p120-catenin differentially regulates cell migration by Rho-dependent intracellular and secreted signals

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Abstract

The adherens junction protein p120-catenin is implicated in the regulation of cadherin stability, cell migration and inflammatory responses in mammalian epithelial tissues. How these events are coordinated to promote wound repair is not understood. We show that p120 catenin regulates the intrinsic migratory properties of primary mouse keratinocytes, but also influences the migratory behavior of neighboring cells by secreted signals. These events are rooted in the ability of p120-catenin to regulate RhoA GTPase activity, which leads to a two-tiered control of cell migration. One restrains cell motility via an increase in actin stress fibers, reduction in integrin turnover and an increase in the robustness of focal adhesions. The other is coupled to the secretion of inflammatory cytokines including interleukin-24, which causally enhances randomized cell movements. Taken together, our results indicate that p120-RhoA-GTPase-mediated signaling can differentially regulate the migratory behavior of epidermal cells, which has potential implications for chronic wound responses and cancer.

Keywords adherens junctions; IL-24; migration; p120-catenin; RhoA GTPase

Subject Categories Cell Adhesion, Polarity & Cytoskeleton

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Introduction

Embryogenesis, tissue remodeling and wound repair largely depend on the coordinated connections between neighboring cells, their attachment to the extracellular matrix and environmental cues. One of the most important and ubiquitous types of adhesive interactions is the one mediated by the classic cadherin adhesion molecules at adherens junctions (AJs) [1]. Alterations in the functional activity or expression of AJ molecules commonly lead to enhanced proliferation, cell migration and invasiveness [2,3]. Among the different AJ proteins, the cadherin-binding protein p120-catenin (p120) is an important regulator of cadherin stability and turnover at the membrane [4]. In addition, cytoplasmic and nuclear pools of p120 have been shown to exert pleiotropic functions that influence cell adhesion, actin dynamics and cell signaling [3,5]. In this regard, one major function of p120 is to control the activities of small Rho GTPases [6,7], which can be regulated by cadherin-dependent [8] or independent mechanisms [9]. Through its ability to inhibit RhoA GTPase (RhoA), p120 acts as a molecular switch to orchestrate the balance between cellular adhesion and migration [6–8,10–12]. More recently, several lines of evidence have underscored a role for p120 as a regulator of inflammatory signals in different mammalian tissues [13–15]. In skin, conditional loss of p120 in epidermis leads to a RhoA-dependent activation of NF-kB signaling and an inflammatory skin disease [13,16]. Whether the involvement of p120 in the regulation of AJs, RhoA, actin cytoskeleton dynamics and inflammation converge to regulate wound repair is not yet understood.

Here, we explore the role of p120 in cell migration using cultured primary mouse keratinocytes (mKer), and uncover that through its ability to regulate RhoA, p120 leads to a two-tiered control of cell migration via intracellular and secreted signals.

Results and Discussion

Loss of p120 induces FAs stability and reductions of individual mKer migration, but an enhancement of randomized collective cell migration

Skin-specific p120 conditional knock-out (cKO) mice exhibit a skin inflammatory disease and impaired umbilical cord-wound healing (Supplementary Fig S1A) [13]. The fact that most mice died prenatally, or were too weak, precluded the possibility to perform wound-healing experiments in vivo. Therefore, we turned to in vitro studies to explore the role of p120 in mKer migration. To this end, we isolated mKer from the backskin of p120lox/lox (p120L/L) mice, and ablated the expression of p120 in vitro via adenoviral transduction of Cre recombinase. Immunoblot analyses confirmed the reduction in p120 levels in p120L/L mKer when compared to p120L/L controls (Fig 1A). Immunofluorescence analysis of the actin cytoskeleton showed an increase in stress fibers, consistent with the documented
Figure 1. Loss of p120 leads to opposite behaviors in individual and collective cell migration.

