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ORIGINAL ARTICLE

Periostin Is Needed For Maintenance of Ovarian Cancer Stem Cells: Role in Carcinogenicity and Metastasis

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ABSTRACT

Background: *As a crucial constituent of tissue microenvironment, extracellular matrix (ECM) proteins can modulate tissue microenvironment and regulate the behavior of the surrounding cells. Periostin is usually absent or expressed in low levels in most adult tissues; however, it is often highly expressed during inflammation, injury and in multiple tumors as recently reported. Alterations in glycosylation regulate the development and progression of cancer, serving as important biomarkers and providing a set of specific targets for therapeutic intervention.* ***Results:*** *In current study, evidence showed that Periostin is overexpressed in ovarian cancer in a tumor type specific glycosylated form that affects Notch levels and activation. Moreover, ovarian cancer cell lines that constantly express glycosylated periostin showed enhanced Notch 1, 3 levels as well as Notch downstream effectors; Hes1, and NRARP. Our experiments in mice xenograft models showed increased carcinogenicity and metastatic potential in tumors produced by cells expressing glycosylated periostin forms.* ***Conclusions:*** *These results indicate that glycosylated periostin overexpression can in turn alter the carcinogenic potential of tumor cancer stem cells (CSC).*

INTRODUCTION

Ovarian cancer is the fifth most common cancer affecting women and the most serious. It is a ubiquitous malignancy that affects women of wide age range starting at reproductive age. It usually arises from epithelial cells in the ovaries or fallopian tubes [(1)]. The most common type of ovarian carcinoma is epithelial ovarian carcinoma (EOC), which accounts for about 90% of all ovarian cancer cases, and is associated with a very high mortality rate. That is a combined effect of the insidious onset of the disease with early nonspecific symptoms plus the lack of sensitive screening tests [(2, 3)]. Glycosylation changes are common to multiple malignancies. Glycans are expressed by most cancer cells, either at abnormal levels or having structure differences from glycans found in normal cells [(4)]. However, its functional effects are still not known. The bisecting GlcNAc is one example which is found in various hybrid and complex N-glycans [(5)]. GnT-III is considered a main glycosyltransferase in N-glycan biosynthetic pathways. It has been reported that this enzyme is associated with multiple biological events such as cell growth and differentiation, cell adhesion, migration, cell, and tumor invasion [(6)].

Cancer research is currently more attracted towards studying the tumor microenvironment (TME). It was shown that tumors need external signals for their maintenance and expansion [(7)]. Therefore, it is needed to have an extensive knowledge of the cross-talk between stromal tumor cells including fibroblasts, macrophages, endothelial cells and adipocytes and their microenvironment. Studying interactions between cancer cells and cancer stem cells constitutes a main contributor to this cross-talk. TME investigations also involve extracellular matrix proteins (ECM), signaling molecules, soluble factors, and other factors needed for tumor growth and tissue invasion, evasion of the immune system, and treatment resistance and recurrence[(8)].

Notch signaling pathway is one of the most critical pathways involved in CSCs and was reported to affect CSCs from different cancer types including melanoma, breast cancer, pancreatic, and lung cancer. Atypical activation of Notch signaling, leading to high levels of Notch-1, Notch-2, accompanied by their ligand Jagged-1, is intensely linked to the CSC phenomenon [(9)].

Multiple studies suggested an important collaboration between Wnt and Notch pathways in breast tumorigenesis [(10)]. Notch signaling has been proved to be a main player in the control of cell fate, progenitor cells during development, maintenance of stem cells and carcinogenesis. Notch also interacts with many other pathways such as NF-κB and STAT3. Dysregulation of Notch signaling can lead to malignant transformation of normal stem cells and/or gaining self-

renewing ability in some progenitor cells during tumorigenesis.

Within the Notch pathway, Notch 3 amplification has been correlated with tumor recurrence, chemoresistance and poor prognosis [11, 12]. In mammals, there are 4 Notch receptors (Notch1–Notch 4) that have discrete tissue expression patterns and are thought to operate in specific cellular contexts [13].

Periostin (POSTN, previously known as osteoblast-specific factor-2 is a 90-kDa secretary protein expressed in the periosteum in bone tissues as well as an extracellular matrix (ECM) protein. POSTN has similar homology to the insect cell adhesion molecule fasciclin I. POSTN belongs to the superfamily of TGF-β-inducible proteins and promotes integrin dependent cell adhesion and motility [14, 15].

