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p130Cas controls the susceptibility of cancer cells to TGF-β-induced growth inhibition

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ABSTRACT

Transforming growth factor-beta (TGF-β) suppresses the initiation of tumorigenesis by causing arrest at the G1 phase of the cell cycle. The loss of the antiproliferative function of TGF-β is a hallmark of many cancers. Here we report that p130Cas plays a role in determining the cellular responsiveness to TGF-β-induced growth inhibition in some cancer cells. An analysis of the tyrosine phosphorylation levels of p130Cas revealed higher levels of phosphorylation in cancer cell lines (MCF7 and A375) than in corresponding normal cell lines (MCF10A and MEL-STV). In contrast to normal cells, the cancer cells showed resistance to not only TGF-β-induced Smad3 phosphorylation and p21 expression, but also growth inhibition. However, silencing p130Cas using siRNA was sufficient to restore Smad3 phosphorylation and p21 expression, as well as the susceptibility to TGF-β-induced growth inhibition. Interestingly, the stable overexpression of p130Cas accelerated TGF-β-induced epithelial–mesenchymal transition. Our results suggest that elevated expression and tyrosine phosphorylation of p130Cas contributes to the resistance to TGF-β-induced growth inhibition, and thus to the initiation and progression of human cancers that harbor an active integrin signal.

1. Introduction

p130Cas (Crk-associated substrate, 130 kDa), the product of the breast cancer anti-estrogen resistance 1 gene, is an adaptor protein that is a tyrosine-phosphorylated protein by *v*-Src-dependent cellular transformation and the *v*-Crk oncogene [1,2]. p130Cas has multiple protein–protein interaction domains, and has a great influence on actin cytoskeleton regulation, cell migration, cell survival, and apoptosis, and especially on the cooperation of integrin with receptor tyrosine kinases, including EGF, estrogen, and VEGF receptors [3–6]. Extensive work has shown that p130Cas is critical for the initiation of tumorigenesis and maintenance of the proliferative capacity of human cancers [7]. The overexpression of p130Cas promotes mammary tumorigenesis and metastasis [7–9], and is correlated with poor prognosis in breast cancer patients [10,11]. Recent studies have extended this notion to the transforming growth factor (TGF)-β signaling pathway [12,13].

TGF-β signals are transduced by receptor serine/threonine kinases (RS/TKs) and intracellular effectors called Smads [14], which are implicated in a variety of cellular functions, such as proliferation, apoptosis, and differentiation [15,16]. In response to TGF-β stimulation, Smad2 and Smad3 are phosphorylated at the C-terminal $S^{422}SXS^{425}$ motif by the TGF-β type I receptor (TβRI) [14]. Phosphorylated Smad2/3 forms a complex with Smad4, and then translocates into the nucleus, in which the complex leads to the transcription of target genes that include cell cycle inhibitor p21 [17,18]. As a consequence, TGF-β induces growth arrest at the G1 phase in most cell types,including normal epithelial, endothelial, and hematopoietic cells, as well as primary fibroblasts of embryonic origin [19,20]. Thus, the TGF-β/Smad cascade has been regarded as a tumor suppressor pathway, and reducing the responsiveness of cancer cells to its tumor suppressor activity is critical for tumorigenesis in epithelia [21].

The loss of the growth-inhibitory function of TGF-β is a hallmark of many cancers. It has been well known that TGF-β also has pro-oncogenic effects, including enhanced epithelial-mesenchymal transition (EMT) and increased invasiveness, coupled with a resistance of the cells to TGF-β-induced growth inhibition and an increased production of TGF-β [21]. Several studies suggest that this transition of the TGF-β signal toward oncogenesis may result from changing thebalance between canonical and noncanonical pathways [13,21]. However, the detailed molecular mechanism

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has not been firmly investigated. Recently, we reported that integrin signaling counteracts the inhibitory effects of TGF-β on cell growth, and that p130cas acts as an important mediator for integrin-dependent TGF-β suppression through its direct interaction with Smad3 [12]. This inhibitory role of p130Cas in the TGF-β signaling pathway was further confirmed in another study, in which it was shown that p130Cas is required for mammary tumor growth and TGF-β-mediated metastasis through the regulation of Smad2/3 activity [13]. Here, we tried to determine whether the p130Cas-mediated control of TGF-β signaling contributes to changing the net cellular readout of the TGF-β signal from tumor suppression to tumor promotion,contributing to the initiation and progression of human cancers.

