + Vehicle)
- G-actin + anti-CTTN IP (siGαq + MCP1)
- G-actin + anti-CTTN IP (siGα11 + Vehicle)
- G-actin + anti-CTTN IP (siGα11 + MCP1)
- G-actin + anti-CTTN IP (siGαq & 11 + Vehicle)
- G-actin + anti-CTTN IP (siGαq & 11 + MCP1)

H

Wound closure (%)

- siControl
- siGαq
- siGα11

Vehicle

MCP1

* p < 0.05 vs. vehicle control or siControl; ** p < 0.05 vs. siControl + MCP1.

pCortactin levels, and cortactin-WAVE2 interactions as described in Figures 5B, 3A, 1A, and 2A, respectively. Equal amounts of protein from the same cell extracts were also analyzed by Western blotting for Gαq and Gα11 levels to show the efficacy of the siRNA on its target molecule level. (F) Equal amounts of protein from cell extracts prepared as for C were analyzed for actin polymerization as described in Figure 1C. (G) All of the conditions were same as for C, except that after quiescence, the cells were treated with vehicle or MCP1 for 6 h and stained for F-actin. (H) All of the conditions were same as for C, except that after quiescence, the cells were subjected to wound-healing migration assay in response to vehicle or MCP1. The bar graphs in A–E and G–H represent mean ± SD values of three experiments. * p < 0.05 vs. vehicle control or siControl; ** p < 0.05 vs. siControl + MCP1.
anti-rabbit or goat anti-mouse secondary antibodies conjugated with HRP were used at 1:4000–1:5000 dilution. Blots were developed using ECL reagent, and signal intensities were quantified by densitometry using ImageJ software.

**G-actin polymerization**
Actin polymerization activity was measured by using the Pyrene-Actin Polymerization Kit (Cytoskeleton, Denver, CO) following the manufacturer’s instructions. Briefly, cell extracts containing equal amounts of protein from control and various treatments were immunoprecipitated with anti-cortactin antibody. The cortactin was eluted from the beads with 0.2 M glycerine (pH 2.6) and neutralized by the addition of an equal volume of 20 mM Tris-HCl (pH 8.5). To measure actin-polymerization activity, stock pyrene-actin was diluted to 2.3 μM with actin buffer (5 mM Tris-HCl, pH 8.0, and 0.2 mM CaCl$_2$) containing 0.2 mM ATP and 0.5 mM DTT and stored on ice for 60 min to depolymerize the actin oligomers. The actin monomers were collected by centrifugation at 14,000 rpm for 30 min at 4ºC. The pyrene-actin monomers were mixed with the eluted cortactin in a 96-well plate containing polymerization buffer (25 mM KCl and 1 mM MgCl$_2$, pH 7.0). The kinetics of actin polymerization was measured by fluorescence intensity generated by the pyrene release for 1 h in a SpectraMax Gemini XS spectrofluorometer at 355/405-nm excitation/emission.

**Statistics**
All experiments were repeated three times, and the data are presented as mean ± SD. The treatment effects were analyzed by one-way analysis of variance followed by Tukey’s post hoc test, and p < 0.05 was considered statistically significant.

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**REFERENCES**
Ando K, Obara Y, Sugama J, Kotani A, Koke N, Ohkubo S, Nakahata N (2010). P2Y2 receptor-Gq/11 signaling at lipid rafts is required for UTP-induced cell migration in NG 108-15 cells. J Pharmacol Exp Ther 334, 809–819.

Arai H, Charo IF (1996). Differential regulation of G-protein-mediated signaling by chemokine receptors. J Biol Chem 271, 21814–21819.

Artyom WV, Zhang Y, Seiler-Mosiewitsch F, Yamada KM, Mueller SC (2006). Dynamic interactions of cortactin and membrane type 1 matrix metalloproteinase at invadopodia: defining the stages of invadopodia formation and function. Cancer Res 66, 3034–3043.

Ayala I, Baldassarre M, Giaichetti G, Caliedi G, Tette S, Luini A, Buccione R (2008). Multiple regulatory inputs converge on cortactin to control invadopodia biogenesis and extracellular matrix degradation. J Cell Sci 121, 369–378.

Bach TL, Chen QM, Kerr WT, Wang Y, Lian L, Choi JK, Wu D, Kazanietz MG, Koretzky GA, Zigmond S, et al. (2007). Phospholipase cβ3 is critical for T cell chemotaxis. J Immunol 179, 2223–2227.

Bajpai AK, Blaskova E, Pakala SB, Zhao T, Glasgow WC, Penn JS, Johnson DA, Rao GN (2007). 15(S)-HETE production in human retinal microvascular

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endothelial cells by hypoxia: Novel role for MEK1 in 15(S)-HETE induced angiogenesis. Invest Ophthalmol Vis Sci 48, 4930–4938.

