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Wnt- β -Catenin Pathway Regulates Vascular Mimicry in Triple Negative Breast Cancer

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Abstract

The Wnt-beta-catenin pathway (WP) regulates different aspects of cell fate, migration and polarity. Inappropriate deregulation of this pathway leads to oncogenic and metastatic changes. The Triple Negative (TN) subset of BC (the absence of hormone receptors and absence of amplification/overexpression of HER2 receptors) exhibits aggressive clinical behavior and has poor clinical outcome. We have recently identified WP as one of the key signature pathways in TNBC associated with metastasis [1,2]. Here we report for the first time that WP controls metastasis-associated phenotype of Vascular Mimicry (VM) in TNBC. We have established 2D and 3D models to study VM in this subset of BC and characterized different TNBC cell lines in terms of VM and identified the role of WP in the regulation of VM. To establish the role of WP in the regulation of VM in TNBC, we have used (1) a pharmacological inhibitor of beta-catenin which is a functional readout of WP and (2) signaling modulators of WP. Since PI3K-pathway is an upstream regulator of WP, we have also used inhibitors of PI3K-pathway to test our hypothesis. Mechanistically (1) sulindac sulfide (pharmacological inhibitor of beta-catenin) and XAV939 (modulator of WP) mediated decrease in beta-catenin, (2) XAV939 mediated increase in axin and (3) LY294002 or SF1126 (pan PI3K inhibitors) mediated decrease in pGSK3beta caused an abrogation of VM in various TNBC cell lines including BT20, HCC1937, SUM149, MDA-MB231 and MDA-MB468. Finally we obtained genetic proof-of-concept using beta-catenin SiRNA. SiRNA-mediated downregulation of WP abrogated VM demonstrating the involvement of WP in VM. Functionally, our data show that VM in brain specific metastatic TNBC cells is mediated via activation of WP.

Keywords: Wnt/beta-catenin pathway; Beta-catenin; Vascular mimicry; TNBC

Introduction

Triple Negative (TN; negative for hormone receptors, and HER2 amplification/overexpression) or basal-like subtypes of Breast Cancer (BC) represents the most challenging diagnosis among BC patients as it confers a poor clinical outcome mostly due to the aggressive nature of the frequently occurring metastasis [3-5]. Despite recent reports that indicated the involvement of certain genes/signaling molecules related to metastatic pathways [6-10] in TNBC (includes both TNBC and basal-like hereof), there remains an unmet need for an in-depth study to identify major pathways associated with metastatic driver pathways in this subtype of BC.

Vascular (vasculogenic) Mimicry (VM) of solid tumor is an endothelium-independent matrix-embedded, blood-perfused non-angiogenic micro-circulatory phenomenon. VM of tumor cells refer to the characteristic plasticity of aggressive cancer cells forming *de novo* vascular networks, which thereby function (1) to perfuse rapidly growing tumors, transporting fluid from leaky vessels and/or (2) to connect with the constitutional endothelial-lined vasculature [11].

Recent discoveries suggest that this alternative mechanism of channel formation is derived from tumor cells [12] which contribute to establish tumor blood supply [13]. BC cells trans-differentiates to drive VM [14-17]. VM is also reported to represent non-angiogenic pathway in breast-cancer metastasis [18]. Studies of aggressive BC have reported VM in the absence of endothelial cells as well as in the absence of central necrosis in the tumor, which indicated the presence of viable tissue without traditional intra-tumoral vasculature [19]. Shirakawa et al. [16] reported a haemodynamic connection between VM in inflammatory breast cancer and angiogenesis. Their data provided an evidence for the vascular phenotype of these tumor cells [20]. TNBC is a highly aggressive subtype of BC characterized by high malignancy and poor survival rates. Malignancy in TNBC is associated with micro-

vascular proliferations. In a study comparing the VM in three subsets of BC, the presence of VM was more profoundly observed in TNBC than in luminal and HER2+ subtypes [21]. VM has also been observed in TNBC cell lines like MDA-MB-435 and BT-549 [22].

Wnt/ β -catenin signaling plays essential roles in embryonic development as well as tissue homeostasis in adults [23]. The protein β -catenin is the most essential component of the Wnt growth factor signaling pathway and intercellular junctions. This ligand-driven signaling pathway regulates several cellular phenotypes related to the development and the progression of various cancers including breast [24-26]. Studies by Jonsson et al., stressed the need to identify elements that selectively drive the oncogenic activity of β -catenin in BC [27]. WP deregulations (via expression of Wnt-ligands, or secreted Wnt antagonists or APC inactivation) have been observed in BC [28]. Although the upregulation of WP in TNBC subtypes as well as its association with poor clinical outcomes has just started to emerge, the mechanism of involvement of WP in metastasis-associated phenotypes still remains either rudimentary or a matter of controversy since confirmatory reports relating to the mechanism of WP's involvement

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in breast tumorigenesis had been mostly obtained from mouse models [29]. Recently, we have identified a differential upregulation of Wnt- β -catenin signaling at the mRNA and protein levels in TNBC specimens and TNBC cell lines as compared to *HER2*-amplified and hormone receptor positive breast tumors [1,2,30-32]. Since this pathway plays a critical role in the integrin-directed migration and invasion of TNBC cells [32,33] and migration is an instrumental event in the regulation of invasion of the tumor cells through the extra-cellular matrix, we hypothesized that the upregulation of WP in this sub-type of BC is functionally connected to the control of metastasis-associated phenotypes of tumor cells. This hypothesis is strengthened by our identification of MMP7 (key target-gene of this pathway), as a selective-biomarker of TNBC subtype [34]. The involvement of WP in the genesis, and the progression of TNBC begun to unfold from the works of Reis-Filho's team [29] and ours [1,2,31] in the context of BC heterogeneity, subtype-specific genomic events and outcome. Although WP's involvement in the control of various phenotypes of tumor cells has been reported in the context of HMGA2 induction and proliferation in metastatic TNBC [35], its role in the context of specific tumor cell phenotypes is unclear.

Since TNBC is an aggressive form of BC subtype and VM is associated with the aggressiveness as well as the poor outcome in many solid tumors including BC, we hypothesized that the upregulation of WP in this BC subtype may have a functional relationship with VM. Here we report for the first time that WP is functionally related to VM in TNBC. With the help of pharmacological and genetic tools, we demonstrate that perturbation of WP (1) at different nodes and (2) via its upstream regulatory PI3K (Phosphatidylinositol-3-Kinase)-pathway, can regulate VM in TNBC cells. Our data showed that mechanistically, (1) sulindac sulfide (pharmacologic inhibitor of β -catenin) and XAV939 (signaling modulator of WP) mediated decrease in β -catenin, (2) XAV939 mediated increase in axin (component of β -catenin destruction complex) and (3) LY294002 or SF1126 (both are pan-PI3K inhibitors) mediated decrease in pGSK3 β caused an abrogation of VM in various TNBC cell lines. In the context of metastasis in TNBC, considering the involvement of VM in mediating the aggressive nature of this highly angiogenic subset of BC, we finally tested the effect of downregulation of WP on VM in brain metastasis specific EGFP tagged MDA-MB231BR cells using a real time confocal microscope. Our data clearly indicate that VM in brain specific metastatic TNBC cells of MDA-MB231 is controlled by WP.

