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MCPIP1 regulates fibroblast migration in 3D collagen matrices downstream of MAP kinases and NF-κB

Jie Chao[1,2], **Xiaoniu Dai**[2], **Tiffany Peña**[1], **David A. Doyle**[1], **Timothy M. Guenther**[1], and **Mark A. Carlson**[1,3,4]

[1]Department of Surgery, University of Nebraska Medical Center, Omaha, Nebraska, 68198, USA

[2]Department of Physiology, School of Medicine, Southeast University, Nanjing, Jiangsu, 210009, China

[3]Department of Surgery, VA Nebraska–Western Iowa Health Care System, Omaha, Nebraska, 68105, USA

[4]Department of Genetics, Cell Biology and Anatomy, University of Nebraska Medical Center, Omaha, Nebraska, 68198, USA

Abstract

The fibroblast-populated 3D collagen matrix has been used to model matrix contraction, cell motility, and general fibroblast biology. MCPIP1 (monocyte chemotactic protein-induced protein 1) has been shown to regulate inflammation, angiogenesis, and cellular motility. In the present study, we demonstrated induction of MCPIP1 in human fibroblasts embedded in the stress-released 3D collagen matrix, which occurred through activation of mitogen-activated protein kinases, phosphoinositide 3-kinase, and NF-κB. Furthermore, MCPIP1 induction was associated with inhibition of fibroblast migration out of the nested collagen matrix. MCPIP1 induction or ectopic expression also upregulated p53. RNA interference of p53 prevented the inhibition of migration produced by induction or ectopic expression of MCPIP1. Our findings suggest a new role for MCPIP1 as a molecular switch that regulates fibroblast migration in the nested collagen matrix model.

Introduction

Recently we observed (Chao *et al.*, 2014) that foreskin fibroblasts preconditioned in a rigidly anchored collagen matrix migrated out of that matrix when it was re-embedded (“nested”) (Grinnell *et al.*, 2006) in cell-free, anchored collagen, while fibroblasts preconditioned in a stress-released matrix had relatively poor motility under the same conditions (5% serum). This observation provided us with an opportunity to study mechanoregulation of fibroblast

Correspondence: Mark A. Carlson, Surgery 112, VA Medical Center, 4101 Woolworth Ave, Omaha, NE 68105, USA. Phone: 001-402-995-5371. Fax: 001-402-995-5370. ; Email: macarlso@unmc.edu

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motility in the fibroblast-populated 3D collagen matrix (FPCM) model (Grinnell, 1994). A relevant signaling molecule was MCPIP1 (MCP-1-induced protein 1, also know as ZC3H12A), a 66 kDa protein identified in human peripheral blood monocytes and cardiomyocytes stimulated with MCP-1 (monocyte chemotactic protein-1) (Liu *et al.,* 2015; Zhou *et al.,* 2006). The known functions of MCPIP1 include: downregulation of inflammation through induction of apoptosis genes (Skalniak *et al.,* 2013; Zhou *et al.,* 2006); induction of angiogenesis in endothelial cells (HUVECs) (Niu *et al.,* 2008); inhibition of Toll-like receptor signaling and macrophage activation (Huang *et al.,* 2012); upregulation of adipogenesis independent of PPARγ (Younce *et al.,* 2009); RNAse activity against viral DNA (Lin *et al.,* 2013; Suzuki *et al.,* 2011); inhibition of JNK and NF-κB (Liang *et al.,* 2008; Liu *et al.,* 2013); and protection against LPS-induced shock (Huang *et al.,* 2013). MCPIP1-deficient mice developed a severe inflammatory syndrome with T-cell activation, increased cytokine production, and a 50% eight-week mortality (Miao *et al.,* 2013).

MCPIP1 also was noted to promote migration in HUVECs (Niu *et al.,* 2008), and MCP-1 knock-out mice demonstrated delayed wound re-epithelialization and angiogenesis (Low *et al.,* 2001). So it seemed logical to test whether MCPIP1 participated in mechanoregulation of fibroblast healing functions, such as proliferation, contraction, and migration. Herein we report data demonstrating that MCPIP1 induction after stress-release of the FPCM inhibits fibroblast migration, working through a signaling pathway involving the mitogen-activated protein (MAP) kinases, NF-κB, and p53. This brake on fibroblast migration appears to be a new function for MCPIP1, and implicates this protein as a participant in the wound healing process.

Results

Mechanoregulation of migration in the restrained nested matrix; upregulation of MCPIP1 in the stress-released FPCM

Fibroblast migration was assayed using serum-treated attached or stress-released collagen matrices populated with green fluorescent protein (GFP)-expressing human foreskin fibroblasts (HFFs) (Chao *et al.,* 2014) in the restrained nested matrix (Supplementary Fig. S1A) (Miron-Mendoza *et al.,* 2010). Fibroblast migration out of the nested stress-released matrix was decreased relative to the nested stressed (attached) matrix (Fig. 1A–B and S4F). Immunoblotting demonstrated that the MCPIP1 protein was upregulated after matrix release (Fig. 1C–D). The MCPIP1 signal reached a maximum at ~1 h and remained elevated for several days. Immunocytochemistry of MCPIP1 in attached *vs.* released matrices also demonstrated induction of this protein in the released state (Fig. 1E). The specificity of the MCPIP1 fluorescence in the released matrix was particularly impressive while focusing up and down through the 3D microscopy specimen.