Berk BC (2001). Vascular smooth muscle growth: autocrine growth mechanisms. Physiol Rev 81, 999–1030.

Berridge MJ, Irvine RF (1989). Inositol phosphates and cell signalling. Nature 341, 197–205.

Blyce NS, Klotz KN, Berson M, Gebicke-Harter PJ, van Calker D (1997). Adenosine A1 receptor-mediated activation of phospholipase C in cultured astrocytes depends on the level of receptor expression. J Neurosci 17, 4956–4964.

Boring L, Gosling J, Cleary M, Charo IF (1998). Decreased lesion formation in CCR2−/− mice reveals a role for chemokines in the initiation of atherosclerosis. Nature 394, 891–897.

Bryce NC, Clark ES, Jaques LJ, Currie JD, Webb DJ, Weaver AM (2005). Cortactin promotes cell motility by enhancing lamellipodial persistence. Curr Biol 15, 1276–1285.

Carr MW, Roth SJ, Luther E, Rose SS, Springer TA (1994). Monocyte chemotactant protein 1 acts as a T-lymphocyte chemotactant. Proc Natl Acad Sci USA 91, 3652–3656.

Charo IF (1999). CCR2α from cloning to the creation of knockout mice. Chem Immunol 72, 30–41.

Clowes AW, Clowes MM, Fingerle J, Reidy MA (1989). Regulation of smooth muscle cell growth in injured artery. J Cardiovasc Pharmacol 14(Suppl 6), S12–S15.

Craig MJ, Lobberg RD (2006). CCL2 (Monocyte Chemotactant Protein-1) in cancer bone metastases. Cancer Metastasis Rev 25, 611–619.

Deshmane SL, Kremlev S, Amini S, Sawaya BE (2009). Monocyte chemotactant protein-1 (MCP-1): an overview. J Interferon Cytokine Res 29, 313–326.

Eissler T, Haussler A, De Kimpe L, Van Lint J, Pfizenmaier K (2010). Cortactin promotes cell motility by enhancing lamellipodial persistence. Curr Biol 15, 1276–1285.

Enrich C, Tebar F (2008). Protein kinase Cdelta and calmodulin regulate epidermal growth factor receptor recycling from early endosomes through Arp2/3 complex and cortactin. Mol Biol Cell 19, 17–29.

Lee JS, Yang EJ, Kim IS (2009). The roles of MCP-1 and protein kinase δ in membrane migration in human eosinophilic leukemia EoE-1 cells. Cytokine 48, 186–195.

Llado A, Timpson P, Vila de Muga S, Moreto J, Pol A, Grewal T, Daly RJ, Enrich C, Tebar F (2008). Protein kinase Cdelta and calmodulin regulate epidermal growth factor receptor recycling from early endosomes through Arp2/3 complex and cortactin. Mol Biol Cell 19, 17–29.

Llado A, Timpson P, Vila de Muga S, Moreto J, Pol A, Grewal T, Daly RJ, Enrich C, Tebar F (2008). Protein kinase Cdelta and calmodulin regulate epidermal growth factor receptor recycling from early endosomes through Arp2/3 complex and cortactin. Mol Biol Cell 19, 17–29.

MacGrath SM, Koleske AJ (2012). Cortactin in cell migration and cancer at a glance. J Cell Sci 125, 1621–1626.

Martinez-Quiles N, Ho H-Y, Kirschner MW, Ramesh N, Geha RS (2004). Erk1/Src phosphorylation of cortactin acts as a switch on-off mechanism that controls its ability to activate N-WASP. Mol Cell Biol 24, 5269–5280.

Matsumura M, Tanaka N, Kuroki T, Ichihashi M, Ohba M (2003). The eta isoform of protein kinase C inhibits UV-induced activation of caspase-3 in normal human keratinocytes. Biochem Biophys Res Commun 303, 350–356.

Mellor H, Parker PJ (1998). The extended protein kinase C superfamily. Biochem J 332, 281–292.

Miki H, Sasaki T, Takai Y, Takenawa T (1998a). Induction of filopodium formation by a WASP-related actin-depolymerizing protein N-WASP. Nature 391, 93–96.

Miki H, Suetsugu S, Takenawa T (1998b). WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. EMBO J 17, 6932–6941.

Mitchison TJ, Cramer LP (1996). Actin-based cell motility and cell locomotion in clathrin-caveolin-independent endocytosis. Traffic 11, 1079–1091.

Mitchison TJ, Cramer LP (1996). Actin-based cell motility and cell locomotion in clathrin-caveolin-independent endocytosis. Traffic 11, 1079–1091.

Nunes JM, Nickerson SP, Revesz IA, Koleske AJ, Parker PJ (1998). The extended protein kinase C superfamily. Biochem J 332, 281–292.