2. Materials and methods

2.1. Cell culture and transfections

Human keratinocyte (HaCaT), human breast cancer (MCF-7), and human melanoma cells (SK-MEL-2, SK-MEL-28 and A375) were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum. MEL-STV immortalized melanocytes were cultured in DMEM with 5% fetal bovine serum [22]. MCF10A normal breast epithelial cells were cultured in DMEM/F12 supplemented with 5% horse serum, 10 μg/ml insulin, 20 ng/ml EGF, 0.5 μg/ml hydrocortisone, and 100 ng/ml cholera toxin (all from Sigma). All transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or GeneExpresso 8000 (InnoVita, Gaithersburg, MD, USA) according to the manufacturer's instructions. HaCaT cell clones stably expressing p130Cas were obtained by transfection with pcDNA3.0-p130Cas, followed by selection for 1–2 weeks in G418 (GIBCO BRL). TGF-β1 was purchased from R&D Systems (Minneapolis, MN).

2.2. Immunoprecipitation and western blot analysis

Cells incubated with or without TGF-β1 (2 ng/ml) were lysed in modified RIPA buffer (50 mM Tris–HCl at pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10 mM NaF, 1 mM PMSF, 1 mM sodium orthovanadate, 10 μM leupeptin, 1.5 μM pepstatin and 10 μg/ml aprotinin). For the analysis of endogenous Smad3–Smad4 and Smad3–p130Cas interaction, the lysates were immunoprecipitated with anti-Smad3 N Ab [17], followed by western blotting with anti-Smad4 mAb (Santa Cruz Biotechnology) or anti-p130Cas mAb (BD Transduction Laboratories, San Jose, CA). The tyrosine phosphorylation of p130Cas was analyzed by immunoprecipitation with anti-Cas2 Ab, followed by immunoblotting with anti-phosphotyrosine mAb (4G10, Upstate Biotechnology, Lake Placid, NY). Anti-Cas2 was obtained from Dr. Hismaru Hirai. Western blot analysis was carried out using anti-Smad3 Ab (Zymed Laboratories, South San Francisco, CA), anti-phospho-Smad3 Ab (a kind gift of Edward B. Leof, Mayo Clinic Cancer Center, MN), anti-tubulin mAb (Sigma), and anti-p21 mAb (BD Transduction Laboratories).

2.3. Silencing of endogenous p130Cas with small interfering RNA (siRNA)

Cells were depleted of p130Cas using siRNA corresponding to nucleotides 2366–2384 (CCCACAAGCUGGUGUUACU dT dT) of human p130Cas (Bioneer, Seoul, Korea) [17]. Cells were transiently transfected with siRNA for p130Cas in Opti-MEM I medium (Invitrogen) using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. Scramble siRNA (Bioneer) was transfected as a negative control. Four hours later, the Opti-MEM I medium was replaced with complete culture medium. TGF-β1 was added to the cultures 24–36 h after the transfection, and the effects of the down-regulation of p130Cas were assessed at 48–60 h.

2.4. Immunofluorescence

Cells were treated withTGF-β, fixed, permeabilized, blocked, and incubated with anti-E-Cadherin (BD Bioscience Transduction Laboratories, Lexington, KY) and anti-β-Catenin (BD Bioscience Transduction Laboratories), followed by staining with Alexa Fluor-conjugated secondary Ab (Invitrogen). Actin was visualized by Alexa 488-conjugated phalloidin (Invitrogen). Images were obtained using an Olympus confocal microscope FV1000 (Olympus, Tokyo, Japan) with FV10-MSASW software.

