The role of integrin α2 in basal-like cell positioning and collective cancer invasion

Internship report

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Abstract:

The primary cause of death in breast cancer cases is often metastasis, the spread of cancer cells systemically and the formation of secondary tumors in distant organs of the body. The invasion of breast tumor cells into the surrounding tissue is an initial step in the metastatic cascade. In addition to the various breast cells, the tissue constitutes of an intricate network of proteins and other elements known as the extracellular matrix (ECM). The breast cancer tumor cells can recognize and adhere to specific components of the ECM, with collagen I being the predominant element in the breast ECM. The typical mode of spread into the surrounding ECM is collective invasion, where breast cancer tumors advance as a cohesive multicellular group. This collective invasion is orchestrated by few cells known as the leader cells, possessing unique characteristics. Leader cells emerge from a specific subset of cells called basal-like cells, already present at the boundary between the tumor and the ECM before their transition into leaders. The reason in which these basal-like cells localize at the tumor-ECM interface remains elusive. In this study we uncover the role of collagen I and its receptor integrin alpha 2 (ITGα2) in the positioning of basal-like cells and their transitioning into leaders guiding the collective movement. We propose that ITGα2 regulates the interplay between the remodeling of the ECM and the paracrine signaling, whereby ITGα2 induces the expression of the TGF-β ligand Inhβa causing the formation of leader cells and initiating collective invasion in collagen I.

Keywords: Basal-like cells, ECM, ITGα2, collective invasion, TGF-β, Inhβa, Yap

Introduction:

Cancer invasion is defined as the migration of cancer cells into surrounding tissue. This is a fundamental step for the spread of cancer cells to distant organs, i.e. metastasize. Invasion is usually conceived as a single step process, however, only now it has become clear that most epithelial tumors, including breast cancers, invade collectively as a cohesive multicellular unit (Friedl & Gilmour, 2009).

In many breast cancers, cells that exhibit basal cells characteristics (e.g. expression of cytokeratin 14) localized at the tumor-Extra Cellular Matrix (ECM) interface, have an advantage in becoming leader cells of collective invasion (Cheung et al., 2013). To initiate and guide collective invasion, the leader cells extend polarized cytoskeletal protrusions to the front while maintaining cell-cell junctions with the follower cells (Cheung et al., 2013). The leaders resemble the elongated mesenchymal cells undergoing single cell migration such as the movement displayed by fibroblast during fibrosis. For the cells to become detached and migrate, they have to undergo a transition from non-motile epithelial cells into mobile mesenchymal cells through a process called Epithelial-Mesenchymal Transition (EMT) (Vilchez Mercedes et al., 2021). Nonetheless, despite the fact that the leader cells display mesenchymal features, they are considered to be in partial EMT as the leaders still maintain junctions with the follower cells to invade collectively (Pearson, 2019).

The transition of basal-like-cells into leaders rely on various extracellular stimuli. Biochemical and physical signals are sensed by the cells which induces transcriptional changes within the cell, regulating proliferation, survival, and invasion. The translation of the extracellular biochemical and physical signals into downstream signaling transducing pathways inducing cellular changes is termed mechano-transduction (Pruitt et al., 2014). In the cases of breast cancer, the tissue surrounding the tumor is characterized with increased fibrillar collagen I density and mechanical stiffness/tension (Conklin et al., 2011). In such a stiff and dense collagen I tissue, most invasion take place. These biophysical collagen signals are sensed by the cancer cells and are now known to induce leader cell function in basal-like cells. The formed leader cells do not only sense the ECM but also induces further modification of the ECM, and therefore creating a loop forming more leader cells directing the invasion. Whereas such bi-directional cell-ECM interactions are established to be supportive of the tumor progression. However, how mechanical changes in the ECM are sensed by cancer cells and which program is activated for leader cell formation remains unclear.
Yes-associated protein 1 (Yap) is an oncogene that is known in many cancer models to aid tumors with their proliferation, survival, and invasion (Fresques & LaBarge, 2020; Overholtzer et al., 2006; Quinn et al., 2021). When changes in collagen I tension are sensed by the cell, is activated and acts as a transcriptional co-activator. Piloting a transcriptional program that allows remodeling of the ECM. The newly modified ECM is further mechano-transduced by the cells leading to a positive feedback loop that allows persistent cancer tissue penetration. Mechano-sensing is thus essential in the invasive behavior of breast tumors. The predominant and most well studied fibrillar collagen I receptor is integrin-alpha2-beta1 (ITGα2β1) (Elango et al., 2022; Helfrich et al., 1996; Quail & Joyce, 2013). However, the role of ITGα2 in mediating collagen mechano-transduction during collective invasion and its link with Yap remains to be elucidated.

In addition to the extracellular matrix, paracrine factors play an important role in the formation of leader cells. One of the most well-established soluble factors that induce EMT and regulated single cell invasion is the transforming growth factor-beta (TGF-β) family. Moreover, besides TGF-β role in collective invasion, it is known to play an important part in cellular proliferation, differentiation, and survival of cells (Anzai et al., 2020; Binamé et al., 2008; Lee & Massagué, 2022).
The TGF-β pathway is activated through binding of the ligand to TGF-β type-I and type II receptors. This binding lead to the phosphorylation and conformational changes of the receptor. Furthermore,

phosphorylation of the downstream transcriptional factors Smad2/3. Smad2/3 associates with Smad4 and is transported into the nucleus (Xu et al., 2009). This is followed by transcriptional regulation of TGF-β target genes that include EMT marker to aid basal-like cells undergo their transition into leader cells. TGF-β signaling contributes to migration via the binding of the phosphorylated Smad3/4 to transcription factors like Snail and Slug. These are EMT markers that are responsible for the repression of tight junctions and E-cadherins (Vincent et al., 2009). In breast cancer, this pathway has to be activated in collagen.

Here, we used in vitro studies of the MMTV-PyMT breast cancer organoid model cultured in a three-dimensional Collagen I ECM to investigate the relationship between ITGα2, Yap, and TGF-β signaling in basal-like cell positioning, formation of leader cells, and collective invasion. The interaction between ITGα2 and Yap was studied by immunostaining of Yap and assessing its nuclear localization in breast cancer organoids expressing wildtype ITGα2 or a knock-out of this gene (CRISPR/Cas 9 gene deletion). This was followed by identifying the effect of ITGα2 KO on yap targets by qPCR. Furthermore, pharmacological TGF-β stimulators (Activin A) were used to observe the interplay that occurs between TGF-β signaling and the receptor ITGα2.
Lastly, to further investigate the factors affecting basal-like cell positioning, immunostaining of cytokeratin 14 (basal cell marker) was performed and positioning of basal-like cells was observed in ITGα2 Knock-out relative to the wildtype.

Materials and methods:

Antibodies and reagents

The following reagents were used during this study: Activin A (Cat#AF338, R&D Systems) and Cilengitide (Cat#HY16141/CS1211) suspended in water for Cilengitide and BSA for Activin A, respectively.
In immunofluorescence the following primary antibodies were used: rabbit anti-human Keratin 14 (Cat # 905301, BioLegend, 1:400), rat anti-mouse Keratin 8 (Cat# 531826, DSHB, 1:50), mouse anti-human YAP (Cat# 101199, Santa-Cruz, 1:100). Abcam). The secondary antibodies included Alexa fluor 488/568/647-conjugated goat anti-rabbit or rat (1:500), all obtained from Invitrogen. To visualize the nucleus and F-actin, 4',6-diamidino-2-phenylindole (DAPI, Cat#D9564, Sigma, 1:500) and Alexa-fluor-568-conjugated phalloidin (Thermo Fisher, 1:250) were used, respectively.