Materials and Methods

Cell lines, reagents, drug treatments and antibodies

All breast tumor cell lines, except SUM149 (Aster and Partners in human tissue research) were obtained from ATCC. Dr. Patricia Steeg (Ph.D. Head, Women's Cancers Section, Laboratory of Molecular Pathology, NCI, Bethesda, MD20892) kindly provided us the EGFP-labeled MDA-MB231BR cell line. Authors acknowledge Professor Victoria L. Seewaldt, Department of Medicine, Duke University Medical Center, Durham, North Carolina, USA, for kindly providing us with DKAT cells. Antibodies against pGSK3 β (Glycogen Synthase Kinase 3 β), GSK3 β , pAKT, AKT (Cell Signaling Technology, MA), axin (Santa Cruz Biotechnology, CA) and β -catenin (Abcam Inc, Cambridge, MA) were used. TN cell lines were cultured according to standard protocol. Cells were treated with sulindac sulfide (Sigma-Aldrich, Inc.), Wnt signaling modulators (Cellagen Technology, CA), LY294002 (Calbiochem) and SF1126 (Kindly provided by Semaphore).

Cell culture and biochemical analysis

TNBC cell lines were all cultured according to standard protocols. Normalized cell lysates (20-40 μ g protein) were resolved by 10% SDS-PAGE for Western blot analysis [36,37].

Vascular mimicry: Tubular network formation assay was performed as described by Mirshahi et al. [38]. VM was induced either by plating the cells on matrigel (2D VM) or covering (on top) the already matrigel plated cells with matrigel (sandwich) (3D VM). Briefly, cell suspensions (control and treated) were plated (1 ml/well) onto the surface of matrigel coated tissue culture plate and incubated at 37°C for different time points. Vascular mimicry was photographed using an Olympus inverted phase contrast photomicroscope. Tubular network formation was semi-quantified by counting the number of tubules in randomly chosen fields and by counting the average VM length and intersecting nodes [39]. For 3D VM, cells (in the presence or absence of drugs) were placed on matrigel at zero hour. Following their attachment on the matrigel, cells are topped with 10% matrigel (in the presence or absence of drugs). VM was recorded at 24 hours. Typical cord formation in HUVEC (Human Umbilical Vein Endothelial Cells) cells has been tested using HUVEC cells (procured from ATCC; passage 8) for the comparison. HUVEC cords were stained with hematoxylin and PAS.

Real-time imaging of live cells: Time-lapse images are acquired with a Nikon confocal system with cell culture chambers. Cells were placed on special dish with cover slip which was coated with matrigel. Cells those were placed on the dish and not the matrigel and cells on the matrigel were simultaneously recorded both for the non-treated and the treated conditions and bright-field images were acquired with a Nikon camera at 10 minutes intervals.

Transient transfection of β -catenin siRNA: Silencing of β -catenin in TNBC cells was carried out using siRNA. Cell lines were transiently transfected with human-specific β -catenin siRNA (Invitrogen Inc.) using Lipofectamin 2000 (Invitrogen Inc.) as described earlier [2]. In brief, cells were grown in 6 well tissue culture plates to 60-70% confluency and then transfected with siRNA plasmids using lipofectamine. Cells were harvested at 72 hours. β -catenin, pGSK3 β , and GSK3 β protein levels were determined by Western Blot [36]. For the study of VM, transiently transfected (both control and β -catenin siRNA transfected cells) cells were plated on matrigel.

Statistical analysis: Students' T-test was used for testing the significance of the differences observed between treated groups and vehicle treated controls.

Results

Two-dimensional VM and three-dimensional VM in TNBC cell lines:

In order study the role of WP in VM, we standardized VM both in 2 and 3 dimensional configuration in different TNBC cell lines. TNBC cells exhibited both 2D and 3D VM (Figure 1). The TNBC cells responded to VM stimulation at different time points. The earliest response was observed around 2-3 hours in BT20, Hs578t and MDA-MB231 (Figure 1A). By 24 hours, SUM149, DKAT, MDA-MB231BR and MDA-MB468 cells demonstrated 2D VM. BT20 cell line showed most pronounced 3D VM effect at 24 hours (Figure 1D). MDA-MB468 was least sensitive to VM. The time line of VM response of different TNBC cell lines is represented in Supplementary Figure 1 (S1). TNBC cells exhibited VM which was initiated as early as 5-6 hours and

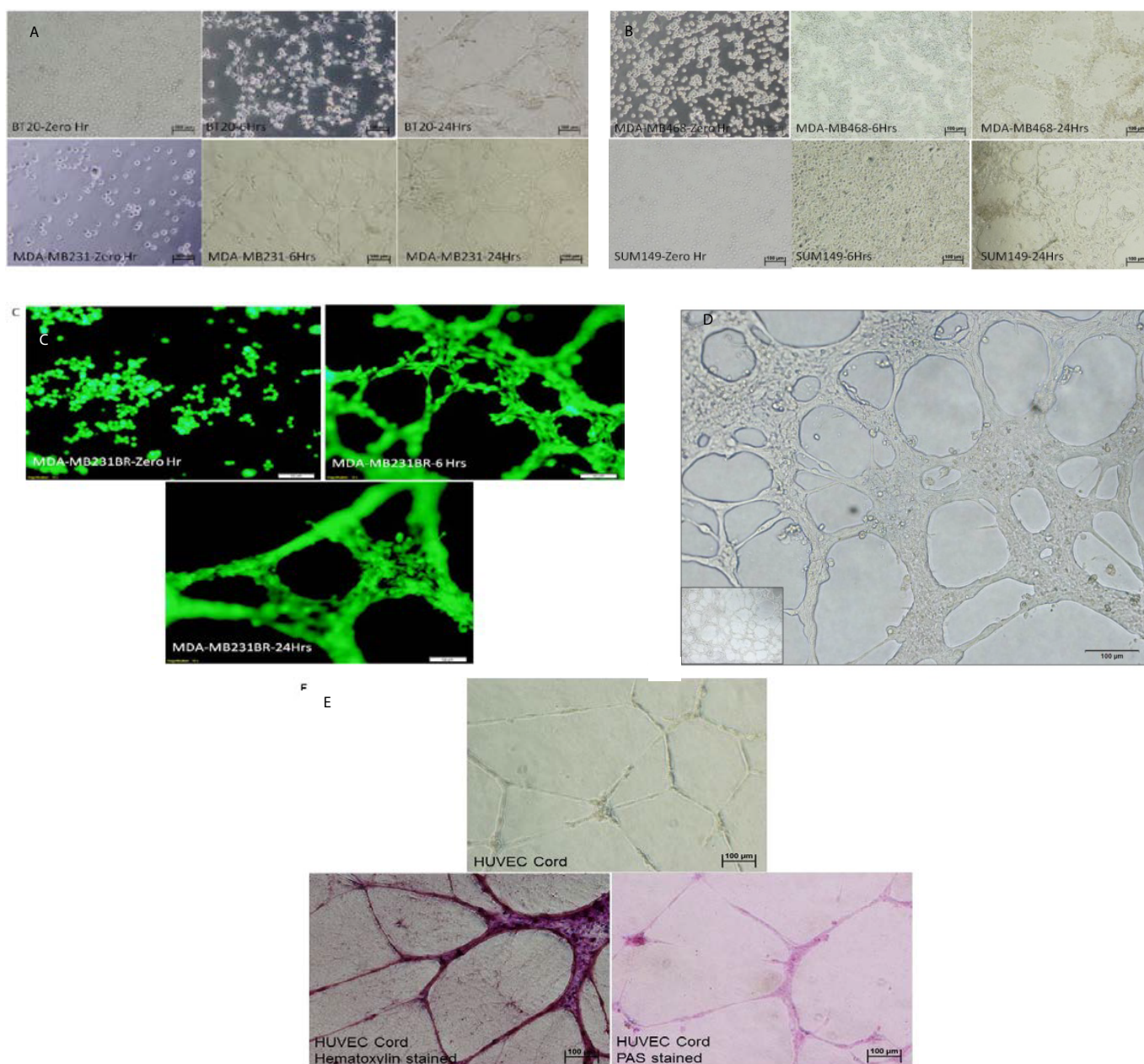


Figure 1: 2D and 3D VM in TNBC cells

A: Two-dimensional VM recorded at 6 and 24 hours of time points in BT20 (upper panel), MDA-MB231 (lower panel)

B: Two-dimensional VM recorded at 6 and 24 hours of time points in MDA-MB468 (upper panel), SUM149 (lower panel).