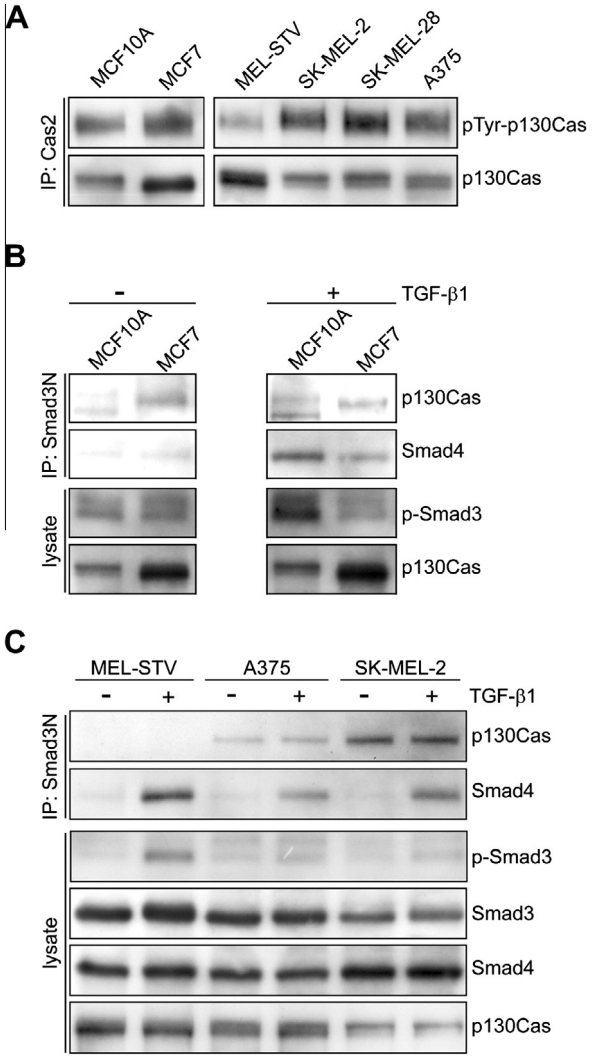

Fig. 1. Expression and phosphorylation levels of p130Cas and Smad3 in normal and cancer cell lines. (A) Levels of total (p130Cas) and tyrosine-phosphorylated p130Cas (pY-p130Cas) were determined by immunoprecipitation using anti-Cas2 Ab, followed by western blotting with anti-Cas mAb or anti-phosphotyrosine mAb (4G10) in normal cells (MCF10A and MEL-STV) and their corresponding cancer cells (MCF7, SK-MEL-2, SK-MEL-28 and A375). (B) MCF10A and MCF7 cells were treated with or without TGF-β (2 ng/ml) for 30 min, and the cell lysate was subjected to immunoprecipitation and western blotting for the indicated proteins. (C) MEL-STV, A375, and SK-MEL-2 cells were treated with or without TGF-β for 30 min, and the cell lysate was subjected to immunoprecipitation and western blotting for the indicated proteins.

2.5. ^{3}H-Thymidine incorporation assays

Cells were seeded to a density of 2×10^5 cells/well in six-well plates, and then incubated for 24 h with or without TGF-β1. Cells were labeled for the last 3 h with 4 μCi of ^{3}H-thymidine, fixed in 10% tricholoacetic acid, and lysed in 0.2 M NaOH. ^{3}H-thymidine incorporation into the DNA was measured using a scintillation counter.

2.6. Statistical analysis

Quantitative data are presented as the mean ± SEM. The differences between mean values were compared statistically by Student's *t*-test. Comparisons were performed using Graphpad Prism (GraphPad Software). A p value of <0.05 was considered statistically significant.

3. Results

3.1. Comparison of phosphorylation levels of p130Cas and Smad3 in the normal and their corresponding cancer cells

We previously reported thatintegrin-stimulated, tyrosine-phosphorylated p130Cas inhibits the TβR-I-dependent phosphorylation of Smad3, and reduces its transcriptional activity [12]. This led to the inhibition of p15 and p21 expression, and facilitated cell cycle progression from the G1 to the S phase [12]. In this regard, the antiproliferative function of TGF-β is lost in cancer, sometimes as a result of mutations that directly inactivate components of the TGF-β/Smad signaling pathway [19,21]. In addition, the enhanced expression of integrin-coupled signaling effectors, including FAK, Src, and p130Cas, or their tyrosine phosphorylation, has been implicated in the induction and proliferation of a wide variety of human cancers [3,10,23–27]. This prompted usto test whether TGF-β-resistance in some cancers could reflect the negative regulation of TGF-β/Smad signaling by p130Cas. Consistent with this idea, analysis of the tyrosine phosphorylation levels of p130Cas revealed higher levels of phosphorylation in some cancer cell lines (human breast (MCF7) and skin (SK-MEL-2, SK-MEL-28 and A375) cancer cells) than in corresponding normal cell lines (MCF10A and MEL-STV, Fig. 1A). We further analyzed the level of TGF-β-induced Smad3 phosphorylation, Smad3–Smad4 interaction, and p130Cas–Smad3 interaction in these cells by co-immunoprecipitation and western blot analysis. MCF7 breast cancer cells showed lower levels of phospho–Smad3 and Smad3–Smad4 interaction and higher levels of p130Cas–Smad3 interaction than MCF10A normal breast epithelial cells (Fig. 1B). Likewise, A375 or SK-MEL-2 melanoma cells showed lower phospho–Smad3 and Smad3–Smad4 interaction, and higher levels of p130Cas–Smad3 interaction than MEL-STV immortalized human melanocytes (Fig. 1C). These data suggest that the resistance of MCF-7 and A375 cancer cells to TGF-β signals might result from the elevated tyrosine phosphorylation of p130Cas.