POSTN is up-regulated in wide array of cancers, such as colon, breast, pancreatic, gastric, head and neck, thyroid cancer, non-small cell lung cancer and neuroblastoma[16-20]. POSTN mRNA levels has been reported to be upregulated in ovarian tumors [21], which was also correlated with clinical late stage and tumor recurrence [22]. POSTN can be secreted by EOC cells and is abundant in malignant ascites of ovarian cancer patients[22]. Also, recombinant purified POSTN supports the adhesion and migration of ovarian epithelial cancer cells through interacting with the integrin receptors αVβ3 and αVβ5 [23]. Moreover, POSTN also increases tumor angiogenesis and decreases tumor cell apoptosis in EOC [24]. Neutralizing monoclonal antibody against POSTN was inhibited anchorage-independent growth and survival of POSTN-expressing cells and also suppressed cancer cell migration and invasion (25). Periostin physically associates with the Notch1 precursor and maintains the Notch1 protein level under the stress conditions, which in turn up-regulates Bcl-xL expression inhibiting cell death [26].

There has been a strong cocensus that tumors consist of seldom dividing cells named cancer stem cells. Cancer stem cells origins can result from transformed adult stem cells, transformed progenitor cells or may be differentiated cells that have gained stem cell characteristics. They function in a similar manner to adult stem cells and show similar properties [27]. Therefore, they are characterized by their ability to renew and give rise to a progeny with a high proliferative potential leading to asymmetric division, where one daughter cell acquires the characteristics of the parent cell while the other cell divide several times to form the main bulk of the tumor [28].

Similar to normal stem cells, CSCs are known to constitute a small fraction of the total tumor cell mass, which is capable of self-renewal and multipotent differentiation, but instead gives rise to a reproducible tumor phenotype. CSCs gained specific significance regarding drug resistance, relapse, residual disease, and tumor dormancy [29].

METHODOLOGY

Plasmids:

Notch reporter plasmid, 12XCSL-d1EGFP was purchased from (Addgene plasmid # 47684). Periostin expression and mutagenesis; the retroviral periostin expression plasmid has been previously described [23]. PCR-based site-directed mutagenesis was carried out using the QuickChange XL Site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA). The mutagenesis primers replaced the single glycosylation site from an Asn to a Gln. Mutation was confirmed by DNA sequencing. Periostin was purified from cell culture supernatants using anti- FLAG tag resin (Sigma-Aldrich).

Cell culture and transfection assay

The EOC cell lines OVCAR3, SKOV3, and the primary patient cell line OVCA26 (supplemental Fig. 1) were grown in RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% FBS (Gemini Bioproducts, West Sacramento, CA) and 100 mg/mL streptomycin (Invitrogen). The OVCA26 patient derived cell line was produced under an approved IRB protocol by Dr. Martin Cannon at The University of Arkansas for Medical Sciences. The cell lines were maintained at 37oC in a humidified atmosphere with 5% CO2. Lentiviral transduction to establish stable POSTN expression was performed as described previously [4]. Spheroids were generated under stem cell selective media (Stem Cell Technologies, Vancouver, British Columbia, Canada) in ultralow adhesion dishes for 3 weeks. Studies evaluating purified periostin included 3 μg purified periostin supplemented in growth media.

Proliferation assay

CellTiter 96 AQueous One Solution Cell Proliferation Assay was used following manufacturer's instructions. Different numbers of cells were grown in 96 well plates, serum starved for 24 hours. The medium was replaced with medium supplemented with EGF (20 ng/mL) and cells were incubated for 24 hours at 37°C for 48–72 hours in a humidified, 5% CO2 atmosphere. 20μl per well of CellTiter 96AQueous One Solution Reagent CellTiter were added, and absorbance was read at 690 nm.

Real-time reverse transcription-PCR analyses

Total RNAs were isolated from culture cells or spheroids using Trizol reagent (Invitrogen) following manufacturer instructions. The RNA samples were subjected to reverse transcription using Superscript III (Invitrogen). The target and control RNAs were amplified using (SYBR supermix; Promega). Primers used are listed in table S1. The crossing threshold values assessed by the real-time PCR were evaluated for the transcripts and normalized with RPL4 mRNA. The mRNA levels were expressed as fold change relative to control with ± SEM value.

Sphere formation assay

Spherical colony formation assay was performed using CSC Complete Recombinant Medium MammoCult TM Human Medium (Stem cell Technologies, USA). Cells were plated at 103 cells per well in 6-well ultra-low attachment plates (Corning Inc., Corning, NY) and cultured for 14 days. The morphology of the cells was assessed and pictures were taken under a light microscope every day. Round cell clusters larger than 100 mm were counted as spheres.