Organoid culture

MMTV PyMT breast cancer organoids were cultured in 20–25 µL drops of 3D basement membrane extracts (BME) (Cat# 3533-005-02, RGF type 2 Path Clear, Bio-Techne). Two types of media were utilized: either 3+ medium, comprising FCS-free culture medium with Advanced DMEM/F12 media (Cat# 12634010, Life Technologies supplemented with 1% glutamax Cat# 35050 038, Life Technologies)), 1% penicillin-streptomycin (P0781 100ML, Sigma Aldrich), and 1% HEPES (Cat# 11560496, Fisher Scientific), or culture medium, which is 3+ medium supplemented with 1X B27 (Cat# 17504001, Fisher Scientific), 1.25 mM N-acetyl cysteine (Cat# A9165 5G, Sigma Aldrich)), 2.5 nM bFGF2 (Cat# 100 18B Peprotech), and 100 µg/mL primocin (Cat# ant pm 2, BioConnect). Organoid cultures were maintained at 37 °C, with 5% CO2 in the culture medium. When passaging, the organoids were collected from BME drops by mechanical and enzymatic disruption of the gel, incubating with 200–600 µL trypsin EDTA (Cat# T3924, Sigma Aldrich) for approximately 20-30 seconds in a 37 °C water bath. Once the organoids were dissociated into single cells and small clusters, the organoids were gently resuspended by pipetting 10-20 times using a P10 tip (no filter) fitted on a P1000 tip. The suspended cells were then washed and centrifuged for 4 minutes at 1100 RPM. The pellets were suspended in BME and polymerized for at least 20-30 minutes before adding culture medium.

Invasion assays

Confluent- and medium-sized organoids, cultured in BME gels, were harvested by resuspension in cold media comprising DMEM/F12 supplemented with HEPES, Pen-Strep, and 1% glutamax. The organoids were centrifuged for 4 minutes at 260G (4°C), and the supernatant was then discarded. To break down the remaining BME, Dispase (10mg/ml, Cat#17105-041, Life Technology) was used, with a 15-minute incubation at 37°C. Following incubation, the organoids underwent thorough washing through four rounds. Subsequently, the organoids were embedded in 40 µL drops of Collagen I (2mg/ml). Non-pepsinized rat-tail Collagen (Cat#354236, Corning) was prepared at a final concentration of 2.0mg/ml. The Collagen I mixture was created using 10X phosphate buffer saline (PBS) (Gibco), NaOH (final pH of 7.5), and dH2O, following the manufacturer's instructions. Collagen I was pre-polymerized for 2 hours on ice. After pre-polymerization, organoids (25% v/v) were added to the Collagen and incubated for 5 minutes at room temperature before plating on pre-warmed plates (Costar) and incubating for 30 minutes at 37°C for polymerization. Subsequently, the desired growth medium was added, and plates were incubated for 3-5 days to allow organoids to invade the collagen I before further processing.

Brightfield imaging and quantification of invasion

Brightfield images of the organoids embedded in collagen I were taken using the EVOS M5000 microscope using the 2X and 10x objective (2x objective NA= 0.08, 10x objective, NA=0.4). Quantifications of the percentage of invasive organoids was done by manually counting the number of organoids using ImageJ. The criteria in selecting an invasive organoid were the presence of one or more protrusive strands extending from the organoid into the ECM. To obtain a percentage, the number of invasive organoids was divided by the number of the total organoids multiplied by a 100%.

Fixation, immunofluorescence, and confocal imaging

Organoids Embedded in collagen I were fixed using 4% paraformaldehyde, incubated for 15 mins at room temperature. This was followed by 3 washing steps using 1x PBS (Cat#D8537, Sigma). The fixed gels containing organoids were stored in 4°C until immunostaining was performed. When immunofluorescence staining was performed, the fixed gels were harvested and placed in a properly labelled Eppendorf tube. The gels were blocked using 10% normal goat serum (Gibco) with 0.3% Triton-X (Sigma-Aldrich) in 1X PBS and incubated for 60 minutes with gentle shaking at room temperature. Next, the primary antibodies were diluted in a solution containing contained 0.1% Bovine Serum Albumin (BSA; Sigma Life Sciences) with 0.3% Triton in 1X PBS. Incubation of the samples with the primary antibodies was done overnight while shaking at 4°C. Following that, the primary antibodies were removed, and the samples were washed for 4 rounds each lasting 10-15 minutes using 1X PBS. The secondary Alexa Fluor-conjugated antibodies were applied on the samples and incubated overnight with gentle shaking at 4°C. Finally, the samples were washed for 4 rounds each lasting 10-15 minutes using 1X PBS and the gels were imaged using confocal microscopy (LSM 880 Zeiss) with a 40X magnification objective (NA=1.1) on a glass bottom WillCo dish (WillCo Well BV).

Quantifications of confocal imaging

Single snap shots or z-stacks of the collagen I embedded organoids, and the midsections were selected for all the quantifications (basal-like cells positioning and Yap distribution). For the positioning of basal-like cells in the ITGα2 knock-out organoids, the number of K14-positive cells were counted manually using ImageJ. To obtain a percentage of K14-positive cells located at the organoid-ECM border, the number of K14-positive cells at the rim was divided by the total number of K14-positive cells present within the whole organoid and multiplied by 100%.
Since protrusive cells are only seen in WT organoids and not in Intga2 KO organoids, our analysis focused specifically on non-invasive K14-positive cells which are positioned at the organoid-ECM interface in both WT and KO organoids. This selection ensures a like-for-like comparison, allowing for accurate assessment and interpretation of the observed differences between WT and ITGα2 KO organoids. Dead cells (condensed/fragmented nuclei, based on the DAPI channel) and cells whose nuclei are out of focus nuclei were excluded during this analysis. Using ImageJ, contours surrounding the nucleus and the cytosol (excluding the nucleus) were drawn manually. The mean gray values (intensity) of the nuclear and cytosolic levels of yap were measured. The values obtained were corrected by subtracting the background signals from the average of two regions per sample. The corrected intensity values were used to calculate a ratio by dividing nuclear over cytosolic yap levels of the different cells.