3.2. Effect of p130Cas knockdown by siRNA on TGF-β-induced Smad3 phosphorylation and growth inhibition

We next determined whether the elevated expression and phosphorylation of p130Cas affects the resistance to TGF-β-induced Smad3 phosphorylation observed with MCF-7 and A375 cancer cells. It has already been shown in a previous report that the knockdown of endogenous p130Cas, even when incomplete, leads to increases in TGF-β-induced Smad3 phosphorylation, p21 expression, and growth inhibition in normalcells [12]. Consistent with this, TGF-β functioned as a powerful inducer of Smad3 phosphorylation in MCF10A normal breast epithelial cells, whereas this effect of TGF-β was largely absent in MCF7 cells (Fig. 2A). Similar differences in the effects of TGF-β on Smad3 phosphorylation were observed when A375 cells were compared with MEL-STV cells (Fig. 2B), suggesting that TGF-β alone is not sufficient to induce Smad3 phosphorylation in A375 and MCF7 cancer cells. After the knockdown of p130Cas, however, a dramatic induction of Smad3 phosphorylation was observed with both MCF7 (Fig. 2A) and A375 (Fig. 2B) cancer cells.

We next investigated whether the knockdown ofp130Cas also modulates the expression of the CDK inhibitor p21, a major transcriptional target of the TGF-β pathway. In contrast to MCF10A and MEL-STV cells, MCF7 and A375 cancer cells were insensitive to TGF-β-induced p21 expression, whereas silencing p130Cas in these cancer cells was sufficient to restore TGF-β-induced p21