A Immunoblotting of p120 expression in p120<sup>L/L</sup> and p120<sup>ΔΔ</sup> mKer. Actin was used as a loading control.
B Immunofluorescence of paxillin (green) and actin (red).
C Histogram of FRAP experiments for zyxin-RD in a FA region of interest in p120<sup>L/L</sup> and p120<sup>ΔΔ</sup> mKer. Solid lines are best-fit single exponential curves.
D Quantification of FRAP mobile fraction and half-life (t<sub>1/2</sub>) of zyxin-RD in p120<sup>L/L</sup> and p120<sup>ΔΔ</sup> mKer.
E Representative individual cell migration trajectories of p120<sup>L/L</sup> and p120<sup>ΔΔ</sup> mKer.
F Quantification of the length and speed of migrated trajectories from individual p120<sup>L/L</sup> and p120<sup>ΔΔ</sup> mKer. n = 5, each 30 cell tracks.
G Brightfield images of p120<sup>L/L</sup> and p120<sup>ΔΔ</sup> mKer during scratch wound-healing assay at t = 0 h and t = 12 h.
H Representative cell trajectories of p120<sup>L/L</sup> and p120<sup>ΔΔ</sup> mKer in collective cell migration after scratching.
I Length, speed and area of wound closure of p120<sup>L/L</sup> and p120<sup>ΔΔ</sup> mKer. n = 4, each 50 cell tracks.

Data information: Results are expressed as means ± s.e.m. **P < 0.01, ***P < 0.001, ****P < 0.0001. In all cases, the endpoint of each track is denoted with a circle.
activation of RhoA [13]. In addition, staining of the FA protein paxillin showed that p120L/L mKer displayed more robust FAs (Fig 1B), in terms of number, area and fluorescence intensity (Supplementary Fig S1B). This was accompanied by changes in the expression of integrins as evaluated by FACS analyses (Supplementary Fig S1C), cell attachment to different substrates (Supplementary Fig S1D) and cell spreading (Supplementary Fig S1E). Interestingly, fluorescence recovery after photobleaching (FRAP) analyses (Supplementary Movie S1) evidenced a decrease in the mobile fraction of the FA protein zyxin, and an increase in the half-time (t1/2) of recovery after photobleaching in p120Δ/Δ mKer (Fig 1C and D), indicating a reduction in FAs turnover. Similar results were observed for vinculin, another FA protein (Supplementary Fig S1F). These features are dependent on the cell context, since loss of p120 in some transformed cell lines can also increase [8] or decrease FAs [17]. Whether these alterations in FAs are a consequence of altered microtubule dynamics in the absence of p120 remains to be explored [18,19]. Our results suggest that absence of p120 in mKer leads to an increase in FA stability, supporting the existence of a crosstalk between AJs and FAs [20,21].

To determine whether these features affected the migration of p120-null mKer, we performed time-lapse microscopy analyses. The migrated distances and speed of individual cells were significantly reduced in p120Δ/Δ mKer (Fig 1E and F). We next analyzed the status of downstream effectors of integrin signaling, such as focal adhesion kinase (FAK). When activated, FAK phosphorylates several targets including paxillin, thereby increasing cell motility [22]. Immunoblot analyses revealed no differences in the levels of FAK and its active form (pFAK Y397), paxillin and phospho-paxillin and vinculin when compared to controls (Supplementary Fig S1G). Globally, these results indicate that the absence of p120 leads to a decrease in the migratory behavior of individual p120-null cells.

Having analyzed the effects of loss of p120 on individual cell migration, we explored the consequences of its loss in the migratory behavior of epidermal sheets in vitro, which resembles the more physiological scenario of wound healing in skin. To this end, we performed in vitro wound-healing assays by scratching mKer monolayers or by employing the Oris cell migration system (Fig 1F and G, Supplementary Fig S2A–C), in the presence of mitomycin C to prevent cell proliferation. We first performed our analyses in the presence of 0.3 mM calcium to allow AJ establishment without inducing differentiation. Strikingly, the migratory behavior of p120-null cells was significantly increased (Fig 1G and H, Supplementary Movie S2), as quantified by the length and speed of migration (Fig II). In addition, a significant decrease in FA number was observed in p120Δ/Δ cells at the leading edge at the last time point analyzed, together with a significant increase in pFAK at FAs (Supplementary Fig S1H and I). The increase in pFAK was also observed by immunoblot starting from the initial time points, with no differences in FAK total levels (Supplementary Fig S1J). These results suggest an increased integrin-mediated signaling under these conditions, probably by alterations in the crosstalk between AJs and FAs. Hence, we surmised that the enhancement in cell migration could be a consequence of the reduced levels of E-cadherin at the membrane in the absence of p120 [12,13]. p120-null mKer fail to bring cells together and migrate as a cohesive epithelial sheet (Supplementary Movie S2), which is required to integrate actin networks across the epithelial layer [23] and could explain the observed differences in cell migration. To test this hypothesis, we performed in vitro wound-healing assays in 0.06 mM low calcium conditions to prevent AJ formation. The results showed that the increase in the migratory behavior of p120-null cells was still maintained when compared to controls (Supplementary Fig S2A–C, and Supplementary Movie S3). Overall, these results indicate that loss of p120 in individual mKer decreased cell migration; however, this behavior is modified when cells are grown at confluence. These differences may reside in a combination of alterations in mechanical forces and the production of secreted factors that could modify the behavior of neighboring cells.

