Fidgetin-Like 2: A Microtubule-Based Regulator of Wound Healing

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Wound healing is a complex process driven largely by the migration of a variety of distinct cell types from the wound margin into the wound zone. In this study, we identify the previously uncharacterized microtubule-severing enzyme, Fidgetin-like 2 (FL2), as a fundamental regulator of cell migration that can be targeted *in vivo* using nanoparticle-encapsulated small interfering RNA (siRNA) to promote wound closure and regeneration. *In vitro*, depletion of FL2 from mammalian tissue culture cells results in a more than twofold increase in the rate of cell movement, in part due to a significant increase in directional motility. Immunofluorescence analyses indicate that FL2 normally localizes to the cell edge, importantly to the leading edge of polarized cells, where it regulates the organization and dynamics of the microtubule cytoskeleton. To clinically translate these findings, we utilized a nanoparticle-based siRNA delivery platform to locally deplete FL2 in both murine full-thickness excisional and burn wounds. Topical application of FL2 siRNA nanoparticles to either wound type results in a significant enhancement in the rate and quality of wound closure both clinically and histologically relative to controls. Taken together, these results identify FL2 as a promising therapeutic target to promote the regeneration and repair of cutaneous wounds.

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INTRODUCTION

Although wound healing is orchestrated largely by the migration of diverse cell types from the wound edge into the wound zone, the intracellular mechanisms that drive cell migration have not yet been effectively harnessed for therapeutic purposes. A potentially ideal target in this regard is the microtubule (MT) cytoskeleton. MTs are highly dynamic, polar polymers of tubulin with a fast growing plus-end and a slower growing minus-end. Within cells, MTs are organized into extremely plastic higher order arrays with their plus-ends generally facing the cell periphery and minus-ends oriented toward the nucleus. Although MTs are not required for cell migration *per se*, there is growing evidence that they control and coordinate multiple parameters of cell movement including actin-based membrane protrusions (Wehrle-Haller and Imhof, 2003; Gardel *et al.*, 2011), the assembly and organization of integrin-based focal adhesion complexes (FAs) (Kaverina *et al.*, 1998; Stehbens and Wittmann, 2012), and the distribution and delivery of Golgi-derived and endosomal vesicles to the leading edge (Watanabe *et al.*, 2005; Kaverina and Straube, 2011).

We recently showed that the migration of *Drosophila melanogaster* cells was strongly enhanced by the RNA interference–mediated depletion of the MT regulatory protein Katanin (Zhang *et al.*, 2011). Katanin is a conserved member of the ATPases associated with diverse cellular activities (AAA) protein superfamily (Roll-Mecak and McNally 2010; Sharp and Ross 2012), which displays the capacity to sever and depolymerize MT plus-ends *in vitro* (McNally and Vale, 1993). In *Drosophila*, Katanin localizes to the cell cortex and normally suppresses cell movement by locally destroying MTs (Zhang *et al.*, 2011). Although Katanin does not regulate the migration of most mammalian cells, our findings raised the intriguing possibility that a related but as of yet undetermined MT-severing enzyme could be similarly targeted to promote cell migration and wound healing in mammals.

We show here that a related but presently uncharacterized protein, Fidgetin-like 2 (FL2), can be targeted to strongly enhance the wound healing behavior of mammalian cells, and in particular skin cells, both *in vitro* and *in vivo*. We previously reported that Fidgetins function as MT severing and depolymerizing enzymes (Zhang *et al.*, 2007; Mukherjee *et al.*, 2012). Small interfering RNA (siRNA)-mediated

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Abbreviations: FA, focal adhesion complex; FL2, fidgetin-like 2; GFP, green fluorescent protein; MT, microtubule; NPsi, nanoparticle siRNA; siRNA, small interfering RNA

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depletion of FL2 induces a more than twofold increase in the rates of *in vitro* cell movement and *in vivo* wound closure. When functioning normally, FL2 localizes to regions of the cell cortex, suppresses MT growth, and regulates the size and distribution of FA complexes, likely through its impact on MT dynamics. Finally, we present an innovative nanoparticle-based topical siRNA delivery system that has allowed us to efficiently reduce the expression of FL2 in murine wounds and accelerate wound healing.