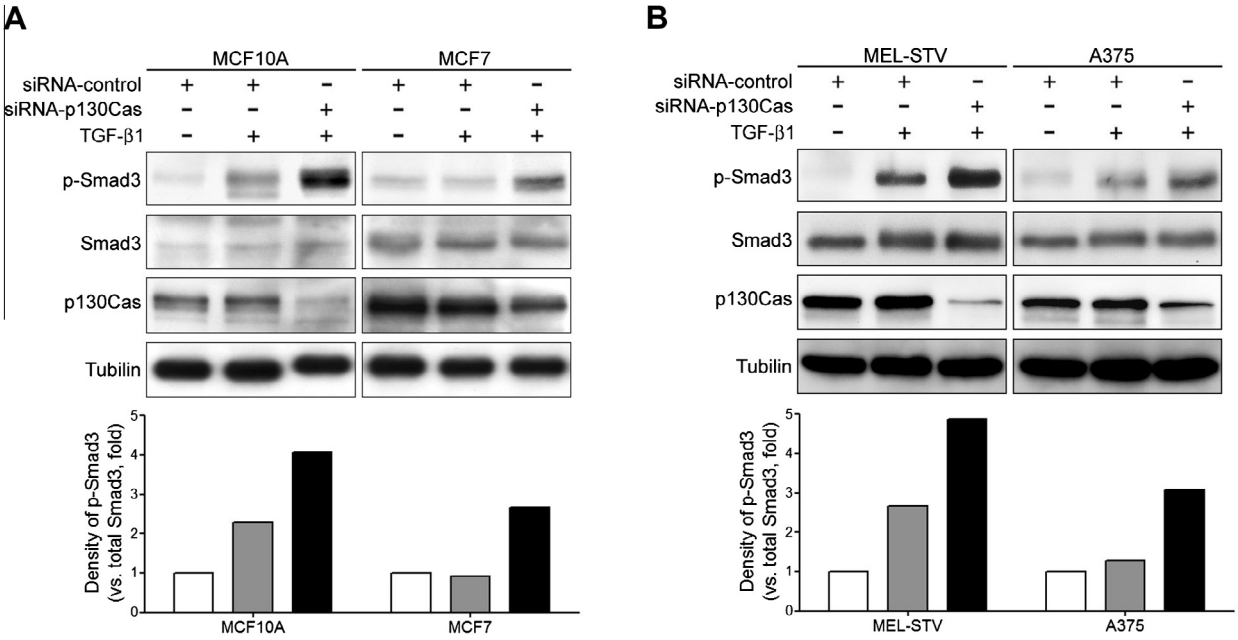

Fig. 2. Silencing of p130Cas increases TGF-β-induced Smad3 phosphorylation. (A and B) Cells were transfected with control or p130Cas siRNA as described in the Section 2. The resultant transfectants were treated with TGF-β for 30 min and then subjected to western blotting for the indicated proteins.