Effect of MCPIP1 RNAi on the mechanoregulation of FPCM contraction, matrix cell number, and fibroblast migration

In order to determine whether MCPIP1 induction associated with FPCM stress-release was biologically relevant, the effect of MCPIP1 knockdown on FPCM contraction, matrix cell number, and fibroblast migration was determined in attached *vs.* released matrices. The

efficiency of MCPIP1 RNA interference (RNAi) in the attached *vs.* released FPCM (72 hr after transfection, 24 hr after release) was near complete by immunoblotting (Fig. 2A). RNAi of MCPIP1 had minimal effect on contraction in the floating collagen matrix assay ("dermal equivalent" (Grinnell and Petroll, 2010)); see Fig. 2B–C. RNAi of MCPIP1 did not affect the decrease in matrix cell number (Fig 2D) known to occur after matrix stress-release (Carlson and Longaker, 2004). Using attached or stress-released matrices populated with GFP-expressing HFFs nested into restrained cell-free collagen, it was observed that MCPIP1 knockdown disinhibited migration out of the released, nested matrix (Fig. 2E–F). That is, the decrease in fibroblast migration (the inhibition) precipitated by release of the nested matrix was prevented (disinhibited) if MCPIP1 expression was blocked.

Effect of MCPIP1 ectopic expression on the mechanoregulation of FPCM contraction, matrix cell number, and fibroblast migration

In order to corroborate the findings in Fig. 2, an analogous set of experiments was performed using plasmid-expressed Flag-tagged MCPIP1 (Fig. 2G–L). Confirmation of MCPIP1-Flag expression after plasmid transfection in the FPCM is shown in Fig. 2G and Supplementary Fig. S1B. Addition of the MAT-Tag-Flag sequence (see Methods) added 15 amino acids to the 599 amino acid sequence of MCPIP1, but no shift was seen on the immunoblots of MCPIP1 vs. MCPIP1-Flag. Subsequent ectopic expression of MCPIP1-Flag had no effect on contraction in the floating collagen matrix assay (Fig. 2H–I). Expression of MCPIP1-Flag also did not affect the decrease in matrix cell number which occurred after matrix stress-release (Fig. 2J). In experiments analogous to those in Fig. 2E–F, expression of MCPIP1-Flag inhibited GFP-HFF migration out of the attached matrix nested into restrained, cell-free collagen (Fig. 2K–L). Ectopic expression of MCPIP1-Flag in monolayer fibroblasts actually increased migration in a scratch assay (Supplementary Fig. 5A–B), i.e., the opposite effect to that observed in the 3D culture model.

Effect of FPCM release on phosphorylation of MAP kinases and Akt

Previous reports have indicated that activation of MAP kinases and the phosphoinositide 3-kinase (PI3K)/Akt pathway both stimulate fibroblast migration (Clement *et al.,* 2013; Li *et al.,* 2004). In order to see if there was a link between these kinase pathways and MCPIP1-associated inhibition of cellular migration, phosphorylation of these kinases in the attached vs. released FPCM was evaluated first (Fig. 3). Within 5 min of matrix stress release, there was increased phosphorylation of Erk, which tapered off by 6 h (Fig. 3A, C). Within 5–10 min of release, p38 demonstrated increased phosphorylation, reaching a peak around 30–60 min and then tapering off (Fig. 3A, B). JNK also demonstrated a burst of activation from 5 to 30 min after release (Fig. 3D, E). The data of Fig. 3 was consistent with previous work of other investigators, who demonstrated Erk and p38 activation after stress-release of the FPCM under similar but not identical conditions (Lee *et al.,* 2000).

Akt also demonstrated a burst of activity 1 h after release, with tapering thereafter (Fig. 3D, F). Of note, previously-published reports demonstrated Akt was dephosphorylated during a longer time course (>6 h) of FPCM stress-release (Carlson *et al.,* 2004; Tian *et al.,* 2002; Xia *et al.,* 2004). A long time course of Akt activity after matrix release was repeated for corroboration purposes in Supplementary Fig. S2A–B, which again demonstrated rapid and

transient Akt phosphorylation, followed by gradual dephosphorlyation evident at 6–12 h (consistent with previously-published data).

Effect of pharmacological inhibition of MAP kinases or Akt on MCPIP1 induction and fibroblast migration after FPCM release

Pharmacological inhibition of the above kinases was used to determine whether the pathways of interest (JNK, ERK, p38, and PI3K/Akt) regulated (1) the expression of MCPIP1 and (2) fibroblast migration out of the nested matrix (Fig. 3G–L). Justification for the 30 min point was the relatively large increase in MCPIP1 expression in the released matrix present at this time; the 24 h time point was chosen because both MCPIP1 upregulation and migration inhibition were evident 24 h after release (Fig. 1). Treatment of FPCMs with commercially-available small molecules U0126 (MEK inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), or LY294002 (PI3K inhibitor) at the manufacturer-recommended dose decreased the stress-release-induced phosphorylation of the respective target kinase (see Supplementary Figure S2C–F).

Inhibitor pre-treatment for 2 hr prior to FPCM stress-release diminished the induction of MCPIP1 at both 30 min and 24 h after stress-release (Fig. 3G–J). At the 30 min time point, the MCPIP1 signal still appeared to be increased in the stress-released matrix in the presence of each kinase inhibitor, but this reached significance only with the p38 inhibitor (SB203580). At the 24 h time point, induction of MCPIP1 expression after stress-release was still blunted by each kinase inhibitor, but effects were less pronounced compared to the 30 min time point (Fig. 3J).

ANOVA and unpaired t-testing performed on the MCPIP1/actin expression ratios for the attached matrix in Fig. 3J (i.e., attached vehicle *vs.* attached SP600125 *vs.* attached U0126 *vs.* attached SB203580 *vs.* attached LY294002) revealed that the attached LY294002 ratio (indicated with an asterisk over that bar) was different from the attached vehicle ratio. So while kinase inhibition may have decreased MCPIP1 expression in the attached FPCM, our assay detected this only for the PI3K inhibitor. This observation might decrease the relevance of the decrease in MCPIP1 induction observed in the LY294002-treated released matrix; the absolute effect of the PI3K inhibitor in the released matrix, however, still was large compared to the effect in the attached matrix.