Tumor Xenografts in mouse model

NOD/SCID female mice were purchased from Jackson Laboratories and housed in pathogen- free conditions. The studies reported here were preapproved and supervised by the Institutional Animal Care and Use Committee (IACUC). Cells (5 × 106) were washed with PBS three times, resuspended in the same buffer, and injected subcutaneously into the dorsal flanks of 12 week old mice. Tumors were measured with calipers twice a week and the volumes were calculated using formula: 0.5 X (length X width2). The tumors were resected 6-8 weeks after cell transplantation and the portions of the tumors were preserved in 5% formalin for immunohistochemistry and stored at -80° C in RNase inhibitors for real-time PCR or directly frozen for Western Blot analyses.

Immunofluorescence

Cells grown on glass cover-slips (VWR) were rinsed in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde in PBS pH 7.4 for 20 min at room temperature, washed twice with ice cold PBS and permeabilized in ice-cold acetone. To stain surface bound proteins, permeabilization step was omitted. Cells were incubated with blocking buffer (10% serum, 0.01 % Triton X-100, in PBS, pH 7.4) for 1 hour, washed with PBS and treated with primary antibodies in PBS-T containing 1% BSA for 1-2 hr. at room temperature or overnight at 4°C. Cover-slips were incubated in the appropriate AlexaFluor conjugated secondary antibodies after washing with PBS-T. The nuclei were counterstained with DAPI (0.1-1 µg/ml PBS). The cover slips were mounted on microscope slides in Vectashield mounting medium (Vector Labs) for detection of Immunofluorescence.

Data Analysis and Statistical Methods

Statistical analyses were done using the parametric unpaired, two-tailed independent sample t- test with 99% confidence intervals.

RESULTS

POSTN is Over-Expressed in Serous Ovarian Adenocarcinoma and Has a Unique Glycoform:

We have observed an abundance of POSTN in membrane fractions isolated from ovarian cancer tissues after 100,000 x g fractionation indicating a strong association with the plasma membrane of ovarian cancer cells.

POSTN mRNA was tested in epithelial ovarian cancer tissue samples. Transcript levels were four to twenty five folds upregulated in five out of ten tissue samples compared to normal ovarian tissue, and was completely absent in normal fallopian tube tissue (Fig. 1A). To examine the glycosylation status of POSTN in ovarian cancer cells, it was overexpressed in different cell lines including OVCAR3, Lec10, and Pro5 cell lines. OVCAR3 is an ovarian cancer cell line while the others are not. Lectin blot of lysates from the 3 cell lines using biotinylated E-PHA showed that only lysates from OVCAR3 cells was bound to E-PHA (Fig. 1B upper panel lane 4) despite all lysates were positive for POSTN (Fig. 1B lower panel). Since E-PHA binds only to bisected glycan structure, then POSTN produced in OVCAR3 cells have the unique bisected glycan, which is the same form present in ovarian cancer tissue.