Olivo-Marin JC, Dautry-Varsat A, Gerthoffer WT (2007). Mechanisms of vascular smooth muscle cell migration. Circ Res 100, 607–621.

Parsons JT (2003). Cortactin interacts with WIP in regulating Arp2/3 complex and cortactin. Mol Biol Cell 14, 1276–1285.
of NFATc1-mediated cyclin D1-CDK6 activity and modulates vascular smooth muscle cell division and migration leading to inward blood vessel wall remodeling. J Biol Chem 287, 36291–36304.

Singh NK, Wang D, Kundumani-Sridharan V, Quyen DV, Niu J, Rao GN (2011). 15-Lipoxygenase-1-enhanced Src-Janus kinase 2-signal transducer and activator of transcription 3 stimulation and monocyte chemoattractant protein-1 expression require redox-sensitive activation of epidermal growth factor receptor in vascular wall remodeling. J Biol Chem 286, 22478–22488.

Smrcka AV, Sternweis PC (1993). Regulation of purified subtypes of phosphatidylinositol-specific phospholipase C beta by G protein alpha and beta gamma subunits. J Biol Chem 268, 9667–9674.

Stupack DG, Cho SY, Klemke RL (2000). Molecular signaling mechanisms of cell migration and invasion. Immunol Res 21, 83–88.

Suetsugu S, Miki H, Yamaguchi H, Obinata T, Takenawa T (2001). Enhancement of branching efficiency by the actin filament-binding activity of N-WASP/WAVE2. J Cell Sci 114, 4533–4542.

Takahashi K, Suzuki K (2009). Membrane transport of WAVE2 and lamellipodia formation require Pak1 that mediates phosphorylation and recruitment of stathmin/Op18 to Pak1-WAVE2-kinesin complex. Cell Signal 21, 695–703.

Urano T, Liu J, Zhang P, Fan Yx, Egile C, Li R, Mueller SC, Zhan X (2001). Activation of Arp2/3 complex-mediated actin polymerization by cortactin. Nat Cell Biol 3, 259–266.

Vicente-Manzanares M, Webb DJ, Horwitz AR (2005). Cell migration at a glance. J Cell Sci 118, 4917–4919.

Wang W, Liu Y, Liao K (2011). Tyrosine phosphorylation of cortactin by the FAK-Src complex at focal adhesions regulates cell motility. BMC Cell Biol 12, 49.

Weaver AM, Karginov AV, Kinley AW, Weed SA, Li Y, Parsons JT, Cooper JA (2001). Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation. Curr Biol 11, 370–374.

Webb BA, Zhou S, Eves R, Shen L, Jia L, Mak AS (2006). Phosphorylation of cortactin by p21-activated kinase. Arch Biochem Biophys 456, 183–193.

Weed SA, Karginov AV, Schafer DA, Weaver AM, Kinley AW, Cooper JA, Parsons JT (2000). Cortactin localization to sites of actin assembly in lamellipodia requires interactions with F-actin and the Arp2/3 complex. J Cell Biol 151, 29–40.

Wu H, Reynolds AB, Kanner SB, Vines RR, Parsons JT (1991). Identification and characterization of a novel cytoskeleton-associated pp60src substrate. Mol Cell Biol 11, 5113–5124.

Xu L, Warren M, Rose W, Gong W, Wang J (1996). Human recombinant monocyte chemotactic protein and other CC chemokines bind and induce directional migration of dendritic cells in vitro. J Leukoc Biol 60, 365–371.

Yamaguchi H, Condeelis J (2007). Regulation of the actin cytoskeleton in cancer cell migration and invasion. Biochim Biophys Acta 1773, 642–652.

Yamaguchi H, Wyckoff J, Condeelis J (2005). Cell migration in tumors. Curr Opin Cell Biol 17, 559–564.

Yamazaki D, Suetsugu S, Miki H, Kataoka Y, Nishikawa S, Fujiwara T, Yoshida N, Takenawa T (2003). WAVE2 is required for directed cell migration and cardiovascular development. Nature 424, 452–456.

Zeng H, Zhao D, Mukhopadhyay D (2002). KDR stimulates endothelial cell migration through heterotrimeric G protein Gq/11-mediated activation of a small GTPase RhoA. J Biol Chem 277, 46791–46798.

Zhang X, Yuan Z, Zhang Y, Yong S, Salas-Burgos A, Koomen J, Olashaw N, Parsons JT, Yang XJ, Dent SR, et al. (2007). HDAC6 modulates cell motility by altering the acetylation level of cortactin. Mol Cell 27, 197–213.

Zhang Y, Zhang M, Dong H, Yong S, Li X, Olashaw N, Kruk PA, Cheng JQ, Bai W, Chen J, et al. (2009). Deacetylation of cortactin by SIRT1 promotes cell migration. Oncogene 28, 445–460.