C: Two-dimensional VM recorded at 6 and 24 hours of time points in MDA-MB231BR cells.

D: Three-dimensional VM recorded at 24 hours of time point in BT20.

E: Typical cord formation in HUVEC (Human Umbilical Vein Endothelial Cells) cells has been presented for comparison. HUVEC cords were stained with hematoxylin and PAS.

matured by 24 hours. Typical cord formation in HUVEC cells stained with hematoxylin and PAS has been presented for comparison (Figure 1E).

Downregulation of Wnt-beta-catenin pathway following sulindac sulfide blocked VM in TNBC cell lines:

In order to know the involvement of WP in the regulation of VM, we used sulindac sulfide to decrease the cellular levels of beta-catenin

and its effect on VM in different TNBC cell lines. Wnt-beta-catenin signaling increases the half-life of beta-catenin and therefore the absolute level of beta-catenin in responding cells to mediate canonical Wnt signaling [40]. The model described by Staal et al. [40] states that the changes in beta-catenin stability set the threshold for Wnt signaling. We hypothesized that upregulation of the WP in this subtype of BC is functionally associated with the control of VM in tumor cells. We used cell line based models to test our hypothesis. Our primary

validation strategy was focused on testing the effect of inhibition of (1) different nodal points of WP and (2) the signaling pathway that control WP on VM in TNBC cells. We have considered three nodal points for this pathway, (1) β -catenin, (2) axin and (3) GSK3 β . β -catenin levels were decreased by sulindac sulfide as well as β -catenin siRNA. Axin1 and Axin2 were stabilized by XAV939. Phospho-GSK3 β levels were decreased by pan PI3K inhibitors. Our *in vitro* phenotypic experiments focused on β -catenin because β -catenin is the functional as well as biochemical readout of WP, and it can be pharmacologically (by sulindac sulfide) [41,42] and genetically (by siRNA) attenuated. Sulindac and its derivatives suppress β -catenin's expression and β -catenin's transcriptional activities in breast cancer and colorectal tumor cells [43-45]. Figure 2A (upper panel bar diagrams) showed that sulindac sulfide administration (25-100 μ M) caused a decrease in cellular levels of β -catenin. A dose and time-dependent loss of β -catenin was reported as early as 24 hours after treatment with sulindac sulfide or sulindac sulfone (ranges 120-600 μ M) via three different ways of induction of β -catenin degradation [46]. We observed a similar decrease in the levels of β -catenin in TNBC cells in our study and this supported our use of sulindac sulfide in the subsequent experiments. Taken collectively the sulindac sulfide mediated decrease in the level of β -catenin in BT20 and SUM149 TNBC cell lines blocked VM. VM was tested in presence of drug for 6 hrs in BT20, MDA231 and SUM149 cells (Figures 2B-D). Our result demonstrates that WP regulates VM in TNBC cells.

Wnt- β -catenin signaling modulators blocks VM in TNBC cell lines:

Sulindac sulfide is not a specific inhibitor of WP, two specific Wnt

signaling modulators of WP, WntC59 and XAV939 were also used in this study. As Wnts are secreted protein ligands, they need membrane-bound enzymes that are required for their post-translational modification in order to enable their transport, secretion and activity. Hence, the first Wnt signaling modulator we used, Wnt-C59, (PatentWO2010101849) is a compound that prevents palmitoylation of Wnt proteins by Porcupine (a membrane-bound Oacyltransferase), and thereby blocks Wnt secretion and activity (IC_{50} < 0.11 nM in Wnt-Luc reporter assay for Wnt pathway inhibition) [47]. We chose Wnt-C59 because it is more potent and selective, and has better chemical/physical properties, suitable for *in vitro/in vivo* studies. The PORCN inhibitor WntC59 blocked progression of mammary tumors in MMTV-WNT1 transgenic mice downregulating Wnt/ β -catenin target genes [48]. Our result shows that WntC59 treatment blocked VM in TNBC cells (Figure 3Bi).

APC and Axin are among key components of the β -catenin destruction complex that promotes β -catenin degradation (Figure 4C). The levels of Axin are controlled by tankyrases, members of the PARP-family of poly-ADP-ribosylation enzymes. Bao et al. examined the effect of inhibiting tankyrases, by XAV939 in breast cancer cells with normal APC and showed that application of the small molecule tankyrase inhibitor, XAV939 or siRNA-mediated abrogation of tankyrase expression increases Axin1 and Axin2 protein levels and attenuates Wnt-induced transcriptional responses in several breast cancer lines [49,50]. XAV939 treatment in our study also increased Axin and decreased β -catenin in MDA-MB231 cells (Figure 3A). Figure 3A shows that XAV939 treatment decrease β -catenin levels and increase Axin levels in cells. XAV939 treatment blocked VM in TNBC cells (Figure 3Bii). These data collectively substantiate our