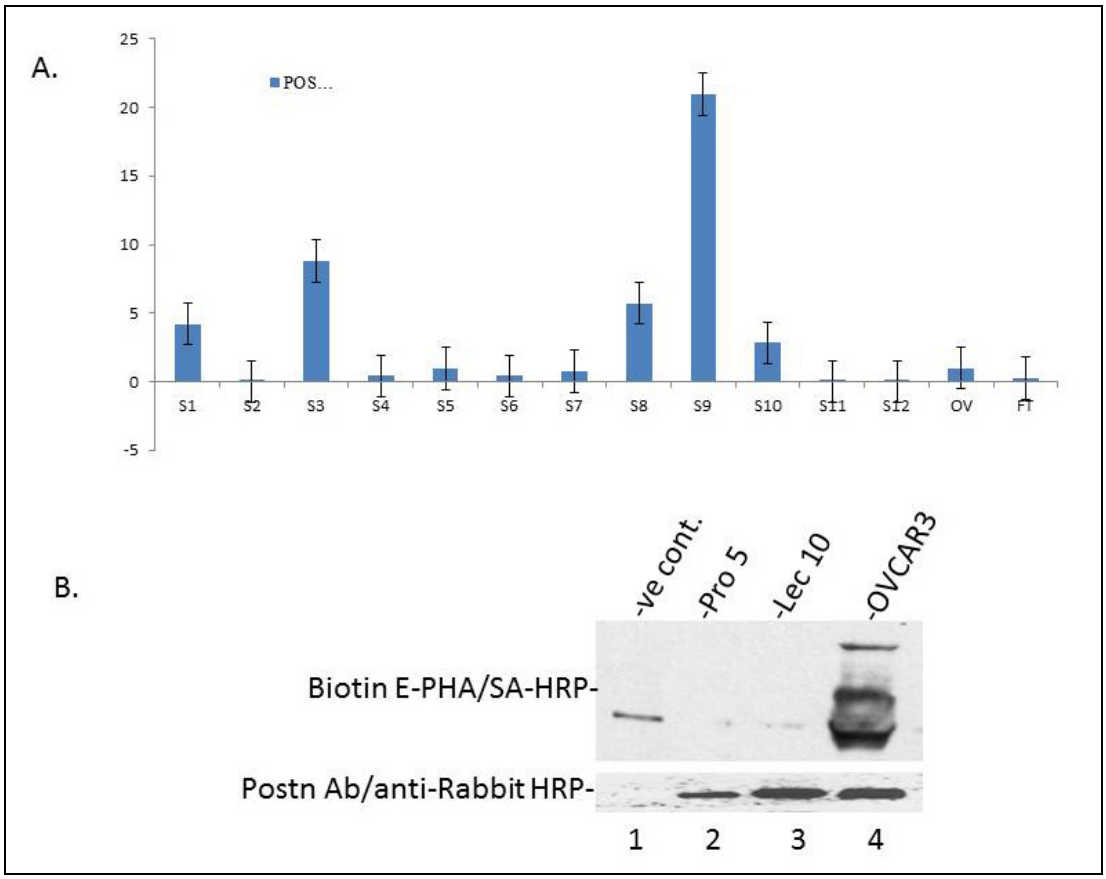

Fig. 1: POSTN is Upregulated in Ovarian Cancer. (A) POSTN expression levels were significantly increased in ovarian cancer tissues from patients compared to normal ovary and fallopian tube. Data are means ± SE, n=3 experiments, P< .0001. (B) Lectin blot and Western blot analysis of purified periostin obtained from the supernatants of OVCAR3 cells, CHO Pro5 cells, and Lec10 cells.

Periostin is expressed by Ovarian Cancer Cells only under Stem Cell Selective Conditions

It was previously reported that Notch1 is first synthesized as a 300-kDa precursor protein and that following the glycosylation step, Notch1 is cleaved by a furin like convertase (site 1 cleavage; S1 cleavage) to form a heterodimer receptor composed of a 230-kDa extracellular domain (N1EC) and a 120-kDa transmembrane domain (N1TM); and then the mature heterodimeric Notch1 is trafficked to the cell surface[13].

Ovarian cancer cell lines grown in 2-D do not express POSTN. However, when grown in stem cell selective media forming spheroids, ovarian cancer cells began to express POSTN (Fig. 2A). Spheroids stain for POSTN (green) while the cells attached to the dish (boxed region) do not (Fig. 2A).

Ovarian Cancer Glycoform of Periostin Increases Notch Levels and Activity

Based on induced expression of POSTN in spheroids, we examined whether expression of POSTN can induce Notch expression in 2-D culture conditions. We chose to use SKOV3 cells for these experiments due to the lower endogenous levels of Notch I expression in these cells compared with OVCAR3 cells. SKOV3 cells stably expressing POSTN induce Notch 1 in a glycosylation-dependent manner (Fig. 2B). Expression of wild-type POSTN with the 6 intact N-linked glycosylation sequon resulted in induced expression of Notch 1 (Fig. 2B lane 2); while expression of a mutant POSTN with the asparagine mutated to glutamine shows no induction of Notch 1 (Fig. 2B lane 3). These results indicate that glycosylation of POSTN is required for induction of Notch 1 in SKOV3 cells.

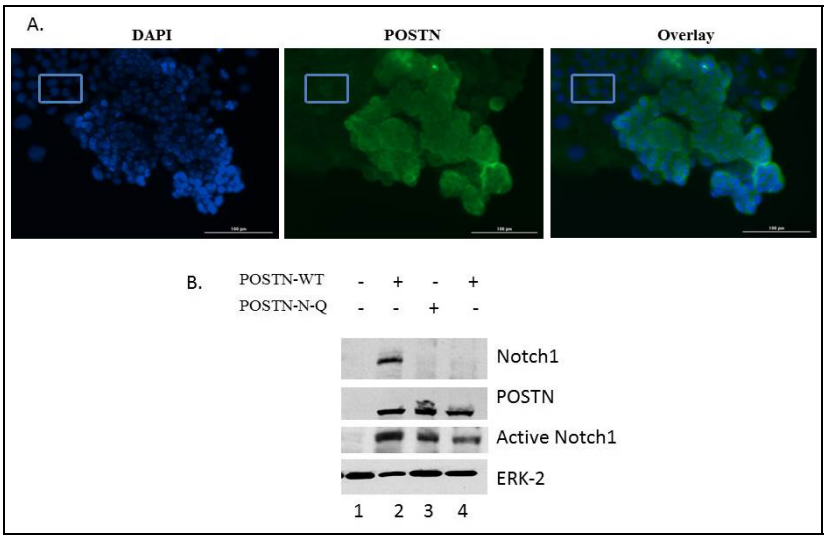

Fig. 2: POSTN is Expressed by Ovarian Cancer Spheroids. (A) Immunofluorescence staining of periostin in OVCAR3 spheroids growing on glass coverslips, bar= 100 µm. Dapi staining highlights adherent and non-adherent cells. Boxed region highlights adherent cells. (B) Western blot analysis of SKOV3 cells grown in 2D cell culture stably expressing POSTN-WT or POSTN- N-Q.

