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Activin and TGFβ regulate expression of the microRNA-181 family to promote cell migration and invasion in breast cancer cells

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ABSTRACT

MicroRNA-181 (miR-181) is a multifaceted miRNA that has been implicated in many cellular processes such as cell fate determination and cellular invasion. While miR-181 is often overexpressed in human tumors, a direct role for this miRNA in breast cancer progression has not yet been characterized. In this study, we found this miRNA to be regulated by both activin and TGFβ. While we found no effect of miR-181 modulation on activin/TGFβ-mediated tumor suppression, our data clearly indicate that miR-181 plays a critical and prominent role downstream of two growth factors, in mediating their pro-migratory and pro-invasive effects in breast cancer cells miR-181 acts as a metastamir in breast cancer. Thus, our findings define a novel role for miR-181 downstream of activin/TGFβ in regulating their tumor promoting functions. Having defined miR-181 as a critical regulator of tumor progression *in vitro*, our results thus, highlight miR-181 as an important potential therapeutic target in breast cancer.

1. Introduction

The TGFβ ligands are multitasking cytokines that play important roles in embryonic development, cell proliferation, motility, invasion and apoptosis, extracellular matrix production and modulation of immune function [1–5]. TGFβ, the founding member of this family, and its receptors are expressed everywhere in the body and deregulation of the TGFβ signaling pathways has been implicated in multiple human diseases [6]. TGFβ plays a dual role in cancer: it limits proliferation in epithelial cells and early-stage cancer cells, whereas in late stage cancer, it accelerates cancer progression and metastasis [2,7–11]. In the cancer niche, TGFβ can be produced and secreted into the extracellular environment by both cancer cells and host cells, such as lymphocytes, macrophages and dendritic cells. In cancer patients, high levels of TGFβ at tumor sites correlate with high histological grade, risk of metastasis, poor response to chemotherapy, and poor patient prognosis [8]. TGFβ interacts with and signals through two transmembrane serine/threonine kinase receptors (TβRI/ALK5 and TβRII), which then activate the Smad family of transcription factors (Smad2 and 3) [1,2,12].

Another member of the family, activin was initially isolated from gonadal fluid [13,14] based on its ability to induce FSHβ secretion and regulate the anterior pituitary function [15–17]. Activin was later shown to regulate cell growth, apoptosis and differentiation in a variety of tissues, including breast cancer [4,18–21]. Similar to TGFβ, activin initiates its signaling through ligand binding to the activin type II receptors at the cell surface, leading to the recruitment and phosphorylation of the type I receptor (ALK4) [19,22]. The activated ALK4 in turn phosphorylates the two intracellular Smad2 and Smad3, the main activin/TGFβ downstream mediators and further lead to their association with the common partner Smad4 [23].

Activin and TGFβ signaling is not limited to the canonical Smad pathway, as they have also been reported to transduce their signal through non-Smad signaling pathways [2,11,18,19,22–25]. While the role of TGFβ in mammary gland and breast cancer has been well characterized, the role and function of activin in this tissue remain largely unknown. In breast tissue, activin and its receptors are expressed during lactation [26] and activin was suggested to participate in mammary epithelium development [27]. In breast cancer, activin can act as a tumor suppressor by inducing cell growth arrest [18,28], apoptosis [29] and by inhibiting telomerase activity [30,31]. However, even though circulating levels of activin have been correlated to bone metastasis in breast cancer [32] and that inhibiting activin was shown to prevent cancer-induced bone destruction *in vivo* [33], a direct role for activin in promoting breast cancer cell invasion and metastasis has yet to be demonstrated.

MicroRNAs (miRNAs) are a novel class of small non-coding RNAs which have eluded researchers for decades stealthily regulating many of the major biological processes in eukaryotic cells by regulating their target genes post transcriptionally. In the past decade, our understanding of miRNA has grown tremendously from an observed oddity in worms [34] to the establishment of a fully recognized new class of regulatory molecules. They are a novel class of small (19-25nt) non-coding RNAs which play important roles in development.

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Bioinformatics approaches suggest that miRNAs represent 1% all human genes and yet over a third of the transcriptome is regulated by these miRNA [35]. It clearly became apparent that miRNA play central and critical role in human diseases, including cancer. Half of the known miRNAs are located on fragile sites of the chromosomes suggesting that they could play major roles in cancer [36]. Cancer-specific chromosomal rearrangement studies have shown that half of the breakpoints coincide with fragile chromosomal sites [37]. Half of the miRNA-encoding genes are located in chromosomal regions that are altered during tumorigenesis [38]. Both TGFβ and activin have been shown to regulate miRNAs *in vitro* [39,40] although very little work has been done on the latter regulation. The role of miRNAs in the progression of breast cancer (BC) is emerging only recently. Several miRNAs have been implicated in several steps of breast cancer progression (reviewed in [2]). For instance miR-31 has been shown to target several genes involved in breast cancer metastasis [41] and miR-200 has been shown to target ZEB2, a transcription factor involved in EMT [42]. We also recently found

