TGF-β/Smad signaling through DOCK4 facilitates lung adenocarcinoma metastasis

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The mechanisms by which TGF-β promotes lung adenocarcinoma (ADC) metastasis are largely unknown. Here, we report that in lung ADC cells, TGF-β potently induces expression of DOCK4, but not other DOCK family members, via the Smad pathway and that DOCK4 induction mediates TGF-β's prometastatic effects by enhancing tumor cell extravasation. TGF-β-induced DOCK4 stimulates lung ADC cell protrusion, motility, and invasion without affecting epithelial-to-mesenchymal transition. These processes, which are fundamental to tumor cell extravasation, are driven by DOCK4-mediated Rac1 activation, unveiling a novel link between TGF-β and Rac1. Thus, our findings uncover the atypical Rac1 activator DOCK4 as a key component of the TGF-β/Smad pathway that promotes lung ADC cell extravasation and metastasis.

[*Keywords*: DOCK180 proteins; TGF-β signaling; lung adenocarcinoma metastasis; tumor cell extravasation; Rac1; cell motility]

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The cytokine TGF-β plays an important, albeit complex, role in epithelial tumorigenesis (Derynck et al. 2001; Elliott and Blobe 2005; Padua and Massague 2009; Jakowlew 2010). During early stages of tumorigenesis, TGF-β typically functions as a tumor suppressor. At later stages, however, as tumors grow and progress, TGF-β produced by both tumor and stromal cells within the tumor microenvironment as a natural response to hypoxic and inflammatory conditions can act as a potent promoter of multiple steps of the metastatic process. These include not only local motility/invasion and entry of cancer cells into the blood stream (intravasation) but also their exit from the blood vessels (extravasation) and survival at the distant organ sites (Ma et al. 2008; Massague 2008; Padua et al. 2008; Giampieri et al. 2009; Padua and Massague 2009; Labelle et al. 2011; Valastyan and Weinberg 2011; Calon et al. 2012; Yuan et al. 2014). The relevance of TGF-β signaling for disease progression has been particularly recognized in tumors where cancer cells retain the core TGF-β signaling components, as is often the case in breast and lung cancers (Kang et al. 2003; Elliott and Blobe 2005; Massague 2008; Padua and Massague 2009). Indeed, in lung adenocarcinoma (ADC), the most common subtype of lung cancer with a high mortality rate, increased TGF-β1 expression correlates with tumor progression and poor patient survival, and various experimental model systems support a prometastatic role for TGF-β in these tumors (Lund et al. 1991; Hoffman et al. 2000; Hasegawa et al. 2001; Gibbons et al. 2009; Nemunaitis et al. 2009; Toonkel et al. 2010; Provencio et al. 2011; Vazquez et al. 2013). However, a major remaining challenge is the identification of TGF-β target genes that drive the different steps of metastasis, especially since TGF-β modulates gene expression in a highly cell- and context-specific manner (Padua and Massague 2009; Mullen et al. 2011; Massague 2012). While some progress has been made in the context of breast cancer metastasis (Michl et al. 2005; Padua et al. 2008; Gregory et al. 2011; Sethi et al. 2011; Shibue et al. 2013), the genes and mechanisms that mediate the prometastatic effects of TGF-β in lung ADC, particularly those mediating the extravasation step, remain largely unknown.

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To explore molecular mechanisms that could mediate the prometastatic effects of TGF-β in lung ADC, we took a candidate gene approach and started by scrutinizing members of the DOCK180-related protein superfamily. The DOCK180 family, of which a total of 11 mammalian members have been identified (termed DOCK1/DOCK180 to DOCK11) emerged as a novel class of Rac/Cdc42 GTPase guanine nucleotide exchange factors (GEFs) (Cote and Vuori 2002; Meller et al. 2005). This class of proteins has been implicated in diverse cell type-specific processes (Laurin and Cote 2014), with some of its members (i.e., DOCK1, DOCK3, and DOCK10) playing distinct roles in the progression and/or metastasis of diverse tumor types—including melanoma, breast cancer, and glioblastoma—by engaging in different protein–protein interactions (Gadea et al. 2008; Sanz-Moreno et al. 2008; Feng et al. 2012; Laurin et al. 2013). Whether any of the DOCK proteins play a role in the progression and/or metastasis of lung ADC has not been previously investigated. Our findings presented here demonstrate that the DOCK4 family member plays a critical role in mediating TGF-β's prometastatic effects in lung ADC. We found that in lung ADC cells, expression of DOCK4, but not other DOCK180 family members, is rapidly and robustly induced by TGF-β in a Smad-dependent manner and that DOCK4 induction is essential for TGF-β-driven lung ADC metastasis. Blockade of TGF-β-induced DOCK4 attenuates the ability of lung ADC cells to extravasate into distant organ sites, resulting in a marked reduction in metastatic burden in mice. At the cellular level, TGF-β-induced DOCK4 elicits lung ADC cell protrusive activity, motility, and invasion and intriguingly does so via Rac1 activation. So far, Rac1 has been linked to TGF-β via noncanonical, non-Smad pathways (Zhang 2009). Thus, our findings identify the atypical Rac1 activator DOCK4 as a novel, key component of the TGF-β/Smad pathway that promotes lung ADC cell extravasation and metastasis.

Results

TGF-β potently induces expression of DOCK4 via the Smad pathway

Upon examining the expression profiles of all 11 DOCK180 family members in the human lung ADC cell line A549 (subjected or not subjected to TGF-β treatment) by real-time quantitative PCR (qPCR), we found that all members, with the exception of DOCK2, were expressed in A549 cells. Strikingly, however, only DOCK4 mRNA levels, but not those of any other DOCK180 family member, were robustly up-regulated by TGF-β treatment (Fig. 1A). In addition, we noted *DOCK4* to be the most strongly up-regulated gene among all Rho family GEFs when analyzing a publically available data set comprising gene expression profiles of TGF-β-treated A549 cells (NCBI, Gene Expression Omnibus [GEO] GSE17708) (Supplemental Fig. 1A). Importantly, TGF-β-induced up-regulation of DOCK4 was also seen at the protein level (Fig. 1B). The increase in DOCK4 protein levels was observed in not only the *KRAS* mutant A549 cell line but also several other lung ADC cell lines carrying either a *KRAS* mutation (H441) or *EGFR* mutations (HCC4006, H1975, and PC9) as well as a *KRAS* and *EGFR* wild-type lung ADC cell line (H1793) (Fig. 1B). All of the above-mentioned cell lines displayed increased Smad3 phosphorylation levels in response to TGF-β (Fig. 1B). Interestingly, no increase in DOCK4 expression by TGF-β was observed in any of the TGF-β-responsive breast cancer and melanoma cell lines that we examined (Supplemental Fig. 1B; data not shown), suggesting that the effect of TGF-β on DOCK4 expression is tumor type-dependent. Notably, a recent study also implicated the WNT/TCF pathway in lung ADC metastasis (Nguyen et al. 2009). However, we did not detect any change in DOCK4 protein levels upon treatment of A549 cells with WNT3A (Supplemental Fig. 1C), implying that *DOCK4* is not likely a target gene of the WNT/TCF pathway in lung ADC metastasis.