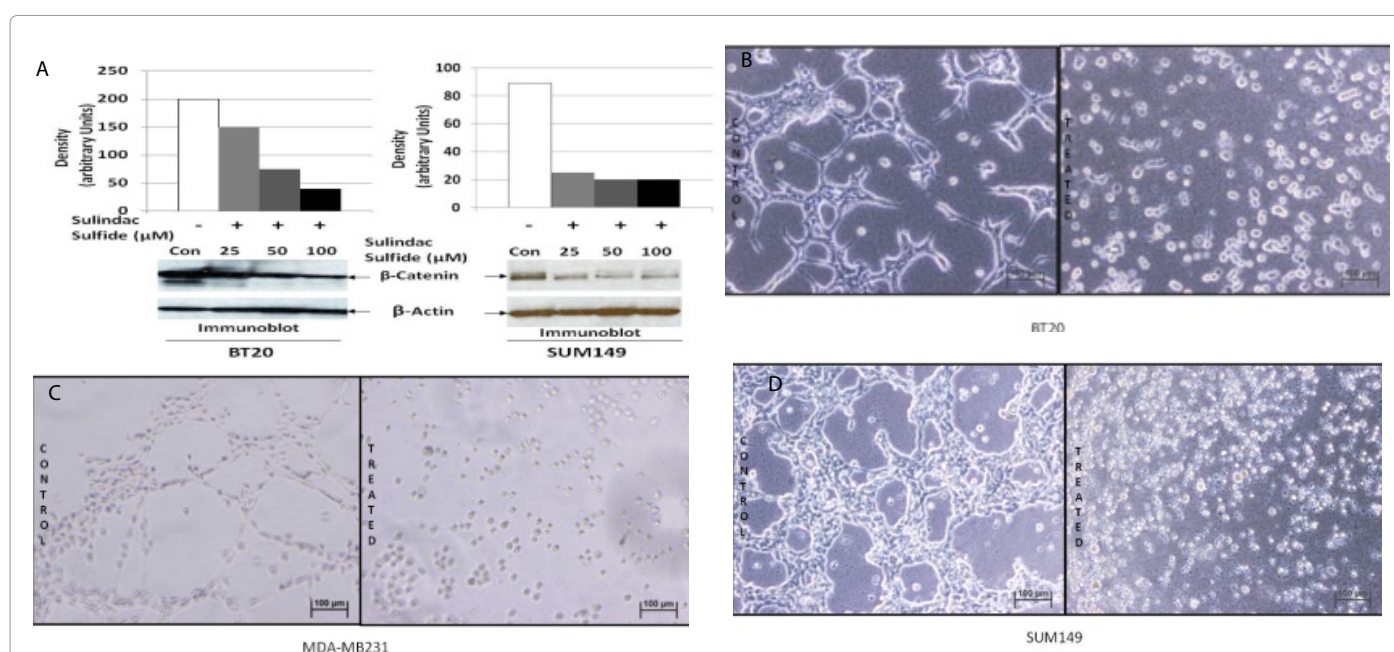


Figure 2: Sulindac sulfide blocked VM in TNBC cells

A: PIK3CA mutated and PTEN null TNBC cell lines, BT20 and SUM149 respectively were treated with three doses of sulindac sulfide for 24 hours and β -catenin protein was determined by Western blot. β -actin was used as loading control. Upper bar diagram showed the relative densitometric expressions of β -catenin in both cell lines.

B: Pharmacological attenuation of β -catenin in MDA-MB468 TNBC cell line is presented.

C: Pharmacological attenuation of β -catenin in MDA-MB231 TNBC cell line is presented.

D: Pharmacological attenuation of β -catenin in SUM149 TNBC cell line is presented.

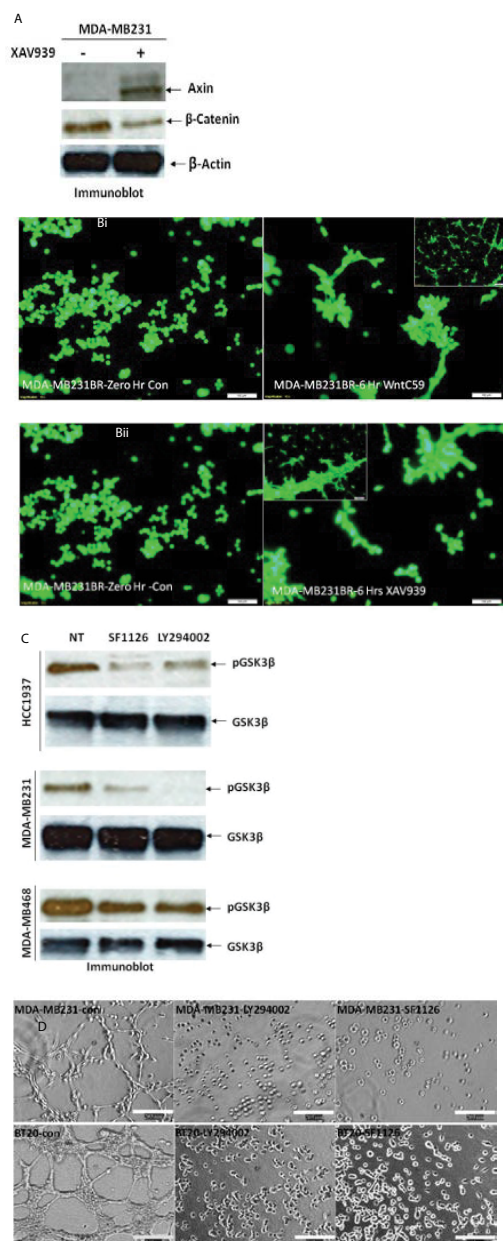


Figure 3: Wnt signaling modulators blocked VM in TNBC cells

A: XAV939 decreased cellular levels of beta-catenin by increasing axin in MDA-MB231 cells. The treatment with XAV939 (10 μ M) (A) caused an increase in axin protein expression as well as a decrease in beta-catenin protein. β -actin was used as loading control.

B: Wnt signaling modulators (WntC59: 10 nM) (i) and (XAV939: 5 μ M) (ii) blocked VM at 6 hours in EGFP-MDA-MB231BR cells. XAV939 (10 μ M) mediated decrease in beta-catenin protein blocked VM in EGFP-MDA-MB231BR cells (B i). The blockade of VM by XAV939 was observed at both 5 μ M and 10 μ M doses. WntC59 blocked VM in EGFP-MDA-MB231BR cells (B ii). The same zero hour control for EGFP-MDA-MB231BR was used for this data (B i & ii) and EGFP-MDA-MB231BR VM standardization data in figure 1C. Insets show respective photomicrographs at 4X magnification

C: LY294002 (25 μ M) and SF1126 (25 μ M) decreased cellular levels of pGSK3 β in HCC1937, MDA231 and MDA-MB468 TNBC cells. Total GSK3 β was used as the specific loading control.

D: LY294002 and SF1126 blocked VM in MDA-MB231 and BT20 TNBT cells. Panels showed images of VM in different cell lines.

previous observations that WP has a regulatory role in mediating VM in TNBC.

Inhibitors of the PI3K pathway blocks VM in TNBC cell lines:

We tested the effect of pan PI3K (Phosphatidylinositol-3-Kinase) inhibitors in order to examine the effect of PI3K pathway on VM in TNBC. PI3K pathway regulates WP via AKT mediated phosphorylation of GSK3 β (Glycogen Synthase Kinase 3 β) (Figure 4C). Both inhibitors of PI3K pathway, LY294002 and SF1126 decreased the levels

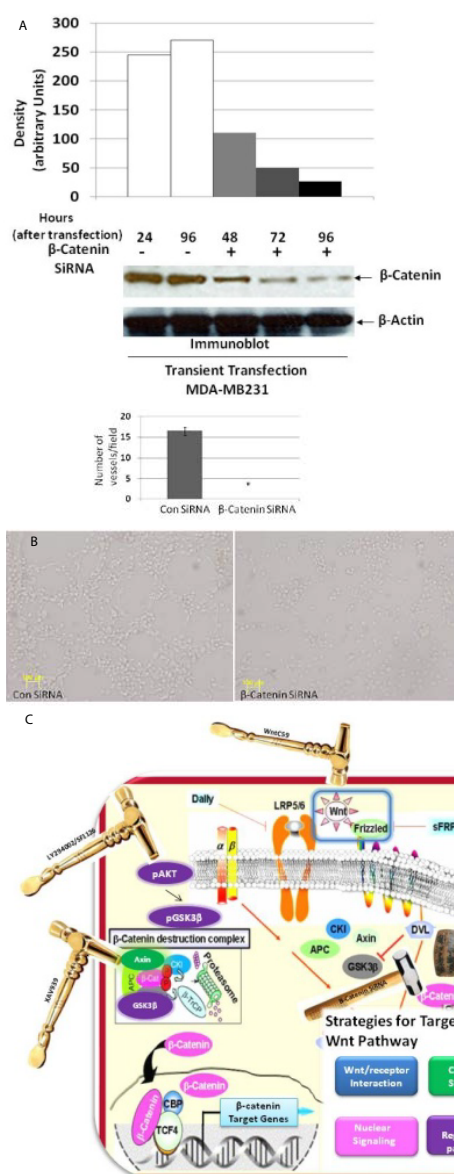


Figure 4: SiRNA mediated downregulation Wnt- β -catenin pathway blocked VM in TNBC cells