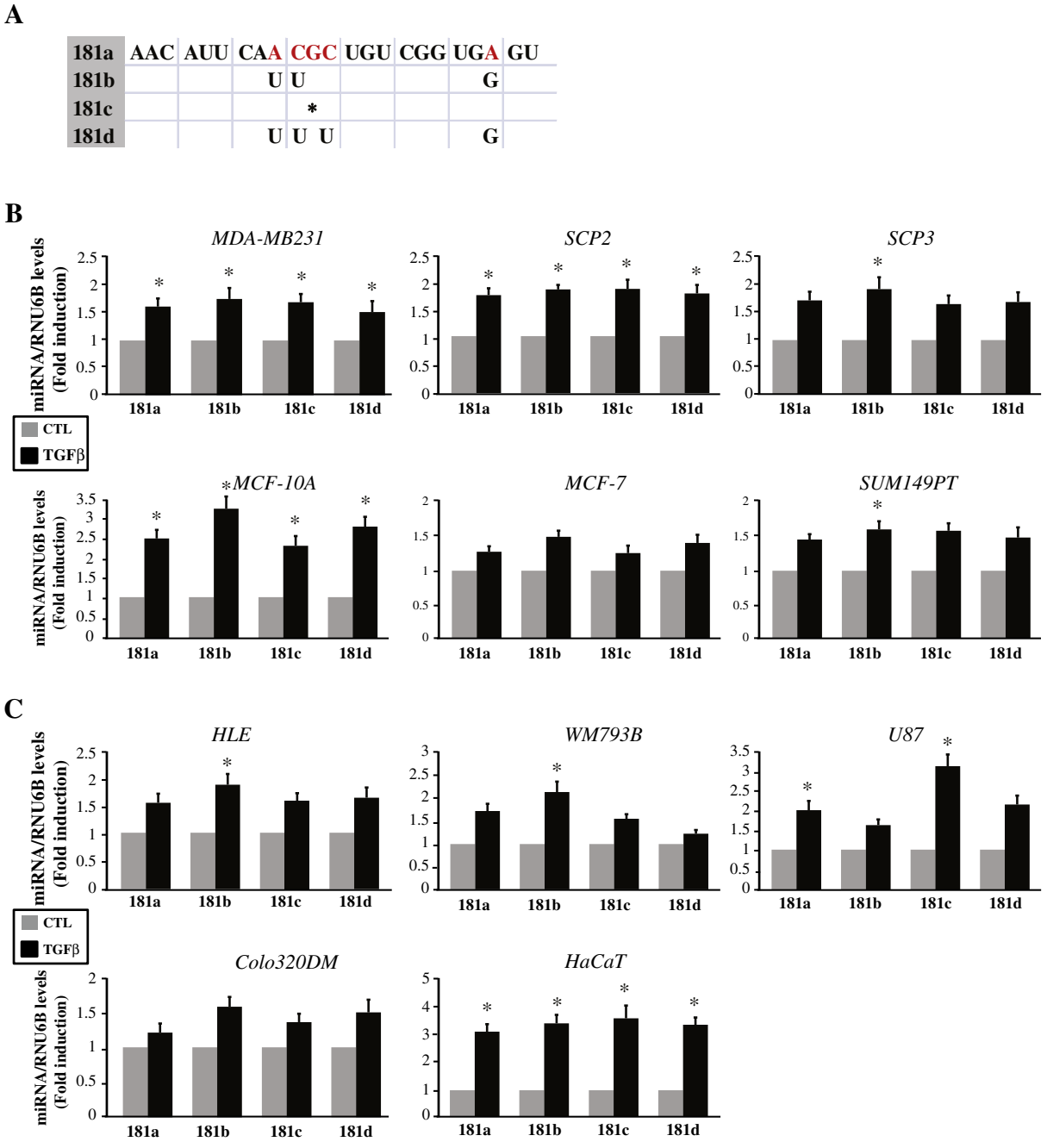


Fig. 1. miR-181 is a conserved target of activin/TGFβ signaling. A, The different miR-181 family members share a common seed and differ in other miRNA regions. B, Breast cancer cells were serum-starved overnight and stimulated or not with 100 pM TGFβ for 24 h and miR-181 expression levels were analyzed by RT-real time PCR. C, Liver (HLE), melanoma (WM793B), glioma (U87), colorectal (Colo320DM) and squamous cell carcinoma (HaCaT) cell lines were treated or not with 100 pM TGFβ1 for 24 h and miR-181 expression levels were analyzed by RT-real time PCR. D, Breast cancer cells were serum-starved overnight and stimulated or not with 500 nM activin A for 24 h and miR-181 expression levels were analyzed by RT-real time PCR. E, Liver (HepG2 and HLE), melanoma (WM1617) and colorectal (Colo320DM) cell lines were treated or not with 500 nM activin A for 24 h and miR-181 expression levels were analyzed by RT-real time PCR. Data is graphed as the geometric mean of RNU6B-normalized fold inductions of miR-181 family members in response to TGFβ for 3 independent experiments. The error bars are geometric standard deviations. For statistical analysis the z-test was performed on the logarithmic values and ligand-treated conditions were compared to the non-treated control (* $p < 0.05$).

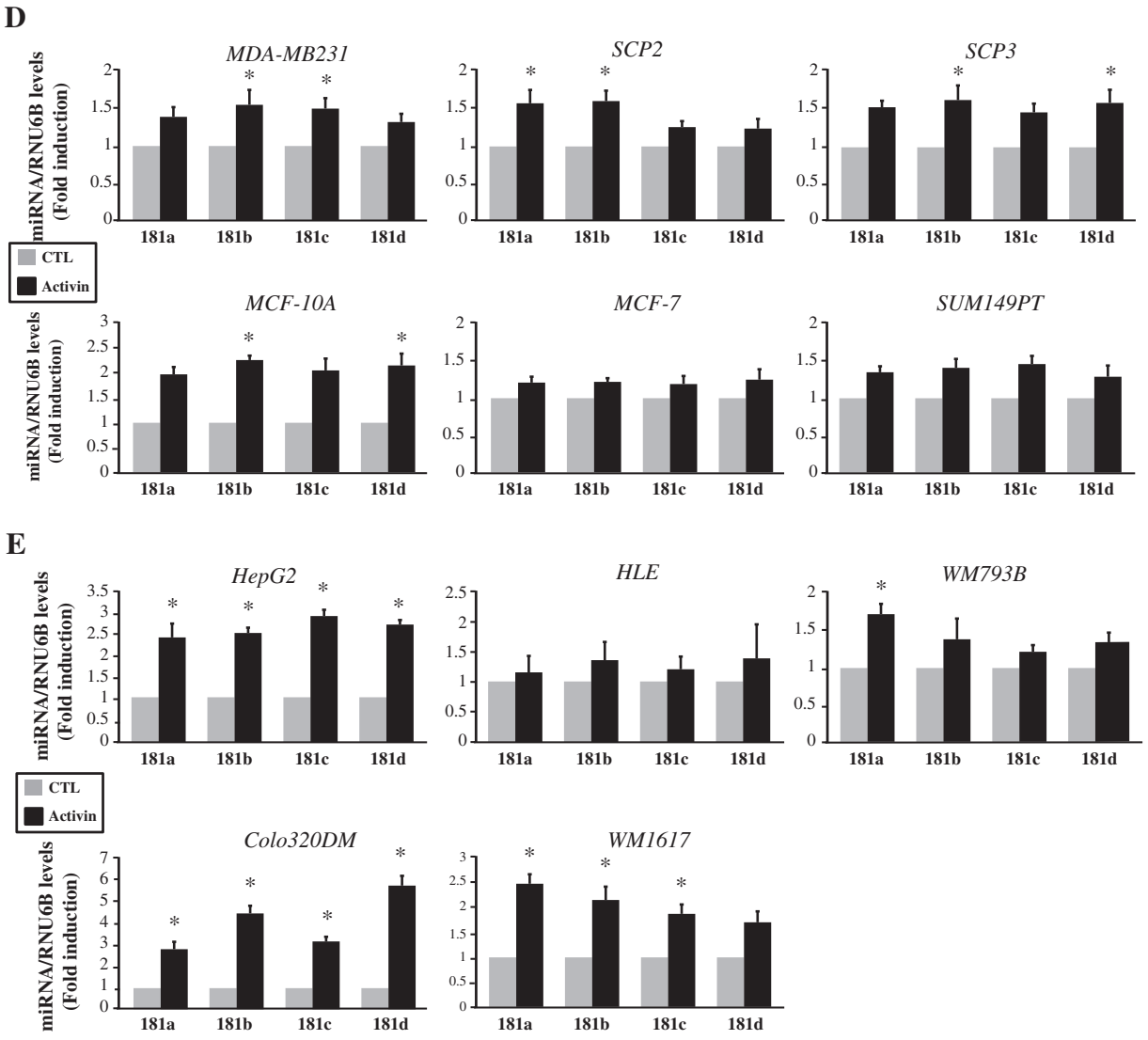


Fig. 1 (*continued*).

TGFβ-mediated down regulation of miR-584 to be critical for breast cancer cell actin skeleton reorganization and cell motility [43].