A key pathway in the regulation of TGF-β-induced gene expression is the canonical Smad pathway, albeit noncanonical non-Smad pathways have been implicated as well (Padua and Massague 2009; Zhang 2009). To explore whether the Smad pathway is responsible for TGF-β-induced DOCK4 up-regulation, we used two previously described Smad4 shRNAs (shSmad4#1 and shSmad4#2) that we confirmed to be effective in reducing Smad4 protein levels (Fig. 1C; Supplemental Fig. 1D). Smad4 is an essential component of the Smad pathway; upon TGF-β stimulation, it forms complexes with receptor-phosphorylated Smad2/Smad3 proteins, which translocate into the nucleus and regulate gene transcription (Padua and Massague 2009). Stable A549 and HCC4006 cell lines expressing shSmad4#1, shSmad4#2, or control shRNA (shCtrl) were generated and subjected to TGF-β treatment. As expected, in shCtrl-expressing cells, TGF-β triggered a robust increase in DOCK4 expression. This increase, however, was largely prevented in shSmad4#1- and shSmad4#2-expressing cells (Fig. 1D, E; Supplemental Fig. 1D,E). Moreover, when we used the Smad3 inhibitor SIS3 or the TGF-β type I receptor inhibitor SB432542 to interfere with the TGF-β/Smad pathway, we similarly found that SIS3 and SB432542 blocked the TGF-β-induced increase in DOCK4 expression (Fig. 1F–H; Supplemental Fig. 1F). Notably, DOCK4 mRNA up-regulation was also seen upon acute TGF-β treatment (3 and 7 h) and was entirely abrogated when cells were treated with the transcription inhibitor actinomycin D (Supplemental Fig. 1G). These data indicate that TGF-β transcriptionally up-regulates DOCK4 in lung ADC cells via the canonical Smad pathway.

To evaluate whether DOCK4 is a direct target of the TGF-β/Smad pathway, we first tested whether new protein synthesis is required for TGF-β-induced transcriptional activation of DOCK4. We found that treatment of A549 cells with the protein synthesis inhibitor cycloheximide (CHX) did not prevent TGF-β-induced up-regulation of DOCK4 mRNA (Supplemental Fig. 1H), indicating that increased DOCK4 expression is not a secondary effect of TGF-β/Smad signaling activation. We next searched for potential Smad-binding elements (SBEs) in the *DOCK4*

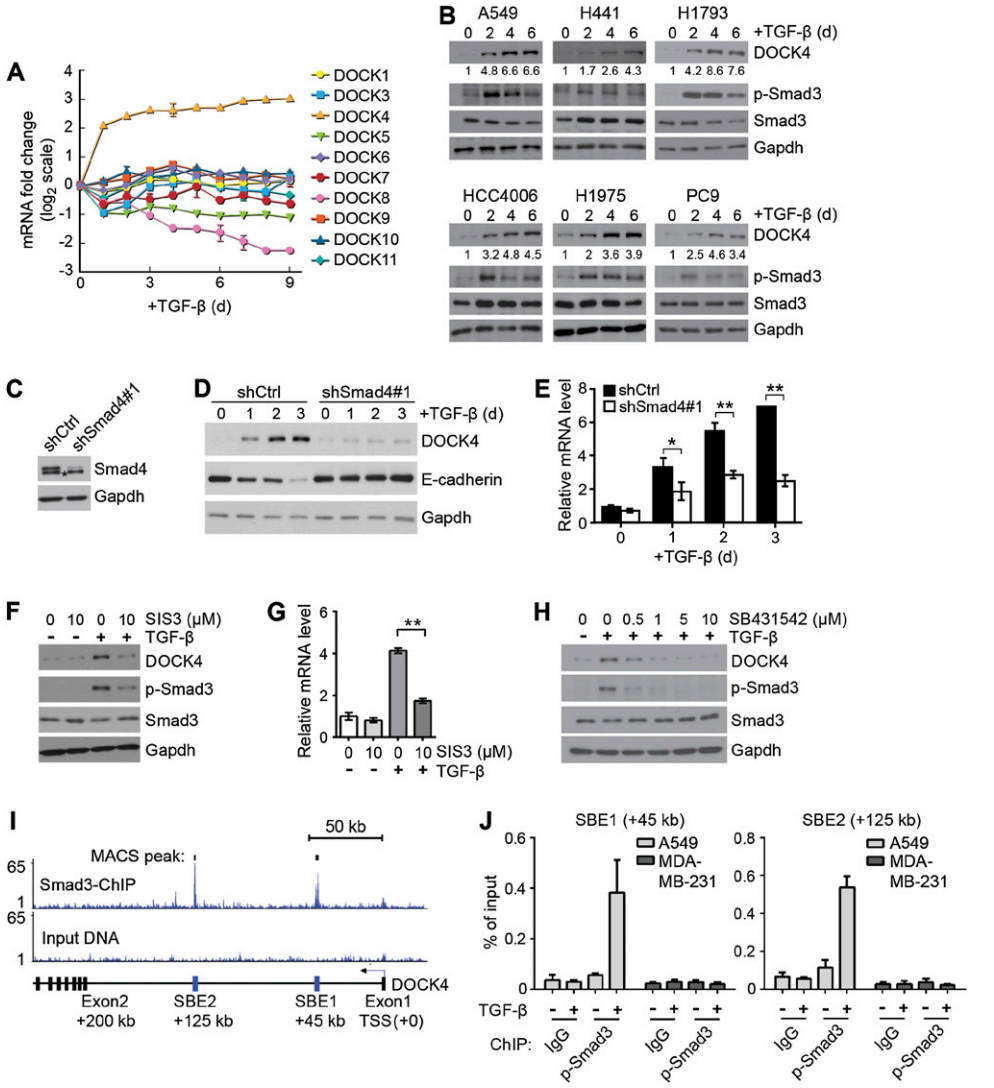

Figure 1. TGF-β induces DOCK4 expression in human lung ADC cells via the Smad pathway. (*A*) qPCR analysis of DOCK180 family member mRNAs in A549 cells treated with 2 ng/mL TGF-β. (*B*) Western blot analysis of DOCK4, p-Smad3, and Smad3 in human lung ADC cell lines treated with TGF-β. DOCK4 levels were normalized to Gapdh and then to a value of 1.0 for day 0. (*C*) Western blot analysis of Smad4 in A549 cells stably expressing shCtrl or shSmad4#1. (*) Nonspecific band. (*D*,*E*) Western blot analysis of DOCK4 and E-cadherin (*D*) and qPCR analysis of DOCK4 mRNA (*E*) in shCtrl- and shSmad4#1-expressing A549 cells treated with TGF-β. (*F*,*G*) Western blot analysis of DOCK4, p-Smad3, and Smad3 (*F*) and qPCR analysis of DOCK4 mRNA (*G*) in A549 cells treated with 10 μM p-Smad3 inhibitor SIS3 and/or 2 ng/mL TGF-β for 24 h. (*H*) Western blot analysis of DOCK4, p-Smad3, and Smad3 in A549 cells treated with 0–10 μM TGF-β type I receptor inhibitor SB431542 and 2 ng/mL TGF-β for 24 h. qPCR data in *A*, *E*, and *G* were normalized to Gapdh and are presented as mean ± SD. $n = 3$. (*) $P < 0.05$; (**) $P < 0.01$ by an unpaired two-tailed Student's *t*-test. (*I*, *top*) ChIP-seq occupancy profile of Smad3 along with input DNA on human DOCK4 locus obtained in A549 cells shown in reads per half-million. MACS peaks depict two validated peaks with a false discovery rate (FDR) <5%. Data were obtained from the GEO database (GSE51509). (*Bottom*) Validated transcript models for *DOCK4* from the hg18 genome assembly. (Black bars) Exons; (blue bars) Smad-binding elements (SBEs) within the corresponding MACS peaks; (TSS) transcriptional start site. (*J*) ChIP assay for p-Smad3 binding to two SBEs in the first intron of *DOCK4*. A549 and MDA-MB-231 cells left untreated or treated with 2 ng/mL TGF-β for 5 h were harvested and processed for ChIP with isogenic IgG or anti-p-Smad3 antibody. The enrichment of the precipitated DNA by p-Smad3 antibody versus the IgG was analyzed by qPCR using primers flanking SBE1 and SBE2. Data are shown as fold of DNA enrichment and presented as mean ± SD. $n = 3$.