Our data indicates that, The presence of the bisecting glycan, detected by the lectin EPHA, was observed only for POSTN purified from OVCAR3 cells (data not shown), so we analysed Notch1 mRNA transcript levels after treating SKOV3 cells with WT or mutant N-Q POSTN or POSTN purified from different cells with varying states of glycosylation to examine the requirement for the presence of the bisecting glycosylation on periostin. When grown in the absence or presence of different purified POSTN glycoforms, SKOV3 cells showed upregulated Notch1 that was up to 3.5 folds only in case of POSTN purified from OVCAR cells (Fig.3A). We also noticed upregulation of Notch3, and downstream Notch effectors, Hes1 and NRARP. Same results were observed when transcript levels from OVCAR3 cells constantly overexpressing WT POSTN were analyzed (Fig. 3B).

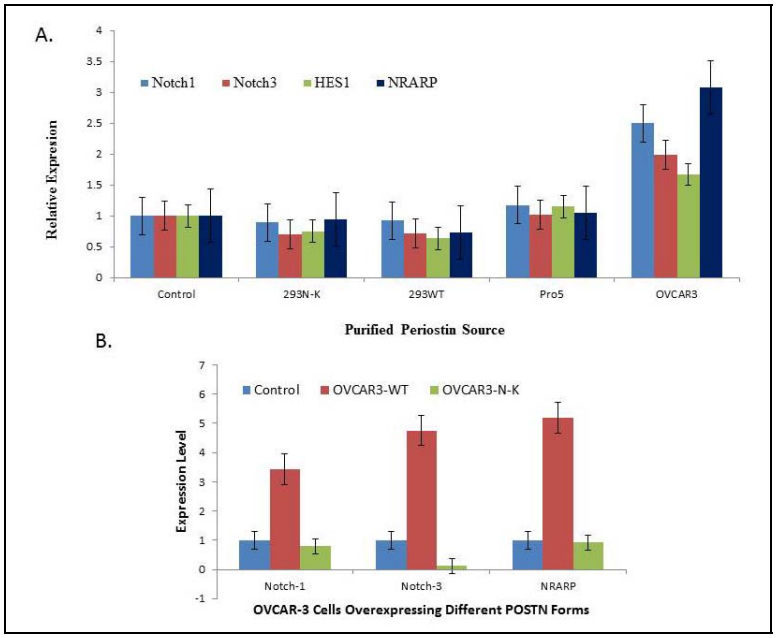

Fig. 3: Effect of WT POSTN Purified From Different Sources on Notch Signaling. (A) SKOV3 cells were grown in 2D culture supplemented without (-) or with POSTN purified from different cell sources for 48 hours. Total RNA was extracted and relative levels of Notch 1, Notch3, Hes1, and NRARP expression normalized to RPL4 is shown. (B) Total RNA extracted from OVCAR3 cells expressing WT or NQ POSTN and levels of Notch1, Notch3, and NRARP are shown. Error bars represent the ± SEM from three different readings.

As reported before, using EDTA stimulates release of the Notch extracellular domain (30). This allows for γ-secretase cleavage of the residual transmembrane fragment, which results in Notch activation. After stimulation with EDTA, OVCAR3 cells expressing WT POSTN and Notch1 reporter construct showed increased reporter GFP fluorescence indicating Notch activation and release of Notch1 (NICD) (Fig.4A) upper panel right side. On the other hand, no NICD cleavage was detected in the POSTN N-Q expressing cells (Fig.4A lower panel Rt.). To confirm these findings, OVCAR3 cells were fixed and immunostained with NICD specific antibody that showed intense fluorescent signal in OVCAR3 POSTN WT cells (fig. 4A upper panel left and middle) but not N-Q cells (Fig 4A lower panel left and middle).

Same results were noticed when another ovarian cancer cell line OVCA-26 cells expressing WT or N-Q POSTN were allowed to form spheroids under stem cell selective conditions for 3 weeks (Fig.4B). The POSTN WT spheroids showed strong immunostaining for Notch1 NICD (Fig. 4B upper panel red) contrary to POSTN N-Q spheroids that showed very less staining (Fig.4B lower panel).

