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P38 delta MAPK promotes breast cancer progression and lung metastasis by enhancing cell proliferation and cell detachment

Masayuki Wada[1], **Daniel Canals**[1,2], **Mohamad Adada**[1,2], **Nicolas Coant**[1], **Mohamed F Salama**[1,4], **Kristi L Helke**[5], **J. Simon C. Arthur**[6], **Kenneth R Shroyer**[7], **Kazuyuki Kitatani**[8], **Lina M Obeid**[1,2,3], and **Yusuf A Hannun**[1,2]

[1]Department of Medicine, Stony Brook University, Stony Brook, NY, 11794, USA

[2]Stony Brook Cancer Center, Stony Brook University

[3]Northport VA Medical Center, Northport, NY, 11768, USA

[4]Department of Biochemistry, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt

[5]Department of Comparative Medicine, Medical University of South Carolina, Charleston, South Carolina

[6]MRC Protein Phosphorylation Unit, School of Life Sciences, University of Dundee, United Kingdom

[7]Department of Pathology, Stony Brook University

[8]Tohoku Medical Megabank Organization and Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, Sendai, Japan

Abstract

The protein p38 mitogen-activated protein kinase delta isoform (p38δ) is a poorly studied member of the MAPK family. Data analysis from The Cancer Genome Atlas (TCGA) database revealed that p38δ is highly expressed in all types of human breast cancers. Using a human breast cancer tissue array, we confirmed elevation in cancer tissue. The breast cancer mouse model, MMTV-PyMT (PyMT), developed breast tumors with lung metastasis; however, mice deleted in *p38δ* (PyMT/$p38\delta^{-/-}$) exhibited delayed primary tumor formation and highly reduced lung metastatic burden. At the cellular level, we demonstrate that targeting of *p38δ* in breast cancer cells, MCF-7 and MDA-MB-231 resulted in a reduced rate of cell proliferation. Additionally, cells lacking *p38δ* also displayed an increased cell-matrix adhesion and reduced cell detachment. This effect on cell adhesion was molecularly supported by the regulation of the focal adhesion kinase (FAK) by *p38δ* in the human breast cell lines. These studies define a previously unappreciated role for *p38δ* in breast cancer development and evolution by regulating tumor growth and altering metastatic properties.

Corresponding author: Yusuf A Hannun, MD, Stony Brook Cancer Center, Stony Brook University, Health Science Center, L-4, 182, Stony Brook, NY 11794, Phone: +1-631-444-8067, FAX: +1-631-444-1719, yusuf.hannun@sbumed.org.

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Keywords

p38δ; MAPK; breast cancer; metastasis; MMTV-PyMT; Stat3; p-FAK Ty397

INTRODUCTION

p38 mitogen activated protein kinases (p38 MAPKs) are activated by cellular stress and cytokines, and they are involved in regulating the production of proinflammatory mediators, cell proliferation, differentiation and survival. There are four known isoforms (α, β, γ and δ) (1). Among these isoforms, p38α has been well characterized in inflammatory responses, and it has been evaluated in cancer studies as a therapeutic target. In contrast, the function of p38δ has not been well characterized (2), mainly due to the lack of specific inhibitors and the absence of a distinct phenotype of p38 deficient mice under normal conditions (3).

Studies using a knockout mouse model showed that p38δ is involved in neutrophil recruitment at inflammatory sites following lung injury (4). Moreover, bone marrow derived macrophages prepared from *p38γ* and *p38δ* double knockout mice had reduced TNF-α, IL-1β and IL-10 production upon LPS stimulation via ERK 1/2 pathway (5). p38δ has also been shown to act as either a tumor promoter or tumor suppressor, depending on the cell type (2). For instance, expression of p38δ suppressed cell proliferation and migration in esophageal squamous carcinoma cells (6) but promoted cancer progression in head and neck squamous cell carcinoma (7), cholangiocarcinoma (8) and rat mesothelioma cell proliferation (9). In contrast to those phenotypes, $p38\delta^{-/-}$mice exhibited a significant delay in skin tumor development and reduction of number and size of tumors using the DMBA/TPA skin carcinogenesis model. Moreover, $p38\delta^{-/-}$mice bred with K-RasG12D (LA-1 allele) mouse model showed attenuated lung tumorigenesis (10). Another study showed partial protection of tumor formation in an AOM/DSS colon cancer model (11). Thus, these *in vivo* studies suggest that in some cancers, *p38δ* could play a tumor promoting function (12).

Breast cancer is the most commonly diagnosed cancer, and the most common cause of cancer death in women worldwide. Over a million cases are diagnosed each year (13). In the treatment of breast cancer, physicians use a multidisciplinary approach involving surgery, radiation, chemotherapy and hormonal therapy (14). Although mortality rates has been decreasing since four decades (15), breast cancer still accounts for over than 40000 deaths in the United States (13). This is partly due to the failure of all treatment modalities especially in locally advanced and metastatic disease. Therefore, there is an urgent need to identify new therapeutic targets for breast cancer.

In a previous study, our group found that overexpression of recombinant human p38δ in MCF-7 cells increased IL-6 production (16). In breast cancer patients, elevated serum IL-6 has been associated with tumor stage, tumor growth and metastasis (17). Moreover, downregulation of *p38δ* in MDA-MB-231 breast cancer cells reduced cell motility (18). These latter studies suggest that *p38δ* could play a role in breast cancer progression. To address this possibility, we analyzed the transcription and protein levels of *p38δ* in several datasets of breast cancer. *p38δ* was found to be overexpressed in all types of breast cancer,

independent of their histological or molecular classification. Using the PyMT mouse model and human breast cancer cell lines, we found that loss of *p38δ* had profound effects on cell proliferation and detachment. Moreover, we found that the effects on cell growth were manifested in the initial phases of cancer development, but lost in more advanced cancers and more aggressive cell lines. In contrast, p38δ appears to play a more selective role in the regulation of adhesion and invasion in advanced cancers and in promoting tumor metastasis. All of these results point to p38δ as a key player in breast cancer growth and metastasis.

MATERIAL AND METHODS

Cell culture

MDA-MB-231, MCF-7 and MCF-10A cells were obtained from ATCC. MDA-MB-231 and MCF-7 cells were cultured in RPMI 1640 with 10% FBS. The culturing condition of MCF-10A cells was previously described (19). Cells were tested for mycoplasma on a monthly basis.