promoter region using TRANSFAC and FIMO from the MEME suite (Grant et al. 2011). However, we did not detect any canonical SBEs within 20 kb of the DOCK4 transcriptional start site (data not shown). Since prior studies had shown that a large proportion of Smad-binding sites are found outside of promoter-proximal regions at putative enhancer elements (Kennedy et al. 2011; Morikawa et al. 2011; Schlenner et al. 2012; Gaunt et al. 2013), we considered whether Smad proteins occupy distal SBEs at the *DOCK4* locus. To this end, we analyzed available Smad3

ChIP-seq (chromatin immunoprecipitation [ChIP] combined with deep sequencing) data (GSE51509) obtained from TGF-β-stimulated A549 cells (Isogaya et al. 2014) and found two significant Smad3 peaks in the first intron of *DOCK4* at +45 kb and +125 kb (Fig. 1I). Notably, each of these two sites contains a single SBE. To test whether p-Smad3 directly binds to the two putative SBEs, we designed primers flanking the two putative SBEs and performed anti-p-Smad3 ChIP followed by qRT–PCR (ChIP-qPCR). We found that p-Smad3 binds to both SBEs in a TGF-β-dependent manner (Fig. 1J). Importantly, binding of p-Smad3 to the two SBEs was detected in lung ADC A549 cells, but not in breast cancer MDA-MD-231 cells (Fig. 1J), in which no TGF-β-induced up-regulation of DOCK4 was observed (Supplemental Fig. 1B). Taken together, these data strongly suggest that *DOCK4* is a direct TGF-β/Smad target gene in lung ADC cells.

High DOCK4 expression correlates with Smad activation and poor prognosis in human lung ADC

To extend our findings beyond cells in culture and determine a possible relevance to human lung ADC disease, we examined whether the levels of DOCK4 and p-Smad3 (used as a readout for activity of TGF-β signaling) were correlated in human lung ADC. To this end, we performed immunohistochemistry (IHC) on human lung ADC tissue microarrays (TMAs) using anti-DOCK4-specific and anti-p-Smad3-specific antibodies (Supplemental Fig. 2A; Siebert et al. 2011). We observed that DOCK4 expression was significantly higher in tumor tissues compared with adjacent normal tissues (Supplemental Fig. 2B,C). Moreover and importantly, we observed a strong and significant positive correlation between DOCK4 and p-Smad3 levels in the tumor tissues (Fig. 2A,B), indicating that DOCK4 levels positively correlate with activated TGF-β signaling in human lung ADC.

We further investigated whether DOCK4 expression was correlated with the clinical outcome of human lung ADC. To this end, we analyzed a publicly available microarray data set containing gene expression profiles of 182 human lung ADCs and clinical follow-up information. Both Cox proportional univariate and multivariate analyses revealed an inverse correlation between DOCK4 expression and patient recurrence-free survival (Fig. 2C,D; Supplemental Table 3). Moreover, contingency analyses revealed that high DOCK4 expression is strongly correlated with high frequency of recurrence events and weakly but significantly correlated with advanced tumor stage (stage III or higher) (Supplemental Table 4). Notably, no such correlations were observed for DOCK3 expression; DOCK3 is the most closely related to DOCK4 family member (Supplemental Fig. 3A,B; Supplemental Table 3). Also, upon analyzing publicly available breast cancer data sets, no inverse correlation between DOCK4 expression and patient recurrence-free survival was observed (Supplemental Fig 3C,D), consistent with our findings that TGF-β does not induce DOCK4 expression in breast cancer cell lines. Together, these results suggest that DOCK4 is a potential prognostic factor that predicts disease relapse in human lung ADC patients.

DOCK4 is required for TGF-β's prometastatic effects in lung ADC

Based on our above findings and previous reports correlating increased TGF-β expression with tumor progression and metastasis in lung ADC (Hasegawa et al. 2001; Nemunaitis et al. 2009; Vazquez et al. 2013), we next asked whether DOCK4 mediates the prometastatic effects of TGF-β in lung ADC. To address this, we first established an experimental model for the analysis of lung ADC metastasis. Because lung ADC typically metastasizes to multiple organs (including bones, the adrenal glands, the brain, and the liver) (Nguyen et al. 2009), with lung ADC cells released from the primary site traveling via the arterial circulation to distant organ sites,

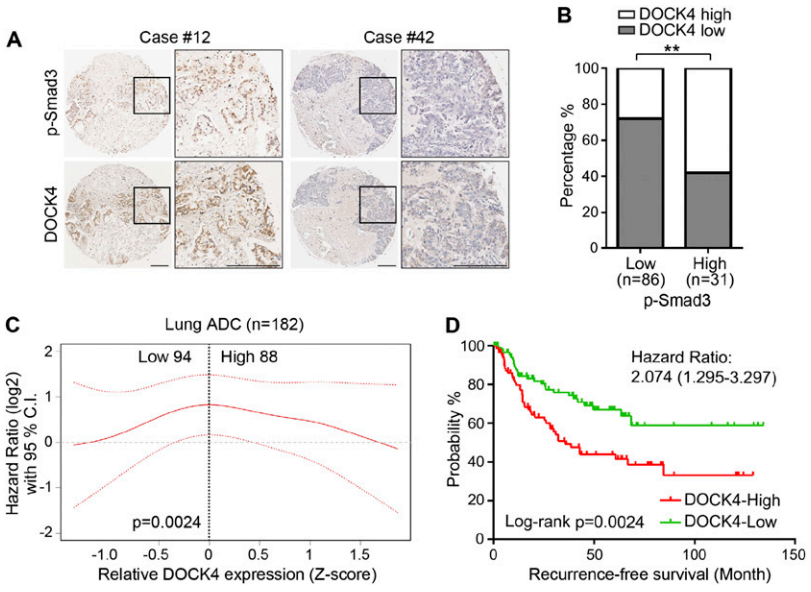

Figure 2. DOCK4 expression is correlated with activity of TGF-β signaling and recurrence-free survival in lung ADC. (*A*) Representative images of IHC stainings for p-Smad3 and DOCK4 in human lung ADC TMAs. Nuclei were counterstained with hematoxylin. Boxed regions are enlarged and shown at the *right*. Bars, 250 µm. (*B*) Percentage of human lung ADC samples displaying low or high DOCK4 expression in the low or high p-Smad3 expression group. (**) $P < 0.01$ by Fisher's exact test. (*C*) Hazard ratio plot in function of DOCK4 expression based on gene expression and recurrence-free survival data for a cohort of 182 lung ADC patients. The dotted line indicates the cutoff that yields the highest hazard ratio with confidence interval 95% to define the low/high DOCK4 expression groups. (*D*) Kaplan-Meier survival curve for the low/high DOCK4 expression groups indicated in *C*. The *P*-value was calculated by log rank test.

we opted to use an intracardiac injection model of experimental metastasis in which cancer cells are injected into the left cardiac ventricle of NOD/SCID-IL2γ (NSG) mice (Fig. 3A). Using this model, we assessed the metastatic potential of both A549 (*KRAS* mutant) and HCC4006 (*EGFR* mutant) cells and evaluated whether pre-exposure of these cells to TGF-β prior to their introduction into the arterial circulation (to "mimic" the source of TGF-β that tumor cells normally experience within the primary tumor microenvironment) increases their metastatic potential. Specifically, A549 and HCC4006 cells engineered to stably express firefly luciferase (A549-luc and HCC4006-luc) were either left untreated or treated with TGF-β for 24 h and placed in the arterial circulation of NSG mice by intracardiac injection. Mice were subsequently monitored for multiorgan metastasis by bioluminescence imaging. While both untreated A549-luc and HCC4006-luc cells formed some metastases in multiple organs—with A549-luc cells preferentially colonizing the liver and bone, and HCC4006-luc cells colonizing the adrenal glands—we noted that the metastatic burden in animals was markedly increased when the A549-luc and HCC4006-luc cells were pretreated with TGF-β prior to injection (Fig. 3B), indicating that TGF-β stimulation enhances the metastatic potential of both lung ADC cell lines. Metastases in the adrenal glands, bones, and the liver were confirmed by ex vivo bioluminescence imaging and histology (Fig. 3C).