The effect of pharmacologic inhibition of kinase activity on fibroblast migration from stress-released matrices nested into restrained cell-free matrices then was evaluated (Fig. 3K–L). Pretreatment of the FPCM for 2 h prior to stress-release with the MEK, p38, or JNK inhibitor enhanced fibroblast migration out of the nested released matrix (i.e., resulted in disinhibition of fibroblast migration). Pretreatment of matrices with the PI3K inhibitor LY294002, however, did not have an effect; that is, fibroblast migration out of the nested released matrix was similar to vehicle treatment, meaning barely detectable. There was no significant effect of any of these inhibitors on fibroblast migration out of the nested attached matrix (Supplementary Fig. S3A–B). The data of Fig. 3 suggested that MAP kinase activation was upstream of MCPIP1 induction in a putative pathway that inhibited fibroblast migration after stress-release of the FPCM.

Involvement of NF-κB in the expression of MCPIP1 and inhibition of fibroblast migration after FPCM release

NF-κB activation has been documented in the contractile FPCM (Carlson *et al.*, 2013; Xu and Clark, 1997; Xu *et al.*, 1998), and NF-κB signaling has been implicated in the induction of both MCP-1 and MCPIP1 in endothelial cells (Qi *et al.*, 2010; Yao *et al.*, 2010). In addition, NF-κB activation can occur secondary to MAP kinase activation (Dhawan and Richmond, 2002; Dong *et al.*, 2012; Troppmair *et al.*, 1998). So it was hypothesized that MCPIP1 induction in the stress-released FPCM occurred as a consequence of MAP kinase-mediated NF-κB activation. During the 5–30 min interval after matrix release, there was an increase in the level of the phosphorylated p65 (p-p65) subunit of NF-κB in the nuclear fraction of the HFFs (Fig. 4A–B), with a concomitant loss of this subunit in the cytoplasm (Supplementary Fig. S4A), consistent with pilot data (Carlson *et al.*, 2013). This increase of p-p65 after matrix release appeared to be abrogated if the matrices were pre-treated with pharmacologic inhibitors of Erk, p38, or JNK; pretreatment with the PI3K inhibitor had less effect (Fig. 4C–D). These results suggested that MAP kinase activation after FPCM release resulted in NF-κB activation.

The MCP-1 promoter has an NF-κB binding site (Yao *et al.*, 2010); a chromatin immunoprecipitation (ChIP) assay was utilized to determine whether a similar site was present on the MCPIP1 promoter (Fig. 4E). Chromatin was cross-linked to protein in attached *vs.* 20 min stress-released FPCMs, and sonicated extracts were processed with a ChIP kit. Subsequent agarose gel electrophoresis of the amplified, immunoprecipitated DNA revealed much greater NF-κB binding in extracts from the released matrix compared to the attached (Fig. 4F). This was consistent with NF-κB binding to the MCPIP1 promoter in extracts from the stress-released FPCM.

In order to determine whether NF-κB activation after FPCM release regulated MCPIP1induction, matrices were pretreated with pharmacologic inhibitors of NF-κB activation (Fig. 4G–I, S1C–D, and S4D–F), including TPCK (N-*p*-tosyl-L-phenylalanine chloromethyl ketone) and SC-514 (4-Amino-[2,3″]bithiophenyl-5-carboxylic acid amide). TPCK, a serine protease inhibitor, will block NF-κB activation by preventing the proteolysis of IκB-α; SC-514, an ATP-competitive inhibitor selective for IκB kinase-2 (IKK-2), will block NF-κB activation by preventing phosphorylation of IκB-α (Ha *et al.*, 2009; Henkel *et al.*, 1993; Karin *et al.*, 2004; Kishore *et al.*, 2003). Compared to vehicle-treated matrices, MCPIP1 induction after release in matrices pretreated with SC-514 or TPCK or was not present at 24 h (Fig. 4G–H and S4D–E, respectively). Furthermore, FPCM pretreatment with either SC-514 or TPCK disinhibited HFF migration in the released matrix that was nested into a restrained, cell-free matrix (Fig. 4I, S1C–D, and S4F). In other words, pharmacologic inhibition of NF-κB allowed fibroblasts to migrate out of the collagen matrix under conditions in which they normally would not. The results thus far suggested that inhibition of fibroblast migration associated with FPCM release involved a pathway with sequential upregulation of MAP kinases, NF-κB, and MCPIP1.

Interaction of MCPIP1 with p53 after release of the FPCM

It has been shown that is p53 upregulated after release of the FPCM (Carlson *et al.,* 2004; Carlson *et al.,* 2009; Carlson *et al.,* 2013). In order to determine whether there was co-localization of MCPIP1 and p53 after release of the FPCM, double-immunocytochemistry for these two antigens was performed (Fig. 5A and S3E). The orange-yellow coloring in the merged images indicated that there was some co-localization of MCPIP1 and p53 in the 1-day released matrix. Immunoprecipitation of either MCPIP1 or p53 followed by immunoblotting for the opposite antigen demonstrated an association of MCPIP1 and p53 in whole cell lysates from the released (but not attached) FPCM (Fig. 5B–C). Control immunoprecipitation experiments that used RNAi to knockdown either MCPIP1 or p53 prior to pull-down confirmed the specificity of the MCPIP1-p53 association (Supplementary Fig. S3C–D). The results from Fig. 5A–C suggested that there was a physical interaction between MCPIP1 and p53 after FPCM stress release.