In this study, we identified miR-181 as a potent regulator of activin and TGFβ signaling in human breast cancer. We found miR-181 to be Smad2/3-dependent downstream target of TGFβ/activin signaling. Furthermore, our data demonstrate that activin, like TGFβ, acts as a potent inducer of cell migration and cell invasion in human breast cancer cells, thus, highlighting a novel function for this growth factor in cancer cells. Moreover, we also found miR-181 to be required for activin/TGFβ-mediated cell migration and invasion, as silencing miR-181 expression significantly antagonize these growth factors pro-invasive effects. Interestingly, while significantly blocking activin/TGFβ-induced cell migration and invasion, modulation of miR-181 endogenous levels did not altered activin and TGFβ tumor suppressive effects in cancer cells, highlighting the therapeutic potential of small antagonists to this microRNA for breast cancer treatment.

2. Materials and methods

2.1. Cell culture and transfection

Human breast carcinoma MDA-MB231, SCP2, SCP3 were grown in DMEM (Hyclone, Logan, UT, USA) supplemented with 10% FBS (Gibco, Grand Island, NY, USA), 2 mM L-glutamine (Hyclone) and penicillin/streptomycin (Hyclone) at 37 °C under a humidified atmosphere of 5% CO_2. MCF7, HuH7, Colo320DM and U87 cells were grown in the same conditions. WM793B cells were grown in RPMI (Hyclone) in similar conditions.

2.2. Transfections

Cells were transfected with different 100 nM miRNA mimics and inhibitors (Genepharma, Shanghai, China) or siRNA (Ambion, Life Technologies, Grand Island, NY, USA) using Lipofectamine™ 2000 reagent (Invitrogen, Grand Island, NY, USA), according to the manufacturer's protocol.

Before treatment, MDA and SCPs cells were serum starved for 24 h and stimulated with 100 pM $TGF\beta_1$ (PeproTech) in DMEM supplemented with 2 mM L-glutamine. SCP2 cells transfected with miRNA mimics or inhibitors were transfected 48 h prior to $TGF\beta_1$ treatment.

2.3. Real-time-PCR

Total RNA was extracted using TRIzol reagents (Invitrogen). Reverse transcription of 250 ng total RNA was carried out using on miScript reverse transcriptase (Qiagen, CA, USA) as the manufacturer's instructions

in a Rotor Gene 6000 PCR detection system (Corbett, San Francisco, CA, USA). miRNA PCR thermoprofile conditions were as follows: 95 °C for 15 min, 40 cycles (94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s).

2.4. Cell viability assay

Following an overnight FBS deprivation, HuH7 or HaCaT cells were resuspended (1×10^5 cells.ml^{-1}) in DMEM supplemented with 2% FBS in the presence or absence of 100 pM TGFβ$_1$ and seeded (1×10^4 cells.ml^{-1}) in 96-well plate for 48 h. After 48 h, cells were incubated for 2 h with Thiazolyl blue tetrazolium bromide (MTT) (5 mg.ml^{-1} in PBS, Sigma-Aldrich, Oakville, ON, Canada). Then a 20% SDS solution in 50% Dimethyl-formamide in H_2O at pH 4.7 was added to stop the reaction and mixed to homogeneity by pipetting. The absorbance at 570 nm was measured on a plate reader (BioTeK PowerWave XS) and a 690 nm measurement was used as a reference.

2.5. Migration assay

Cells were grown in 6-well plates until confluency and serum-starved overnight. A scratch was generated in the cell monolayer in straight lines using a sterile 10 μL tip guided by a ruler. Cells were then stimulated with or without 100 pM TGFβ$_1$ or 500 nM activin A for 24 or 48 h. Lines were drawn under the wells so photos could later be acquired from the same area. Photographs were taken initially and at the end of the ligand stimulation under phase contrast light microscopy (Olympus IX70, ImagePro AMS) and the wound closure was quantified by Image J software (NIH freeware).

2.6. Invasion assay

Cells were serum-starved overnight and seeded 5×10^4 onto a Matrigel-coated 24-well cell culture Transwell insert (8-μm pore size; BD Biosciences). Coating was done with 30 μl of 1:3 water-diluted growth factor reduced (GFR) Matrigel (BD) into each insert of the 24-tranwell invasion plate and incubated for 2 h at 37 °C in the cell culture incubator for Matrigel gelation. Cells were seeded in starvation medium on the top chamber the precoated Transwell Insert and were stimulated or not with 100 pM TGFβ$_1$ for 24 h. The bottom chamber contained 10% FBS in DMEM medium which acted as chemoattractant. After 24 h, cells from the top chamber were removed by cotton swab and invading cells were fixed with 4% formaldehyde for 10 min and then stained with a crystal violet solution for 10 min. Images of the invading cells were photographed using an inverted microscope (Olympus IX70, ImagePro AMS) and total cell numbers were counted and quantified by Image J software (NIH freeware).

3. Results

3.1. Expression of the miR-181 family members is induced by TGF-β and activin in cancer cells of multiple origins

miRNAs are naturally occurring small non-coding RNA molecules that play crucial functions in cells by base pairing to the 3′ untranslated region (UTR) of target mRNAs, resulting in mRNA degradation or translational inhibition. Multiple miRNAs have been implicated in human diseases [44,45]. Of particular interest, the broadly conserved miRNA family miR-181 has been implicated in various human cancers. Elevated levels of miR-181 are observed in the cancer of breast, prostate and