Having established an experimental model for lung ADC metastasis, we next assessed the requirement of DOCK4 in TGF-β-driven lung ADC metastasis. To approach this, we generated a retroviral vector that coexpresses EGFP and a miR-30-based shRNA targeting the 3′ untranslated region (UTR) of DOCK4 mRNA (shDock4#1). This shRNA substantially reduced DOCK4 protein levels

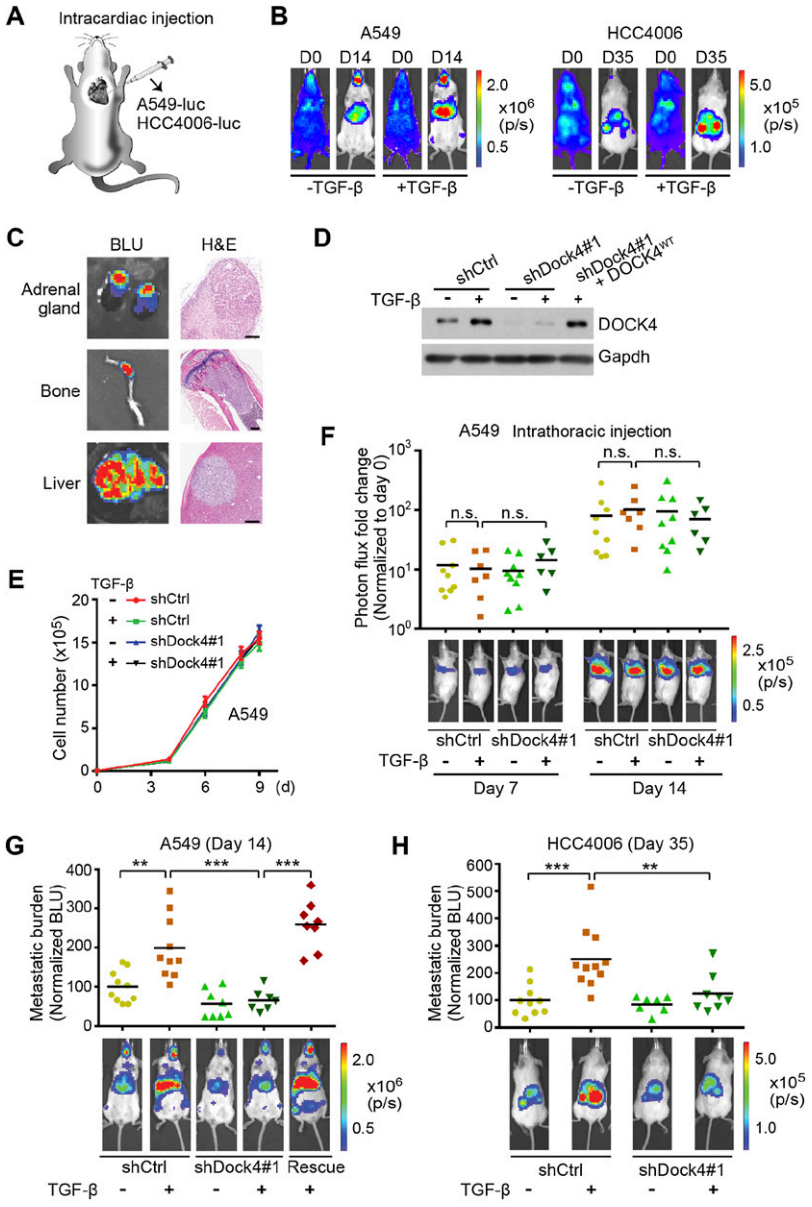

Figure 3. DOCK4 is required for TGF-β-driven lung ADC metastasis. (*A*) Schematic drawing of intracardiac injection. (*B*) Bioluminescent (BLU) images of NSG mice intracardially injected with TGF-β-pretreated (24 h, +) or untreated (−) A549 and HCC4006 cells taken at the indicated days after injection. (*C*) BLU images and H&E staining of metastases in the indicated organs harvested from the mice in *B*. Bars, 200 µm. (*D*) Western blot analysis of DOCK4 in A549 cells expressing the indicated constructs. (*E*) Growth curve of TGF-β-pretreated (24 h, +) or untreated (−) A549 cells stably expressing shCtrl or shDock4#1. Data represent mean ± SD. $n = 3$. (*F*) Analysis of lung tumor growth in NSG mice intrathoracically injected with TGF-β-pretreated (24 h, +) or untreated (−) A549 cells expressing shCtrl or shDock4#1. (*Top*) Dot plots of lung photon flux at days 7 and 14. BLU signals were normalized to day 0. (*Bottom*) Representative images of mice with lung tumors. $n = 6$–9 mice per condition. (*G*,*H*) Analysis of metastatic burden in NSG mice intracardially injected with TGF-β-pretreated (24 h, +) or untreated (−) A549 (*G*) or HCC4006 (*H*) cells expressing the indicated constructs. (*Top*) Dot plots of metastatic burden at day 14 (A549) and day 35 (HCC4006). BLU signals were normalized to day 0 and then to a value of 100 for control conditions (shCtrl, TGF-β$^-$). (*Bottom*) Representative images of mice with metastases. $n = 7$–10 (*G*) and 7–11 (*H*) mice per condition. *P*-values in *F*–*H* were calculated by an unpaired Mann-Whitney test. (**) $P < 0.01$; (***) $P < 0.001$; (n.s.) $P \geq 0.05$.

when stably introduced into A549-luc or HCC4006-luc cells, whereas shCtrl had no effect. Moreover and importantly, shDock4#1 largely blunted induction of DOCK4 expression by TGF-β (Fig. 3D; Supplemental Fig. 4A). Before assessing the metastatic potential of the shRNA-expressing cells, we first scrutinized their proliferative properties, especially since DOCK4 had been reported to display tumor-suppressive activity in osteosarcoma cells (Yajnik et al. 2003). We found that DOCK4 knockdown did not affect the growth rate of A549 or HCC4006 lung ADC cells regardless of them being pretreated with TGF-β (Fig. 3E; Supplemental Fig. 4B). Moreover, when shRNA-expressing cells pretreated or not pretreated with TGF-β were implanted into the lungs of NSG mice via intrathoracic injection, no difference in tumor growth rate was observed among the four experimental groups (Fig. 3F), indicating that DOCK4 knockdown does not affect the ability of these cells to form primary pulmonary tumors. Also, DOCK4 knockdown did not affect the survival of these cells in either monolayer or suspension culture (Supplemental Fig. 4C,D; data not shown).

We then injected the shRNA-expressing cells, pretreated or not pretreated with TGF-β, into the arterial circulation of NSG mice and monitored the mice for metastases. Strikingly, DOCK4 knockdown markedly blunted the pro-metastatic effects of TGF-β in both A549 and HCC4006 cells. Compared with animals injected with TGF-β-pretreated shCtrl-expressing cells, the metastatic burden in animals injected with TGF-β-pretreated shDock4#1-expressing cells was markedly reduced (Fig. 3G,H). Importantly, rescue experiments using DOCK4 cDNA that lacks the 3′ UTR and therefore is resistant to Dock4 shRNA-mediated RNAi (Fig. 3D) demonstrated that the effects of DOCK4 RNAi were specific (Fig. 3G, Rescue). Expression of DOCK4 alone (to levels similar to those induced by TGF-β) did not alter the metastatic potential or growth properties of lung ADC cells and, accordingly, did not affect the metastatic burden in animals (Supplemental Fig 5A–C), implying that pathways parallel to DOCK4 contribute to TGF-β's prometastatic effects (see further below). Of note, we also saw a slight decrease in metastatic burden in animals injected with TGF-β-untreated shDock4#1-expressing cells as compared with animals injected with untreated shCtrl-expressing cells (Fig. 3G,H). We presume that the lung ADC cells, when placed in circulation, can still become exposed to TGF-β signals provided by platelets in the bloodstream (Labelle et al. 2011). Clearly, though, compared with TGF-β-pretreated lung ADC cells, the metastatic efficiency of untreated cells is much lower, supporting the notion that pre-exposure of cells to TGF-β ("mimicking" the source of TGF-β at the primary site) primes these cells for efficient metastasis as they enter the circulation. Together, these data unveil an essential role for DOCK4 in mediating the metastasis-promoting activity of TGF-β in lung ADC cells from circulation to distant organ sites.