In order to determine whether one of these proteins regulated the concentration of the other, RNAi of MCPIP1 or p53 was performed and protein levels were determined with immunoblotting (Fig. 5D–F). Fibroblasts transfected with siRNA against MCPIP1 did not have p53 upregulation 24 h after matrix release (Fig. 5F), even though the knockdown of MCPIP1 did not appear complete (Fig. 5E). Treatment with siRNA against p53 prevented most of the post-release increase in p53 (Fig. 5D, F). Dissimilar to the situation with MCPIP1 RNAi, however, partial knockdown of p53 did not affect the induction of MCPIP1 in the released matrix (Fig. 5D–E). These data suggested that MCPIP1 regulated p53 induction after FPCM release, i.e., that MCPIP1 was upstream of p53 in a putative pathway.

Further exploration of an MCPIP1–p53 relationship was done using ectopic expression of MCPIP1-Flag combined with p53 RNAi (Fig. 6). Expression of MCPIP1-Flag upregulated p53 in the attached FPCM, with no significant effect in the released matrix (Fig. 6A, C); as expected, MCPIP1-Flag inhibited cell migration out of the nested attached FPCM (Fig. 6D–E). Consistent with Fig. 5, RNAi of p53 did not affect induction of MCPIP1 associated with matrix release (Fig. 6A–B), but did result in disinhibition of cell migration that occurred in the nested released FPCM (Fig. 6D–E). Expression of MCPIP1-Flag could not overcome the effect of p53 RNAi on cell migration (Fig. 6D–E). In other words, MCPIP1 could inhibit the ability of fibroblasts to migrate out of the attached matrix that was nested into a restrained, cell-free matrix if p53 was not inhibited. If p53 was knocked down, however, then the fibroblasts could migrate out of either an attached or released matrix, regardless of the state of MCPIP1.

MCPIP1 associated with USP10 (Ubiquitin Specific Peptidase 10) after genotoxic stress, enhancing the latter's deubiquitinase activity, thereby producing deubiquitination of NEMO (IKK-γ) which resulted in inhibition of NF-κB activation (Niu *et al.,* 2013). Genotoxic stress also produced a TANK-MCPIP1-USP10 complex that effected/enhanced deubiquitination of both TRAF6 (E3 Ubiquitin Protein Ligase) and NEMO, which in turn inhibited NF-κB activation (Wang *et al.,* 2015). So in the setting of genotoxic stress, MCPIP1 appears to function as a facilitator of USP10 deubiquitinase, thereby acting as a "brake" (negative feedback mechanism) on NF-κB activation. In order to determine whether MCPIP1-facilitation of deubiquitination was relevant for MCPIP1-induced upregulation of the p53

protein, we performed FPCM experiments with fibroblasts transfected with MCPIP1(ΔZF), an MCPIP1 mutant that does not promote deubiquitination (Liang *et al.*, 2010). Our data suggested that MCPIP1-facilitated deubiquitination is important for induction of p53 by MCPIP1 (Supplementary Fig. 4B–C), which suggested that the increase of the p53 protein after FPCM stress-release was mediated at least in part by a decrease in p53 ubiquitination. Whether MCPIP1 affects the ubiquitination status of p53 through USP10 will need further investigation.

The data of Fig. 6 were consistent with a pathway in which MCPIP1, acting upstream and through p53, effected inhibition of cell migration out of the nested released FPCM. Stress-release of the FPCM also has been shown both to increase apoptosis and inhibit the cell cycle in the resident fibroblasts (Carlson and Longaker, 2004; Carlson *et al.*, 2013; Fluck *et al.*, 1998; Grinnell *et al.*, 1999; Hadjipanayi *et al.*, 2009; Tian *et al.*, 2002), which likely is an effect of p53 upregulation. Although cell survival and proliferation were not the focus of this study, we did demonstrate that the Bax protein increased after FPCM stress-release, and that this induction was abrogated by p53 knockdown (Supplementary Fig. 5C). This finding would be consistent with a p53-modulated increase in apoptosis associated with FPCM stress-release.

Discussion

The data suggested the existence of an MCPIP1-centric network which regulated fibroblast migration in the nested FPCM model (Fig. 6F–G). In this putative network, MCPIP1 in the nested attached FPCM was undetectable, allowing fibroblasts to migrate out of the FPCM and into the acellular neomatrix. If the matrix was released, however, phosphorylation of MAP kinases (Erk, p38, and JNK) ensued and produced NF-κB activation. The p65 subunit of NF-κB then bound to the promoter region of the MCPIP1 gene, followed by increased MCPIP1 expression. This induction of MCPIP1 upregulated p53, possibly through binding events between MCPIP1 and p53 (and perhaps some unspecified scaffolding proteins). Upregulation of p53 produced, through unidentified additional steps, inhibition of fibroblast migration out of the nested released matrix.

While we have implied that MCPIP1 induction after FPCM release was secondary to increased transcription and translation, we have not ruled out other protein turnover mechanisms, such as transcript stability or protein degradation. Our assumption of increased MCPIP1 transcription was based on two observations: (1) p65 NF-κB bound to the MCPIP1 promoter after FPCM release; and (2) inhibition of NF-κB activation abrogated both the induction of MCPIP1 and the inhibition of migration that occurred after matrix release (Fig. 6).

It is conceivable that other signaling events (e.g., involving the Rho GTPases (Raftopoulou and Hall, 2004)) may have contributed to the inhibition of migration associated with FPCM stress-release. Nevertheless, various “molecular switches” have been described in which a change in protein level and/or activity produced a large downstream event (Drees *et al.*, 2005; Milburn *et al.*, 1990; Murphy *et al.*, 2004), so it is not inconceivable that increased MCPIP1 protein contributed to the inhibition of fibroblast migration. The effect of MAP

kinase inhibition on MCPIP1 induction in Fig. 6 was partial, suggesting that the MAPK inhibitors may have had produced other downstream effects (e.g., on myosin light chain kinase (Huang *et al.,* 2004)) that resulted in disinhibition of migration after matrix stress-release.