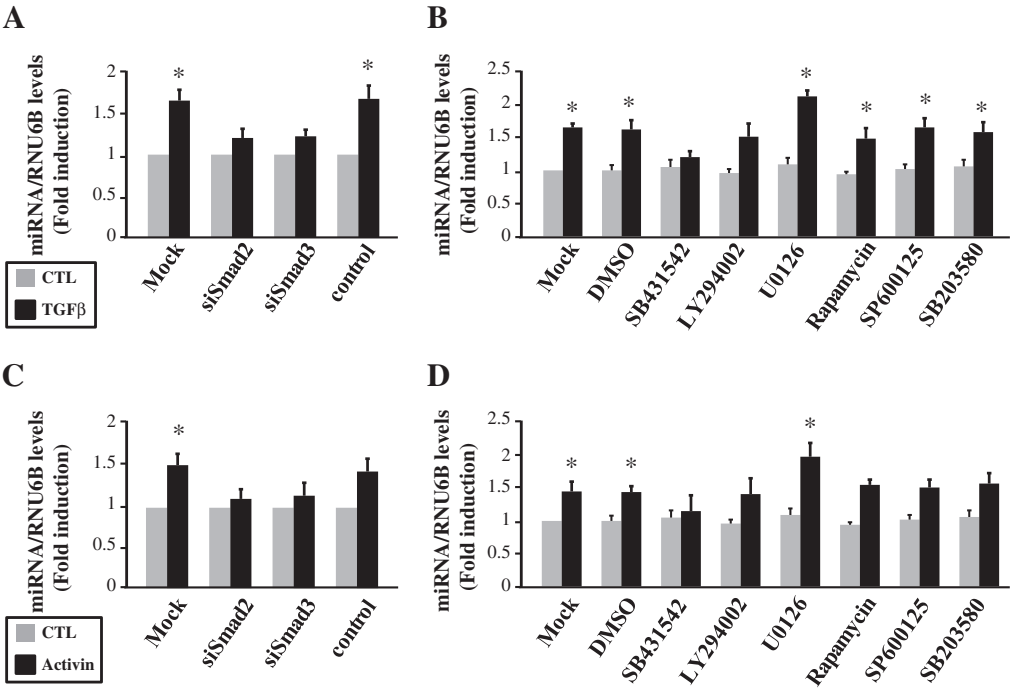


Fig. 2. MiR-181 induction by activin/TGFβ signaling is mediated through the canonical Smad2/3 pathway. A, SCP2 cells were transfected with Smad2, Smad3 or a control siRNA for 24 h then serum-starved overnight and treated or not with 100 pM TGFβ1. miR-181 expression levels were assessed by RT-real time PCR. B, SCP2 cells were serum-started overnight and pre-treated with different chemical inhibitors then treated or not with 100 pM TGFβ1. miR-181 expression levels were assessed by RT-real time PCR. DMSO was used as a vehicle control. C, SCP2 cells were transfected with Smad2, Smad3 or a control siRNA for 24 h then serum-starved overnight and treated or not with 500 nM activin A. miR-181 expression levels were assessed by RT-real time PCR. D, SCP2 cells were serum-started overnight and pre-treated with different chemical inhibitors then treated or not with 500 nM activin A. miR-181 expression levels were assessed by RT-real time PCR. DMSO was used as a vehicle control. Data is graphed as the geometric mean of RNU6B-normalized fold inductions of miR-181 family members in response to TGFβ1 for 3 independent experiments. The error bars are geometric standard deviations. For statistical analysis the z-test was performed on the logarithmic values and ligand-treated conditions were compared to the non-treated control (* $p < 0.05$).

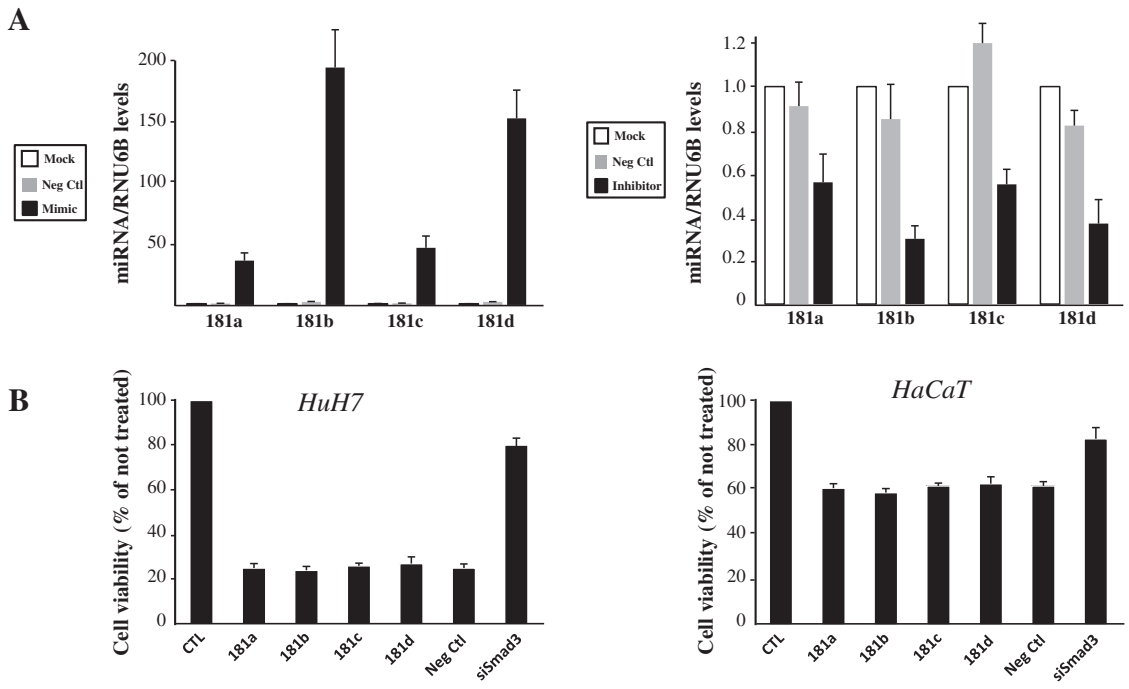
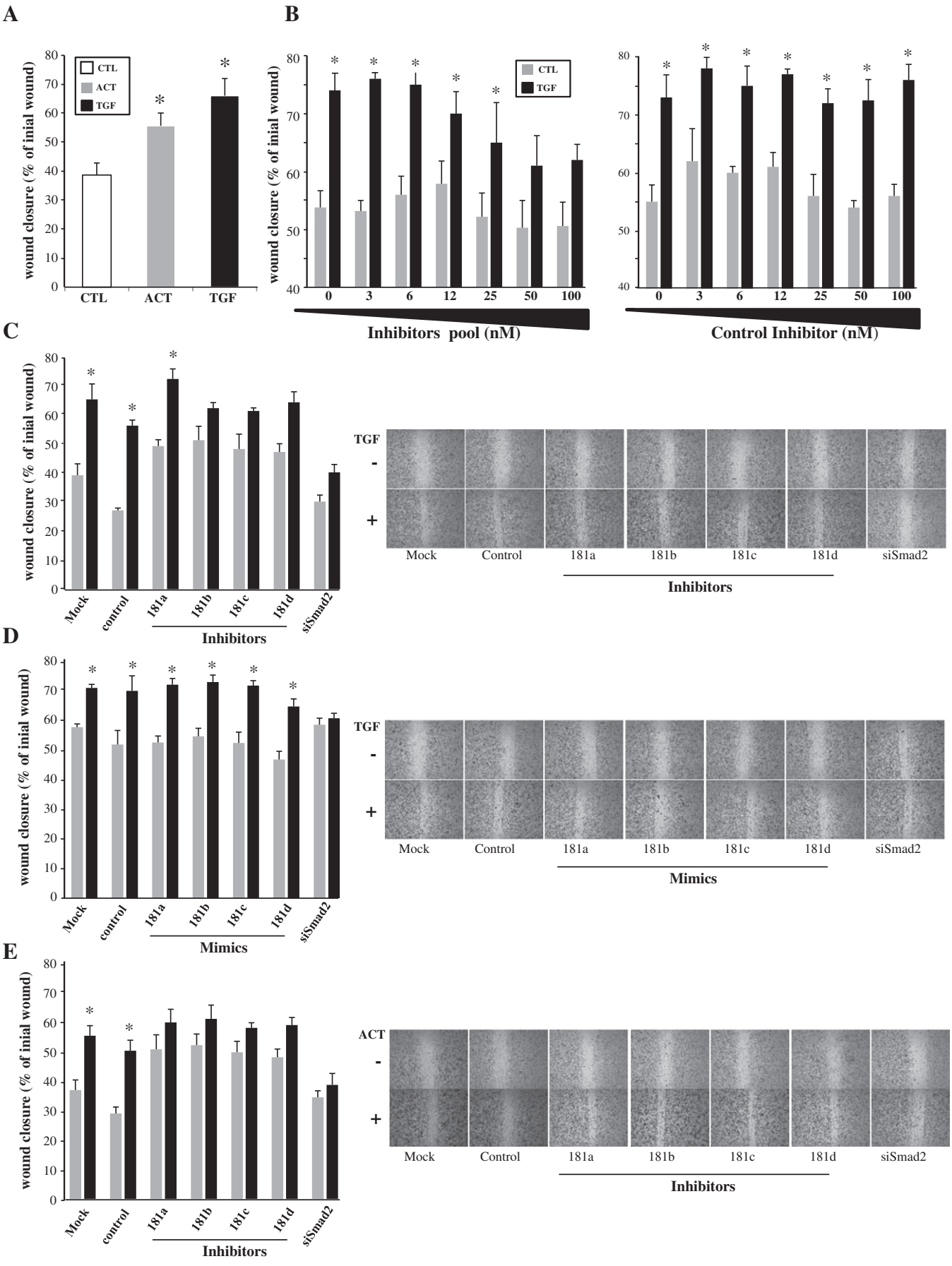