Real-time PCR (qPCR)

To isolate RNA from the collagen I gels containing the organoids, the gels were collected and lysed using the reagent TRIzol™ (Thermo fisher). The isolation of RNA then was continued by utilizing NucleoSpin® RNA kit (Cat#740955.50, Macherey-Nagel). The conversion of the extracted RNA to cDNA was carried out by using the iScript™ cDNA Synthesis Kit (Cat#1708891, Bio-Rad) (500-1000 ng in 20ul of the reaction volume). The qPCR reaction mixture consisted of 10-100X diluted cDNA, 0.4uM forward and reverse primers, and 1X FastStart Universal SYBR Green Master mix (Cat#4913850001, Roche), making a final volume of 15ul.
The qPCR program used for the amplification and detection of the genes of interest was, the denaturation phase: 10 minutes at 95°C followed by a repetition of 40× cycles of 10 seconds at 95°C. The annealing phase: 10 seconds at 55°C, and an elongation phase of 30 seconds at 72°C. finally, the melt peak program used for detecting primer amplification was set at 95°C for 10 seconds.
The relative fold change was analyzed by calculating the ΔCq and using the value obtained in the equation $2^{\Delta Cq}$ to obtain the ΔΔCq. Moreover, to obtain a more reliable comparison of the genes between the different conditions, the GOIs were normalized using four housekeeping genes (HNRNPA, Tuba1, Cyca, PBGD).

Table 1: list of the genes of interest their primer sequences

The gene of interest	Forward primer sequence	Reverse primer sequence
HNRNPA	TGACAGCTATAACAACGGAG	AAAGTTTCCTCCCTTCATCG
Tuba1	AGGATTATGAGGAGGTTGGT	ATAAACATCCCTGTGGAAGC
Cyca	ACTGAATGGCTGGATGGCAA	CAAAACGCTCCATGGCTTCC
PBGD	GCCTACCATACTACCTCCTGGCT	AAGACAACAGCATCACAAGGGTT
Yap1	CCTCGTTTTGCCATGAACCAG	GCTGCTGCTGGTTGGAACTG
Ctgf	TGCGAAGCTGACCTGGAGGAAA	CCGCAGAACTTAGCCCTGTATG
Cyr61	GAAAGGGATCTGCAGAGCTCAG	GCACTGGTGTTTACAGTTGG
Ankrd1	GCTGGTAACAGGCAAAAAGAAC	CCTCTCGCAGTTTCTCGCT
Areg	GGTGGTGACATGCAATTGTC	GCAATCTTGGATAGGTCCTTG
ITGα2	GGAGGCTTTCTAACCTGTGGTC	CCTGAACTGCAGGTGAAAAGC
Smad7	GGGCTTTCAGATTCCCAACTTC	CCATTCCCCTGAGGTAGATCAT
Vimentin	GCACGATGAAGAGATCCAGGA	CATACTGCTGGCGCACATCAC
Inhβa	GGAGATAGAGGACGACATTGGC	ACGCTCCACTACTGACAGGTCA
Snail1	GCGTGTGTGGAGTTCACCTT	GGTACCAGGAGAGAGTCCCA
Slug	CCAAGAAGCCCAACTACAGC	TGGTATGACAGGTATAGGGTAACT

Statistical analysis

Graph pad prism version 9.0 was used for the statistical analysis of the obtained data. Statistical analyses were done only when the experiments were performed three or more times. The qPCR data were tested for significant differences by student t-test after confirming normal distribution by D'Agostino and Pearson omnibus. Differences in P-values less than 0.05 were considered statistically significant.

Results

Yap activity in breast cancer organoids is induced by ITGα2

In this study, in order to replicate collective invasion in vitro, we make use of breast cancer organoids derived from mammary tumors in MMTV-PyMT mice (Christenson et al., 2017). This model replicates various aspects of invasion, tumor growth, and metastasis of invasive ductal carcinoma (IDC). In this research we frequently embedded MMTV-PyMT organoids in 3D collagen I gels or in Basement Membrane Extract (BME). The rationale behind embedding organoids in collagen I is, mimicking the ECM matrix of the breast in which tumors migrate in. Whereas BME mimics the basement membranes and consist mainly of Collagen IV and laminin I and lacks collagen I.
In BME gels, the MMTV-PyMT organoids show a non-invasive, sphere-like phenotype (Kleinman & Martin, 2005). In contrast to BME, when embedding the MMTV-PyMT in 3D collagen I ECM, the organoids display a mesenchymal, finger-like multicellular strands and become highly invasive. This signifies the importance of the collagen I in initiating collective invasion (Nguyen-Ngoc, 2012). So, in order to test whether collagen I-induced invasion depends on ITGα2, the major collagen I receptor, we used MMTV-PyMT organoids with either a wild type or Crispr/Cas9 deleted ITGα2 (KO) gene.

MMTV-PyMT wildtype organoids and ITGα2 KO organoids were seeded in collagen I gels for 1, 3, and 10 days (Figure 1a). The wildtype organoids embedded in collagen I gels, display an invasive phenotype that is characterized by the multicellular invasive strands guided by one or a few extended cytoskeletal protrusions to the front (mesenchymal like) (Figure 1a, arrowheads). Moreover, the percentage of invasion in the wildtype starts at an 88% on day 1 and progresses to 100% as day 10 is reached (Figure 1b). These strands, viewed through the brightfield imaging, elongate, and increase in number throughout the progression of time (Figure 1a, wildtype, arrowheads). On the other hand, the ITGα2 KO organoids remain spherical and non-invasive (Figure 1b). However, approximately 14% of the Day 10 KO organoids become invasive and develop short protrusions (Figure 1a, ITGα2 KO, arrowheads). These results suggest that ITGα2 is necessary for cells to become protrusive and drive an efficient collective invasion in a 3D collagen environment.

It is known that Yap is active in basal-like cells and is a factor driving the transition into leader cells the initiation of collective invasion (Khalil et al., 2024). However, the collagen mechano-sensor that is upstream and recognizes the changes in the surrounding ECM, sending signals to activate Yap, is yet to be identified. In order to investigate whether Yap is regulated and is downstream of the collagen I receptor ITGα2, immunostaining of nuclear yap of the wildtype and KO organoids was carried out. This was followed by analysis of Yap localization by confocal microscopy (Figure 1c).
Yap, when activated, translocates into the nucleus to act as a transcriptional co-activator, the measurement of nuclear Yap intensity in comparison to cytosolic intensity levels in the KO relative to the wildtype would give an indication to the link between Yap and ITGα2.
We investigated the difference in nuclear Yap localization in cytokeratin 14-positive cells located at the organoid rim in WT and ITGα2 KO organoids. Looking at the nuclear levels of Yap in comparison to the cytosolic levels in the wildtype (Figure 1c, Yap insert, wildtype), it appears that the nucleus has a notable increase relative to the cytosol. Which can be further appreciated with the nuclear/cytosolic Yap ratio with a median of 1.1 (Figure 1d). However, the ITGα2 KO organoid displayed in (Figure 1c, Yap insert, ITGα2 KO), show a darker nucleus in contrast to the cytosol as well as to the wildtype. The ITGα2 KO K14-positive basal-like cells at the rim showed a median nuclear/cytosolic ratio of 0.9 (Figure 1d). These results indicate that ITGα2 is important in the regulation of yap localization and therefore its activity.

Next, we tested for expression of known Yap target genes. In the ITGα2 KO MMTV-PyMT organoids, we found a significant downregulation of the genes Ctgf, Yap1, Inhβa relative to the wildtype mRNA

levels (2-fold decrease, Figure 1e). Nevertheless, the Yap targets Cyr61, Ankrd1, and Areg show a significant upregulation in the ITGα2 KO (Figure 1f). This suggests that the collagen I sensor ITGα2 is required for Yap mediated transcription for at least for some of the Yap target genes tested (incl. Inhβa). Since, inhβa is a TGF-β ligand, the downregulation of its mRNA levels indicates that ITGα2 may play an important role in regulating TGF-β signaling.