Previous reports have suggested that activation of oncogenic Ras, MAP kinases, and PI3K all may work to enhance fibroblast migration (Clement *et al.,* 2013; Li *et al.,* 2004; Menezes *et al.,* 2008), in apparent contradiction to our report. These earlier reports all used one or more of the following conditions: (1) transformed cell lines with forced/sustained expression of various signaling proteins; (2) monolayer culture conditions; and (3) variable amounts of soluble growth factors. In contrast, our experimental system (1) did not utilize forced expression to make primary observations (Fig. 1 and 4), (2) did utilize a three-dimensional culture system, and (3) maintained a constant culture medium. These differences in experimental design might explain the discrepancy between the previous studies and our data. For example, we observed transient MAP kinase activation, the effect of which likely is different from activation secondary to forced expression (Marshall, 1995; Sabbagh Jr *et al.,* 2001).

Our intent herein was to study fibroblast migration in a tractable 3D model which has morphologic and physiologic similarities to dermal wounds (Carlson and Longaker, 2004); clearly, these results may not perfectly translate to *in vivo* processes. It is interesting to note that forced expression of MCPIP1 in a monolayer assay of migration actually appeared to promote fibroblast motility; i.e., the opposite effect to what was observed in the 3D collagen matrix. This discordance of experimental results from monolayer vs. 3D culture systems suggests that it may be better to utilize 3D culture systems when studying cells whose natural environment is three-dimensional, not planar (Cukierman *et al.,* 2001).

The FPCM model has been in continual use since the 1970s to study various phenomena related to wound healing, such as cellular migration, cell-mediated matrix contraction, synthesis and secretion of matrix proteins, cell death, cellular proliferation. Derivatives of the FPCM model also have been used to study tissue engineering (Carlson and Longaker, 2004; Grinnell and Petroll, 2010; Harunaga and Yamada, 2011; Kim *et al.,* 2011). Continued progress in wound healing science likely will benefit from the use of (1) culture systems such as the FPCM and (2) *in vivo* models.

Materials and methods

Cell culture

The use of primary dermal fibroblasts derived from discarded human neonatal circumcision specimens was approved by the Research and Development Committee of the Omaha VA Medical Center and by the Institutional Review Board of the University of Nebraska Medical Center. Fibroblasts were cultured from explants of human neonatal foreskins, as previously described (Carlson *et al.,* 2009). The collagen matrix model was utilized, as previously described (Carlson *et al.,* 2009; Carlson *et al.,* 2013; Grinnell *et al.,* 1999). Lentiviral-transduced HFFs with stable expression of GFP was described in a separate report (Chao *et al.,* 2014).

Nested matrix model and cell migration

The nested collagen matrix model was utilized as previously described (Chao *et al.,* 2014; Grinnell *et al.,* 2006; Liu *et al.,* 2015; Miron-Mendoza *et al.,* 2010) with some modifications; refer to Supplementary Fig. S1A and protocol in the Supplementary Information. For the nested attached matrix, a standard FPCM was incubated in the attached state for 72 h with 5% FBS in DMEM; the FPCM then was removed from the culture well, and placed onto a 60 μL aliquot of fresh acellular collagen matrix solution (neomatrix solution) that was centered inside a 12 mm-diameter score on the bottom of a new culture well. A 140 μL aliquot of neomatrix solution then was used to cover the newly-transferred FPCM. The neomatrix was allowed to polymerize for 1 h at 37°C and 5% CO_2, and then 2 mL of DMEM with 5% FBS was added to the well. The same procedure was followed for the nested released matrix, except that the initial incubation of the FPCM was 48 h in the attached stated, followed by detachment, and then 24 h incubation in the released state (see Supplementary Fig. S1A). Cell migration out of the nested FPCM and into the acellular neomatrix was quantified 24 h after nesting with fluorescent microscopy, as described in the Supplementary Information. Cell number per matrix was determined using a Scepter™ Cell Counter (EMD Millipore), as previously described (Chao *et al.,* 2014).

See Supplemental Information for details on reagents, transfections, contraction assays, immunoblotting, immunocytochemistry, immunoprecipitation, ChIP assay, and other methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

3D	three-dimensional
FPCM	fibroblast-populated collagen matrix
HFF	human foreskin fibroblasts
HUVEC	human umbilical vein endothelial cells
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase

MCPIP1	monocyte chemotactic protein-induced protein 1

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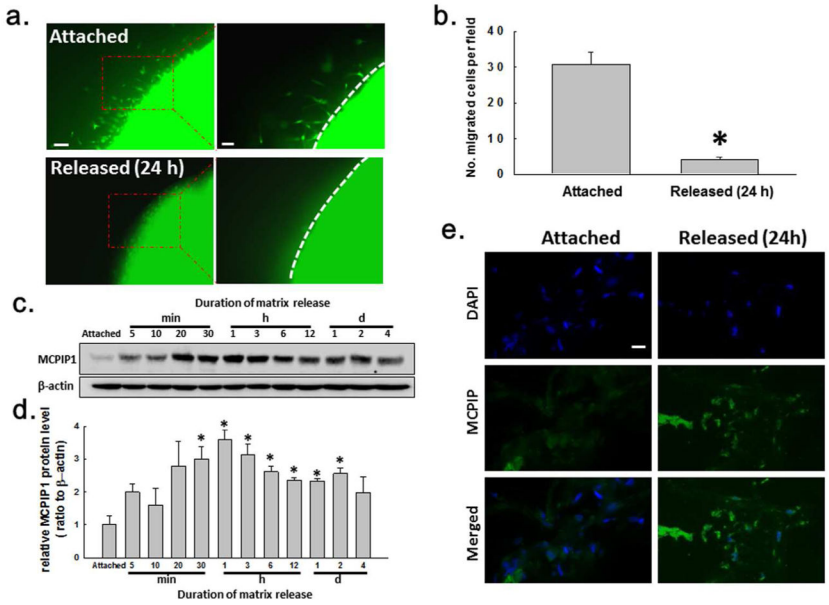