A: MDA-MB231 TNBC cell line, was transiently transfected with beta-catenin SiRNA for three different time points (48-96 hours) and beta-catenin protein was determined by Western blot

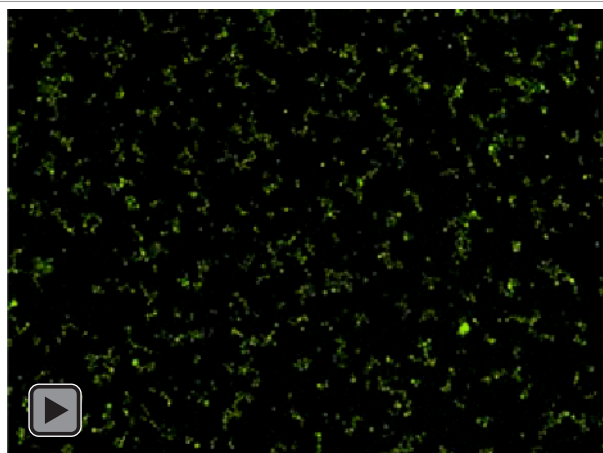
B: Genetic attenuation of beta-catenin in MDA-MB231 blocked VM over 6 hours. Bars represent mean number of semi-quantified vessels counted per randomly chosen field, *P<0.001 (n= 10)

C: Schematics of WP are represented with the tools used in the study.

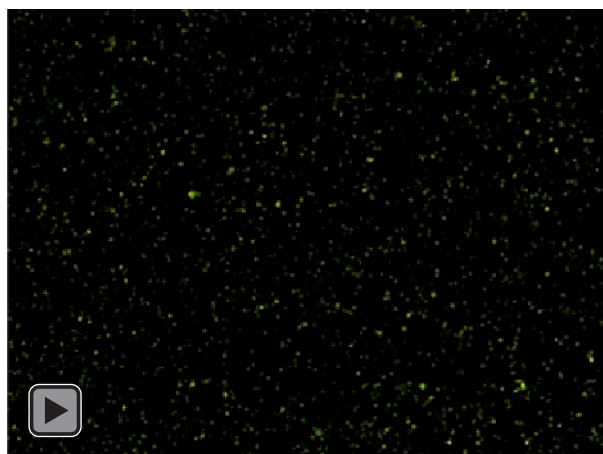
of cellular pGSK3 β in HCC1937, MDA-MB231 and MDA-MB468 cells (Figure 3C). LY294002 and SF1126 mediated decrease in the levels of cellular pGSK3 β blocked VM in MDA-MB231 and BT20 cells (Figure 3D). Treatment with SF1126 and LY294002 (30 minutes; 25 μ M and 25 μ M) significantly decreased the levels of pAKT (phospho-AKT; Ser473) TNBT cells (Supplementary Figure 2).

SiRNA mediated downregulation Wnt- β -catenin pathway blocks VM in TNBC cells:

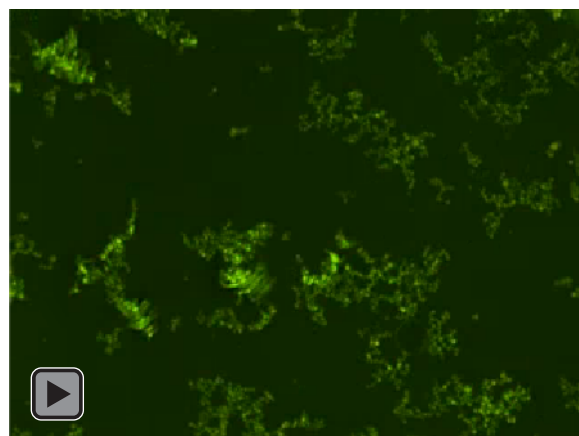
In order to obtain genetic proof-of-concept, we decreased the cellular levels of beta-catenin by beta-catenin SiRNA and tested the effect of SiRNA mediated decrease of beta-catenin on VM in TNBC cell. Figure 4A showed that transient transfection of SiRNA time dependently (upto 96 hours) decreased beta-catenin levels in MDA-MB231. Figure 4B shows that SiRNA mediated decrease of beta-catenin blocked VM in MDA-MB231. Our result shows that beta-catenin, a functional read out of WP is a necessary component of VM in TNBC. The fact that a decrease in the protein levels of beta-catenin following the transient transfection of beta-catenin SiRNA in TNBC cells abrogated the VM response directly implies that VM is mediated by WP.



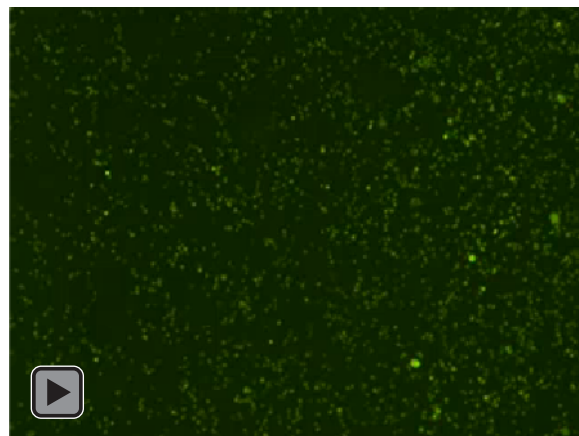
Movie 1:



Movie 2:



Movie 3:



Movie 4:

Figure 5: Downregulation of the Wnt- β -catenin pathway blocked VM in brain metastasis specific MDA-MB231BR cells in Real Time (Four Movies; AVI files). For each control (non-treated) and sulindac sulfide (50 μ M) treated cells, two movies (4X) were acquired simultaneously from each plate, one from cells on the matrigel and the other from cells outside the matrigel. The movie of the non-treated control cells outside the matrigel (M1; "Outside the matrigel"-non-treated control) was used as an internal control of VM and compared with the movie of cells on the matrigel (M2; "On matrigel"-non-treated control). On the other hand, sulindac sulfide treated cells plated on outside the matrigel (M3; "Outside the matrigel"-Sulindac sulfide treated) were compared with the sulindac treated cells on matrigel (M4; "On matrigel"-Sulindac sulfide treated).

Downregulation Wnt- β -catenin pathway blocks VM of brain metastasis specific MDA-MB231BR cells in real time:

Encompassing the critical role of VM in metastasis of TNBC, we tested the effect of sulindac sulfide mediated downregulation of beta-catenin on VM in brain metastasis specific MDA-MB231BR cells in real time. Downregulation of the Wnt- β -catenin pathway blocked VM in brain metastasis specific MDA-MB231BR cells in real time (Figure 5). For each control (non-treated) and sulindac sulfide treated cells, two movies are acquired simultaneously from each plate, one of cells on the matrigel and the other of cells outside the matrigel. The movie of the non-treated control cells outside the matrigel (Figure 5 M1; "Outside

the matrigel"-non-treated control) is used as an internal control of VM and showed no formation of vascular structures in contrast to the cells on the matrigel (Figure 5 M2; "On matrigel"-non-treated control) on the same plate. On the other hand, sulindac sulfide treated cells plated on outside the matrigel (Figure 5 M3; "Outside the matrigel"-Sulindac sulfide treated) did not form any structures after 24 hours, while the sulindac treated cells on matrigel (Figure 5 M4; "On matrigel"-Sulindac sulfide treated) formed rudimentary structures during the early hours of treatment which collapsed with time.