DOCK4 depletion inhibits TGF-β-driven lung ADC cell extravasation

For circulating cancer cells to establish distant metastases, they must leave the circulation in a process called extravasation at distant organ sites and then, after infiltrating the new tissue, must acquire the ability to survive and proliferate in this new microenvironment in order to form macroscopic metastases (Valastyan and Weinberg 2011; Scott et al. 2012). To explore whether depletion of DOCK4 in TGF-β-primed lung ADC cells impacts their extravasation capabilities and/or their ability to survive/proliferate at the distant organ sites, we began by scoring the number and size of metastatic nodules formed in the livers of mice that were intracardially injected with TGF-β-pretreated or untreated shCtrl- or shDock4#1-expressing A549-luc cells (Fig. 4A). In line with our above findings, pretreatment of shCtrl-expressing cells, but not of shDock4#1-expressing cells, with TGF-β prior to injection led to a marked increase in the number of metastatic nodules (Fig. 4A). Interestingly, though, the size of the metastatic nodules that developed in the livers was not significantly different among the four experimental groups (Fig. 4B), implying that TGF-β pretreatment and, importantly, DOCK4 depletion do not alter the capacity of lung ADC cells to grow in the new microenvironment. To substantiate this, we first checked that the liver metastases formed from shDock4#1-expressing cells were not attributable to proliferation of tumor cells that lost shRNA expression. To this end, we extracted EGFP-labeled tumor cells from liver metastases originating from TGF-β-pretreated shCtrl- and shDock4#1-expressing A549-luc cells (referred to as liver mets-derived cells) and assessed DOCK4 levels by Western blot analysis. We found that DOCK4 levels were still efficiently knocked down in these cells (Fig. 4C). Moreover, when we assessed their proliferative properties in vitro, we did not detect any differences between the growth rates of shDock4#1- and shCtrl-expressing liver mets-derived cells (Fig. 4D). In addition, when we injected shDock4#1- or shCtrl-expressing A549-luc cells pretreated or not pretreated with TGF-β into livers of NSG mice and evaluated tumor growth 7 and 14 d following injection, we found that the growth rate of the tumors was similar among all of the experimental groups (Fig. 4E). Thus, DOCK4 depletion in TGF-β-primed lung ADC cells does not affect their ability to survive/proliferate in a new microenvironment.

Hence, we next assessed whether TGF-β/DOCK4 signaling influences the capacity of lung ADC cells to extravasate into distant target organs. To this end, we isolated livers from NSG mice that were sacrificed 20 h after intracardiac injection with TGF-β-pretreated or untreated shCtrl- or shDock4#1-expressing A549-luc cells. Of note, we chose the liver because it is a relatively large and highly vascularized organ. Livers were perfused, and liver sections were immunostained for GFP and CD31 to visualize tumor cells and liver vasculature, respectively (Fig. 4F). The percentages of tumor cells inside (intravascular) and outside (extravascular) the blood vessels were then quantified (Fig. 4G). We found that the fraction of tumor cells that extravasated out of the liver vasculature was markedly increased in the TGF-β-pretreated shCtrl group as compared with the untreated shCtrl group, supporting the notion that TGF-β promotes tumor cell extravasation. However, no such increase was observed

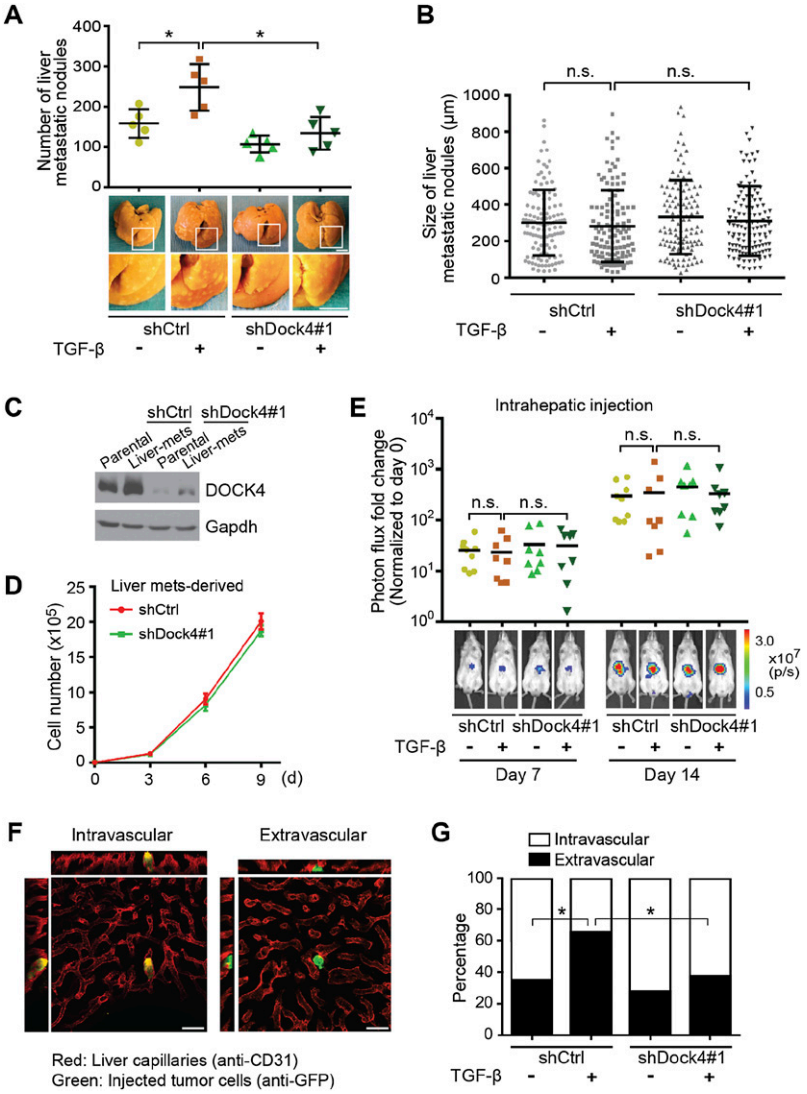

Figure 4. DOCK4 depletion inhibits TGF-β-driven lung ADC cell extravasation but does not affect the ability of lung ADC cells to grow in distant organs. (*A*) Quantification of the number of metastatic nodules in the liver after intracardiac injection of TGF-β-pretreated (24 h, +) or untreated (−) A549 cells expressing shCtrl or shDock4#1. (*Top*) Number of metastatic nodules on the liver surface. $n = 5$ livers per condition. (*Bottom*) Representative images of livers. Boxed regions are enlarged and shown in the *bottom* row. Bars, 1 cm. (*B*) Quantification of size (by measuring diameter) of metastatic nodules in H&E-stained liver sections from the mice in *A*. $n = 120$ per condition. Data in *A* and *B* represent mean ± SD. (*C*) Western blot analysis of DOCK4 protein expression in A549 parental and liver mets-derived cells. Gapdh was used as a loading control. (*D*) Growth curve of shCtrl- and shDock4#1-expressing A549 liver mets-derived cells. Data represent mean ± SD. $n = 3$. (*E*) Analysis of tumor growth in livers of NSG mice intrahepatically injected with TGF-β-pretreated (24 h, +) or untreated (−) A549 cells expressing shCtrl or shDock4#1. (*Top*) Dot plots of liver photon flux at days 7 and 14, normalized to day 0. (*Bottom*) Representative images of mice with liver tumors. $n = 8$–9 mice per condition. *P*-values in *A*, *B*, and *E* were calculated by an unpaired Mann-Whitney test. (n.s.) $P \geq 0.05$. (*F*) Representative confocal images of liver sections depicting tumor cells inside (intravascular) or outside (extravascular) blood vessels obtained from NSG mice 20 h after intracardiac injection with the cells in *A*. Bars, 20 μm. (*G*) Percentage of intravascular or extravascular A549 cells from the experiment shown in *F*. $n = 31$–40 cells per condition. (*) $P < 0.05$ by Fisher's exact test.