Fig. 3. Modulation of miR-181 does not affect antiproliferative effects of TGFβ. A, SCP2 cells were transfected with 100nM miR-181 mimics or inhibitors. Five days post-transfection miR-181 modulations were assessed by RT-real time PCR. Data is graphed as the geometric mean of RNU6B-normalized fold inductions of miR-181 family members for 3 independent experiments. The error bars are geometric standard deviations. B, Hepatocarcinoma (HuH7) and squamous cell carcinoma (HaCaT) cells were transfected with 100 nM miR-181 inhibitors and stimulated or not for 48 h with 100 pM TGFβ1. Cell viability was assessed by the MTT assay. Data is graphed as the arithmetic mean of cell viability (% of non-treated control) for 3 independent experiments. The error bars are the standard error of the mean.

pancreas [46]. miR-181 has been reported to act as a tumor suppressor in glioma [47]. Interestingly, the mir-181 family has also been implicated downstream of TGFβ signaling. Indeed, miR-181b can promote hepatocarcinogenesis downstream of TGFβ by regulating metalloproteinase activities [48] and it was shown that TGFβ could up-regulate the sphere-initiating stem cell-like feature in breast cancer through miR-181 [49]. A recent study in murine breast cancer model revealed that miR-181a was up-regulated by TGFβ [50]. However, the exact role of miR-181 in human cancer remains unclear. Indeed miR-181 was reported to act as a tumor suppressor in leukemia [51] and in glioma [52] but an oncogenic miRNA or oncomir in hepatocarcinoma [48]. To start investigating the role of the miR-181 family in human cancer, we initially examined the regulation by TGFβ of the different miR-181 family members shown in Fig. 1A, miR-181a, miR-181b, miR-181c and miR-181d in different human cancer cell lines of various origins.

We used a panel of human breast cancer cell lines derived from pleural effusions (MCF7 from early breast adenocarcinoma, MDA-231 with greater tumorigenic potential, SCP2, SCP3 having strong bone and lung metastatic tropism respectively [53]). The TGFβ effect on miR-181 expression was assessed by real-time semi-quantitative PCR in cells stimulated or not with TGFβ for 24 h. As shown in Fig. 1B, TGFβ significantly up-regulated the expression of all miR-181 family members to various levels in the different breast cancer cell lines tested. Interestingly, mir-181 regulation by TGFβ does not seem to be dependent on the hormone receptor status of the cells. We next investigated whether the TGFβ effect on miR-181 expression was restricted to breast cancer and, as shown in Fig. 1C, we found that TGFβ could potently induce miR-181 family members' expression in a variety of human cancer cell lines, including liver cancer (HepG2, HuH7 and HLE), melanoma (WM1617, WM793B and WM278), colon carcinoma (Colo320DM) keratinocytes (HaCaT) and glioma (U87). This apparently conserved

Fig. 4. miR-181 is required for activin/TGFβ-induced migration in breast cancer. A, SCP2 monolayer was wounded and cells were stimulated or not with 500 nM activin A or 100 pM TGFβ1. The effect of ligand stimulation was visualized after 24 h using phase contrast microscopy. The area of the wound was measured using ImageJ software and the wound closure was graphed after normalization to the corresponding initial wound size. Data is graphed as the arithmetic mean of 4 independent experiments. The error bars are the standard error of the mean.B, SCP2 cells were transfected with gradually increasing concentrations of pooled miR-181 or negative control inhibitors (from 0 nM to 100 nM) and stimulated or not with 100 pM TGFβ1. The effect ligand stimulation following a gradual inhibition of miR-181 activity was visualized after 24 h using phase contrast microscopy. The area of the wound was measured using ImageJ software and the wound closure was graphed after normalization to the corresponding initial wound size. Data is graphed as the arithmetic mean of 4 independent experiments. The error bars are the standard error of the mean. C, SCP2 cells were transfected with 100 nM of individual miR-181 family inhibitors and stimulated or not with 100 pM TGFβ1. 100 nM siRNA targeting Smad2 was used as a control. The effect ligand stimulation following inhibition of individual miR-181 family members was visualized after 24 h using phase contrast microscopy. The area of the wound was measured using ImageJ software and the wound closure was graphed after normalization to the corresponding initial wound size. Representative photos are shown in the right panel. Data is graphed as the arithmetic mean of 4 independent experiments. The error bars are the standard error of the mean. D, SCP2 cells were transfected with 100 nM of individual miR-181 family mimics and stimulated or not with 100 pM TGFβ1. 100 nM siRNA targeting Smad2 was used as a control. The effect ligand stimulation following inhibition of individual miR-181 family members was visualized after 48 h using phase contrast microscopy. The area of the wound was measured using ImageJ software and the wound closure was graphed after normalization to the corresponding initial wound size. Representative photos are shown in the right panel. Data is graphed as the arithmetic mean of 3 independent experiments. The error bars are the standard error of the mean. E, SCP2 cells were transfected with 100 nM of individual miR-181 family inhibitors and stimulated or not with 500 nM activin A. 100 nM siRNA targeting Smad2 was used as a control. The effect ligand stimulation following inhibition of individual miR-181 family members was visualized after 24 h using phase contrast microscopy. The area of the wound was measured using ImageJ software and the wound closure was graphed after normalization to the corresponding initial wound size. Representative photos are shown in the right panel. Data is graphed as the arithmetic mean of 4 independent experiments. The error bars are the standard error of the mean (* $p < 0.05$).