Discussion

VM represents the trans-endothelial differentiation of tumor cells [11] as it describes the ability of highly aggressive tumor cells to form vessel-like networks by virtue of their trans-differentiation property/plasticity and its occurrence is strongly associated with poor prognosis in several tumor types [51,52]. VM or vascular channel formation *in vitro* [12,53] has been associated with angiogenesis. This matrix embedded, blood-perfused microvasculature participates in tumor progression independent of endothelial cell angiogenesis and is believed to be at least partially ascribed to the multi-potency of glioblastoma stem cells which are capable of trans-differentiation into vascular non-endothelial cells as in the case of GBM and other aggressive cancers [54-56].

In a systemic review and meta-analysis by Cao et al. tumor VM has been shown to be associated with poor prognosis of human cancer patients wherein they reported that VM-positive cancer patients show a poor 5-year overall survival compared with VM-negative malignant tumor cases, particularly in metastatic cancers [57]. Results of this study indicate the need to further investigate the involvement of VM in metastasis. Molecular pathways for VM in tumor cells have been warranted serious scrutiny in the context of (1) potential therapeutic/vascular targets, (2) diagnostic indicators of plasticity/angiogenic drug resistance and (3) the aggressive metastatic phenotypes [11,58]. From the therapeutic point of view, the most important one from the above contexts is the role of VM in anti-angiogenic resistance. Neo vascularization originating from tumor cells has been reported to include VM, which has been suggested to be involved in the resistance to anti-VEGF therapy [59]. Thus, mechanisms of VM can be targeted in addition to anti-angiogenic therapies to achieve better results for patients with failure of anti-angiogenic therapy, such as bevacizumab in TNBC. One of the current chemotherapies against TNBC aims at vascular endothelial cells that orchestrate a significant component of blood vessels. However, it has been increasingly documented that an anti-angiogenic monotherapy did not deliver to the promise for improvement of patient for various reasons. For example, clinical trials using a neutralizing anti-VEGF antibody (bevacizumab, also named Avastin) revealed minimal benefit in BC. Phase III trials of antiangiogenic drugs for metastatic BC have either had only limited success, e.g. the monoclonal anti-VEGF antibody bevacizumab when used with various conventional chemotherapy regimens [60]. Among the reasons to help explain the limited benefits observed thus far include the possibility that anti-angiogenic drugs may secondarily aggravate biologic aggressiveness of the tumors, thereby reducing their overall efficacy after inducing an initial benefit [60]. In randomized phase III trials using anti-vascular endothelial growth factor (VEGF) approaches, Jain et al. reported that adding bevacizumab to chemotherapy failed to increase survival in patients with previously treated and refractory metastatic breast cancer [61]. These facts implicate that there may be an *alternate escape route* that accounts for the treatment failure/malignancy and VM may serve as an additional mechanism by which these highly invasive and genetically deregulated tumor cells obtain oxygen and

nutrients to survive, especially in poorly vascularized regions of the tumor or the tumor cell use this route for the micro-dissemination. Recently, a number of research groups have demonstrated VM as an alternative vascular mechanism, which contributes to the central role of the vascularization as in GBM in which tumor cells participate [62,63]. Reports by Qu et al. indicate VM as an alternative circulatory system, present in multiple malignant tumor types with a poor prognosis wherein VM serves as an adjunct to the existing vasculature system contributing to the metastatic process. In their central hypothesis they argue that when the endothelium-dependent vessels are inhibited by the effective angiogenesis inhibitors, the hypoxia of tumor cells caused by anti-angiogenesis may increase the chance of compensatively VM which provides a convenient route of tumor metastasis. Thus anti-angiogenesis therapy may lead to promotion of tumor metastasis by increasing VM unintentionally [62]. In this context, our result proves that regulation of VM via WP can be an alternate novel approach to counter the anti-angiogenic resistance conditions in TNBC.

Recently, signaling pathways controlling VM in a number of aggressive solid tumors have been reported. Glioblastoma-derived tumor cells have been shown to induce VM through Flk-1 protein activation [52,63] while the involvement of Notch signaling has been observed in melanoma VM [64]. To our knowledge, this is the first report to demonstrate a direct functional role of WP in the regulation of VM in TNBC. Four strategies were employed to understand the role of WP in VM. They are (1) Wnt-receptor interaction, (2) Wnt cytosolic signaling, (3) Wnt nuclear signaling and (4) Wnt-regulating pathway as schematically represented in Figure 4C. We used Wnt C59 to test Wnt-receptor interaction, XAV939 to test Wnt cytosolic signaling, sulindac sulfide and beta-catenin SiRNA to test Wnt nuclear signaling. Additionally pan PI3Kinase inhibitors (LY294002 and SF1126) were used to test Wnt-regulating pathway. Since the activation of the WP leads to an increase of beta-catenin protein via inhibition of its degradation [65] and sulindac sulfide mediated loss of beta-catenin occurs via reactivation of proteosomal degradation [43], we chose sulindac sulfide for the attenuation of the pathway in order to test our hypothesis. Our current findings provide mechanistic insights into the role of WP in the control of VM in TNBC tumor cells, the vascularization that occurs in a number of human cancers including BC, GBM, ovarian and melanoma [12,15,66]. Identification of WP as a key factor regulating VM can offer a novel therapeutic target for TNBC. As a result, multiple anti-vascular approaches, including targeting VM and angiogenesis together with chemotherapy/targeted treatment can be designed as the best possible therapy regimen in combating against this devastating disease.

The armory of "targeted therapy" for the treatment of metastatic TNBC has been inadequate due to the lack of identification of pathway-specific targets [6,67,68] and the absence of a validated targeted-therapy [69,70]. Our recent study pointed out an up-regulation of the WP as a key expression signature of TNBC and revealed for the first time that the high expression of MMP7, a transcriptional target of WP, in a subpopulation of TNBC patients has been associated with the loss of tumor suppressor PTEN, the most common first somatic event associated with basal-like subtype. Our recent data is in agreement with our previous observation [1,2] and a report by Khramtsov et al. which states the association of Wnt signaling in TNBC with poor prognosis and metastasis [71]. Here we report for the first time that the functional upregulation of WP is associated with VM function in TNBC. Our data categorize the activation of the WP with metastasis-associated phenotypes in TNBC, suggesting that the WP pathway can provide attractive pharmacological targets for TNBC.