in the TGF-β-pretreated shDock4#1 group, where, similar to the untreated shDock4#1 and shCtrl groups, the majority of tumor cells remained in the blood vessels (Fig. 4G), indicating that DOCK4 function is required for TGF-β's enhancing effect on tumor cell extravasation. Notably, DOCK4 levels remained high in the TGF-β-pretreated cells for at least 48 h following TGF-β removal (Supplemental Fig. 6), which is well within the time period needed for these cells to extravasate following intracardiac injection. Combined, these data establish a critical role for the TGF-β/DOCK4 signaling axis in the regulation of lung ADC cell extravasation and metastasis in vivo.

DOCK4 mediates TGF-β's enhancing effects on lung ADC cell protrusive activity, motility, and invasion, but not epithelial–mesenchymal transition (EMT), via Rac1 activation

We next sought to gain further insight into the cellular mechanisms by which DOCK4 mediates TGF-β's enhancing effect on tumor cell extravasation and metastasis. Although the cellular underpinnings of tumor cell extravasation still remain poorly understood, increasing evidence indicates that it is a dynamic process involving changes in not only the vascular endothelium but also the tumor cells during their intravascular transit to the sites of metastasis, with tumor cells undergoing changes in cell shape and migratory behavior (Strell and Entschladen 2008; Stoletov et al. 2010; Reymond et al. 2013). TGF-β has been implicated in most of these processes (Giampieri et al. 2009; Valastyan and Weinberg 2011), and, interestingly, recent studies reported that TGF-β-induced EMT not only facilitates tumor cells to intravasate but also helps them to extravasate (Stoletov et al. 2010; Labelle et al. 2011; Tsai and Yang 2013; Yu et al. 2013). Based on these findings, we first explored whether DOCK4 affects TGF-β-induced EMT in A549 and HCC4006 lung ADC cells. To this end, we examined the impact of DOCK4 knockdown on the expression levels of TGF-β-responsive genes known to be involved in EMT (including *E-cadherin*, *Vimentin*, *Snail*, *Slug*, *Twist1*, and *Zeb1/2*) (Valastyan and

Weinberg 2011). In both cell lines, TGF-β induced a down-regulation and/or cytoplasmic translocation of E-cadherin and an up-regulation of Vimentin, Snail, and Slug as well as an EMT phenotype (Fig. 5A; Supplemental Fig. 7A–D). Knockdown of DOCK4 did not affect any of the TGF-β-induced changes in EMT markers and did not prevent the acquisition of a mesenchymal-like phenotype (Supplemental Fig. 7A–D). Also, ectopic expression of DOCK4 did not alter any of these properties (Supplemental Fig. 7E; data not shown). These data indicate that DOCK4 is dispensable for TGF-β-induced EMT in A549 and HCC4006 lung ADC cells and further imply that TGF-β drives EMT and DOCK4 induction via parallel pathways.