A
B
wound closure (% of inial wound)
CTL
ACT
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181d
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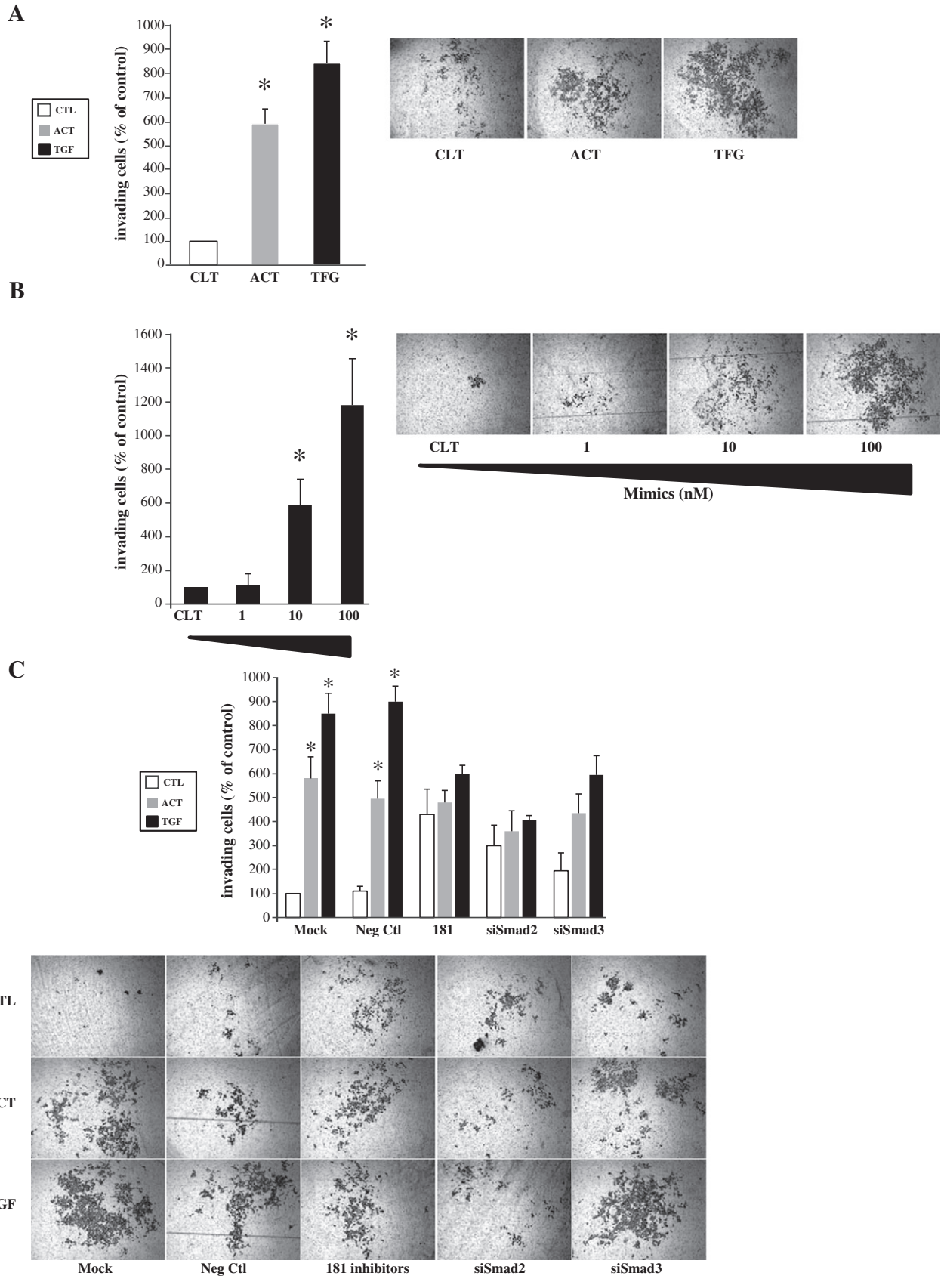
Inhibitors
D
Mimics
E

effect of TGFβ on miR-181 expression suggested an important role for miR-181. We then further examined the regulation of miR-181 downstream of TGFβ signaling was restricted to the TGFβ ligand itself or weather it also included other members of the TGFβ superfamily. For this, we analyze the activin effect on the different miR-181 isoforms. Indeed, activin belongs to the TGFβ family and shares downstream



signaling pathways and molecules (Smad2, 3 and 4) with TGFβ. Interestingly, as shown in Fig. 1D and E, we found that activin could significantly up-regulate miR-181a, b, c and d in a broad range of tissues. Together, these results define the miR-181 family as a downstream target for both activin and TGFβ signaling in human cancer cells of various origins and suggest an important regulatory role for this family of microRNAs in the mediation of the activin/TGFβ responses in cancer cells.

3.2. Activin/TGFβ-induced miR-181 expression is mediated through the canonical Smad pathway and is both Smad2 and Smad3-dependent

The Smad proteins are the main mediators of the activin and TGFβ signaling pathways [23,54]. To assess whether the activin/TGFβ-mediated induction of miR-181 expression is dependent on the Smad pathway, breast cancer cells were transfected or not with specific siRNAs against Smad2, Smad3 or a scrambled sequence as negative control and incubated in the presence or the absence of activin or TGFβ for 48 h. As shown in Fig. 2A and C, both activin and TGFβ significantly induced mir-181d expression, even though activin is slightly less potent. Interestingly, knocking down expression of either Smad2 or Smad3 completely abolished both the activin and TGFβ effects on miR-181d. Similar patterns were observed for the other members of the miR-181 family.

We then investigated whether the activin/TGFβ non-Smad signaling pathways were also involved in the regulation of miR-181, by specifically inhibiting these pathways using chemical inhibitors (LY294002 for PI3Kinase, Rapamycin for the mTOR in the PI3K/Akt pathway, U0126 for MEK1/2 in the MAP Kinase pathway, SB203580 for the p38 and SP600125 for the JNK in the p38/JNK pathway). As a positive control we also used the activin/TGFβ type I receptor kinase inhibitor SB431542. SB431542 was characterized as a potent inhibitor of the activin, TGFβ and nodal type I receptors (ALK4, ALK5 and ALK7 respectively) [55]. As shown in Fig. 2B and D, blocking the type I activin/TGFβ kinase activity completely abolished activin and TGFβ-induced miR-181 expression. Interestingly, blocking the PI3Kinase pathway, using the LY294002 inhibitor also partially antagonized the activin/TGFβ effects on mir-181 expression, suggesting a role for this pathway in the regulation of the miR-181, in addition to the Smads. Interestingly, inhibition of the MEK1/2 pathway using U0126 resulted in an increase of the TGF-β induction of mature miR-181 from 1.6-fold in vehicle-treated condition to 2.2-fold in U0126-treated condition. Taken together, our data indicate that the activin/TGFβ regulation of miR-181 expression is specifically mediated through the Smad pathway and is both Smad2 and Smad3-dependent.