**Loss of p120 leads to the production of soluble factors that promote cell migration**

Prior studies have positioned p120 as a regulator of Rho-dependent inflammatory responses in epidermis [13]. Whether p120-null mKer influence the migratory behavior of neighboring epidermal cells via secreted factors has not yet been tested. To explore this possibility, we employed again in vitro scratch wound-healing assays in p120L/L mKer and stimulated them using the conditioned medium (CM) of either p120Δ/Δ mKer or p120L/L mKer (Fig 2A). Interestingly, the p120Δ/Δ CM was able to significantly increase the migratory behavior of p120L/L mKer at the leading edge (Fig 2A and B). Of note, this was not accompanied with reductions in E-cadherin (Supplementary Fig S2D). We also tested the stimulatory effect of the p120Δ/Δ CM in individual cell migration and observed a significant increase in both length of cell trajectories and speed (Fig 2C and D). Overall, these results suggest that cell migration stimulatory factors were present and accumulated in the CM of confluent layers of p120-null cells. To further test this hypothesis, we seeded p120L/L and p120Δ/Δ mKer in adjacent wells, using culture inserts located in the same plate. As control, we seeded p120L/L/p120Δ/Δ mKer, and p120Δ/Δ/p120L/L mKer in the two different wells (Fig 2E). mKer were first allowed to attach and reach confluence. After that, the inserts were removed, allowing the diffusion of their corresponding CMs and free cells to migrate. The results showed that p120L/L mKer cultured with p120Δ/Δ mKer (p120L/L versus p120Δ/Δ) moved faster to seal the wound, when compared to p120L/L mKer exposed to the CM of p120Δ/Δ mKer (p120L/L versus p120Δ/Δ) (Fig 2E and F). In contrast, p120Δ/Δ mKer exposed to the CM of p120Δ/Δ mKer (p120Δ/Δ versus p120Δ/Δ) were not able to migrate to the same extent as the p120Δ/Δ mKer exposed to p120Δ/Δ CM (Fig 2E and F). Overall, these results suggest a two-tiered control of the migratory properties of cells regulated by p120. One intrinsic, and the other at the level of signaling via production of soluble factors that are able to render neighboring cells with enhanced migratory characteristics.

**Interleukin-24, a soluble factor induced by loss of p120**

To identify which factor/s could be responsible for the stimulation of cell migration in mKer, we performed microarray analyses of total skin samples isolated from p120cKO E18.5 mouse embryos and controls. Different inflammatory cytokines and chemokines were found differentially expressed in the skin of p120cKO
mice compared with controls (Fig 3A). Based on the reported expression of some of these factors and their receptors in mKer, we validated their expression in mKer after ablation of p120 in vitro by RT–PCR analyses (Fig 3B). We found that TNF-α and interleukin-24 (IL-24), also known as melanoma differentiation associated gene-7, were the two major upregulated cytokines in cultured mKer in the absence of p120 (Fig 3B). TNF-α can stimulate the activation of NFκB [24] in agreement with the previous findings reported in mKer.

Figure 2. Loss of p120 leads to the secretion of soluble factors that stimulate cell migration.

A Schematic representation of the experimental procedure. The conditioned medium (CM) of p120L/L or p120Δ/Δ mKer was used to stimulate confluent-scratched epithelial sheets of p120Δ/Δ mKer. Representative cell trajectories of p120Δ/Δ mKer incubated with p120L/L mKer CM or with p120Δ/Δ CM.

B Length, speed and area of wound closure of p120Δ/Δ mKer incubated with p120L/L CM or with p120Δ/Δ CM. n = 4, each 50 cell tracks.

C Representative cell trajectories of individual p120Δ/Δ mKer incubated with p120L/L CM or with p120Δ/Δ CM.

D Length and speed of individual cell migration of p120Δ/Δ mKer incubated with p120L/L CM or with p120Δ/Δ CM. n = 5, each 30 cell tracks.