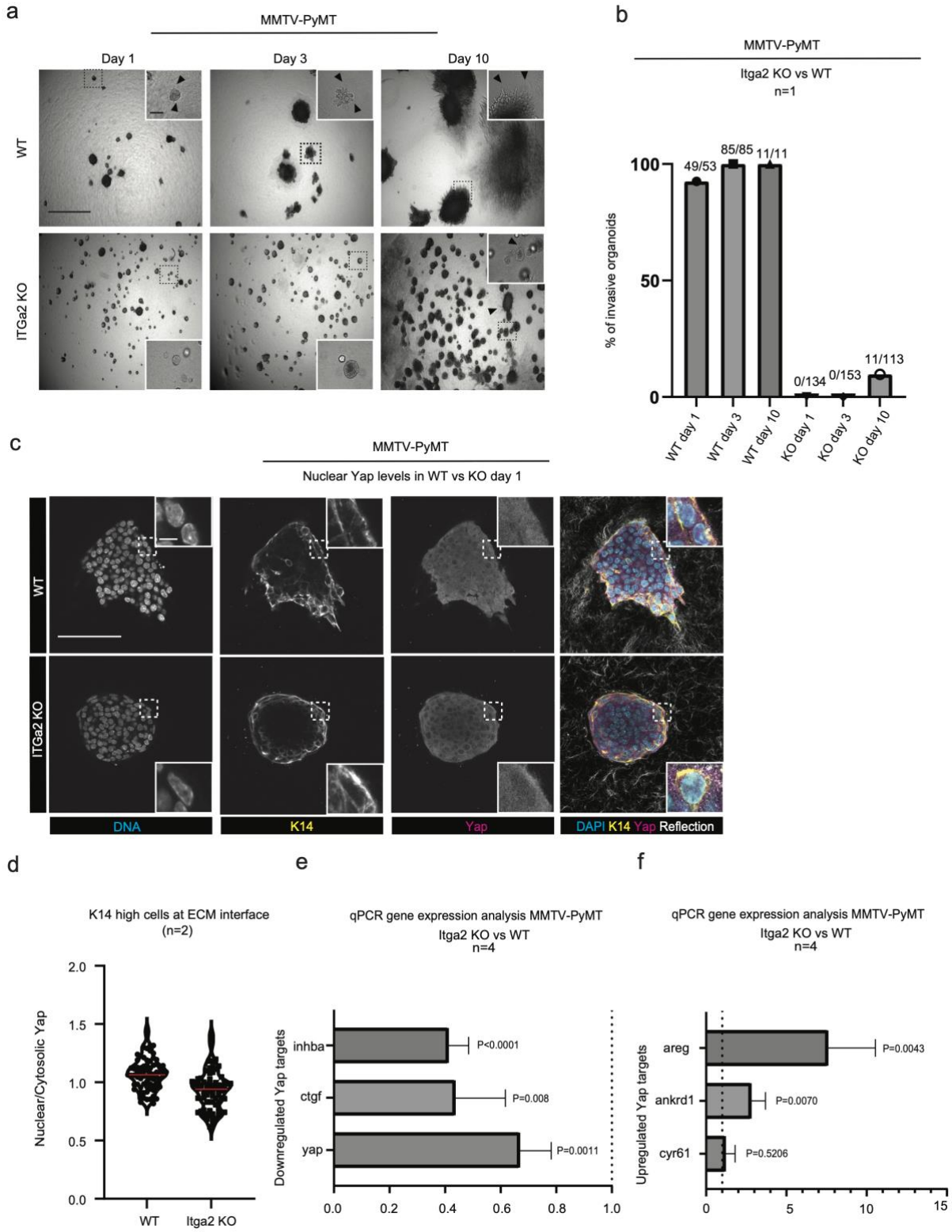


Figure 1. Yap localization and its coactivator activity is induced by ITGα2. (a) Brightfield images displaying the progression of collective invasion in day 1,3, and 10 in the wildtype and ITGα2 KO

organoids, scale bars 1000µm, 400µm for inserts. Wildtype arrowheads: elongation of the protrusive strands. ITGα2 KO arrowheads: the rescue of invasion in some of the organoids is displayed by the reformation of short and inefficient strands. **(b)** Quantifications of the percentage of invasion in the wildtype and ITGα2 KO on days 1,3, and 10, displayed as a bar graph n=1. **(c)** Representative confocal images of Yap and K14 in MMTV-PyMT wildtype and KO organoids cultured in collagen for 1 day. Zoom ins represent K14 positive cells located at the organoid-ECM interface. Scale bar 25µm and 10µm for inserts. **(d)** Analysis of the nuclear/cytosolic Yap ratio in the ITGα2 KO relative to the wildtype displayed as a volcano plot. The wildtype is composed of 73 data points whereas the ITGα2 KO is composed of 69, n=2. **(e,f)** mRNA fold change levels of up- and downregulated Yap target genes in ITGα2 KO all relative to the wildtype, n=4. P-values: Yap= 0.0011, Ctgf= 0.008, Inhβa<0.001, Areg= 0.0043, Ankrd1= 0.007, Cyr61= 0.5206.

ITGα2 induces EMT characteristics through promoting TGF-β signaling

Since the TGF-β receptor ligand Inhβa mRNA levels were significantly down-regulated in response to ITGα2 KO, we hypothesized that ITGα2 may play a crucial role in the regulation of TGF-β signaling. To test this, we examined the expression of known TGF-β targets, Vimentin and Smad7 (Luo et al., 2014 ;Zhang et al., 2007), in the ITGα2 KO MMTV-PyMT organoids relative to the wildtype. Interestingly, Vimentin was 2-fold down-regulated (Figure 2a). This is an indication of the importance of ITGα2 in regulating TGF-β targets. Unlike the downregulation of vimentin, Smad 7, a known TGF-β target did not exhibit a significant down-regulation and seemed to be unaffected from the absence of ITGα2 (Figure 2a). Moreover, after multiple measurements, a lot of variation in the mRNA levels of smad7 took place, however, the difference remains insignificant. The results obtained give an indication on the importance of ITGα2 in regulating some TGF-β transcriptional targets.