Antibodies

p38δ antibody was purchased from R&D systems. FAK, phospho-FAK Tyr397, p38s (α, β and γ), phospho-p38s, Cyclin D1, ERK1/2, phospho-ERK1/2, Stat3 and phospho-Stat3 Ser727 antibodies were obtained from Cell Signaling Technology. β-actin antibody was purchased from Sigma-Aldrich. Ki67 antibody was obtained from Millipore. When molecular weight did not overlap, same western blott membranes were re-used for different antibodies after stripping the previous antibody.

Animals

MMTV-PyMT (FVB/N-Tg(MMTV-PyVT)634Mul/J) mice were purchased from The Jackson Laboratory. The phenotype of *p38δ*-null ($p38\delta^{-/-}$) mouse was previously described (3). $p38\delta^{-/-}$ mice were backcrossed for six generations with FVB mice, and then crossed with MMTV-PyMT mice to obtain PyMT/$p38\delta^{+/+}$, PyMT/$p38\delta^{-/-}$, $p38\delta^{+/+}$ and $p38\delta^{-/-}$ female mice for this study. No obvious health problems were observed in $p38\delta^{-/-}$/FVB background mice. Animals were maintained under standard laboratory conditions and all animal procedures were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina (MUSC) and Stony Brook University (SBU) and followed the guidelines of the American Veterinary Medical Association.

Mouse monitoring and tissue excision

From 4 weeks of age, female mice were palpated and observed visually twice per week to monitor the onset of mammary tumors. Mice were euthanized at different ages (6, 10 and 14 weeks), body weight was measured, whole blood was collected from the heart, and all the mammary glands were dissected and tumor weight was measured to calculate the total tumor burden. Tumors were fixed in 10% buffered formalin or snap frozen in liquid nitrogen for further studies. Lungs from 14 week old mice were inflated with 1.5 ml of 10% buffered formalin via tracheal injection. Once inflated, lungs were checked for surface metastatic foci. Fixed lungs were serially sectioned 5 mm apart, and stained with hematoxylin and

eosin. The number of lung metastatic foci was counted by microscopy and their sizes were measured using NIH Image J software.

Immunohistochemistry

Paraffin-embedded mouse tissues were sectioned (5 μm sections) in the MUSC and SBU Research Histology Cores. All immunochemical studies were performed on deparaffinized and rehydrated sections. After antigen retrieval, sections were incubated with goat serum in PBS to reduce nonspecific staining and then incubated with appropriate antibodies (p38δ or Ki67) overnight at 4^{o}C. Slides were incubated with appropriate secondary antibodies and visualized with DAB Substrate Kit (Vector Lab). Samples were also counterstained with hematoxylin. Ki67 staining was evaluated in five fields per randomly selected tumor section.

siRNA transfection

Lipofectamine RNAiMAX reagent, Opti-MEM medium and *p38δ* siRNAs (siRNA-#1: Cat No142319 and siRNA-#2: Cat No 142320) were obtained from Invitrogen. *p38δ* siRNAs or AllStar siRNA (Qiagen) as a negative control were transfected into MCF-7 or MDA-MB-231 cells (20 nM) according to the manufacturer's protocol. Forty-eight hours after transfection, cells were used for the indicated experiments.

Immunoblotting analysis

Cells on culture dishes were washed with cold PBS and lysed in 1% SDS solution. Sonicated whole cell lysate (15 μg of protein) was used for western blot analysis. Mouse tissues were homogenized on ice using a Polytron homogenizer in RIPA buffer containing 2mM EDTA, protease inhibitor cocktail (Roche), phosphatase inhibitor cocktail-2 and -3 (Sigma), and then sonicated four times for 5 sec each. The homogenates were kept on ice for 10 min, and then centrifuged at 14.000 xg at 4°C. The collected supernatant (20 μg of protein) was used for western blot analysis. Protein concentrations were determined using a Pierce BCA protein assay kit according to the manufacturers instructions. For immunoblotting, proteins were separated by electrophoresis on 4–20% gels and transferred onto a nitrocellulose membrane. The membranes were blocked in PBS containing 0.1% Tween 20 (PBS-T) and 5% skim milk, and then incubated overnight at 4°C with appropriate antibodies in PBS-T and 1% BSA. Membranes were incubated with appropriate HRP-conjugated secondary antibodies (Santa Cruz) and developed using Pierce ECL or ECL Dura kit followed by exposure to X-ray films.

MTT assay

Forty-eight h after transfection with *p38δ* or AllStar siRNA, MCF-7 and MDA-MB-231 cells were harvested and seeded (0.5×10^5 cells per well) in 6 well plates and incubated overnight. The medium was changed to fresh medium and then incubated for different time points (0, 24, 48, 72 and 96 h). Medium was then replaced with fresh medium containing MTT (0.5 mg/ml) at each time point and incubated for 2.5 h. Medium was then aspirated, DMSO was added into each well, and plate was incubated for 10 min at RT. Absorbance was measured using spectrophotometer.

Adhesion assay

Forty-eight hours after siRNA transfection, MDA-MB-231 cells were harvested (1×10^5 cells) and then plated on fibronectin (5 μg/ml) coated 12 well plates. At each time point (0, 30, 45, 60, 90 and 120 min), plates were washed 5 times to remove unbound cells, followed by addition of fresh medium and incubated until the end of the assay. The number of adherent cells was evaluated using MTT.

Migration and Invasion assay

Forty-eight hours after siRNA transfection of MDA-MB-231, harvested cells (1×10^5 cells) were plated on pre-coated transwell devices (Thermofisher). For migration assays, the transwell was pre-coated with rat-tail collagen and for invasion was pre-coated with matrigel as previously reported (20, 21).

Crystal violet staining

p38δ or control siRNA transfected MDA-MB-231 were washed with PBS, fixed in 4% paraformaldehyde, and stained with 0.02% crystal violet (22).

Cell detachment assay

Forty-eight h after siRNA transfection of MDA-MB-231 cells, culture medium was replaced with fresh medium with or without PMA (100 nM) and incubated for additional 18 h. Plates were washed twice with PBS and then trypsinized to harvest and count adherent cells. Cell were counted using Invitrogen Countess cell counter.

Statistical analysis

Statistical analyses were performed by one-way Anova with Bonferroni's Multiple Comparison Test or Mann-Whitney U test for nonparametric data using Graphpad Prism Software (San Diego, CA).