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References

- Dey N, Barwick BG, Moreno CS, Ordanic-Kodani M, Chen Z, et al. (2013) Wnt signaling in triple negative breast cancer is associated with metastasis. *BMC Cancer* 13: 537.
- Dey N, Young B, Abramovitz M, Bouzyk M, Barwick B, et al. (2013) Differential Activation of Wnt- β -Catenin Pathway in Triple Negative Breast Cancer Increases MMP7 in a PTEN Dependent Manner. *PLoS One* 8: e77425.
- Bilici A, Arslan C, Altundag K (2012) Promising therapeutic options in triple-negative breast cancer. *J BUON* 17: 209-222.
- Dreyer G, Vondorp T, Smeets A, Forceville K, Brouwers B, et al. (2013) Triple negative breast cancer: clinical characteristics in the different histological subtypes. *Breast* 22: 761-766.
- Pires MM, Hopkins BD, Saal LH, Parsons RE (2013) Alterations of EGFR, p53 and PTEN that mimic changes found in basal-like breast cancer promote transformation of human mammary epithelial cells. *Cancer Biol Ther* 14: 246-253.
- Carey LA (2011) Directed therapy of subtypes of triple-negative breast cancer. *Oncologist* 16 Suppl 1: 71-78.
- Moulder SL (2010) Does the PI3K pathway play a role in basal breast cancer? *Clin Breast Cancer* 10 Suppl 3: S66-71.
- Hoeflich KP, O'Brien C, Boyd Z, Cavet G, Guerrero S, et al. (2009) In vivo antitumor activity of MEK and phosphatidylinositol 3-kinase inhibitors in basal-like breast cancer models. *Clin Cancer Res* 15: 4649-4664.
- Cancer Genome Atlas Network (2012) Comprehensive molecular portraits of human breast tumours. *Nature* 490: 61-70.
- Prat, A., C.M. Perou (2010) WITHDRAWN: Deconstructing the molecular portraits of breast cancer. *MolOncol*
- Seftor RE, Hess AR, Seftor EA, Kirschmann DA, Hardy KM, et al. (2012) Tumor cell vasculogenic mimicry: from controversy to therapeutic promise. *Am J Pathol* 181: 1115-1125.
- Maniotis AJ, Folberg R, Hess A, Seftor EA, Gardner LM, et al. (1999) Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Am J Pathol* 155: 739-752.
- Fox SB, Generali DG, Harris AL (2007) Breast tumour angiogenesis. *Breast Cancer Res* 9: 216.
- Paulis YW, Soetekouw PM, Verheul HM, Tjan-Heijnen VC, Griffioen AW (2010) Signalling pathways in vasculogenic mimicry. *Biochim Biophys Acta* 1806: 18-28.
- Basu GD, Liang WS, Stephan DA, Wegener LT, Conley CR, et al. (2006) A novel role for cyclooxygenase-2 in regulating vascular channel formation by human breast cancer cells. *Breast Cancer Res* 8: R69.
- Shirakawa K, Shibuya M, Heike Y, Takashima S, Watanabe I, et al. (2002) Tumor-infiltrating endothelial cells and endothelial precursor cells in inflammatory breast cancer. *Int J Cancer* 99: 344-351.
- Liu T, Sun B, Zhao X, Gu Q, Dong X, et al. (2013) HER2/neu expression correlates with vasculogenic mimicry in invasive breast carcinoma. *J Cell Mol Med* 17: 116-122.
- Francesco Pezzella (2000) Evidence for novel non-angiogenic pathway in breast-cancer metastasis. *Breast Cancer Progression Working Party. Lancet* 355: 1787-1788.
- Martine Piccart (2007) *Breast Cancer Management and Molecular Medicine: Towards Tailored Approaches*. Springer.
- Shirakawa K, Tsuda H, Heike Y, Kato K, Asada R, et al. (2001) Absence of endothelial cells, central necrosis, and fibrosis are associated with aggressive inflammatory breast cancer. *Cancer Res* 61: 445-451.
- Liu TJ, Sun BC, Zhao XL, Zhao XM, Sun T, et al. (2013) CD133+ cells with cancer stem cell characteristics associates with vasculogenic mimicry in triple-negative breast cancer. *Oncogene* 32: 544-553.
- Zantek ND, Walker-Daniels J, Stewart J, Hansen RK, Robinson D, et al. (2001) MCF-10A-NeoST: a new cell system for studying cell-ECM and cell-cell interactions in breast cancer. *Clin Cancer Res* 7: 3640-3648.
- Yi YW, Hong W, Kang HJ, Kim HJ, Zhao W, et al. (2013) Inhibition of the PI3K/AKT pathway potentiates cytotoxicity of EGFR kinase inhibitors in triple-negative breast cancer cells. *J Cell Mol Med* 17: 648-656.
- Nusse R, Varmus HE (1992) Wnt genes. *Cell* 69: 1073-1087.
- Schlang T, Matsuda Y, Lienhard S, Huber A, Hynes NE (2007) Autocrine WNT signaling contributes to breast cancer cell proliferation via the canonical WNT pathway and EGFR transactivation. *Breast Cancer Res* 9: R63.
- Turashvili G, Bouchal J, Burkadze G, Kolar Z (2006) Wnt signaling pathway in mammary gland development and carcinogenesis. *Pathobiology* 73: 213-223.
- Jönsson M, Borg A, Nilbert M, Andersson T (2000) Involvement of adenomatous polyposis coli (APC)/beta-catenin signalling in human breast cancer. *Eur J Cancer* 36: 242-248.
- Zardawi SJ, O'Toole SA, Sutherland RL, Musgrove EA (2009) Dysregulation of Hedgehog, Wnt and Notch signalling pathways in breast cancer. *Histol Histopathol* 24: 385-398.
- Geyer FC, Lacroix-Triki M, Savage K, Arnedos M, Lambros MB, et al. (2011) β -Catenin pathway activation in breast cancer is associated with triple-negative phenotype but not with CTNNB1 mutation. *Mod Pathol* 24: 209-231.
- Dey N (2009) Cell line based model to study Wnt-beta catenin pathway in the control of tumor phenotypes in triple negative subset of breast cancer. *AACR Annual Meeting*, Denver, CO, USA.
- Kragh JF Jr, San Antonio J, Simmons JW, Mace JE, Stinner DJ, et al. (2013) Compartment syndrome performance improvement project is associated with increased combat casualty survival. *J Trauma Acute Care Surg* 74: 259-263.
- Dey N (2012) Wnt- β -catenin pathway controls metastasis-associated phenotypes of tumor cells in TN subset of breast cancer: A proof of concept study in 24th EORTC-NCI-AACR Symposium on 'Molecular Targets and Cancer Therapeutics', Dublin, Ireland.
- Dey N (2009) Role of Wnt-beta-catenin pathway in the control of tumor cell migration in the triple negative subset of breast cancer. *Advances in Breast Cancer Research*, AACR, San Diego.
- Abramovitz M (2009) Identification of matrix metalloproteinase 7 (MMP7) as a selective biomarker of the triple negative breast cancer subtype. in *AACR Annual meeting*, Colorado, Denver.
- Wend P, Runke S, Wend K, Anchondo B, Yesayan M, et al. (2013) WNT10B/ β -catenin signalling induces HMGA2 and proliferation in metastatic triple-negative breast cancer. *EMBO Mol Med* 5: 264-279.
- Dey N, De PK, Wang M, Zhang H, Dobrota EA, et al. (2007) CSK controls retinoic acid receptor (RAR) signaling: a RAR-c-SRC signaling axis is required for neurogenic differentiation. *Mol Cell Biol* 27: 4179-4197.
- Dey N, Howell BW, De PK, Durden DL (2005) CSK negatively regulates nerve growth factor induced neural differentiation and augments AKT kinase activity. *Exp Cell Res* 307: 1-14.
- Mirshahi P, Rafii A, Vincent L, Berthaut A, Varin R, et al. (2009) Vasculogenic mimicry of acute leukemic bone marrow stromal cells. *Leukemia* 23: 1039-1048.
- Jiang J, Liu W, Guo X, Zhang R, Zhi Q, et al. (2011) IRX1 influences peritoneal spreading and metastasis via inhibiting BDKRB2-dependent neovascularization on gastric cancer. *Oncogene* 30: 4498-4508.
- Staal FJ, Noort Mv Mv, Strous GJ, Clevers HC (2002) Wnt signals are transmitted through N-terminally dephosphorylated beta-catenin. *EMBO Rep* 3: 63-68.
- O'Connor R, O'Leary M, Ballot J, Collins CD, Kinsella P, et al. (2007) A phase I clinical and pharmacokinetic study of the multi-drug resistance protein-1 (MRP-1) inhibitor sulindac, in combination with epirubicin in patients with advanced cancer. *Cancer Chemother Pharmacol* 59: 79-87.