What yet remains to be fully understood is the manner in which ITGα2 initiates and regulates the TGF-β cascade. Based on the results shown previously (Figure 1e), the downregulation of the ligand Inhβa suggest that the link between ITGα2 and TGF-β signaling and its targets, in collective invasion, is through promoting the expression of Inhβa. To further investigate this hypothesis, we treated the MMTV-PyMT wildtype and ITGα2 KO organoids embedded in collagen I ECM for three days with Activin A. Activin A comprises of the homodimer Inhβa, when secreted, binds to the TGF-β receptor and initiates the activation of the downstream signaling cascade. This was performed to observe the effect that Activin A has on collective invasion in addition to its effect on the TGF-β related genes. By brightfield microscopy, we observed that when treating the wildtype collagen cultures with Activin A, the invasive phenotype increased when compared to the BSA control (solvent) treatment (Figure 2b, wildtype, arrowheads). However, in the ITGα2 KO organoids, Activin A seem to have no phenotypical effect on the KO between the treatments, i.e. Activin A vs BSA after 3 days of culture in collagen I (Figure 2b, ITGα2 KO, arrowheads). These results show that Activin A treatment promote the development of invasive protrusions. To further investigate whether Activin A induces TGFb signaling and whether this is dependent on Itga2 expression, we conducted real-time PCR to assess TGF-β-related gene expression under various conditions: wildtype Activin-treated, KO Activin and BSA-treated (control), all compared to the wildtype BSA control (Figure 2c).
The mRNA levels in wildtype Activin treatment show an up-regulation of most TGF-β-related genes, except for Inhβa and vimentin (Figure 3c). Consistent with Figure 1e, the KO control treatment (BSA) exhibits a significant down-regulation, approximately 2-fold, in Inhβa and Ctgf. Notably, the genes Smad7 and Snail1 (EMT marker) also appear down-regulated (1.5-fold) compared to the wildtype BSA control. Despite vimentin's lower mRNA expression in Figure 1e, it seems unaffected in the KO, possibly influenced by BSA treatment. Similarly, the EMT marker remains unchanged after knocking out ITGα2 (Figure 2c).

Adding Activin A to KO organoids results in an upregulation of mRNA expression levels in all TGF-β-related genes, excluding Inhβa, which remains unaffected (Figure 3c). This suggests that ITGα2 regulates and interacts with the TGF-β pathway and partial EMT through the expression of its ligand Inhβa.

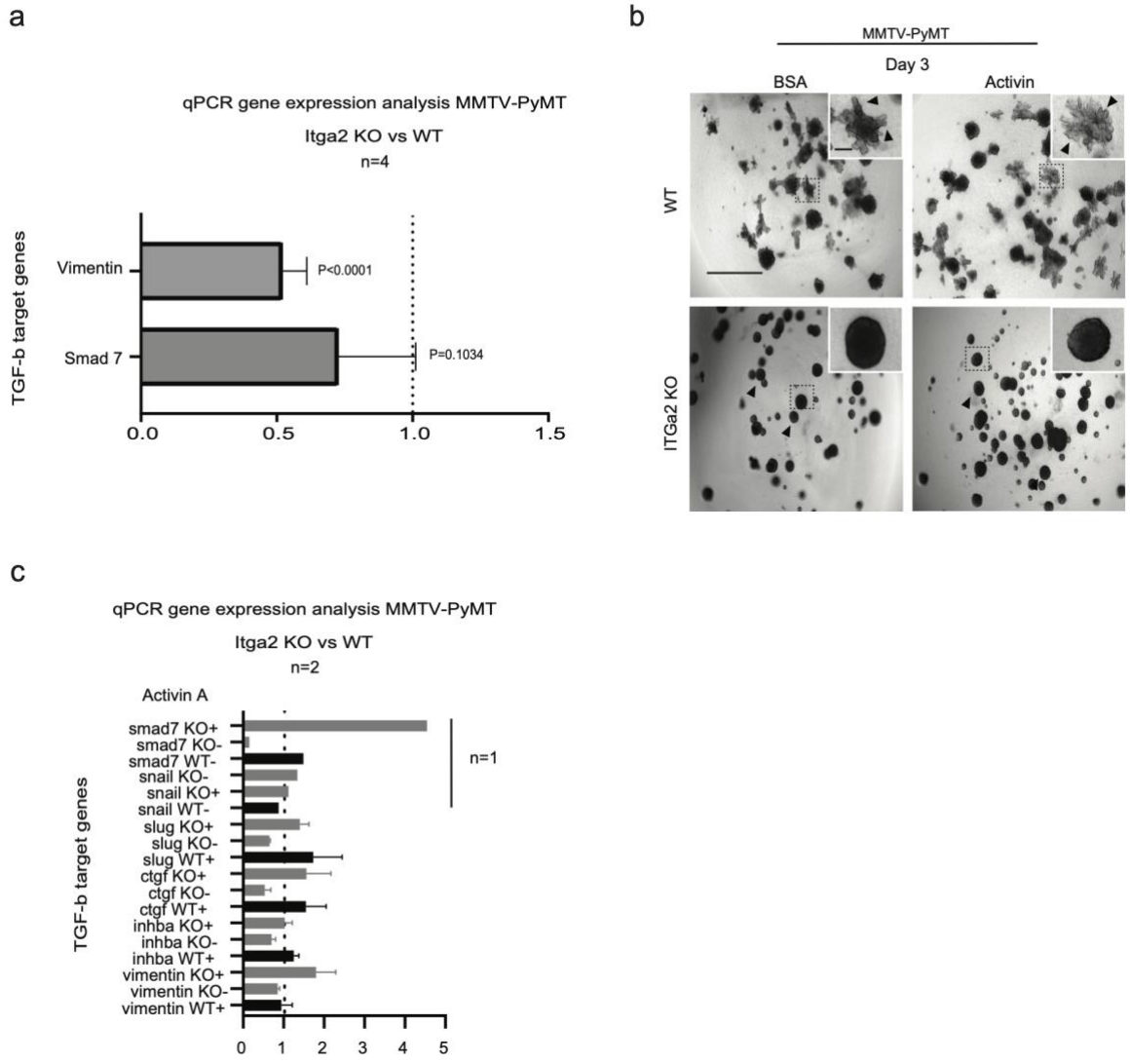


Figure 2. ITGα2 induces TGF-β through the expression of its ligand inhβa. (a) mRNA fold change levels of the TGF-β target genes smad7 and vimentin in ITGα2 KO all relative to the wildtype, n=4. P-values: Smad7=0.1034, Vimentin<0.0001. **(b)** Representative brightfield images of the MMTV-PYMT WT and ITGα2 KO organoids treated with 20ng/µl Activin A ligand or an equal volume of 0.1% BSA in PBS (control), grown for three days in Collagen I, scale bar 1000µm, inserts 400µm. Wildtype arrowheads: the effect of Activin A treatment on the elongation of the strands. **(c)** mRNA fold change levels of the TGF-β related genes, n=2. The genes Smad7 and Snail1 were only performed once, n=1.

Preliminary trial of targeting ITGαv and ITGα5 in ITGα2 KO breast cancer organoids

Previous data in our group has shown that the basal-like-cells (cells that have an advantage of becoming leader cells), lose their ability to position at the organoid-ECM interface when deprived of the collagen I receptor ITGα2. The basal-like cells become randomly distributed within the organoid body. This implies that when basal-like-cells are deprived from sensing the surrounding matrix, the positioning in addition to the invasive phenotype are lost. However, aforementioned (Figure 1a), the invasive property of ITGα2 KO organoids in some of the organoids is recovered after 10 days. This must be accompanied with the relocation of basal-like-cells to the organoid-ECM rim.

We still do not know why this relocation happens. We hypothesize that the secretion and accumulation of other ECM components such as but not limited to fibronectin and vitronectin, that can be recognized by their specific integrins (ITGαv and ITGα5), is a factor aiding the cells in rescuing invasion(Akiyama, 1996). A substantial upregulation in the gene encoding for fibronectin is shown through previously performed single-cell mRNA sequencing of the MMTV-PyMT organoids (Khalil et al., 2024). This is the rationale behind targeting the receptors ITGαv and ITGα5.
To investigate this, we embedded the MMTV-PyMT wildtype and ITGα2 KO organoids in collagen I for 6 and 10 days and observed their invasive status. These organoids were treated with Cilengitide, an ITGαv and ITGα5 small molecule inhibitor.