RESULTS

FL2 is a regulator of cell migration *in vitro*

Our interest in FL2 as a potential therapeutic target for wound healing initiated from a siRNA-based screen for MT severing or depolymerizing enzymes that regulate human cell motility. This screen targeted all of the known or putative MT severing or depolymerizing enzymes encoded by the human genome, and the effect of siRNA treatment on the migration of cultured U2OS cells (human osteosarcoma cells) was measured using a standard *in vitro* scratch assay. Of all targets analyzed in this screen, depletion of the previously uncharacterized protein, FL2, had the most pronounced effect on cellular behavior. A decrease in FL2 mRNA and protein levels following siRNA treatment was confirmed using quantitative PCR and western blotting, respectively. (Supplementary Figure S1A and S1C online). In particular, FL2 siRNA treatment induced a statistically significant ~2.5-fold increase in the rate of scratch closure (Figure 1a and b; Supplementary Movie S1 online) largely due to enhanced cell migration into the cell-free scratch zone. Quantification of the movement trajectories of isolated individual cells indicated that Fl2 siRNA treatment caused cells to move significantly faster and more directionally compared with controls (Figure 1c and d). The increased rate of cell migration caused by FL2 knockdown was rescued to control levels when FL2 siRNA–treated cells were induced to express an exogenous green fluorescent protein (GFP)–FL2 construct (which lacks the 3′ UTR sequences targeted by our siRNA), ruling out off-target effects (Supplementary Figure S2A online). Finally, a qualitatively similar enhancement of cell migration after FL2 siRNA treatment was observed in a human keratinocyte cell line (HaCaT) and a mouse fibroblast cell line (L929; Figure 1d; Supplementary Figure S3 online). Thus, FL2 normally functions to suppress cell motility in a variety of cell types relevant to mammalian cutaneous wound healing.

It should be noted that a small but statistically significant increase in the proliferation rates of FL2-depleted U2OS and L929 cells (but not HaCaTs) was also observed in our studies (Supplementary Figure S4 online). However, we do not believe that this can account for the enhancements of cell migration described above as migration measurements (Figure 1d; Supplementary Figure S2A and S3A online) were performed on isolated cells that did not undergo mitosis during the analysis time-course and were not pushed forward by a proliferating cell mass (e.g., at the scratch edge). The potential mechanisms by which FL2 influences the proliferation of some cell types will be explored in a separate study.

FL2 localizes to and regulates the organization of MTs at the cell cortex

As a first step toward understanding the mechanistic basis of FL2's impact on cell motility, we examined its subcellular distribution using indirect immunofluorescence. Multiple monoclonal antibodies raised against different regions of FL2 showed a strong enrichment at the cortex of U2OS cells with similar staining patterns observed in HaCaTs and L929s. This cortical labeling was generally discontinuous and most pronounced at the leading edge/lamellipodium of polarized cells where it often became particularly concentrated at sites where individual microtubule ends contacted the cortex (Figure 1e). siRNA treatment significantly reduced the intensity of cortical FL2 immunostaining, supporting the specificity of this localization pattern (Supplementary Figure S5 online). Moreover, exogenously expressed GFP–FL2 acquired a similar cortical localization (Supplementary Figure S6 online). By western blot, our antibodies primarily labeled a tight doublet running between 65 and 75 kD (Supplementary Figure S1B online). The predicted molecular mass of FL2 is 66 kD. This doublet was substantially reduced after FL2 siRNA treatment strongly supporting the hypothesis it corresponds to native FL2 and that FL2 protein levels are reduced by siRNA treatment (Supplementary Figure S1C online).

We next tested the hypothesis that FL2 impacts cell motility via the MT cytoskeleton. Quantitative immunofluorescence performed in U2OS cells, which are particularly suited for this kind of microscopy, owing to their size and extremely flat morphology, revealed that the depletion of FL2 significantly increased cellular MT polymer levels ~40% relative to controls ($P<0.005$)—an expected outcome if FL2 normally functions by destabilizing MTs. This siRNA phenotype was rescued by exogenously expressed GFP–FL2 (Supplementary Figure S2B online). Depletion of FL2 also induced the formation of dense MT bundles just beneath the cell edge consistent with the protein's cortical localization (Figure 2a and b). In addition, cells with reduced FL2 displayed a decrease in acetylated MTs (Figure 2c and d). Acetylation is a posttranslational modification that usually marks a slow-growing stable MT subpopulation (Hammond *et al.*, 2008), thus suggesting that FL2 normally acts on dynamic, non-acetylated MTs. This is in contrast to Katanin that preferentially severs acetylated MTs (Sudo and Baas, 2010).

In a parallel line of study, we examined how the loss of FL2 affects MT dynamics and organization by immunostaining for EB1, a protein that specifically labels growing MT-plus ends. MT-associated EB1 staining normally acquires a comet-like appearance because the protein associates with tubulin subunits as they are incorporated into the MT end and then dissociates shortly thereafter (Vaughan, 2005). Immunofluorescence analysis showed a significant increase in the average number of EB1 comets at the cell edge following FL2 knockdown, further supporting the hypothesis that FL2 locally targets dynamic MTs (Figure 3a and b). Moreover, the mean length of EB1 comets was significantly increased after FL2 knockdown, suggesting an increase in the MT growth rate (Figure 3b). Tracking of GFP–EB1 comet movement in living cells is standardly used to directly assess