3.3. Silencing and overexpression of the miR-181 using antagomirs and mimics, respectively

Several generations of chemically-modified oligonucleotides have been developed in order to deplete endogenous RNA. Initial generations of antisense oligonucleotides (ASO) were developed to target mRNA. These first generation ASO targeted and paired endogenous mRNAs leading to DNA–RNA hybrid that recruited RNAse H to cleave the mRNA [56]. In order to functionally characterize miR-181, we used chemically-modified inhibitor oligonucleotide sequences that bind to and irreversibly sequester endogenous miR-181 thereby decrease the intracellular miR-181 activity. We used double-stranded oligonucleotide mimic sequences that were processed as miRNA duplexes by cell machinery thereby elevating miR-181 activity. We were able to decrease miR-181 potently with a remaining 40% decrease 5 days post-transfection Fig. 3A–B. We were also able to have a strong increase of miR-181 levels with an 8 to 10-fold increase 5 days post-transfection.

3.4. miR-181 modulation does not affect TGFβ anti-proliferative effects

Previous reports have indicated that miRNA modulation affected cell proliferation [57]. Moreover, both activin and TGFβ exert strong anti-proliferative effects in breast cancer [18,58] but also in other target tissues, such as hepatocarcinoma [21,59], pituitary tumors [17] or keratinocytes [31,60]. Thus, we examined the functional relevance of activin/TGFβ-induced miR-181 expression in mediating the activin and TGFβ anti-proliferative effects. For this, we used miRNA mimics and inhibitors to respectively increase and decrease miR-181 levels *in vitro*. Inhibition of miR-181 with individual miR-181 family member inhibitors did not affect TGFβ anti-proliferative effects as assessed by MTT (Fig. 3B) in HuH7 cells and HaCaT keratinocytes. However a partial disruption of this effect was observed following knockdown of Smad3 in all model cell lines tested indicating that Smad3 was a downstream mediator of TGFβ anti-proliferative effects. Similar results were obtained in other cell types (i.e. breast cancer) and in response to activin (data not shown). This suggests that miR-181 is not involved in mediating TGFβ anti-proliferative effects.

3.5. miR-181 modulation affects activin/TGFβ-induced cell migration

As miR-181 is strongly induced by activin and TGFβ signaling in breast cancer, we further assessed the functional role of miR-181 in the mediation of other effects of TGFβ in breast cancer. TGFβ exerts a dual role in breast cancer, acting as a tumor suppressor in early carcinoma and as a tumor promoter in advanced malignant tumors (reviewed in [2]). Such a role for actin in the other hand has yet to be demonstrated. To further investigate the role of miR-181 downstream of activin and TGFβ in breast cancer, we first examined the effects of these two growth factors on cellular migration. For this we used a model cell line representing an aggressive, highly metastatic human breast cancer cell line, SCP2, in which we previously found TGFβ to exert strong pro-migratory and pro-invasive effects [61]. As shown in Fig. 4A, TGFβ significantly promoted cell migration. Interestingly, activin also strongly stimulated cell migration, to a level similar to what observed for TGFβ. We did not observed any synergistic effect between activin and TGFβ on cell migration (data not shown), presumably due to the fact that TGFβ and activin signal through and compete for the same Smad molecules. Our data highlight for the first time a pro-migratory role for activin in human breast cancer cell migration [62]. Activin was reported to promote migration in prostate [63] and dendritic cells [64]. There is no definitive

Fig. 5. miR-181 is required in activin/TGFβ-induced invasion in breast cancer. A, SCP2 cells were seeded in starvation medium on a Transwell cell culture insert coated with growth factor reduced Matrigel and stimulated or not with 500 nM activin A or 100 pM TGFβ1 for 48 h. The invading cells were visualized and photographed on the bottom side of the filter of the insert after crystal violet staining using phase contrast microscopy. Representative photos are shown in the right panel. Invading cells were counted using ImageJ software. Data is graphed as the arithmetic mean of 2 independent experiments. The error bars are the standard error of the mean. B, SCP2 cells were transfected with increasing concentrations of pooled miR-181 mimics from 0 nM to 100 nM and seeded in starvation medium on a Transwell cell culture insert coated with growth factor reduced Matrigel. The invading cells were visualized and photographed after 48 h on the bottom side of the filter of the insert after crystal violet staining using phase contrast microscopy. Representative photos are shown in the right panel. Invading cells were counted using ImageJ software. Data is graphed as the arithmetic mean of 2 independent experiments. The error bars are the standard error of the mean. C, SCP2 cells were transfected with 100 nM of pooled miR-181 inhibitors. Transfections with siRNA targeting Smad2 and Smad3 were used as controls. Trasnfected SCP2 cells were seeded in starvation medium on a Transwell cell culture insert coated with growth factor reduced Matrigel and stimulated or not with 500 nM activin A or 100 pM TGFβ1 for 48 h. The invading cells were then visualized and photographed on the bottom side of the filter of the insert after crystal violet staining using phase contrast microscopy. Representative photos are shown in the right panel. Invading cells were counted using ImageJ software. Data is graphed as the arithmetic mean of 2 independent experiments. The error bars are the standard error of the mean (* $p < 0.05$).

demonstration that activin has tumor promoting potential in the mammary gland and little is known about the role of activin in oncogenic progression in breast cancer [62].

To then address the functional relevance of activin/TGFβ-induced mir-181 expression downstream of these growth factors induction of cell migration, we knocked-down miR-181 family members' expression in cells stimulated or not with TGFβ and cell migration was assessed using the scratch/wound healing assay, as previously described [61]. We initially tested the effect of increasing concentrations of pooled miR-181 antagomir on TGFβ-induced promigratory response. We observed a gradual decrease of TGFβ-induced promigratory response between 6 nM and 50 nM of pooled antagomirs. This was not paralleled in the negative control antagomir transfections (Fig. 4B). We then tested the individual antagomirs of the miR-181 family members and observed that they all modulated TGFβ-induced migration but to different extents. Blocking expression of miR-181b, c, d and to a lesser extend miR-181a, slightly but significantly attenuated the TGFβ pro-migratory effect in SCP2 cells. miR-181a down-regulation was recently shown to decrease mouse 4T1 cell migration [50]. We also observed the TGFβ pro-migratory response to be Smad2-dependent (Fig. 4C). The sense sequences of the same miR-181 family members had no detectable potentiating effect on the TGFβ-induced migration (Fig. 4D). Interestingly, our results also indicate that activin also potentiate breast cancer cell migration, even though to a lesser extent than TGFβ (Fig. 4E). To our knowledge, this is the first demonstration for such a role of activin in breast cancer. Together, our findings highlight a novel function for activin in regulating cell migration in breast cancer cells and indicate that up-regulation of miR-181 expression by these growth factors is a prerequisite step for the induction of cell migration in human breast cancer.