RESULTS

Database analysis reveals that p38 delta is increased in breast cancer with a poor prognostic outcome

Data analysis of The Cancer Genome Atlas (TCGA) database showed that *p38δ* is overexpressed in a large set of human breast cancers. To evaluate the *p38δ* expression in different breast cancers, histopathological and molecular subtype classification were analyzed from the TCGA database. The specific role of *p38δ* in breast cancer was highlighted when it was compared with other p38 isoforms. For example, the levels of *p38α* mRNA were higher than *p38δ* mRNA in normal breast tissue. However, there was no difference in *p38α* expression between normal tissue and breast cancer. In contrast, the expression of *p38δ* mRNA was significantly increased in all of the histopathological types of breast tumor that are listed in the TCGA database, compared to normal breast tissue (Supplementary Table S1*), and in all molecular subtypes of breast cancer (Supplementary Table S2). In order to study the biological significance of such elevation in *p38δ* mRNA, we analyzed the outcomes in breast cancer patients with high *p38δ* using Kaplan-Meier plot

analysis (www.kmplot.com/analysis/). The analysis showed that all types of breast cancer patients with high *p38δ* levels are associated with poor prognosis, especially ER positive/Her2 negative types and luminal A (supplementary figure S1). Patient survival rates in other types of cancer, such as ovarian and lung are not affected by *p38δ* expression levels (Fig. 1).

Murine MMTV-PyMT recapitulates the profile expression of *p38δ* in breast cancer

Next, we employed immunohistochemistry staining for p38δ to evaluate its protein expression in human breast cancer tissue compared to non-cancer tissues in a human breast tissue array (US Biomax Inc). Normal tissues showed that p38δ was limited to the ductal epithelium (upper panels, Fig. 2A) with no other positive staining detected. However, invasive ductal carcinoma (IDC) sections showed significantly increased staining for p38δ (lower panels, Fig. 2A).

In order to evaluate the oncogenic role of p38δ during breast cancer progression, we tested whether p38δ protein was also increased in the murine breast cancer model MMTV-PyMT. This transgenic mouse develops spontaneous tumors in breast at about 6 weeks of age with metastasis in the lungs at around the 14^{th} week. Similar to the human tissues, enhanced p38δ protein staining was also observed in tumor regions (Fig. 2B). The *p38δ* mRNA expression was also carried out by qPCR analysis on tissue samples prepared from 14-week old mice. *p38δ* mRNA levels were increased in breast tumor and lung tissue metastases prepared from MMTV-PyMT mice compared to normal tissue from Wild Type (WT) mice (Fig. 2C). These data suggest that MMTV-PyMT breast cancer model mouse recapitulates the increase in *p38δ* seen in human breast cancer and can thus serve as a useful model to evaluate the function of p38δ.

Loss of *p38δ* reduces tumor volume and breast cancer cell proliferation in MMTV-PyMT

To investigate the effect of *p38δ* on breast tumor development and on the metastatic potential, $p38\delta^{-/-}$ mice were crossed with MMTV-PyMT mice (Fig. 3A). Breast tumor onset was evaluated by palpation and visual inspection starting at 4 weeks of age in both PyMT/$p38\delta^{+/+}$ and PyMT/$p38\delta^{-/-}$ mice. PyMT/$p38\delta^{-/-}$ mice showed a longer latency in tumor formation compared to PyMT/$p38\delta^{+/+}$ (Fig. 3B). At 10 and 14 weeks of age, mice were sacrificed and the tumor burden was quantified. Tumor volumes at 10 and 14 weeks in PyMT/$p38\delta^{-/-}$ were significantly smaller than PyMT/$p38\delta^{+/+}$ mice (Fig. 3C). The average weight of total mammary gland and percent of tumor burden in PyMT/$p38\delta^{-/-}$ mice showed about 50% reduction compared with PyMT/$p38\delta^{+/+}$ mice (Fig. 3C).

Of note, tumors from PyMT control *and* $p38d^{-/-}$ did not show morphological nor pathological differences in tumor sections. Therefore, the difference in mass could be due to differences in cell proliferation rates. To evaluate whether this could be involved in generating smaller number of tumors and smaller tumor size, cell proliferation of mammary tumors was quantified by Ki67 staining in sections from PyMT/$p38\delta^{+/+}$and PyMT/$p38\delta^{-/-}$ mice at 10 weeks of age. The PyMT/$p38\delta^{-/-}$ mice showed lower Ki67 staining than PyMT/$p38\delta^{+/+}$ mice (Fig. 3D). As mentioned earlier, PyMT cancers follow a progressive sequence of markers during tumor progression. In that context, p38δ could have effects on cancer growth during one stage but not in another. To investigate whether the role of p38δ in tumor

growth involves early or late phases of PyMT cancer progression, mice at early (6 weeks), middle (10 weeks) and late (14 weeks) stages were analyzed for proliferation markers (cyclin D1) and the effect of targeting *p38δ* was evaluated. Based on the results depicted in Fig 3E (quantified in 3F) the effect of *p38δ* deletion on cancer growth was pronounced in early and intermediate stages, but not at late stages. These results disclose an *in vivo* effect of *p38δ* on breast cancer proliferation.

p38δ knockdown regulates cell growth in human breast cancer cells

Based on the previous results, *p38δ* regulates tumor size by regulating cell growth rate in tumor cells. To determine if the effect of *p38δ* on cell growth can be cell autonomous, we evaluated two well established breast cancer cell lines: MCF-7 and MDA-MB-23. First, we confirmed that these cell lines had higher levels of *p38δ* when compared to a non-tumorigenic cells. As shown in Figure 4A, MCF-7 and MDA-MB-231 exhibited higher *p38δ* expression compared with non-cancer cells MCF-10A.

To elucidate the effect of *p38δ* on cell proliferation in MCF-7 and MDA-MB-231, *p38δ* was knocked down by using siRNA technology. Two different sequences of *p38δ* siRNA were used to assure specificity. After 48h of siRNA transfection, knock down of *p38δ* was confirmed by immunoblotting (Fig. 4B). As shown in Figure 4C, both *p38δ* siRNAs significantly reduced cell proliferation in both cell lines starting at 24 h after transfection (Fig. 4D). Together with the previous results, we concluded that high levels of *p38δ* positively regulated cell proliferation in breast cancer cells.