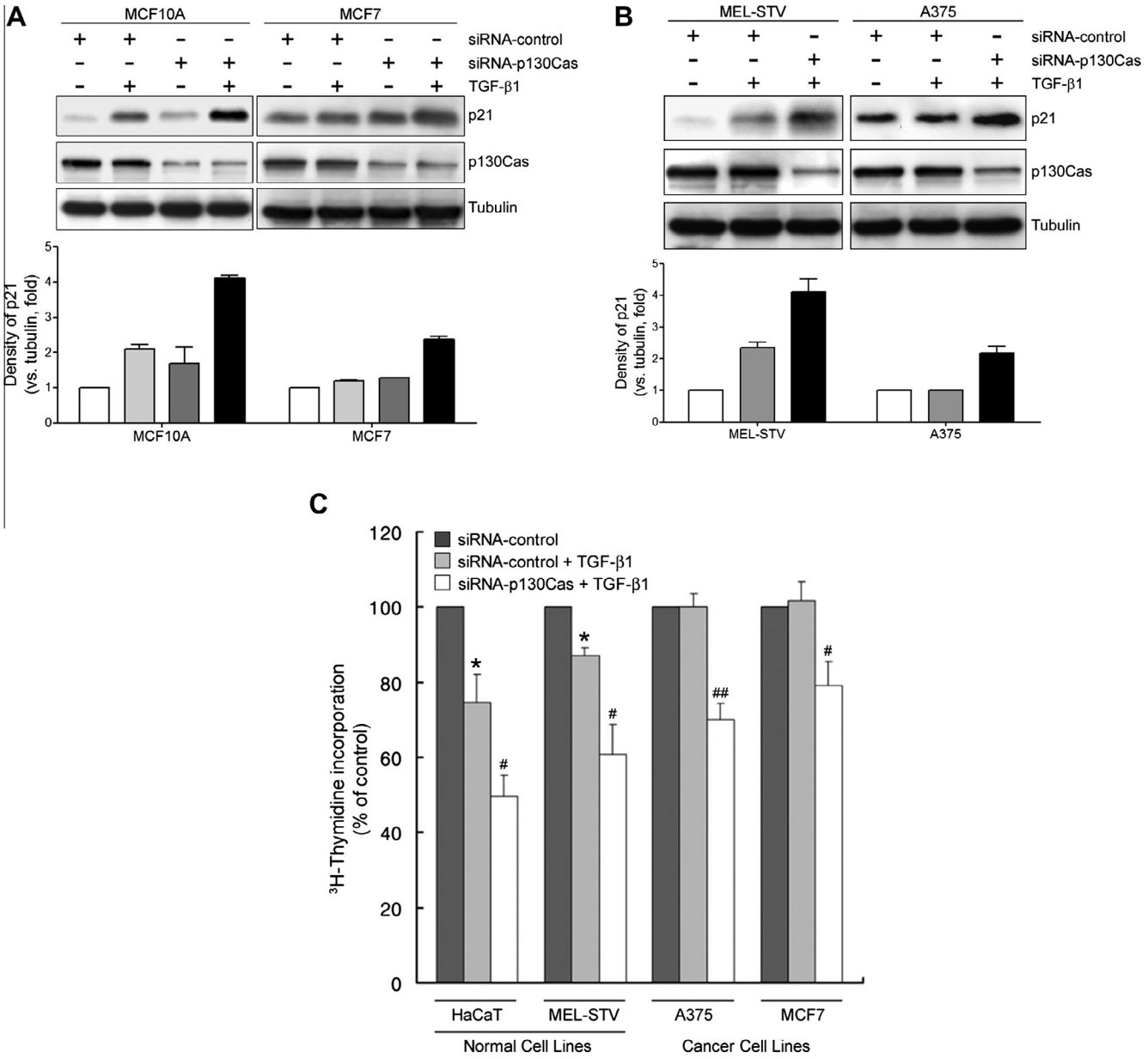

Fig. 3. Silencing of p130Cas restores the sensitivity of cancer cells to TGF-β-induced p21 expression and cell cycle arrest. (A–C) Cells were transfected with control or p130Cas siRNA as described in the Section 2. The resultant transfectants were treated with TGF-β for 24 h and then subjected to western blotting for the indicated proteins (A and B) and ^{3}H-thymidine incorporation assays (C). ^{3}H-thymidine incorporation in (C) was calculated as a percentage of that seen in control-siRNA-transfectants not treated with TGF-β. Data are shown as the mean ± SEM from three independent experiments. $^{*}P$ <0.05 versus siRNA-control; $^{\#}P$ <0.05; $^{\#\#}P$ <0.01 versussiRNA-control treated with TGF-β.

expression (Fig. 3A and B). Consistently, both MCF7 and A375 cells appear resistant to TGF-β-induced growth inhibition (Fig. 3C). After knockdown of p130Cas, however, the susceptibility to TGF-β-induced growth inhibition was restored in both cancer cells (Fig. 3C). These results suggest that elevated levels of p130Cas make a key contribution to the resistance of cancer cells to TGF-β-induced growth inhibition.

3.3. Effect of p130Cas on TGF-β-induced epithelial-mesenchymal transition (EMT)

TGF-β is proposed to suppress tumor growth in early tumorigenesis but to promote pro-oncogenic activities such as tumor cell motility and invasion in late stages through the induction of epithelial-mesenchymal transition (EMT) [28,29]. To assess whether p130Cas affects the TGF-β-induced EMT, we used HaCaT keratinocytes stably transfected with an empty vector (HaCaT-Vector) or p130Cas (HaCaT-p130Cas). In the absence of TGF-β, HaCaT-Vector cells had an epithelial-like morphology with E-cadherin, actin cytoskeleton, and β-catenin arranged in a cortical pattern at cell–cell junctions. Upon TGF-βtreatment, HaCaT-Vector cells acquired spindle-shaped fibroblast-like morphology with the delocalization of E-cadherin and β-catenin, as well as the formation of actinstress fibers (Fig. 4). Interestingly, stable overexpression of p130Cas in HaCaT cells accelerated the morphological changes induced by TGF-β (Fig. 4), suggesting that p130Cas promotes TGF-β-induced EMT.

4. Discussion

We have previously provided evidence that under physiological conditions, p130Cas interacts with Smad3 and reduces its

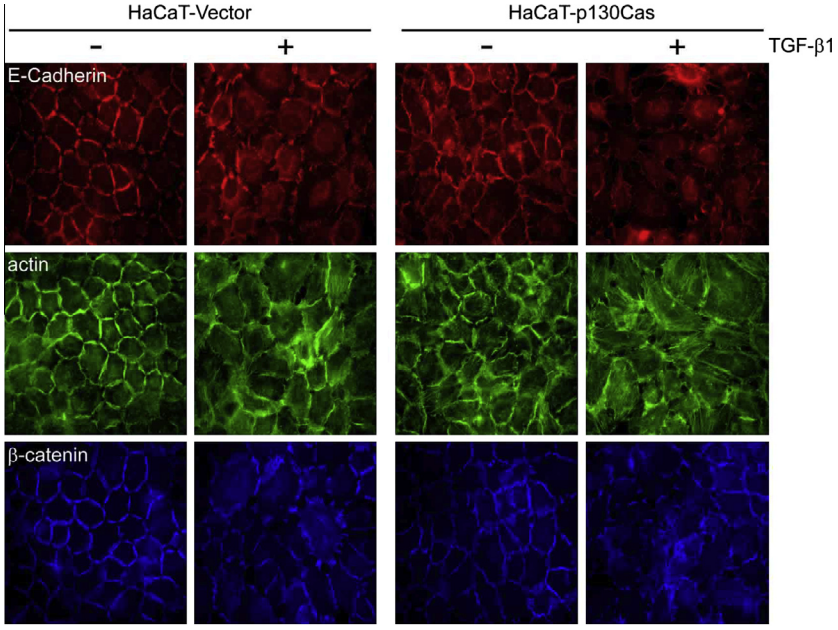

Fig. 4. p130Cas promotes epithelial–mesenchymal transition (EMT). Confluent HaCaT human keratinocytes stably transfected with empty vector (HaCaT-Vector) or p130Cas (HaCaT-p130Cas) were treated with TGF-β for 48 h and then subjected to immunofluorescent staining for the indicated proteins.