When treating the wildtype MMTV-PyMT organoids with Cilengitide, on day 6, invasion strands (number and length) seem to be remarkably decreased compared to its solvent control BSA (Figure 3a). By day 10, the difference in invasion between the treatment and water control becomes less obvious. This aspect becomes more evident in Figure 3b, where the invasion percentage remains unaffected when applying the Cilengitide inhibitor to the wildtype organoids. In ITGα2 KO organoids, on day 6, Cilengitide treatment seem to have no effect on invasion relative to the water control. Nonetheless, on day 10, Cilengitide treatment appear to have a decreasing effect on the reformation of the short and inefficient invasive strands (Figure 3a). This can be further appreciated when observing the 2% decrease in invasion obtained from the brightfield analysis (Figure 3c). These findings indicate that the ECM receptors ITGαv and ITGα5 is influencing the ability of some ITGα2 KO organoids to restore their invasive phenotype.
To restore the ability of ITGα2 KO organoids to protrude and invade, basal-like cells must move to the interface between organoids and the extracellular matrix (ECM). Since Cilengitide affects the rescue of invasion in the KO (Figure 3a, 3c), we wondered if inhibiting ITGαv and ITGα5 could influence basal-like cell positioning. For that, we immunostained K14 in different KO conditions at day 6 and day 10, followed by confocal imaging. Analyzing the distribution of K14-positive cells in day 6 KO organoids, Cilengitide disrupted the relocation of basal-like cells to the organoid rim by approximately 20% compared to the water control (Figure 3d). Similarly, at day 10, basal-like cells treated with the inhibitor still haven't fully relocated to the rim and we found that there is a reduction of 20% of these basal-like cells that are located at the organoid-ECM rim when treated with Cilengitide. These findings indicate that the repositioning of basal-like cells to the rim is influenced by inhibiting the receptors ITGαv and ITGα5, thereby restricting their capacity to start collective invasion and formation of strands.

Figure 3

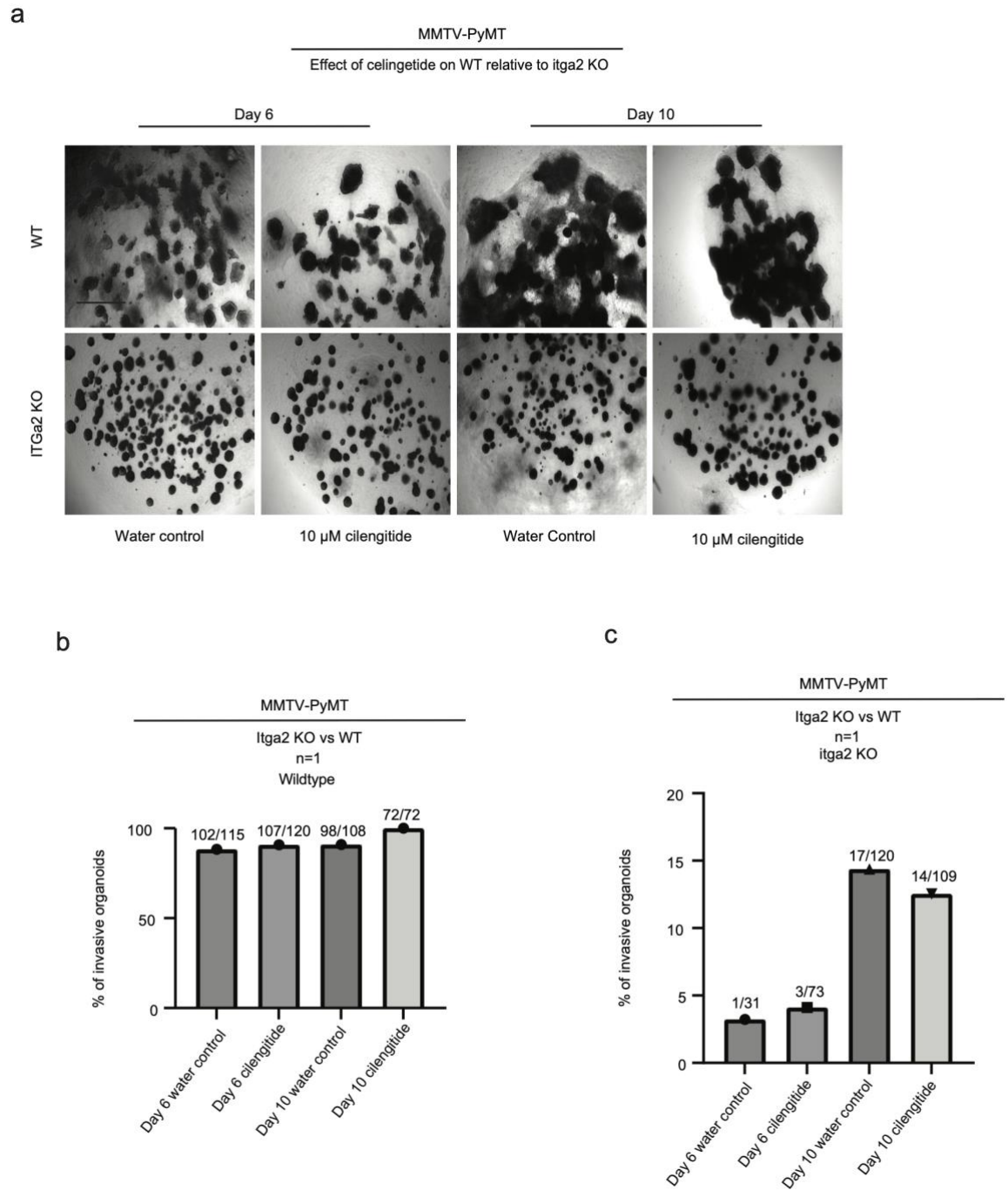
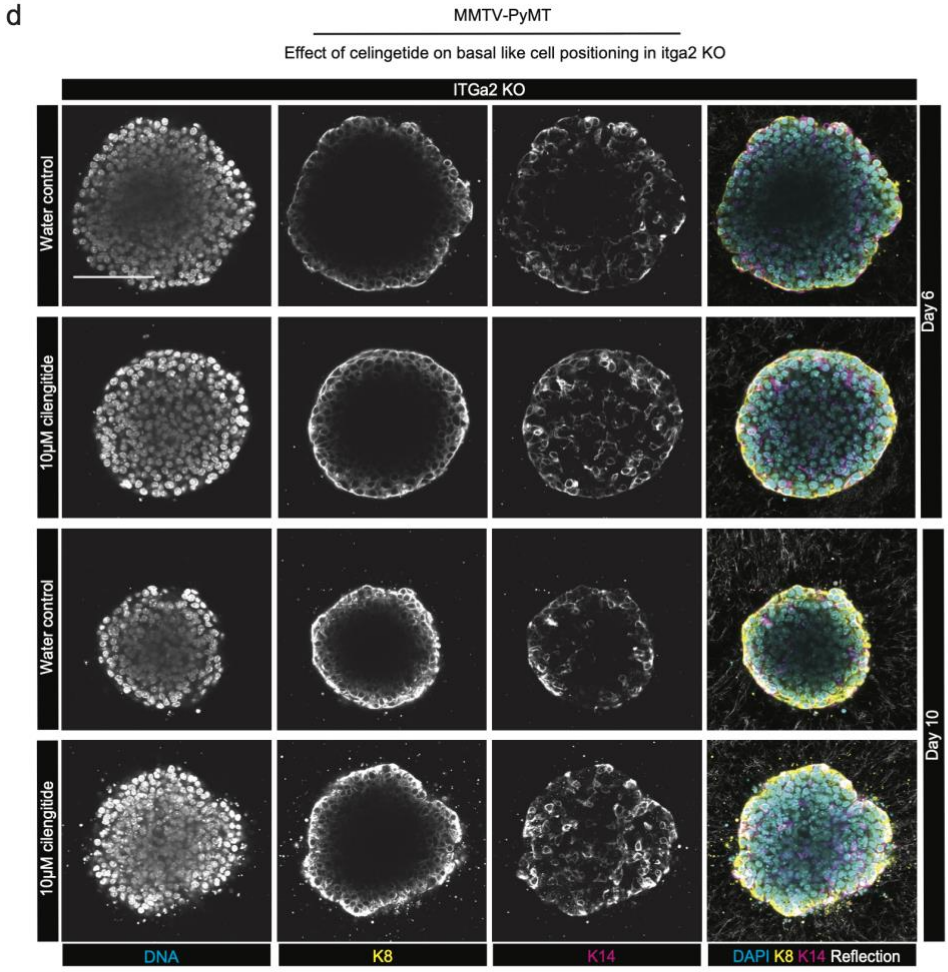