The loss of p*38δ* results in decrease in lung metastasis in the MMTV-PyMT mouse

Breast cancer becomes lethal in humans because of metastatic behavior. Since higher *p38δ* expression was shown to be associated with a poor outcome of breast cancer patients, we reasoned that it may not only result from its effect on cell growth, but also from the potential effect of p38δ on metastasis. With progression beyond 14 weeks, MMTV-PyMT mice develop lung metastasis (23). Therefore, to evaluate the effects of loss of *p38δ* on lung metastasis, the number and size of micrometastases in the lung were measured by microscopy (Fig. 5A). As depicted in Fig. 5B&C, the number and size of metastatic lesions were considerably reduced in PyMT/$p38\delta^{-/-}$ mice. Reduction in growth of the primary tumors was approximately 50%; whereas, the reduction in metastatic burden exceeded 90%. Together, these data strongly suggested that loss of *p38δ* not only effected growth of the primary tumor, but also strongly effected the metastatic ability of the tumors.

The effects of knockdown of *p38δ* in MDA-MB-231 on cell adhesion and motility

Metastatic cells require tumor detachment from the primary tumor, often losing cell-cell and cell-matrix contact, followed by migration through the basement membrane. One possibility could be that loss of *p38δ* could affect cell adhesion and/or cell migration. If loss of *p38δ* affects cell adhesion, then the key proteins in the cell adhesion pathway, such as focal adhesion kinase (FAK) should be altered. FAK is recruited on early focal adhesion sites (FA), where it is activated by phosphorylation (p-FAK) as a part of the maturation process of FAs. As shown in Fig 6., tumor breast sample from PyMT showed a dramatic decrease in p-FAK, which was partially recovered in PyMT/$p38\delta^{-/-}$ mice (quantified in Fig. 6B). To

further confirm that p-FAK levels are regulated by p38δ, we tested the effect of *p38δ* knockdown in metastatic breast cancer line MDA-MB-231 (Fig. 6C). The results showed that p-FAK was negatively regulated by p38δ in this cell line.

Based on these results, we hypothesized that the higher levels of p-FAK on PyMT/*p38δ*$^{-/-}$ could prevent the detachment of tumor cells from cancer tissue, reducing the chance of metastatic cells to escape to surrounding tissues. To investigate the effects on cell adhesion upon *p38δ* depletion, we carried out an adhesion assay using *p38δ* siRNA. Both sequences of *p38δ* siRNA resulted in faster attachment of cells to fibronectin compared to control siRNA-transfected cells (Fig. 7A). We also employed a cell detachment assay to evaluate whether loss of *p38δ* affects the attachment of cells. PMA pre-treatment can promote p-FAK dephosphorylation and cell detachment and increases invasiveness in MDA-MB-231 cell towards serum (22). We stimulated MDA-MB-231 cells with PMA to induce dell detachment form the extracellular matrix and evaluated the effect of loss of *p38δ*. As depicted in Fig 7B (quantified in 7C) loss of *p38δ* significantly protected cancer cells from detachment.

Since p38δ promoted cell detachment, we also evaluated the effects of p38δ on cell motility using wound-healing and invasion assays. Knockdown of *p38δ* in MDA-MB-231 cells decreased cell migration (Fig. 7E) and invasion (Fig 7D). Taken together, these results demonstrate important role of p38δ in cell migration, invasion, and increased number of metastatic lesions.

DISCUSSION

In this study, we identified p38δ MAPK as a key player in the regulation of both early tumor growth and development of subsequent metastasis in breast cancer. The data demonstrate that *p38δ* is overexpressed in breast cancer, resulting in a statistically significant correlation of *p38δ* with a poor outcome. This was recapitulated *in vivo* using the murine mammary tumor model, MMTV-PyMT. Moreover, knocking out *p38δ* resulted in inhibition of tumor growth and decreased number and size of metastatic lesions. A role for p38δ was defined in the regulation of cell growth using human breast cancer cell lines MCF7 and MDA-MB-231. Another role for p38δ was also defined in the regulation of adhesion and detachment of cancer cells, one of the early steps in metastatic dissemination. Taken together, these results define important and novel roles for p38δ in breast cancer and raise the possibility that p38δ may emerge as a novel therapeutic target.

The more impressive finding from this work was the identification of a role for p38δ in promoting breast cancer metastasis. Our data revealed a dramatic effect of *p38δ* deletion on the generation and size of metastatic lesions. At the cell level, knock down of *p38δ* resulted in significant effects on cell adhesion/detachment. FAK is a multi-functional regulator of proliferation, adhesion, migration, and invasion (24, 25). High levels of p-FAK have been linked to an increased number of distant metastasis. However, the relationship between p-FAK and cancer outcome is controversial. It has been reported that high tissue staining levels of p-FAK had better outcome than low levels in epithelial ovarian cancer (26). Here, we found that in breast cancer cells, down regulation of *p38δ* were associated with increased

level of p-FAK, decreased cell detachment and migration. PyMT/*p38δ*$^{-/-}$ recapitulated these relationships. PyMT/*p38δ*$^{+/+}$ mice presented elevated levels of *p38δ* and decreased levels of p-FAK; whereas, tumors from PyMT/*p38δ*$^{-/-}$ recovered p-FAK levels closer to that of non-tumor tissue, with fewer lung metastatic lesions. Contrary to this finding, elevated mRNA levels of *FAK* have been reported in cancer tissue (27) and the *FAK* conditional knockout mice bred with MMTV-PyMT mice suppressed tumor progression and metastasis (28–31). This is seemingly in opposition to our results. However, there are some possible explanations, FAK itself has many distinct functions (some of them independent of the phosphorylation site), and p38δ could regulate some of these functions to enhance metastasis. Moreover, FAK participates to assemble focal adhesions, and its phosphorylation status requires cycles of phosphorylation/dephosphorylaition (32). An elevated level of p-FAK could indicate a rapid turnover, which could be used by cancer cells to attach to new environments. However, for *p38δ*$^{-/-}$ tissues an elevated p-FAK cannot necessarily be translated to a migratory phenotype but to maintain tissue integrity. In *p38δ*$^{+/+}$ tumors, loss of p-FAK will lead to cell detachment that would facilitate cells to escape to the primary tumor and enhance tumor spread and metastasis. However, at this point, we cannot rule out other roles for p38δ that supersede its effects on FAK.

Another result to emerge from this study is the regulation of cell proliferation and tumor volume by p38δ in breast cancer At the cellular level, depletion of *p38δ* in MCF-7 and MDA-MB-231 cells resulted in a decreased cell growth. Interestingly, this regulation seemed to occur only in early tumor stage (6–10 weeks), but not in more advanced tumors (14 weeks) in the PyMT cancer model. In this regard, it is well appreciated that the PyMT model shows progressive transition towards more aggressive behavior including subsequent metastasis with time (33). Accordingly, MCF-7 mimics the earlier stages and MDA-MB-231 is more representative of the late stages of more aggressive tumors since MCF-7 is an ER+, PR+ cell line with low invasiveness phenotype; whereas, MDA-MD-231 is a triple negative, invasive, metastatic, *p53* and *KRas* mutant cell line (34).