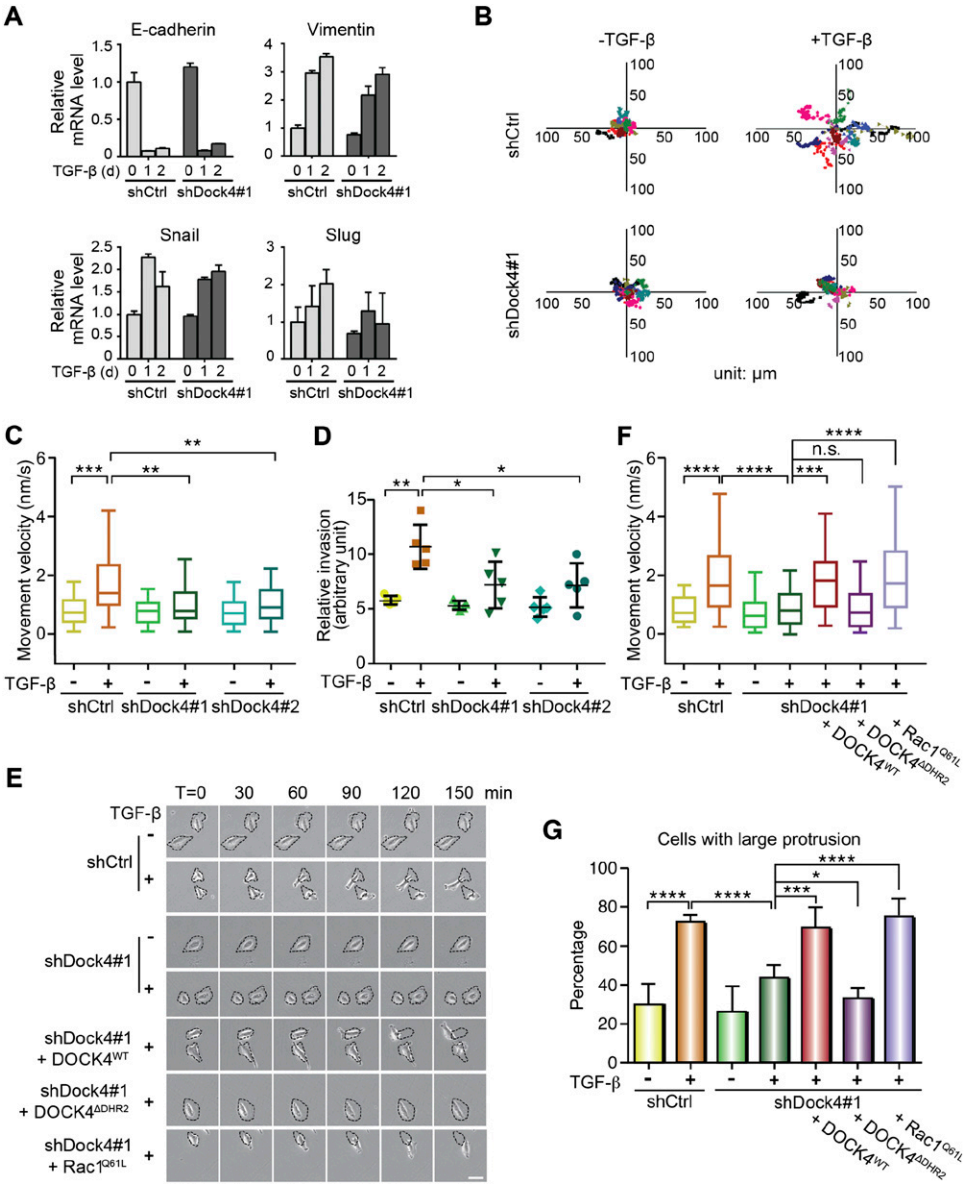

Figure 5. DOCK4 mediates TGF-β's enhancing effects on lung ADC cell protrusive activity, motility, and invasion, but not EMT, via Rac1 activation. (*A*) qPCR analysis of mRNAs of EMT markers in shCtrl- and shDock4#1-expressing A549 cells treated with TGF-β. Data represent mean ± SD. $n = 3$. (*B*) Representative movement trajectories of single shCtrl- or shDock4#1-expressing A549 cells left untreated or pretreated (for 24 h) with TGF-β obtained over 4.5 h. (*C*) Quantification of movement velocity of shDock4#1- and shDock4#2-expressing A549 cells. $n = 33$–44 cells per condition. (*D*) Quantification of Matrigel invasion assays of shDock4#1- and shDock4#2-expressing A549 cells. Data represent mean ± SD. $n = 5$ transwells per group. (*E*) Representative images of single A549 cells expressing the indicated constructs, left untreated or pretreated with TGF-β for 24 h, obtained from live-cell imaging at the indicated time points. Bars, 50 μm. (*F*) Quantification of movement velocity of the cells in *E*. $n = 36$–56 cells per condition. Data in *C* and *F* are presented as Tukey box plots. *P*-values in *C*, *D*, and *F* were calculated by an unpaired Mann-Whitney test. (*G*) Percentage of cells with a large protrusion over a 30-min time interval. $n = 6$ fields containing 104–151 cells per condition. Data represent mean ± SD. *P*-values were calculated using an unpaired two-tailed Student's *t*-test. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$; (****) $P < 0.0001$; (n.s.) $P \geq 0.05$.

We next assessed whether DOCK4 knockdown influences the migratory and invasive behavior of the lung ADC cells. Since the majority of circulating tumor cells appears to consist of single cells (Yu et al. 2013), we tracked the movement of single cells pretreated or not pretreated with TGF-β. We observed that TGF-β pretreatment of shCtrl-expressing A549 or HCC4006 cells greatly enhanced the motility of these cells and that this enhancement was abrogated upon inhibition of the TGF-β/Smad pathway (Fig. 5B,C; Supplemental Fig. 8B–E). Importantly, while DOCK4 knockdown using two independent shRNAs (shDock4#1 and shDock4#2) did not affect the basal levels of A549 or HCC4006 cell motility, it impeded, similarly to as seen for TGF-β/Smad inhibition, the TGF-β-induced increase in cell motility (Fig. 5B,C; Supplemental Fig. 8A–E). We further examined the invasive potential of these cells using a Matrigel-coated Boyden chamber assay, given that tumor cells must invade the basement membrane surrounding the blood vessels to enter the parenchyma of their target organs (Reymond et al. 2013). While TGF-β treatment of shCtrl-expressing A549 cells resulted in a robust increase in invasion through Matrigel, only a very modest increase was observed when shDock4#1- or shDock4#2-expressing cells were treated with TGF-β (Fig. 5D). Thus, DOCK4 function is essential for TGF-β's stimulating effects on lung ADC cell motility and invasion. Of note, a role for DOCK4 in the migration of distinct cell types had been reported previously (Hiramoto et al. 2006; Kawada et al. 2009; Kobayashi et al. 2014). Interestingly, a closer scrutiny of the morphology of the shRNA-expressing cells in our live-cell imaging experiments revealed that TGF-β-pretreated shCtrl-expressing cells displayed higher membrane protrusive activity than the untreated shCtrl-expressing cells, with TGF-β-pretreated cells extending a large forward protrusion (Fig. 5E,G). In contrast, while shDock4#1- and shDock4#2-expressing cells did undergo EMT when exposed to TGF-β, they hardly extended forward protrusions (Fig. 5E,G; data not shown), indicating that DOCK4's function is important for TGF-β-promoted mesenchymal cancer cell protrusive activity.

Finally, we asked whether DOCK4 exerts its cellular effects by acting on the Rac1 signaling pathway or, potentially, other pathways. On the one hand, Rac1 activation has been implicated in cell motility and protrusion extension during transendothelial migration (Reymond et al. 2013); on the other hand, however, we found that TGF-β induces DOCK4 expression via the canonical Smad pathway, and so far Rac1 has been mainly linked to TGF-β via noncanonical pathways (Zhang 2009). Hence, we first determined whether Rac1 activation is mediated via the TGF-β/Smad/DOCK4 pathway in lung ADC cells. While TGF-β triggered a robust increase in Rac1 activity in shCtrl-expressing A549 cells, we found that this increase was greatly reduced in both shSmad4#2- and shDock4#1-expressing A549 cells (Supplemental Fig. 9A,B,D). Of note, DOCK4 knockdown did not affect TGF-β-induced activation of Rap1 or Cdc42 in A549 cells (Supplemental Fig. 9C). Furthermore, we found that the DHR2 domain of DOCK4, which is conserved among all DOCK180 family members and catalyzes the exchange of GDP for GTP on Rac1 (Cote and Vuori 2002; Meller et al. 2005), is essential for TGF-β-elicited Rac1 activation in A549 cells. Indeed, introduction of $DOCK4^{WT}$, but not a $DOCK4^{\Delta DHR2}$ mutant lacking 77 amino acids within the DHR2 domain (Kawada et al. 2009), in shDock4#-expressing cells restored the levels of TGF-β-induced Rac1 activation to that seen in control cells (Supplemental Fig. 9A,B). Thus, DOCK4 links the canonical TGF-β/Smad pathway to Rac1 activation in lung ADC cells. These findings prompted us to investigate whether DOCK4 exerts its cellular effects via activation of the Rac1 pathway. We first examined the ability of $DOCK4^{\Delta DHR2}$ to rescue the impaired protrusive activity and cell motility observed in the TGF-β-treated shDock4#1-expressing cells. While $DOCK4^{WT}$ was able to fully rescue these phenotypes, $DOCK4^{\Delta DHR2}$ failed to do so (Fig. 5E–G), indicating that DOCK4's Rac-GEF activity is essential for its function in mediating TGF-β's effects on cell motility and protrusion formation. Of note, expression of $DOCK4^{WT}$ alone did not affect the motility or protrusive activity of these cells (Supplemental Fig. 8F), consistent with our findings that it does not affect their metastatic potential. We next examined whether concomitant expression of an activated mutant form of Rac1 ($Rac1^{Q61L}$) with shDock4#1 could rescue the DOCK4 RNAi-produced phenotypes and found that this is indeed the case. Cells coexpressing shDock4#1 and $Rac1^{Q61L}$ extended protrusions and displayed increased cell motility upon TGF-β treatment (Fig. 5E–G). Finally, we investigated whether knockdown of Rac1 phenocopies the effects of DOCK4 down-regulation on TGF-β-promoted cell motility and protrusive activity. To this end, we took advantage of two previously described shRNAs, shRac1#1 and shRac1#2 (Akunuru et al. 2011), with shRac1#1 being more effective than shRac1#2 (Supplemental Fig. 9E). We found that both shRac1#1 and shRac1#2 interfered with a TGF-β-induced increase in cell motility and protrusive activity, with shRac1#1 being more effective than shRac1#2 (Supplemental Fig. 9F; data not shown), as expected. Thus, DOCK4 links the canonical TGF-β/Smad pathway to the Rac1 pathway in the regulation of lung ADC cell shape and migratory behavior, processes fundamental to tumor cell extravasation and metastasis. Notably, a previous study reported that DOCK3 forms a complex with NEDD9 and that this complex regulates Rac1 activation to drive mesenchymal movement in melanoma cells (Sanz-Moreno et al. 2008). However, we found that DOCK4, while effectively activating Rac1, does not interact with NEDD9 (Supplemental Fig. 10A,B), further supporting the notion that these two proteins have distinct modes of regulation and likely serve distinctive cell type-specific functions.