3.6. miR-181 modulation affects Activin/TGFβ-induced cell invasion

Recent studies associated miRNA modulation with invasive potential such as miR-10b in hepatocarcinoma [65] and miR-183 in osteosarcoma [66]. Moreover, a recent study by Wang et al. [48] showed that TGFβ promoted liver cell invasion by increasing miR-181 in hepatocarcinoma, through down-regulation of tissue inhibitor of metalloproteinase 3 (TIMP3) leading to an increase activity of MMP2 and MMP9. As TGFβ is pro-invasive in breast cancer, this prompted us to investigate whether miR-181 modulation affected invasive potential in our SCP2 breast cancer model. Using Transwell/Matrigel assays, in SCP2 cells, we found TGFβ to potently induced cell invasion (Fig. 5A). The invasive potential of the cells was assessed by the increase in the number of cells digesting through the Matrigel and reaching the insert filter. Quantification was performed using ImageJ. Moreover, our results also indicate that activin strongly promotes invasion of these breast cancer cells, further expanding on the new roles played by this growth factor in breast cancer. No synergistic effects between activin and TGFβ were observed on cell invasion (data not shown). Interestingly, gradual overexpression of miR-181 led to an increased in the basal invasion rate of SCP2 cells, indicating that overexpression of miR-181 also is sufficient to mimic the activin and TGFβ effects on breast cancer cell invasion (Fig. 5B). Moreover, our results also clearly indicate that miR-181 is required for activin and TGFβ to induce cell invasion through the Matrigel, as miR-181 silencing completely blocked these growth factor effects on cell invasion. Inhibition of activin/TGFβ-mediated cell invasion was similar to what observed following gene silencing of the canonical Smad pathway, clearly indicating that miR-181 is critical to the cell invasion process (Fig. 5C). It was intriguing to observe that treatment of the cells with the miR-181inhbitor also led to an increase in basal cell invasion. This increased cell invasion is possibly due to an off-target effect of the inhibitor. MicroRNAs are known to regulate multiple intracellular targets and it is conceivable that miR-181 regulates the expression of other target genes that involved in the maintenance of cell invasion under basal conditions. Collectively, our data show that both activin and TGFβ are potent inducer of cell invasion in breast cancer cells and that their effects require the up-regulation of miR-181 gene expression.

4. Discussion

In this study, we describe a novel role for the microRNA miR-181 as a potent-mediator of breast cancer cell migration and invasion. Early works had shown miR-181 to be a tumor suppressor in glioblastoma [47]. The role of miR-181 in the context of breast cancer remained to be characterized. We found miR-181 gene expression to be dependent and regulated by the two growth factors, activin and TGFβ in multiple cancer cell lines of various origins. Furthermore, our data also indicate that up-regulatoin of miR-181 by activin/TGFβ is required for these growth factors to mediate cell migration and cell invasion in breast cancer and suggest an important role for miR-181 in the metastatic process of this type of cancer.

Activin has been reported to inhibit cell cycle through the p38 pathway [18]. Activin induces migration in mast cells however at higher concentrations than TGFβ [67]. In colon cancer, restoration of activin signaling reveals its pro-migratory role similar to TGFβ [68]. The effect of activin on breast cancer cell migration was unclear. Al-Hajj et al. [69] described that the $CD44^{pos}/CD24^{pos}$ subpopulation was non tumorigenic and that $CD44^{pos}/CD24^{pos}$ subpopulation was tumorigenic in immunocompromised mice. Activin signaling has been shown to mediate the interconversation of noninvasive $CD44^{pos}/CD24^{pos}$ cells into their $CD44^{pos}/CD24^{neg}$ invasive counterparts [70]. Recent studies have shown that miRNA could modulate cancer cell migration *in vitro* in glioma [71], liver [72] and breast [73] cells.

To our knowledge, this is also the first report of a role for activin in mediating cell migration/invasion in breast cancer. Activin has been studied in the context of embryo development. Until recent studies including those in colon [74] and prostate cancer [75] few studies had focused on its role in cancer. Only one paper focused on miRNAs downstream of activin signaling [40]. We observed a conservation of the regulation of miR-181 by TGFβ through different cellular contexts suggesting an important role for miR-181 across tissues. Interestingly, we report a novel role of activin in regulating miRNA in several model cells lines. This is indeed the first report of activin-mediated miR-181 induction. This miRNA regulation provides new insight into the role of activin in different cancer models. We observed that the miR-181 induction was more potent in cells with more pronounced anti-proliferative TGFβ responses such as HuH7 and HaCaT cells where the induction was >6-fold and >3-fold respectively as opposed to ~2-fold in SCP2 cells where the prometastatic response of the cells to TGFβ is accompanied by a milder transcriptional response. Interestingly we observed that miR-181 was induced by both activin/TGFβ, two members of the TGFβ superfamily.

We showed that the regulation of miR-181 by activin/TGFβ in this breast cancer model is dependent on the canonical Smad2/3 pathway but also requires the PI3 kinase pathway. Such a role for the PI3K pathway, downstream of TGFβ, in association with the Smads has been documented previously. Indeed, TGFβ signals through the PI3 K pathway to regulate cell growth inhibition [76] and induction of EMT [77]. We observed that inhibiting activin/TGFβ-induced miR-181 activity with 3′O-methyl-modified antisense RNA sequences was effective and lasted up to 5-days. The inhibition of miR-181 activity resulted in impaired activin/TGFβ-induced pro-migratory responses. This is also the first report of activin promigratory effect in breast cancer. The incomplete blockade of acivin/TGFβ-induced pro-migratory responses by miR-181 inhibiton suggests that other miR-181-independent pathways might be mediating pro-migratory effects. Increasing miR-181 levels with miRNA mimics did not significantly increase TGFβ pro-migratory effects. These effects of miR-181 modulation however did not affect the tumor-suppressive responses of TGFβ as assessed by the anti-proliferative effects of TGFβ in any of the model cell lines tested. Together our findings highlight miR-181 knockdown as a possible

strategy to inhibit pro-migratory and pro-invasive effects of activin/TGFβ signaling without interfering with the tumor suppressive arm of the pathway. Early clinical studies [78,79] concluded that increased TGFβ signaling led to increased metastasis prompting the industry to develop antagonists for the TGF-β signaling pathway. Models of TβRII knockouts showed surprisingly minimal phenotype [80–82] suggesting that there were compensatory mechanisms to the endogenous TGFβ growth inhibitory role. TβRII knockout however has a marked effect when combined with oncogene activation or tumor suppressor gene attenuation suggesting that the tumor suppressor arm of TGFβ is not compensated for by other signaling pathways [83]. Indeed the study by Forrester et al. indicated that TβRII knockout increased lung metastases in their model of oncogene-induced mammary carcinoma. Disrupting TGFβ signaling necessarily also affected the tumor microenvironment and increases the number of myeloid immune suppressor cells which contributes to tumor growth and vascularization [84,85]. These observations show that blocking TGFβ signaling too broadly has deleterious effects. It is in this context that miR-181 has a potential therapeutic value as it is downstream of TGFβ signaling and does not seem to be involved in the tumor suppressor arm of TGFβ signaling.

miRNA therapeutics is a growing field with potential application in liver cancer treatment [86]. miR-181 could act as a potential therapeutic metastatic miRNA target in breast cancer. Indeed although numerous approaches have been undertaken over the past decade to disrupt TGFβ signaling at different levels of the signaling cascade [2], none of the methods were effective as they all resulted in some alteration of the beneficial tumor suppressor arm of the signaling cascade. This study indicates that modulating miR-181 downstream of activin/TGFβ signaling not only decreases pro-migratory and pro-invasive effects of TGFβ signaling but also does not affect the tumor suppressor arm of TGFβ.

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