Our studies show the important effects of p38δ *in vivo*, and cell studies recapitulate the majority of these effects. This suggests that p38δ may play key cell-autonomous roles in breast cancer. However, we cannot rule out additional roles for p38δ *in vivo* through effects on cytokines, tumor microenvironment, and circulating cells that can contribute to or influence tumor growth and metastasis (35, 36). Assessing the relative contribution of tumor versus host will require additional tissue-specific knock-outs.

The results from this study also carry therapeutic implications for p38δ as a novel target in breast cancer. Our data clearly demonstrate that p38δ is elevated in human breast cancer as well as in a murine model, and that its higher expression correlates with worse prognosis. Defining roles for p38δ in tumor growth and even more in metastasis suggests that it functions to promote tumor progression and could be a novel therapeutic target. Moreover, in the TCGA database, *p38δ* is also overexpressed in lung, liver, thyroid, ovarian and bladder cancer (data not shown). Consequently, p38δ may emerge as an important factor for many cancer types.

In summary, the present findings suggest that p38δ functions as a promoter of breast tumor progression and metastasis. We propose that p38δ as a potentially therapeutic target to delay breast cancer development and progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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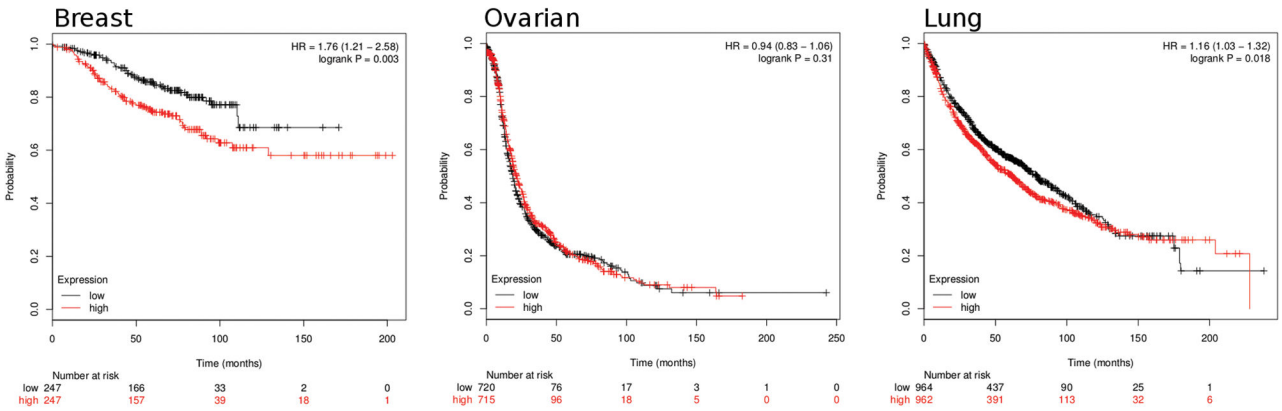

Figure 1. Kaplan-Meier Survival analysis based on p38 levels

Datasets from breast, ovarian and lung cancers were analyzed for survival outcome depending on high or low gene expression of p38δ (HR, hazard ratio). The log rank test was used for statistical analysis. Breast cancer showed significant differences on outcome, where ovarian and lung did not show differences or very moderate.