42. Witta SE, Gustafson DL, Pierson AS, Menter A, Holden SN, et al. (2004) A phase I and pharmacokinetic study of exisulind and docetaxel in patients with advanced solid tumors. *Clin Cancer Res* 10: 7229-7237.
43. Rice PL, Kelloff J, Sullivan H, Driggers LJ, Beard KS, et al. (2003) Sulindac metabolites induce caspase- and proteasome-dependent degradation of β -catenin protein in human colon cancer cells. *Mol Cancer Ther* 2: 885-892.
44. Han A, Song Z, Tong C, Hu D, Bi X, et al. (2008) Sulindac suppresses β -catenin expression in human cancer cells. *Eur J Pharmacol* 583: 26-31.
45. Clapper ML, Coudry J, Chang WC (2004) β -catenin-mediated signaling: a molecular target for early chemopreventive intervention. *Mutat Res* 555: 97-105.
46. Dihlmann S, von Knebel Doeberitz M (2005) Wnt/ β -catenin-pathway as a molecular target for future anti-cancer therapeutics. *Int J Cancer* 113: 515-524.
47. Proffitt KD, Virshup DM (2012) Precise regulation of porcupine activity is required for physiological Wnt signaling. *J Biol Chem* 287: 34167-34178.
48. Proffitt KD, Madan B, Ke Z, Pendharkar V, Ding L, et al. (2013) Pharmacological inhibition of the Wnt acyltransferase PORCN prevents growth of WNT-driven mammary cancer. *Cancer Res* 73: 502-507.
49. Bao R, Christova T, Song S, Angers S, Yan X, et al. (2012) Inhibition of tankyrases induces Axin stabilization and blocks Wnt signalling in breast cancer cells. *PLoS One* 7: e48670.
50. Huang SM, Mishina YM, Liu S, Cheung A, Stegmeier F, et al. (2009) Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature* 461: 614-620.
51. Kirschmann DA, Seftor EA, Hardy KM, Seftor RE, Hendrix MJ (2012) Molecular pathways: vasculogenic mimicry in tumor cells: diagnostic and therapeutic implications. *Clin Cancer Res* 18: 2726-2732.
52. Soda Y, Myskiw C, Rommel A, Verma IM (2013) Mechanisms of neovascularization and resistance to anti-angiogenic therapies in glioblastoma multiforme. *J Mol Med (Berl)* 91: 439-448.
53. Kerbel RS (2011) Reappraising antiangiogenic therapy for breast cancer. *Breast* 20: S56-60.
54. Jain RK, Duda DG, Clark JW, Loeffler JS (2006) Lessons from phase III clinical trials on anti-VEGF therapy for cancer. *Nat Clin Pract Oncol* 3: 24-40.
55. Qu B, Guo L, Ma J, Lv Y (2010) Antiangiogenesis therapy might have the unintended effect of promoting tumor metastasis by increasing an alternative circulatory system. *Med Hypotheses* 74: 360-361.
56. Francescone R, Scully S, Bentley B, Yan W, Taylor SL, et al. (2012) Glioblastoma-derived tumor cells induce vasculogenic mimicry through Flk-1 protein activation. *J Biol Chem* 287: 24821-24831.
57. Cao Z, Bao M, Miele L, Sarkar FH, Wang Z, et al. (2013) Tumour vasculogenic mimicry is associated with poor prognosis of human cancer patients: A systemic review and meta-analysis. *Eur J Cancer*.
58. Vartanian AA (2012) Signaling pathways in tumor vasculogenic mimicry. *Biochemistry (Mosc)* 77: 1044-1055.
59. Scully S, Francescone R, Faibish M, Bentley B, Taylor SL, et al. (2012) Transdifferentiation of glioblastoma stem-like cells into mural cells drives vasculogenic mimicry in glioblastomas. *J Neurosci* 32: 12950-12960.
60. Hendrix MJ, Seftor EA, Meltzer PS, Gardner LM, Hess AR, et al. (2001) Expression and functional significance of VE-cadherin in aggressive human melanoma cells: role in vasculogenic mimicry. *Proc Natl Acad Sci U S A* 98: 8018-8023.
61. Folberg R, Maniotis AJ (2004) Vasculogenic mimicry. *APMIS* 112: 508-525.
62. Ping YF, Bian XW (2011) Concise review: Contribution of cancer stem cells to neovascularization. *Stem Cells* 29: 888-894.
63. Chen Y, Jing Z, Luo C, Zhuang M, Xia J, et al. (2012) Vasculogenic mimicry-potential target for glioblastoma therapy: an in vitro and in vivo study. *Med Oncol* 29: 324-331.
64. Vartanian A, Gatsina G, Grigorieva I, Solomko E, Dombrovsky V, et al. (2013) The involvement of Notch signaling in melanoma vasculogenic mimicry. *Clin Exp Med* 13: 201-209.
65. Angers S, Moon RT (2009) Proximal events in Wnt signal transduction. *Nat Rev Mol Cell Biol* 10: 468-477.
66. Sood AK, Fletcher MS, Zahn CM, Gruman LM, Coffin JE, et al. (2002) The clinical significance of tumor cell-lined vasculature in ovarian carcinoma: implications for anti-vasculogenic therapy. *Cancer Biol Ther* 1: 661-664.
67. Conlin AK, Seidman AD (2008) Beyond cytotoxic chemotherapy for the first-line treatment of HER2-negative, hormone-insensitive metastatic breast cancer: current status and future opportunities. *Clin Breast Cancer* 8: 215-223.
68. Elias AD (2010) Triple-negative breast cancer: a short review. *Am J Clin Oncol* 33: 637-645.
69. Corkery B, Crown J, Clynes M, O'Donovan N (2009) Epidermal growth factor receptor as a potential therapeutic target in triple-negative breast cancer. *Ann Oncol* 20: 862-867.
70. Marchiò C, Natrajan R, Shiu KK, Lambros MB, Rodriguez-Pinilla SM, et al. (2008) The genomic profile of HER2-amplified breast cancers: the influence of ER status. *J Pathol* 216: 399-407.
71. Khramtsov AI, Khramtsova GF, Tretiakova M, Huo D, Olopade OI, et al. (2010) Wnt/ β -catenin pathway activation is enriched in basal-like breast cancers and predicts poor outcome. *Am J Pathol* 176: 2911-2920.