a
MMTV-PyMT
Effect of celingetide on WT relative to itga2 KO
Day 6
Day 10
WT
ITGa2 KO
Water control
10 µM cilengitide
Water Control
10 µM cilengitide
b
MMTV-PyMT
Itga2 KO vs WT
n=1
Wildtype
% of invasive organoids
100
50
0
102/115
107/120
98/108
72/72
Day 6 water control
Day 6 cilengitide
Day 10 water control
Day 10 cilengitide
c
MMTV-PyMT
Itga2 KO vs WT
n=1
itga2 KO
% of invasive organoids
20
15
10
5
0
1/31
3/73
17/120
14/109
Day 6 water control
Day 6 cilengitide
Day 10 water control
Day 10 cilengitide

Figure 3 continued



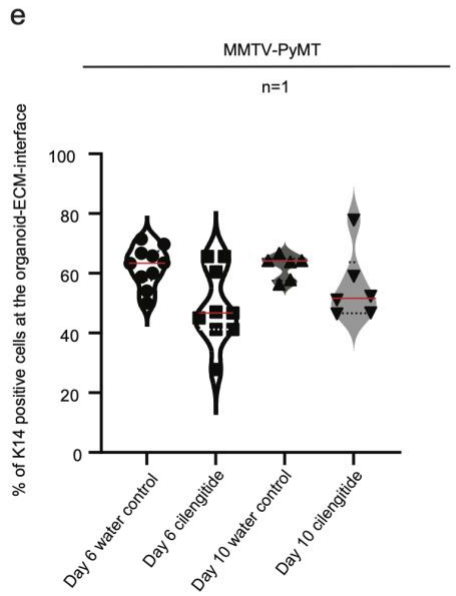


Figure 3. Targeting ITGαv and ITGα5 disrupts the positioning of basal-like cells rescue in ITGα2 KO organoids. (a) Representative brightfield images of targeting the MMTV-PyMT wildtype and ITGα2 with 10µM of the fibronectin receptor inhibitor Cilengitide, scale bar: 1000µm. **(b,c)** Quantifications of the percentage of invasive organoids embedded in collagen I for 6 and 10 days and treated with

Cilengitide relevant to the water control, displayed as a bar graph, n=1. **(d)** Confocal images of K8 and K14 in the MMTV-PyMT ITGα2 KO organoids cultured in collagen I for 6 and 10 days and treated with Cilengitide. Scale bar 25µm. (e) Analysis of the percentage of basal-like cells located at the organoid-ECM interface displayed as a volcano plot, n=1. The day 6 water control and treated are composed of 9 data points each. The day 10 water control and treated are composed of 6 data points each.

Discussion:

We reveal that the fibrillar collagen I receptor ITGα2 is required for basal-like cells to sense the surrounding ECM and attach to it, leading to the positioning of the basal-like cells to the tumor-ECM interface. Besides its role in basal-like cell positioning, we also show that ITGα2 is involved in the formation of leader cells to guide collective invasion. ITGα2 induces leader cell formation by sensing physical and biochemical changes in the surrounding collagen I. This is followed by a signaling pathway leading to the expression of the TGF-β ligand Inhβa. Exogeneous addition of Inhβa (Activin A) to the organoids enhances the TGF-β signaling pathway and leader cell formation through inducing mesenchymal characteristics in the cancer cells. Thus, bidirectional ECM interactions and paracrine signaling cooperate to drive collective invasion.

Basal-like cell positioning at the tumor-ECM interface is a preliminary step for these cells to transform into leader cells and to initiate collective cancer invasion. Previous studies suggest that basal-like cells are created at the ECM interface by luminal to basal transition as a response to ECM stimuli (Cheung et al., 2013; Vishwakarma et al., 2018). However, other studies show that preexisting basal-like cells it was observed that in hypoxia, the chemokine SDF1 plays a role in the positioning of leader cells in collective migration of organoids (Hwang et al., 2019). Here, we propose that the ability of the preexisting basal-like cells to sense the surrounding ECM and adhere to it, is a major reason for the cells to locate at the ECM interface. Previous work in our lab showed that when the MMTV-PyMT organoids were embedded in a non-adhesive ECM (hydrogels), the positioning of basal-like cells at the organoid-ECM rim is lost. Nevertheless, when these organoids are cultured in a three-dimensional collagen I, basal-like cells recognize this ECM and adhere to it, which allows for the positioning at the ECM interface. Integrin alpha 2 is the predominant fibrillar collagen I sensor, thus, we hypothesized that it may be the receptor mediating the interaction between the ECM and basal-like cells. Indeed, the loss of ITGα2 (KO by CRISPR/Cas 9 gene deletion), in 3D collagen I, causes the basal-like cells to be randomly distributed within the organoid body. However, this positioning is reestablished in the ITGα2 KO organoids when cultured in collagen I for 7 days. We speculated that the repositioning of basal-like cells may be due to their ability to secrete, recognize, and finally attach to the newly deposited ECM components. Previously performed single-cell mRNA sequencing show that there is a significant upregulation of the genes encoding for fibronectin (Khalil at al., 2024). ITGαv and ITGα5 are the most well-established fibronectin receptors (Akiyama, 1996). So, we targeted the receptors ITGαv and ITGα5 in MMTV-PyMT wildtype and ITGα2 KO using a small molecule inhibitor (Cilengitide) and found that indeed, when blocking the receptors ITGαv and ITGα5, the basal-like cells are not able to locate to the organoid-ECM interface, after 7 days of culturing. However, to investigate whether this loss of positioning in the KO organoids is consistent, more testing of the inhibitor should take place. Moreover, in order to further test whether other ECM components have an effect on basal-like cell positioning rescue and invasion, introducing fibronectin to the organoid collagen and assessment of basal cells positioning in wildtype and ITGα2 KO organoids should be carried out.