Discussion

Metastasis from lung ADC, the most common subtype of lung cancer, typically occurs rapidly to multiple organs (Hoffman et al. 2000; Provencio et al. 2011). A key factor reported to drive lung ADC metastasis is the cytokine TGF-β (Lund et al. 1991; Hasegawa et al. 2001; Nemunaitis

et al. 2009; Toonkel et al. 2010; Vazquez et al. 2013); however, the genes and mechanisms that mediate the prometastatic effects of TGF-β remain largely unknown. Here we identify DOCK4 as a novel, key target of the TGF-β/Smad signaling pathway that promotes lung ADC metastasis by enhancing the competence of lung ADC cells to extravasate into distant organs. We further present evidence that DOCK4 does so at least in part by stimulating the protrusive activity and motility of mesenchymal lung ADC cells via activation of the Rac1 pathway.

DOCK4 is a member of the DOCK180 family of GEFs, of which a total of 11 mammalian family members have been identified (Cote and Vuori 2002; Meller et al. 2005). While all members, with the exception of DOCK2, are expressed in lung ADC cells, we found that the TGF-β/Smad pathway selectively up-regulates the expression of DOCK4 but not other DOCK family members, supporting the notion that these proteins exhibit different modes of regulation (Laurin and Cote 2014). Notably, high DOCK4 expression levels correlate with activated TGF-β signaling and poor prognosis in human lung ADC. Interestingly, the regulation of DOCK4 by TGF-β appears to be tumor type-dependent. Indeed, no induction of DOCK4 by TGF-β was observed in breast cancer and melanoma cells. Also, binding of p-Smad3 to SBEs within *DOCK4*'s first intron was detected in lung ADC cells but not in breast cancer cells. Finally, no correlation between DOCK4 expression and disease relapse was found in estrogen receptor (ER)-negative breast cancer patients. One possible explanation for these context-dependent observations is that the epigenetic status of lung ADC cells is different from that of breast cancer and melanoma cells. Also, cell type-specific Smad cofactors critical to the regulation of DOCK4 expression in response to TGF-β signaling could be present in lung ADC cells but not in breast cancer and melanoma cells. Future studies will be required to decipher the precise cell/tumor type-specific regulation of DOCK4 expression in response to TGF-β/Smad signaling.

Using xenograft mouse models, we showed that DOCK4 plays a critical role in mediating TGF-β-driven lung ADC metastasis. An intriguing finding from our studies is that while DOCK4 expression is already induced by TGF-β in primary human lung ADC (as indicated by our IHC stainings of human lung ADC TMAs), DOCK4 appears to exert its effects at a later time by enhancing the extravasation capabilities of lung ADC cells. Indeed, we found that DOCK4 knockdown in TGF-β-primed lung ADC cells that are about to enter circulation impedes their ability to form metastatic foci at the distant organ sites and that DOCK4 exerts this effect without affecting the growth properties or survival of the metastasizing cells. Moreover, we observed that the fraction of TGF-β-primed lung ADC cells that extravasated out of the liver vasculature was markedly reduced in the DOCK4 knockdown group compared with the control group. These findings reinforce the view that TGF-β signals produced within the primary tumor microenvironment can influence later stages of metastasis and that a protein induced by TGF-β in tumor cells at the primary site may also act at a later step of the metastatic process (Padua et al. 2008; Calon et al. 2012; Yuan et al. 2014). Our data, however, do not exclude that DOCK4 could also play a role in mediating the enhancing effect of TGF-β on lung ADC cell intravasation. Future studies will be required to determine whether DOCK4 acts on not only the late steps but also the early steps of lung ADC metastasis.

Padua et al. (2008) previously showed that TGF-β in the breast tumor microenvironment primes tumor cells for metastasis to the lungs by driving the expression of angiopoietin-like 4 (ANGPTL4). Interestingly, while ANGPTL4 facilitates tumor cell extravasation in a non-cell-autonomous manner by disrupting endothelial junctions at the metastatic sites, our data indicate that DOCK4 does so in a cell-autonomous manner by promoting the protrusive activity and motility of lung ADC cells. Notably, a recent in vivo study showed that tumor cells with high protrusive activity can migrate and navigate through narrow vessel lumen openings and vessel branch points, a process that could allow them to find optimal sites for extravasation (Stoletov et al. 2010). Combined, these findings indicate that tumor cell extravasation is a highly dynamic and coordinated process involving contributions of both cell-extrinsic and cell-intrinsic factors. With regard to DOCK4, it should be noted that additional factors acting in parallel to DOCK4 in mediating TGF-β-promoted lung ADC cell extravasation and metastasis likely come into play. While depletion of DOCK4 impaired TGF-β-induced cell protrusive activity/motility and extravasation of lung ADC cells, ectopic expression of DOCK4 (at levels similar to those induced by TGF-β) did not enhance the protrusive activity/motility of these cells or their metastatic potential. In light of our findings that DOCK4 is dispensable for TGF-β-induced EMT in lung ADC cells, we envision that TGF-β drives the activation of genes required for EMT and, in parallel, the induction of DOCK4 expression and that the former is a prerequisite for DOCK4's subsequent enhancing effects on the protrusive activity/motility and extravasation potential of lung ADC cells.

Finally, our data unveil that TGF-β/Smad-induced DOCK4 promotes the protrusive activity and motility of mesenchymal lung ADC cells via the activation of the Rac1 GTPase. While Rac1 has been implicated previously in cell motility and protrusion formation in different tumor cell types (Reymond et al. 2013), so far, Rac1 has been mainly linked to TGF-β via noncanonical pathways (Zhang 2009). Here we showed that blockage of the canonical Smad pathway greatly reduces TGF-β-induced activation of Rac1 similarly to depletion of DOCK4. Thus, our findings unveil a previously unrecognized link between TGF-β/Smad and Rac1 signaling and identify DOCK4 as a key player bridging the two pathways. Interestingly, while multiple Rac-GEFs, including other DOCK family members, are expressed in lung ADC cells, our findings imply that they do not compensate for DOCK4 function, as depletion of DOCK4 alone was sufficient to blunt TGF-β-promoted lung ADC cell protrusive activity and motility.

Thus, DOCK4 likely provides specificity in signaling to Rac1 activation downstream from TGF-β to control lung ADC cell protrusion and motility. This is of particular interest given that global and long-term inhibition of Rac1 is well known to exert anti-proliferative effects on not only tumor cells but also normal cells. Therefore, the development of small molecules that specifically inhibit DOCK4's Rac-GEF activity or abrogate the interaction between DOCK4 and Rac1 could present a valid therapeutic strategy in the treatment of lung ADC metastasis.

Materials and methods

Cell lines and human lung ADC TMAs

The lung ADC and breast cancer cell lines used in this study were a gift from M. Wigler and R. Sordella (Cold Spring Harbor Laboratory) and are listed in Supplemental Table 1. The cell lines were grown and transduced with retroviral or lentiviral vectors expressing the indicated shRNAs or cDNAs as described in the Supplemental Material. Human lung ADC TMAs (TMA LC706 and TMA LC10013) used to assess levels of DOCK4 and phospho-Smad3 expression were obtained from US Biomax, Inc., as described in the Supplemental Material.

Animal studies

Five-week-old to 7-wk-old NSG mice (National Cancer Institute) were used for all xenografting studies. Lung ADC cells stably expressing firefly luciferase were injected intracardially for assessment of multiorgan metastasis. Tumor growth in the lungs and liver was assessed using intrathoracic and intrahepatic injections, respectively. Bioluminescent signals were monitored and quantified using the IVIS-200 imaging system (Xenogen). Details of the procedures are described in the Supplemental Material.

RNA and protein analysis, Rac1 activity, imaging, cellular assays, public data set analysis, and statistics

RNA isolation, qPCR, coimmunoprecipitation, Rac activation assays, immunofluorescence and image analysis, cellular assays, ChIP-seq data analysis, lung ADC and ER-negative breast cancer clinical data analysis, and statistic analysis are described in the Supplemental Material.

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