Fig. 1. Effect of FPCM release on fibroblast migration and expression of MCPIP1

(A) Migration of GFP-expressing fibroblasts out of nested matrices was decreased 24 hr after matrix release. Fibroblast migration shown at the interface between the nested matrix and the restrained cell-free matrix. Left scale bar = 200 μm, right scale bar= 80 μm. **(B)** Plot of migration (three separate experiments from panel A). **(C)** MCPIP1 induction after FPCM release. Whole cell lysates from attached or released matrices immunoblotted for MCPIP1 and β-actin. **(D)** MCPIP1 densitometry (four separate experiments from panel C). **(E)** MCPIP1 immunocytochemistry in the attached vs. released FPCM. Blue = DAPI; green = MCPIP1. Scale bar = 20 μm. Data are mean ± S.E.M.; *$p < 0.05$ vs. attached (unpaired t-test).

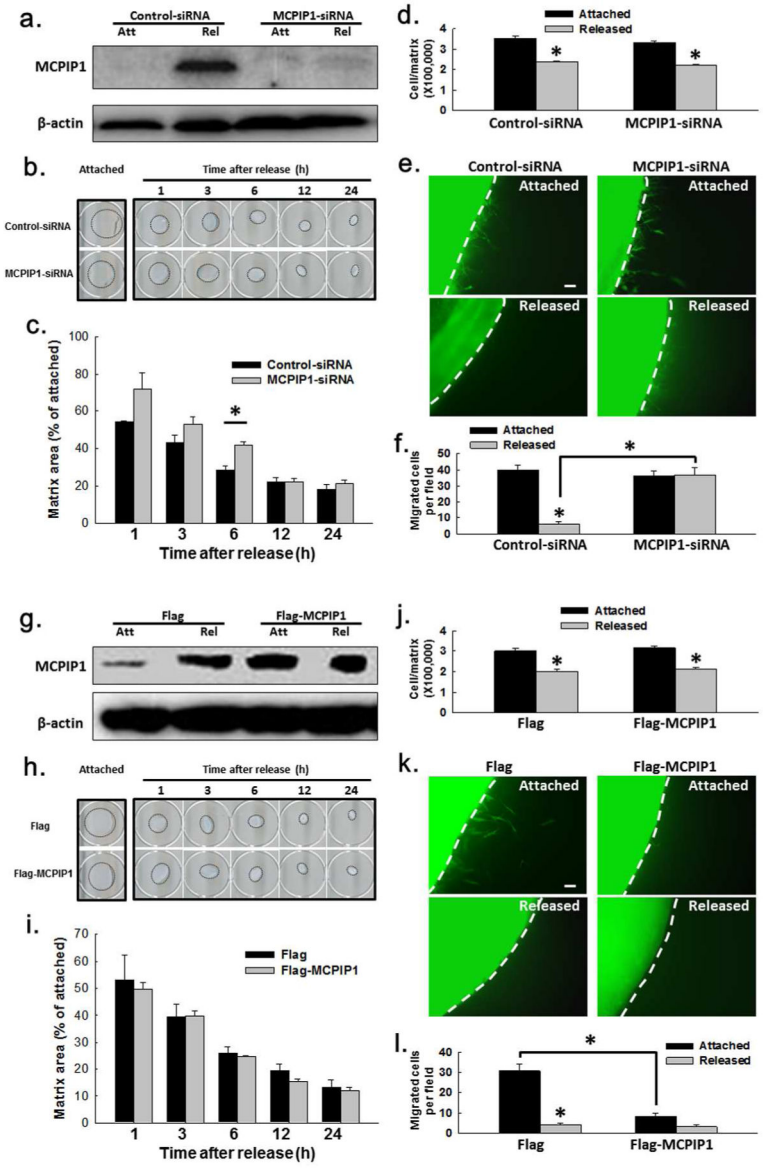

Fig. 2. Effect of MCPIP1 RNAi or ectopic expression on matrix contraction, matrix cell number, and fibroblast migration

(A) Immunoblots of lysates from FPCMs expressing siRNA (MCPIP1 *vs.* nonsense). **(B–C)** Effect of MCPIP1 RNAi on FPCM contraction (well diameter=19mm); plot=three experiments. **(D)** Effect of MCPIP1 RNAi on FPCM cell number, one day post-release. **(E–F)** Effect of MCPIP1 RNAi on migration out of the released, nested FPCM (scale bar=80 μm); plot=three experiments. **(G)** Immunoblots of lysates from FPCMs expressing MCPIP1-Flag *vs.* Flag. **(H–I)** Effect of MCPIP1-Flag expression on FPCM contraction; plot=three experiments. **(J)** Effect of MCPIP1-Flag expression on FPCM cell number, one day post-release. **(K)** Effect of MCPIP1-Flag expression on migration out of the attached, nested FPCM (scale bar=80 μm); plot=three experiments. *$p < 0.05$, unpaired t-test.