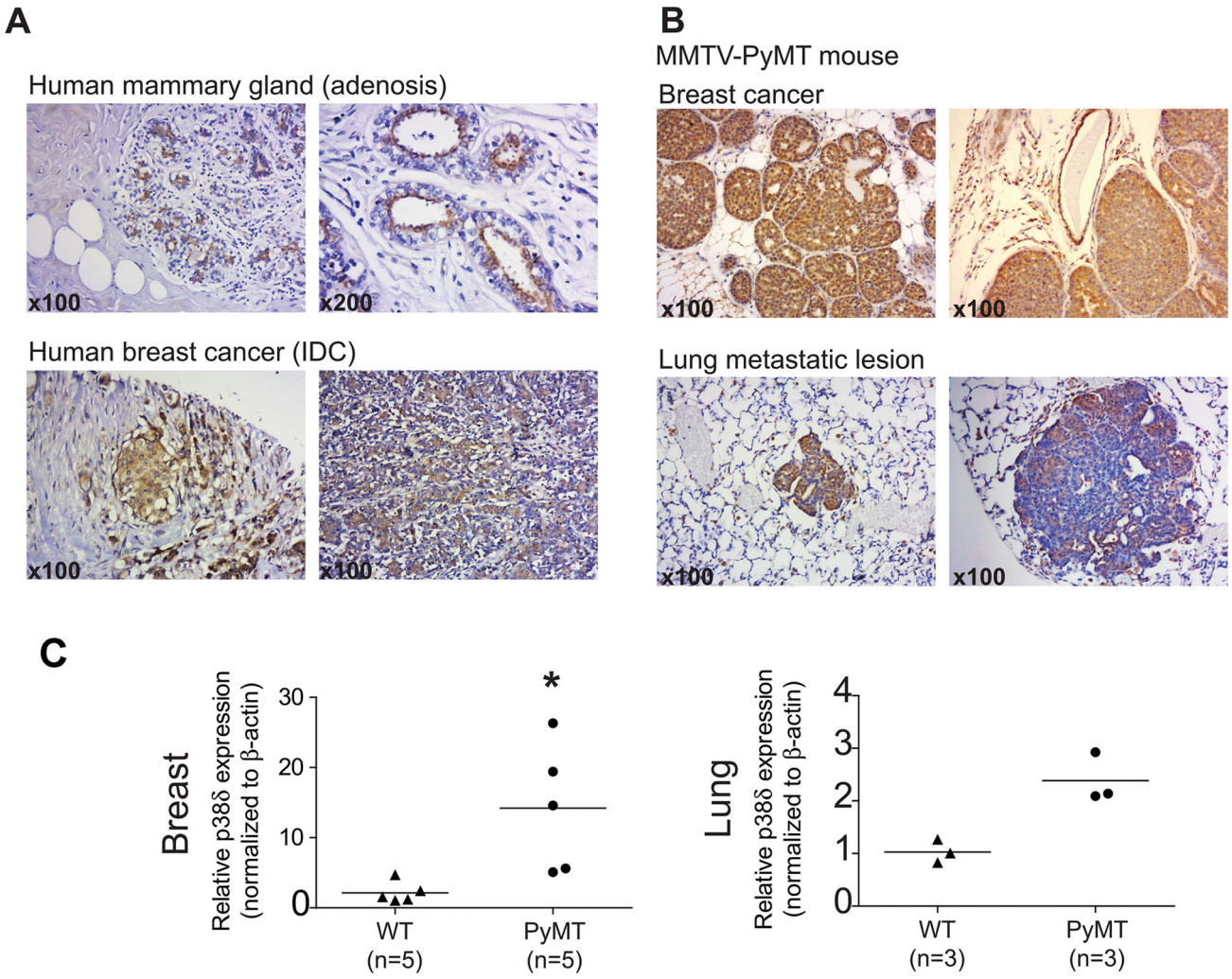

Figure 2. p38δ is highly expressed in human and MMTV-PyMT murine breast tumors

A, p38δ staining in normal mammary gland (upper left ×10 and upper right ×20 magnification), and in invasive ductal carcinoma (IDC): grade 1 (bottom left ×10) and IDC; grade 3 (bottom right ×10). Brown staining indicates p38δ expression. **B,** p38δ staining in MMTV-PyMT breast tumor (upper panels) and metastatic lung tumor colonies (bottom panels). Magnification, ×10. Brown staining indicates p38δ expression. **C,** p38δ mRNA expression in WT and MMTV-PyMT breast tumor (left panel) and lung tissues (right panel). Wild type mice and MMTV-PyMT mice were scarified at 14 weeks, and p38d mRNA was measured by qPCR. *P<0.05.

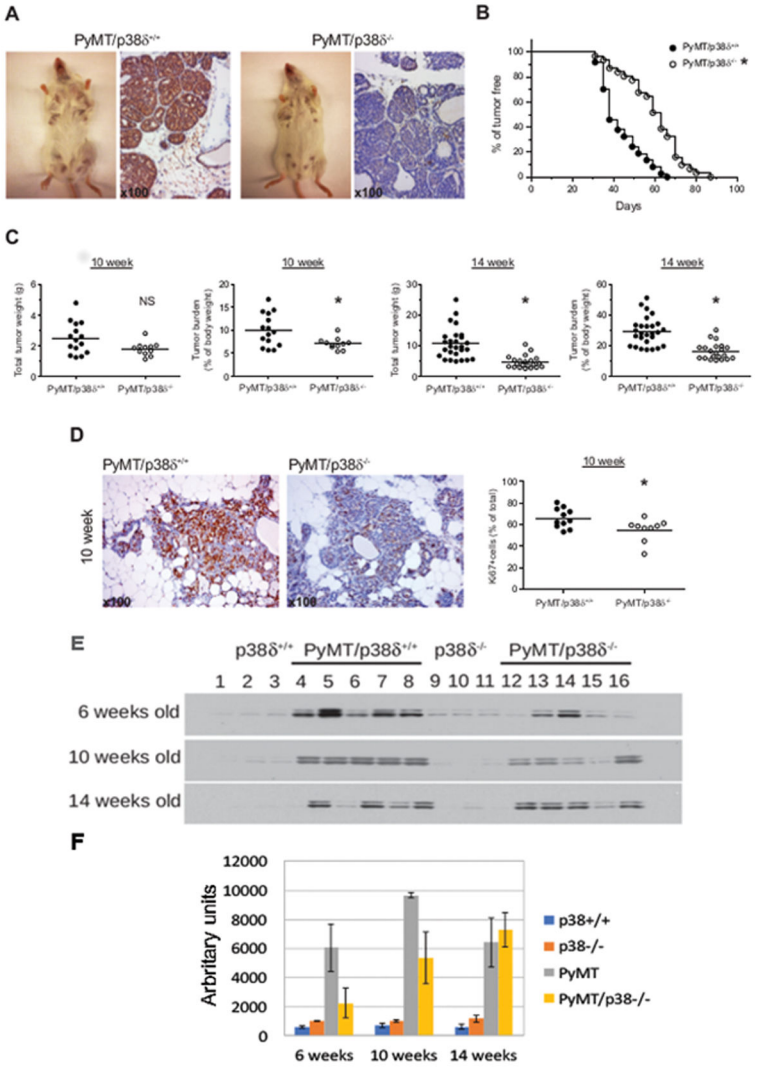

Figure 3. Loss of p38δ delays tumor growth in MMTV-PyMT mouse

A, appearance of breast tumor burden on PyMT/p38δ$^{+/+}$ and PyMT/p38δ$^{-/-}$ mice at 14 weeks old and p38δ IHC staining of tumor breast tissue. **B,** Kaplan-Meier tumor-free curve of the day at first detection of mammary tumor by bi-weekly palpation in each PyMT/p38δ$^{+/+}$ (n = 36) and PyMT/p38δ$^{-/-}$ (n = 31) mice. The data were analyzed by the log rank test. *, P < 0.05.. **C,** total tumor weight (g) and % of tumor burden per body weight of each mice at 10 weeks old of PyMT/p38δ$^{+/+}$ (n = 15) and PyMT/p38δ$^{-/-}$ (n = 11) mice and at 14 weeks old of PyMT/p38δ$^{+/+}$ (n = 27) and PyMT/p38δ$^{-/-}$ (n = 21) mice were measured at the time of euthanasia. *, P < 0.05. **D,** cell proliferation in primary tumors was analyzed by IHC using Ki-67 marker in PyMT/p38δ$^{+/+}$ (n = 11) and PyMT/p38δ$^{-/-}$ (n = 9) mice at 10 weeks old. Brown staining indicates Ki-67 positive cells. Ki67 staining data was analyzed by counting Ki67 positive cells divided by total number of cells in five fields per each tumor section by which randomly selected under blinded setting. Data are mean. *, P < 0.05. **E,** cell proliferation in primary tumors was also analyzed by cyclin D1 expression in p38δ$^{+/+}$,

PyMT/p38δ$^{+/+}$, p38δ$^{-/-}$ and PyMT/p38δ$^{+/+}$ at 6, 10 and 14 weeks old. **F,** Quantification of cyclin D1 on the different genotype groups.