Now that we have established the importance of ITGα2 in the positioning of basal-like cells in collective invasion, the mechanisms in which it mediates collective movement is yet to be fully elucidated. To start collective invasion, the basal-like cells located at the tumor-ECM interface need to transition into leaders that initiate and guide the formation of invasive strands. ITGα2 plays a

crucial role in the adhesion and collective migration of endothelial cells during tumor angiogenesis (Naci et al., 2015). Integrins achieves this by the formation of stabilized actin-rich protrusions for cells to move (Chan et al., 2007). So, mediating the interaction between the surrounding tissue and the actin cytoskeleton, allowing the cells to migrate. Previous studies have shown that the blocking of ITGα2 through monoclonal antibodies causes a direct inhibition of melanoma cancer cell migration in vitro (Etoh et al., 1993). Similarly, our findings show that the KO of ITGα2 in the MMTV-PyMT organoids causes a full inhibition of collective invasion. However, after a prolonged time (10 days) short invasive strands start to emerge from the organoid body, indicating that the breast cancer organoid, although inefficiently, can still invade in an ITGα2 independent way.

The molecular program that is required for the formation of leader cells is not fully understood. However, it is known that in many breast cancer models, the ability of cells to sense the biochemical and physical changes in the ECM and translate the signals into downstream transducing pathways (Mechano-transduction), play a crucial role in the formation of leaders in collective movement. Yap is a transcriptional coactivator that is activated when changes in the surrounding ECM are mechano-sensed. Yap translocates to the nucleus where it activates a transcriptional program inducing the formation of protrusive leaders is activated, allowing for further modifications of the ECM, creating a bidirectional interaction between the ECM and the cells. Here, we suggest that ITGα2 is a major regulator of Yap. Indeed, our data show that the nuclear/cytosolic levels of basal-like cells located at the rim are reduced in response to Itga2 KO. In addition to the protein localization of Yap, we observed a significant downregulation in many of the Yap targets upon the KO of ITGα2. Interestingly, one of the genes exhibiting a significant decrease expression in the ITGα2 KO is Inhβa, a TGF-β ligand. However, the Yap target genes Ankrd1 and Areg, showed a significant upregulation despite KO of ITGα2 compared to the wildtype. We do still do not understand the molecular explanation of this upregulation. One possible explanation is compensatory mechanisms as a consequence of ITGα2 gene deletion. It has been previously reported that, upon the disruption of a protein, upregulation of certain related genes is a method in which cells genetically compensate in order to survive (El-Brolosy et al., 2017). The upregulation of ankrd1 and Areg could potentially provide KO cells with advantages similar to what has been previously shown, where the upregulation of Ankrd1 promotes cell proliferation and tumor progression in gliomas, renal cell carcinoma, and non-small cell lung cancer (Hao et al., 2017; Yin et al., 2017). Thus, the overexpression of ankrd1 and Areg may be a compensation method for the ITGα2 KO cells to proliferate and survive. Nonetheless, our data show at that ITGα2 is important for Yap nuclear localization, and its transcriptional activation of at least some of its targets (Ctgf and Inhβa). To further investigate the link between ITGα2 and mechano-transduction through Yap, more testing of this interplay has to be carried out. One of the proposed experiments is to assess nuclear Yap localization over multiple time periods in the wildtype compared to the ITGα2 KO organoids to determine whether the activation of Yap becomes independent at later time points (including day 10) of ITGα2 and is what causes the formation of the short protrusive strands after 10 days. Furthermore, examining the impact of a Yap inhibitor/activator on the expression of Yap targets in ITGα2 knockout (KO) organoids, relative to the wildtype, allows for a comprehensive investigation of the interaction between Yap and ITGα2.

The TGF-β signaling family is known to regulate various aspects of cellular processes, such as proliferation, differentiation, and migration (Anzai et al., 2020; Binamé et al., 2008; Hao et al., 2019). TGF-β signaling is known to induce EMT in a variety of diseases, including cancer, fibrosis, and chronic obstructive pulmonary disease (Kajdaniuk et al., 2013; Upton & Morrell, 2009). EMT is a process in which non-mobile epithelial cells are transformed into becoming mesenchymal motile cells. In the case of cancer, tumor cells utilize this transformation to gain the ability to become motile and invade as single cells. The bidirectional nature of the interactions between the ECM and the cells through integrins facilitates the regulation of several signaling pathways, including the paracrine TGF-β transducing pathway (Munger et al., 2011). Here, we demonstrate that ITGα2 plays a role in

activating TGF-β signaling in collagen I. We have shown that collagen I through ITGα2 induces the expression of the TGF-β ligand Inhβa and subsequent mesenchymal characteristics. Similarly, Zhang et al,. showed that the overexpression of ITGαv enhances the secretion of TGF-β factors and propose that this increased stemness and EMT of pancreatic cancer cells (Zhang et al., 2019). We found that Vimentin (EMT marker) is downregulated by the loss of ITGα2, whereby Smad7 remained unaffected. The mRNA levels of Smad7 showed a lot of variation throughout the multiple qPCRs, more testing of this gene will help us obtain a clearer picture on the effect ITGα2 has on Smad7 expression upon the loss of ITGα2 and the TGF-β pathway in general. We observed by the addition of an exogenous Inhβa (Activin A), that in the wildtype organoids invasion is increased. Whereas the lack of invasion in the KO organoids is not an indication that Activin has no effect on the downstream TGF-β targets, as it is an early time point for the KO organoids to rescue their ability to invade (day 3). Next, our results show that addition of the TGF-β ligand Activin in the wildtype as well as the ITGα2 KO has a substantial increasing effect on the mRNA levels of most TGF-β related genes, proving the interplay that occurs between TGF-β and ITGα2 in collagen I. However, to have a better understanding of this interaction in collective invasion more experimentation should take place. For example, introducing a TGF-β inhibitor and observing the effect that blocking the TGF-β has on its target genes and collective invasion. Moreover, introducing a knock-out to the TGF-β receptor could aid in gathering a better understanding on its necessity for collagen I mediated collective invasion.

In conclusion, our study highlights the pivotal role of ITGα2 in orchestrating the bidirectional tumor-ECM interaction during collective invasion. We propose a mechanistic link between ITGα2 and mechano-transduction pathways, particularly through the activation of Yap and regulation of TGF-β paracrine signaling to induce leader cell function and collective invasion. While our study provides valuable molecular insights on the regulation of cellular movements, further investigation is needed to fully delineate the interplay between ITGα2 and TGF-β paracrine signaling and its role in driving invasion in vivo and in patients. Our work holds promise for the development of targeted therapeutic strategies aimed at disrupting the invasive behavior of cancer cells and improving patient outcomes by double targeting ECM mechano-transduction and TGF-b mediated paracrine signaling.

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