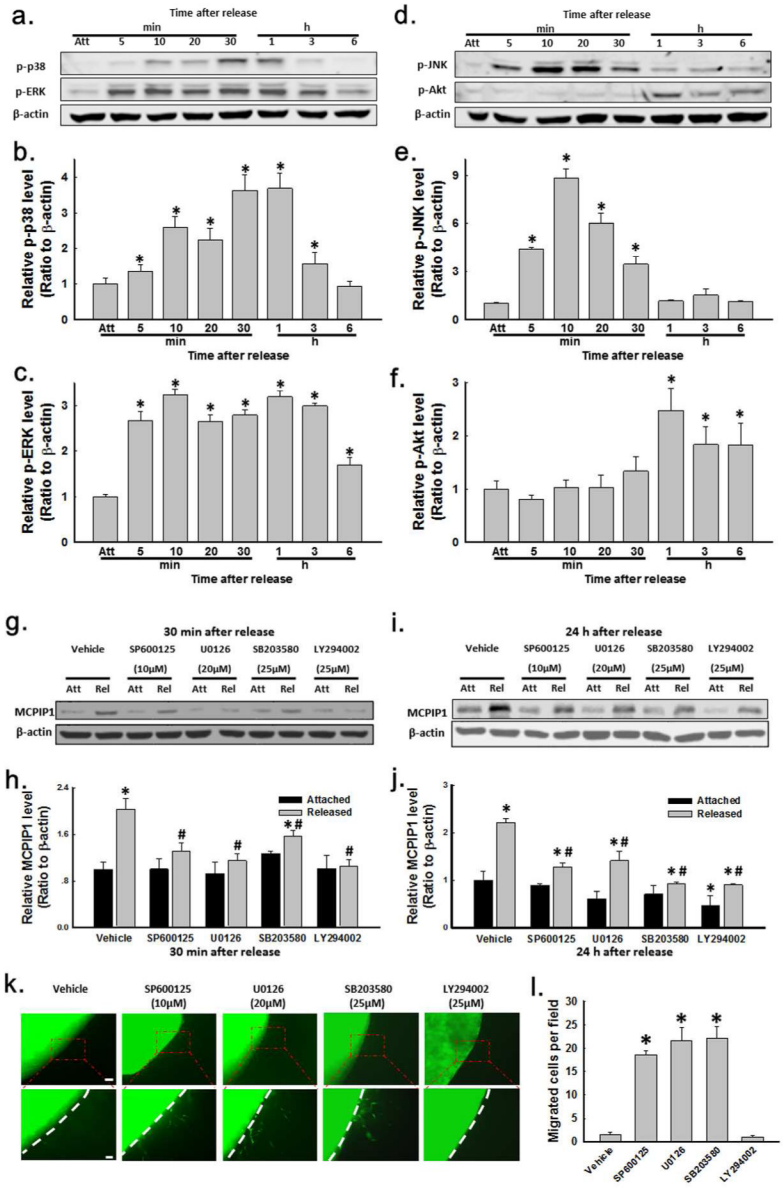

Fig. 3. Kinase activity, inhibition, MCPIP1 expression, and migration in the FPCM

(A–F) p38, Erk1/2, JNK, and Akt phosphorylation after FPCM release (immunoblots of whole lysates), with densitometry of n=4 experiments. **(G–J)** Effect of kinase inhibitors (JNK=SP600125, MEK=U0126, p38=SB203580, PI3K=LY294002) on FPCM MCPIP1, 30 min and 24 h post-release (whole lysate immunoblots), with densitometry (n=4 experiments each). *$p < 0.05$ *vs.* vehicle-attached (unpaired t-test); #$p < 0.05$ *vs.* vehicle-released (unpaired t-test). **(K–L)** Effect of kinase inhibitors on migration out of the released, nested matrix (upper scale bar= 200 μm, lower scale bar =80 μm); plot represents n=3 experiments/ condition. *$p < 0.05$, unpaired t-test.

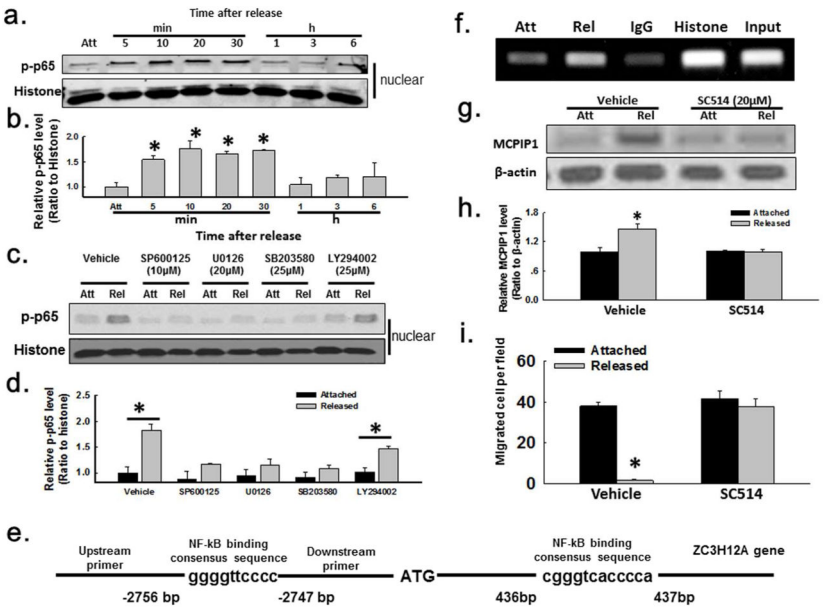

Fig. 4. NF-κB signaling, MCPIP1 induction, and inhibition of migration in the FPCM

(A–B) NF-κB p65 phosphorylation after FPCM release (nuclear fraction immunoblots); n=4 experiments for densitometry. **(C–D)** Effect of kinase inhibitors on p-p65 in attached vs. 20 min-released FPCMs; n=4 experiments for densitometry. **(E)** NF-κB p65 binding sequence, MCPIP1 promoter region. **(F)** ChIP of p65 binding to the MCPIP1 promoter, 20 min after FPCM release. **(G–H)** Effect of NF-κB activation inhibitor (SC-514) on MCPIP1 induction after FPCM release (24 h time point; whole cell immunoblots); n=4 experiments for densitometry. **(I)** Effect of SC-514 resulted on migration out of the nested released FPCM (immunofluorescent images in Supplementary Fig. S1C–D); n=3 experiments. *p<0.05, unpaired t-test.