E Schematic representation of the experimental procedure. p120L/L and p120Δ/Δ mKer were plated in separated wells in culture inserts located in the same plate, after removing the insert cells were allowed to migrate under their combined media, in order to fill in the gap. As a control, p120L/L or p120Δ/Δ were used.

F Quantification of the covered area (in pixels) relative to starting time point (t = 0 versus t = 14 h) of the following populations: p120L/L versus p120Δ/Δ mKer, p120Δ/Δ versus p120Δ/Δ, p120L/L versus p120Δ/Δ, and p120Δ/Δ versus p120Δ/Δ mKer (n = 2).

Data information: Results are expressed as means ± s.e.m. *P < 0.05, **P < 0.01, ****P < 0.0001. In all cases, the endpoint of each track is denoted with a circle.
However, we were intrigued by the increased expression of IL-24. Prior studies have shown that K14-IL-24 transgenic mice present an inflammatory skin disease [25]. In addition, it has been documented that IL-24 levels increase during the early phases of wound repair of human skin [26], and in chronic wounds [27]. However, *in vitro* studies have shown that recombinant IL-24 acts...
as a migration inhibitory factor in human keratinocytes [26,27].
After validating the increased expression of IL-24 by immunoblot (Fig 3C), we analyzed whether IL-24 had an effect in the migratory properties of mKer. To this end, we transfected control p120<sup>L/L</sup>-mKer with a vector expressing mouse IL-24 (p120<sup>L/L</sup>-IL-24 mKer). Immunoblot and RT–PCR analyses confirmed the overexpression of IL-24 (Fig 3D and Supplementary Fig S3A). After performing in vitro scratch wound-healing assays, an increase in cell motility and wound closure was observed in p120<sup>L/L</sup>-IL-24 mKer (Fig 3E). This was also observed when control mKer were stimulated with the CM of p120<sup>L/L</sup>-IL-24 mKer (Supplementary Fig S2E), without signs of apoptosis (Fig 3F). In addition, this CM was also able to rescue the reduced individual cell migration of p120-null cells (Supplementary Fig S2F).

To determine the causal role of IL-24 in the migratory behavior of p120-null cells, we performed loss-of-function studies using a combination of two specific shRNAs against IL-24. Both protein and mRNA levels of IL-24 were significantly reduced in p120<sup>L/L</sup>-IL-24 knockdown mKer (p120<sup>L/L</sup>-mL-24KD), reaching the same levels as in control cells (Fig 3G and Supplementary Fig S3B). Interestingly, p120<sup>L/L</sup>-mL-24KD exhibited a decrease in cell motility (Fig 3H and Supplementary Fig S3E), without signs of apoptosis (Fig 3I). In addition, the CM of p120<sup>L/L</sup>-mL-24KD was not able to enhance cell motility in control mKer (Supplementary Fig S3F), rescuing the stimulatory effect of the p120<sup>L/L</sup>-CM on cell migration.

The differences in the documented roles of IL-24 in cell migration may reside in the levels of recombinant IL-24 used to stimulate human Ker [26,27], or the expression levels of its receptor in that system. However, using loss- and gain-of-function studies, our results place IL-24 as an stimulatory factor of cell migration in mKer [26,27], or the expression levels of its receptor in that system. Since in mKer, loss of p120 is also accompanied by MAPK activation [13], it will be interesting to learn any possible connections between RhoA and IL-24 in that context.

Finally, to explore the causal involvement of p120 in the expression of IL-24, we conducted rescue experiments by transducing p120-null mKer with lentiviruses encoding full-length p120-GFP (p120-FL-GFP), and p120 mutants lacking either the putative Rho GTPase regulatory domain (p120ARDD-GFP) or the E-cadherin-binding domain (p120Acad-GFP) (Fig 4H, I and J). To obtain homogeneous populations of GFP, positive cells were FACS-sorted (Fig S4A). Immunoblot and RT–PCR analyses confirmed similar levels of expression of all constructs (Fig 4I and J). In p120<sup>L/L</sup> mKer, the re-expression of p120 was able to reduce the mRNA and protein expression levels of IL-24 (Fig 4K and L). This was also observed when the p120Acad-GFP mutant was expressed. By contrast, the expression of p120ARDD-GFP was not able to decrease the IL-24 protein or mRNA levels (Fig 4K and L). Overall, these results indicate a role for p120 in the regulation of IL-24 through its Rho-regulatory domain, although further studies are needed to determine how exactly RhoA activation leads to the regulation of IL-24. The fact that p120 re-expression also induces the modulation of other Rho GTPases, including Rac1 [30], technically precluded the possibility to observe a rescue of the migratory properties of p120-null cells (Supplementary Fig S4B). Indeed, even cells expressing low levels of GFP presented the previously documented branching phenotype, associated with increased membrane dynamics and cell migration (Fig S4C) [30]. Interestingly, p120ARDD-GFP did not induce the formation of cell protrusions. In closing, our results show that p120 is involved in the regulation of cell migration by regulating RhoA intrinsic and extracellular signals. p120-null cells fail to seal wounds and bond cells together due to the reduced levels of cadherins at the membrane. Since in human biopsies, it has been documented that IL-24 levels increase at early phases of wound healing and decrease as wounds heal [26], and IL-24 is elevated in chronic wounds [27], it is tempting to speculate that p120 is required to decrease the expression levels of IL-24 to allow cell repair, along with the re-establishment of intercellular junctions to allow tissue remodeling at later stages of wound healing.