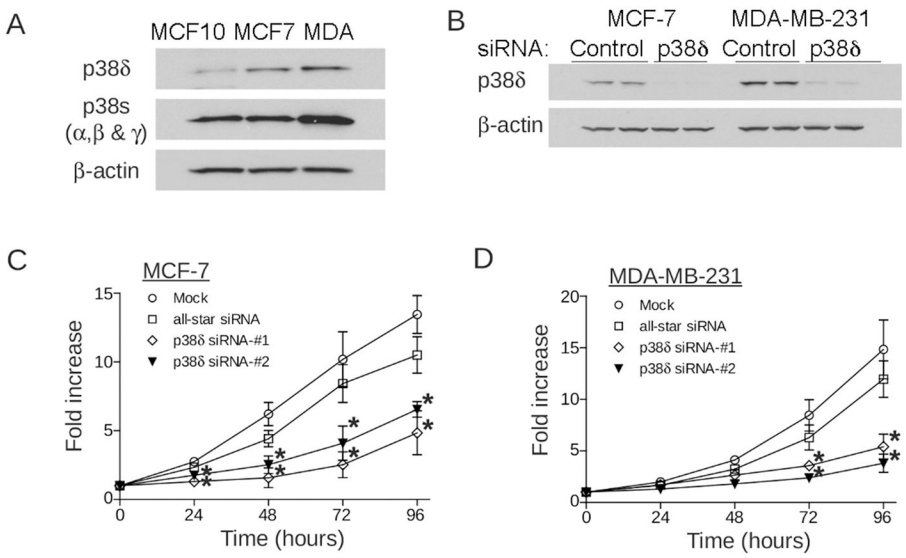

Figure 4. Loss of p38δ reduces cell proliferation in human breast cancer cell lines

A, p38δ was barely detected in MCF-10A, and higher levels were found in breast cancer cells MCF-7 and MDA-MB-231 cells. **B,** p38δ levels were depleted in MCF-7 and MDA-MB-231 after 48h of knock down: non-transfected cell (Mock), control siRNA, p38δ siRNA-#1 and p38δ siRNA-#2. **C,** Loss of p38δ significantly reduced cell proliferation as measured by MTT assay in MCF-7 cells ($n = 3$ independent experiments) and **D,** in MDA-MB-231 cells ($n = 3$ independent experiments). Data are mean ± S.E. *, $P < 0.05$ as comparison with all-star siRNA transfection.

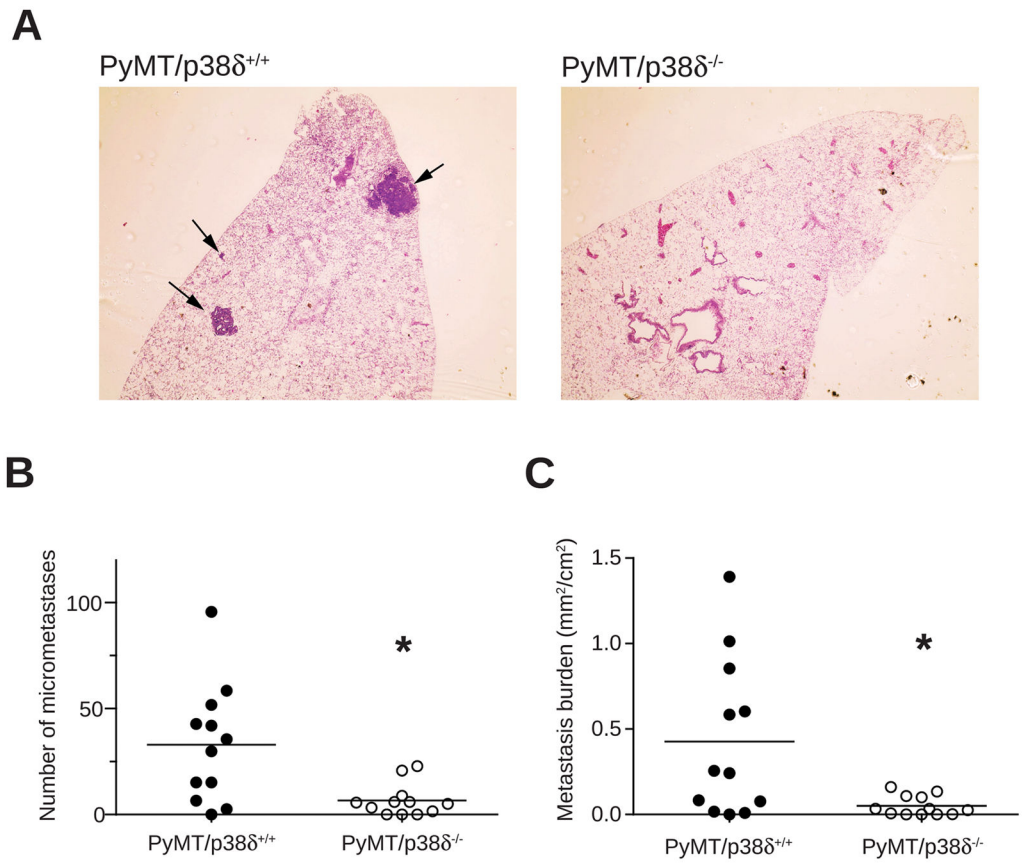

Figure 5. Loss of p38δ dramatically reduced the number of lung metastatic lesions

A, H&E staining of representative lung sections prepared from PyMT/p38δ$^{+/+}$ and PyMT/p38δ$^{-/-}$ mouse at 14 weeks old. Arrows indicate micrometastases. **B**, number of micrometastasis in lung sections (number of metastasis/cm^2) from PyMT/p38δ$^{+/+}$ ($n = 12$) and PyMT/p38δ$^{-/-}$ ($n = 12$) mice at 14 weeks old. **C,** total metastatic burden (mm^2/cm^2) in PyMT/p38δ$^{+/+}$ and PyMT/p38δ$^{-/-}$ mice. Data are mean. *, $P < 0.05$ as comparison between PyMT/p38δ$^{+/+}$ and PyMT/p38δ$^{-/-}$ mice group.