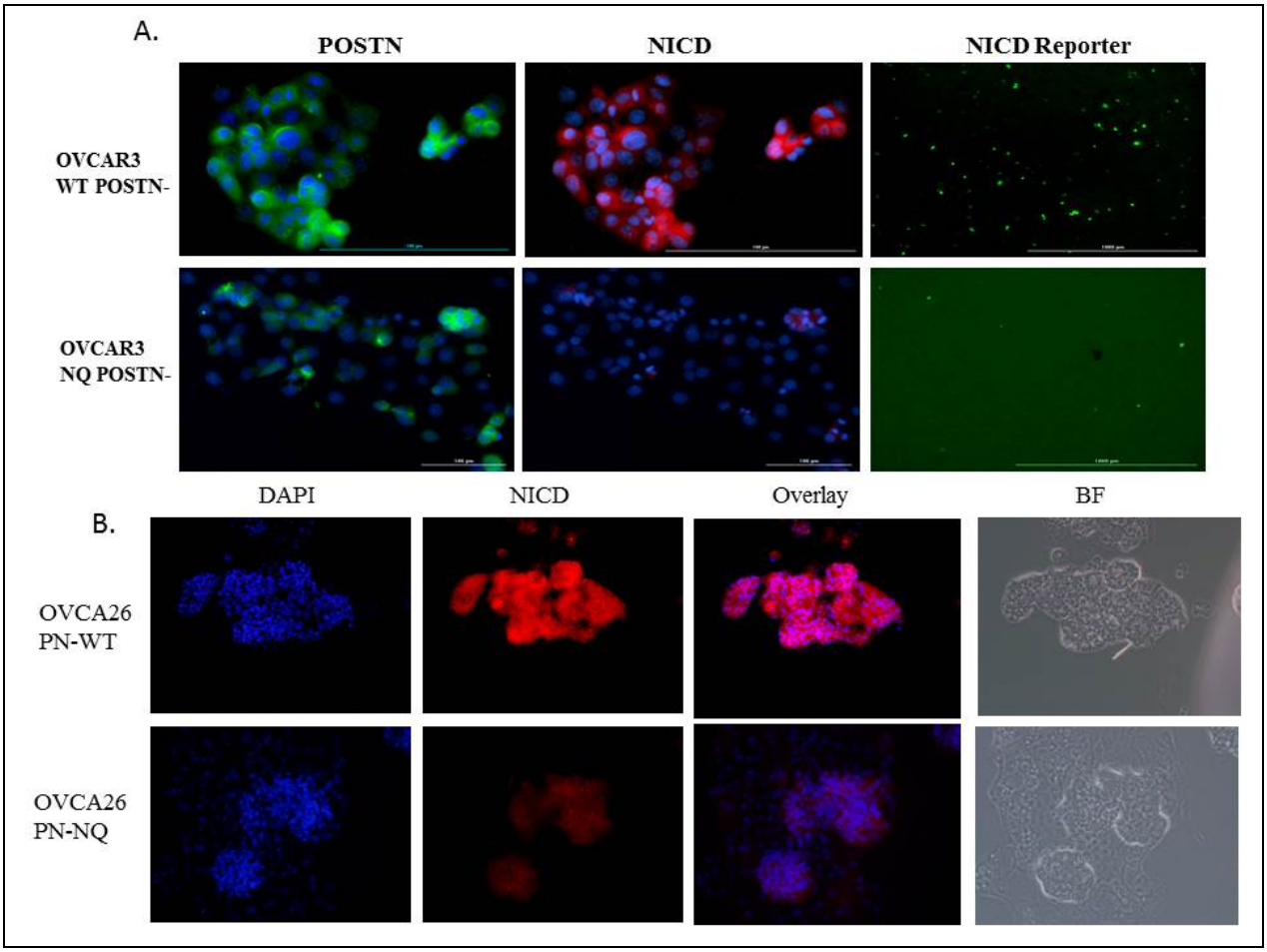

Fig. 4: Notch Activation in OVCAR3 - POSTN WT Cells. (A) Immunofluorescence staining of active NICD in OVCAR3 WT (top) and OVCAR3 NQ (bottom), bar= 100 µm. (B). Immunofluorescence staining of active NICD in OVCA-26WT (top) and OVCA-26NQ spheroids (bottom), bar= 100 µm.

WT POSTN Enhances Cellular Proliferation Under EGF Stimulation:

Epidermal growth factor (EGF) is known to induce proliferation in various kinds of cells. When epidermal growth factor (EGF) was added in different concentrations to OVCAR3 cells producing either WT or mutant POSTN (Fig. 5A), cell proliferation was significantly enhanced in a concentration of 25 ng/ml in the presence of WT POSTN (blue bar). No significant difference was noticed with mutant form (red bar).