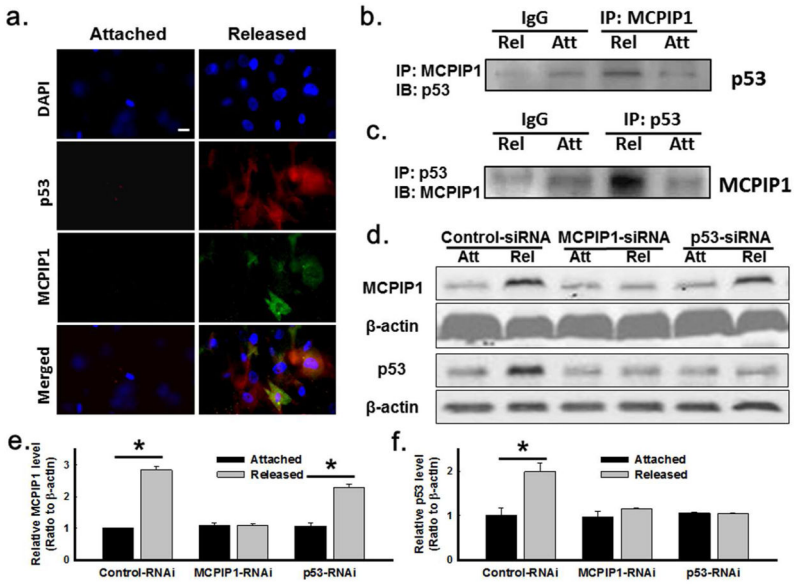

Fig. 5. MCPIP1 and p53 association after FPCM release

(A) MCPIP1 and p53 immunocytochemistry in attached *vs.* 1 day-released FPCM. Blue=DAPI; green=MCPIP1; red=p53; scale bar=20 μm. **(B–C)** MCPIP1 and p53 co-localization after FPCM release (whole lysates from attached *vs.* 1 day-released FPCMs immunoprecipitated for MCPIP1 or p53, then immunoblotted for the other; IgG used as immunoprecipitate control). **(D–F)** Effect of RNAi of MCPIP1 or p53 on p53 or MCPIP1 in 1 day-released FPCM (whole lysate immunoblots); n=4 experiments for densitometry. *p < 0.05 vs. attached (unpaired t-test).

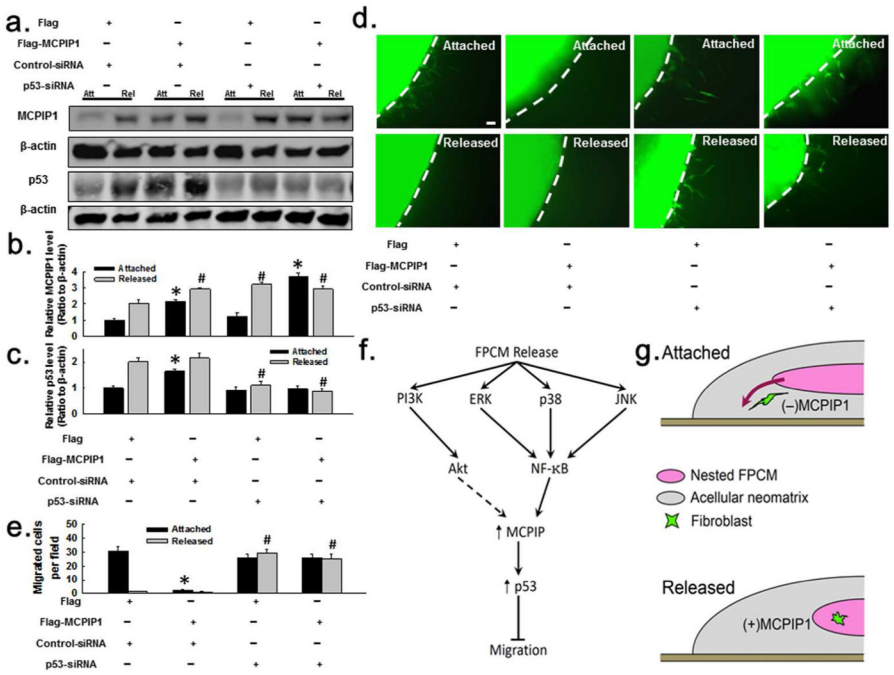

Fig. 6. MCPIP1 regulates cell migration through p53

(A–C) Effect of MCPIP1-Flag expression and/or p53 RNAi on MCPIP1 and p53 in attached *vs.* 1 day-released FPCM (whole lysate immunoblots); n=4 experiments for densitometry. **(D–E)** Effect of MCPIP1-Flag expression and/or p53 RNAi on migration out of attached *vs.* 1 day-released FPCMs (scale bar=80 μm); plot represents n=3 experiments/condition. *$p < 0.05$ *vs.* control-attached (unpaired t-test); #$p < 0.05$ *vs.* control-released (unpaired t-test). **(F–G)** Putative pathway of MCPIP1-centric pathway regulating migration in the FPCM. Stress-release of the FPCM activates PI3K, ERK, p38, and JNK. Activated NK-κB induces expression of MCPIP1, which upregulates p53, which then inhibits fibroblast migration through unknown mechanisms. A pointed arrowhead head = stimulatory effect; flat arrowhead = inhibition.