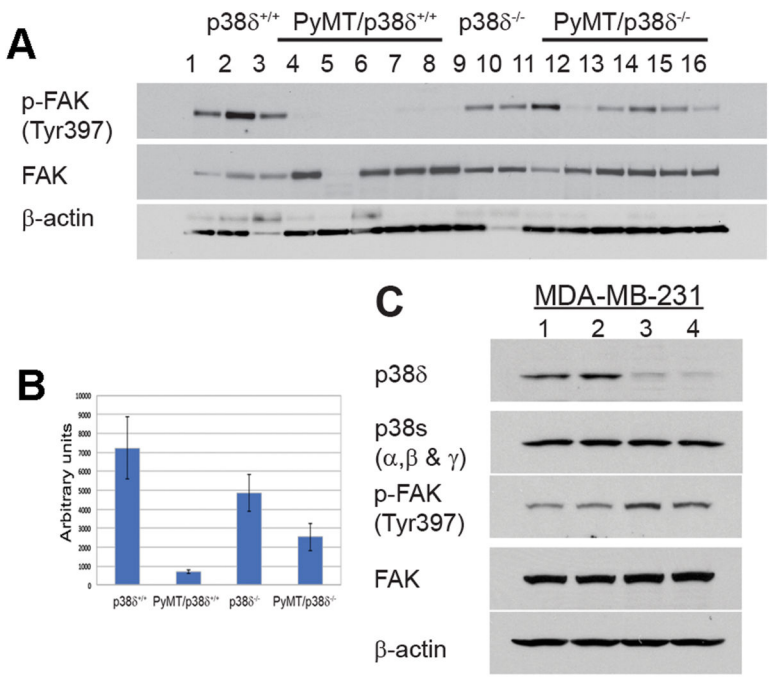

Figure 6. Loss of p38δ increases p-FAK signal

A, p-FAK expression in normal mammary gland and breast tumor homogenate supernatant at 14 week old mice. Normal mammary gland tissues were obtained from p38δ$^{+/+}$ and p38δ$^{-/-}$ mice. Breast tumor tissues were obtained from PyMT/p38δ$^{+/+}$ and PyMT/p38δ$^{-/-}$ mice. Each lane indicates a different animal.

B, p-FAK expression quantification of A using ImageJ software on genotype groups. **C,** p-FAK expression in MDA-MB-231 metastaic breast cancer cells upon knockdown using two different p38δ siRNA sequances. Total p38 isoforms was also shown to highlight specificity on the delta isoform.

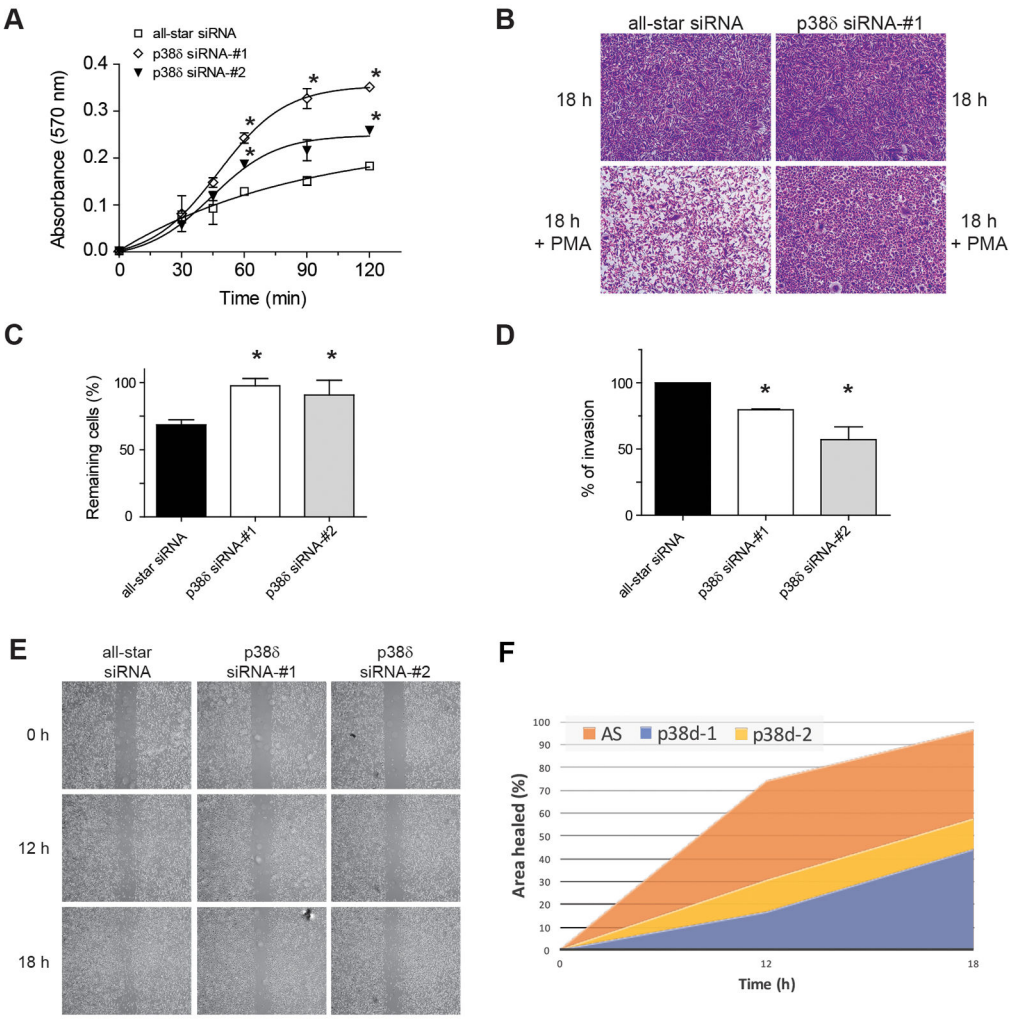

Figure 7. Loss of p38δ increases cell adhesion and reduces cell detachment and cell migration

A, Control and p38δ (p38δ-#1 and p38δ-#2) siRNAs transfected cells were plated on fibronectin coated plates. At different times unbound cells were thoroughly washed and the amount of attached cells were estimated by MTT ($n = 2$ independent experiments carried out in duplicate). Data are mean ± S.E. *, $p < 0.05$ as comparison with control (all-star) siRNA transfection. **B,** crystal violet staining in all-star or p38δ siRNA-#1 transfected MDA-MB-231 cells with or without PMA treatment for 18 h. **C,** Cells were lysed and crystal violet solubilized for quantification. Remaining adherent cells were expressed as percentage of PMA untreated cells ($n = 3$ independent experiments). Data are mean ± S.E. *, $p < 0.05$ as comparison with all-star siRNA transfection. **D.** Trans-well invasion assay of MDA-MB-231 cells. Loss of p38δ significantly decreased invasiveness. **E.** Scratch wound-healing assay. Loss of p38d also decreased cellular migration. **F.** Quantification of E using ImageJ software using the Wound-Healing macro tool.