Glycosylated POSTN (WT) Increased Carcinogenicity and Metastasis of OVCAR3 Cells in Immune- Deficient Mice:

To examine the effect of Glycosylated POSTN on tumor formation in vivo, OVCAR3 cells expressing WT or N-Q POSTN were evaluated in a xenograft model (Fig.5). WTOVCAR3 cells (5 x 106 cells) displayed robust ($P < 0.05$) tumor-initiating capacity and increased tumor volume compared with mutant expressing cells. 5x106 cells in 100mL PBS were injected subcutaneously into the dorsal area of female NOD/SCID mice. Tumor growth was noticed in 5 out of 5 and 4 out of 5 animals at 6 weeks after injection of WT or N-Q POSTN expressing cells respectively. It was striking that tumors formed by WT cells were bigger, more vascular and with more metastasis to peritoneum than mutant cells tumors (Fig. 5).

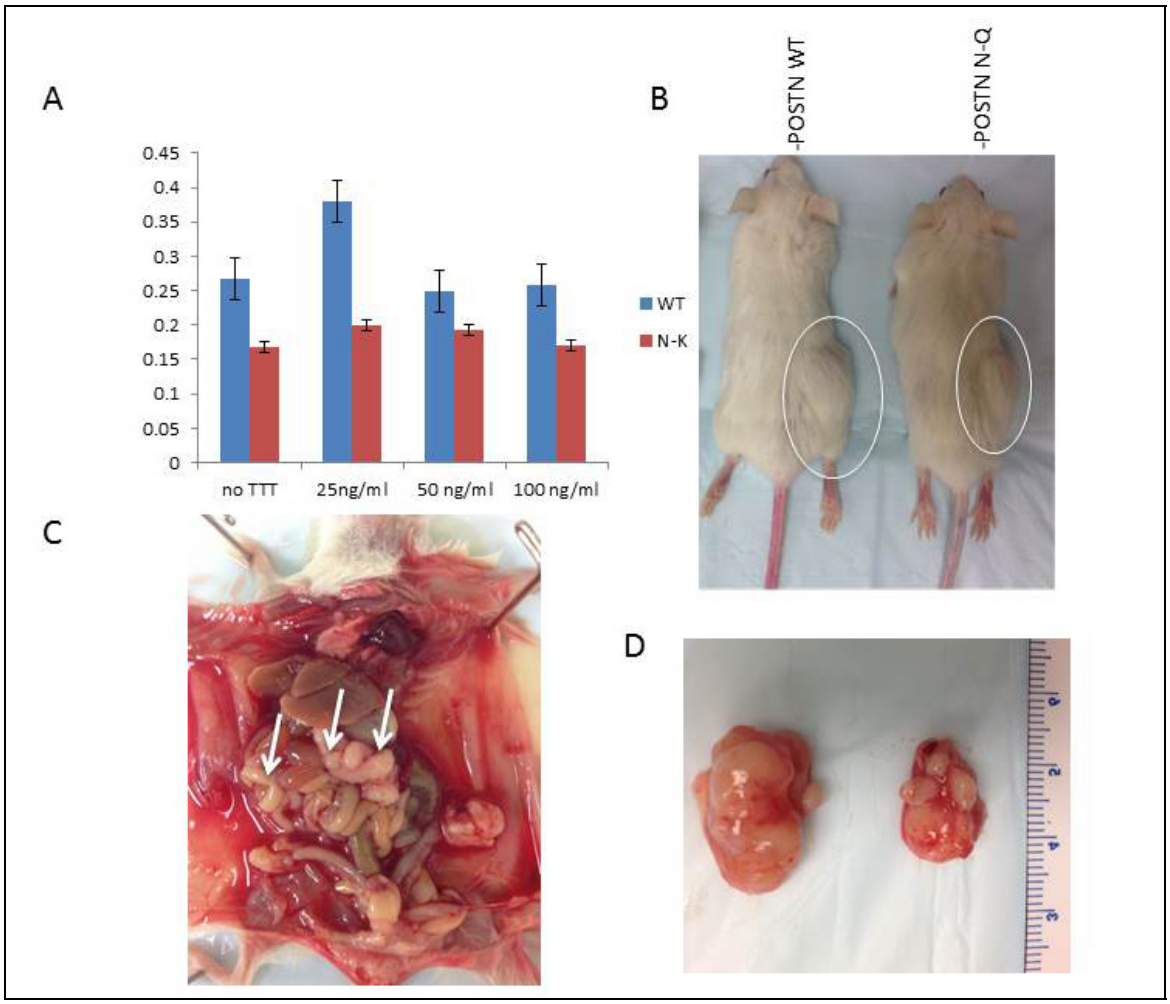

Fig. 5: POSTN Expression Enhances Cellular Proliferation, Tumor Growth and Metastasis. Different concentrations of EGF were added to either WT or NQ POSTN overexpressing cells grown in 2D cultures (A). OVCAR3 stable cells expressing WT or NQ POSTN were injected subcutaneously in the right flanks of NOD/SCID mice. The sizes of selected resected tumors are shown (B, C, D).