Materials and Methods

Cell culture

mKer were isolated from backskins of p120<sup>L/L</sup> P3 mice as previously described [5]. Briefly, the epidermis was separated from the dermis using dispase (Sigma) and trypsin (Gibco). After filtration
in 40-μm cell strainers, cells were cultured in low-calcium medium (LC).

**Cell adhesion assay**

Coverslips were coated with fibronectin (10 μg/ml; Calbiochem, Cat N. 341635), collagen I (10 μg/ml; Invitrogen, Cat N. A10483-01) or vitronectin (10 μg/ml; Chemicon, Cat N. CC080) in PBS overnight at RT. $5 \times 10^3$ mKer were plated in triplicates for 30 min, washed, fixed and stained with DAPI. The experiment was repeated three times ($n = 3$).

**Cell spreading**

$5 \times 10^3$ mKer were plated onto fibronectin-coated plates for 12 h, fixed with 4% PFA and permeabilized with blocking solution (1% BSA, 5% normal goat serum, 5% normal donkey serum, 1% Gelatin and 0.3% Triton X-100 in PBS). Cells were stained with cell mask...
Figure 4. The secretion of IL-24 is regulated by p120-RhoA-dependent signals.

A Immunoblot analysis of IL-24 in p120L/L or p120K/K mKer treated or untreated with a RhoA inhibitor.
B Luciferase assays of the activity of the IL-24 promoter before and after treating p120L/L or p120K/K mKer with the RhoA inhibitor.
C Experimental procedure: The CM of the Rho inhibitor-pretreated p120L/L mKer or p120K/K mKer was collected and added to p120L/L mKer-scratched monolayers.
D Quantification of length, speed and wound-healing closure of p120L/L mKer incubated with p120L/L CM, p120K/K mKer incubated with p120K/K CM, p120K/K mKer incubated with p120L/L CM pretreated with RhoA inhibitor or p120K/K mKer incubated with p120K/K CM pretreated with RhoA inhibitor; n = 4, each 50 cell tracks.
E Immunoblot of RhoA and c-myc in p120L/L mKer overexpressing a dominant active form of RhoA tagged with myc (DA-RhoA-myc).
F RT-qPCR analysis of IL-24 expression levels in p120L/L mKer and p120K/K mKer overexpressing DA-RhoA-myc.
G Control and IL-24 promoter luciferase activity in p120L/L mKer overexpressing DA-RhoA-myc.
H Scheme of the p120-full-length GFP (p120-Fli), p120-ΔEcad-GFP (p120-ΔEcad) and p120-ΔRBD-GFP (p120-ΔRBD).
I Immunoblot of p120 and GFP expression levels in p120L/L mKer expressing the p120 rescue constructs.
J RT-qPCR analysis of p120 mRNA expression levels of p120L/L mKer expressing the p120 GFP constructs.
K Immunoblot of IL-24 expression levels in p120L/L mKer expressing the p120 rescue constructs.
L RT-qPCR analysis of IL-24 mRNA expression levels in p120K/K mKer expressing the p120-GFP constructs.

Data information: Results are expressed as means ± s.e.m. ***P < 0.001, ****P < 0.0001.

Supplementary information for this article is available online: http://embor.embopress.org

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Author contributions

CE designed, performed and analyzed experiments. DM performed time-lapse microscopy experiments and analyses. MPM conceived, designed and supervised the study. CE and MPM wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References