phosphorylation by TβR-I, thereby reducing levels of p15 and p21 and facilitating cell cycle progression from the G1 to S phases [12]. Thus, we have proposed p130Cas as a general inhibitor that functions at an early step in the TGF-β signaling pathway, as opposed to regulating subsets of Smad3-mediatingresponses, such as growth inhibition through the expression of cell cycle regulators (p15 and p21) [12]. However, we did not determine in this report whether the p130Cas-mediated control of TGF-β signaling contributes to the initiation and progression of human cancers. Here, we demonstrate the oncogenic role of p130Cas on the resistance to TGF-β-induced growth inhibition, a hallmark of many cancers, and TGF-β-induced EMT. In normal cells, cell cycle progression was inhibited in the presence of TGF-β by inducing Smad3 phosphorylation and p21 expression. Cancer cells, in contrast, especially breast cancer and melanoma cells, showed high levels of expression or tyrosine phosphorylation of p130Cas (Fig. 1A). A recent tyrosine phosphorylation profiling study consistently revealed increased tyrosine phosphorylation of p130Cas in basal breast cancer cells [30]. Elevated levels of expression or tyrosine phosphorylation of p130Cas were positively correlated with the resistance of MCF7 and A375 cells to TGF-β-induced Smad3 phosphorylation, p21 expression, and growth inhibition. Silencing p130Cas using siRNA was sufficient to restore TGF-β-induced Smad3 phosphorylation and p21 expression, and, in turn, the sensitivity of the cells to TGF-β-induced growth inhibition. Thus, we propose that during mammary tumorigenesis, the aberrant up-regulation of p130Cas diminishes the responsiveness of a cell to the canonical TGF-β signaling by the complex formation, thereby limiting the tumor-suppressive functions of TGF-β, and consequently contributing to the initiation of human cancers.

In addition to the role as a tumor suppressor, TGF-β also has oncogenic activity by promoting invasion and metastasis during tumor progression [21,31]. Several lines of evidence implicate dysregulated activation of the noncanonical TGF-β signaling during the conversion of TGF-β from a tumor suppressor to a promoter of its growth and metastasis [13,32–34]. One of the tumor-promoting effects of TGF-β is the induction of EMT [29,31,35], and many types of carcinoma cell lines undergo partial or complete EMT in response to chronic TGF-β exposure over a period of days [36], which causes a spindle-shaped mesenchymal appearance with increased motility and invasiveness through a loss of the epithelial phenotype, including a reduction in E-cadherin and β-catenin levels and an increase in actin stress fibers [37]. Interestingly, we found using immunofluorescence analysis that the stable expression ofp130Cas accelerated TGF-β-induced EMT (Fig. 4). This study is in general agreement with recent works that showed a role for the p130Cas protein family in EMT [38–42]. The p130Cas protein family member NEDD9 regulated EMT in breast [39,41] and melanoma [42] cancer cells, as well as p130Cas served as a scaffold to facilitate signaling that switches from the epithelial to mesenchymal phenotype in pancreatic cancer cells [40]. As described previously, p130Cas is often overexpressed or hyperphosphorylated, especially during breast cancer and melanoma progression, and integrin β1 signaling, an upstream of p130Cas, is necessary for the noncanonical TGF-β activation and EMT in mammary epithelialcells [43]. Furthermore, although integrins have not traditionally been thought of as proto-oncogenes, there are data that suggest integrins play an important, cooperative role during tumor induction by activated oncogenes. Thus, our findings may provide new insight into the mechanisms underlying the oncogenic conversion of TGF-β signaling from a tumor suppressor function to a pro-metastatic function. Although recent studies have provided the molecular mechanism underlying p130Cas as a regulatory component in TGF-β signaling [12,13], it will be worthwhile in the future to explore the additional details by which p130Cas regulates TGF-β-induced EMT, as well as to develop a useful method to directly control p130Cas activity in vivo.

Acknowledgments

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