DISCUSSION

Tumor stem cells have been proved to be the source of most cancers in addition to metastasis, drug resistance, and tumor recurrence [(28)]. Reported recently, periostin was described as a cofactor used by cancer stem cell to facilitate metastasis [(20)].

Periostin is reported to be part of multiple processes in carcinogenesis including, tumor epithelial Mesenchymal Transition (EMT), extracellular matrix degradation, tumor invasion and distant metastasis, but the mechanism by which it is involved is still unclear. Periostin also regulates E-cadherin in a cell-type-dependent way. The induction of EMT is mediated by the N-terminal region [(20)]. Identified by Abbott et al., Specific N-linked glycan structures were found on the glycoprotein periostin in ovarian cancer tissue samples and patients serum [(31)]. The role of PN in angiogenesis, metastasis, and its main function as an ECM protein, make it a potential target for ovarian cancer treatment.

Our discovery that bisecting glycosylation present on the non-canonical Notch ligand, POSTN, is involved in the induction of Notch expression is novel (Fig. 4B and 4C). We report for the first time that periostin is induced by non-adherent cell growth in ovarian cancer cells and induces Notch expression in a glycosylation-dependent manner (Fig. 2A and 2B). There has been a reported connection of periostin with Notch 1 in breast cancer ErbB2/Neu/HER2 driven tumors[(20)]. Researchers found that tumors derived from periostin null mice had lower levels of Notch 1 and lower levels of the downstream effector Hey1. Our data indicates that the bisecting glycoform of periostin is an important factor for the induction of Notch in ovarian cancer cells. Glycosylation may be forming crucial molecular interactions that either stabilize Notch receptor or promote signaling pathways that contribute to Notch induction.

In the study done by Li Z. Et al. 2015, it was found that POSTN over-expression in colorectal cancers positively correlated with tumor size, differentiation, lymph node metastasis, serosal invasion, CSC ratio, clinical stage and five-year survival rates [(18)]. Our results showed that 50% of EOC patient samples had high POSTN expression (Fig.1). In addition when ovarian cancer was recapitulated in nude mice using OVCAR-3 cells expressing WT POSTN, the resulting tumors were bigger and more vascular (Fig.5).

It was demonstrated that periostin in the stroma is indispensible for metastatic seeding as it regulates the interactions between cancer stem cells and their metastatic niche [(32)]. Since periostin mediates the crosstalk between cancer stem cells and their niche, it is required for maintaining cancer stem cells. Therefore, blocking periostin can prevent metastasis.

The Notch signaling pathway is important for the activation and maintenance of stem cell progenitors in normal tissue development. This pathway is abnormally activated in ovarian cancers with recent studies supporting a role for Notch in ovarian cancer progression and chemoresistance (10, 33). We present data showing that glycosylated POSTN expression (Fig.2) controls Notch receptor levels and can induce Notch activation (Fig. 4). Our data demonstrates that POSTN mutant form leads to lower levels of NICD than WT.

As we showed that periostin is expressed in OVCAR cells grown into spheroids, OVCA-26 spheroids also where able to produce POSTN under stem cell selective conditions (Fig. 4B green) and we were able to demonstrate that its presence enhanced Notch cleavage and release of NICD (Fig. 4 B red). Cells expressing WT POSTN demonstrated higher potential for Notch activation than mutant expressing cells (Fig. 4) Hence, periostin may be a potential cancer treatment target.

Currently, the expression status of periostin protein in ovarian cancer and its relationship to the biological behavior of the disease are still unclear. Our results suggest that monitoring the levels specific glycoproteins such as POSTN that carry bisecting glycans, may be useful for predicting chemoresistance or monitoring for disease recurrence in patients.

In conclusion, tumor specific glycosylation of periostin is crucial to create a CSC-supportive niche and promotes metastatic colonization in ovarian cancer. Periostin influences Notch pathway through its unique cancer specific glycan structure. Previous studies found that periostin was highly expressed in CSC cells. Therefore, it could be a potential biomarker for metastasis and chemotherapy resistance of ovarian cancer tumors. However, the underlying genetic mechanism of periostin in ovarian cancer CSC is still unclear, and needs further investigation. Our results provide important insights into the regulation of Notch signaling epithelial ovarian cancer, and into periostin role as a regulator of Notch expression and activation in ovarian cancer microenvironment. Further work is still needed to decipher the molecular mechanisms involved.

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