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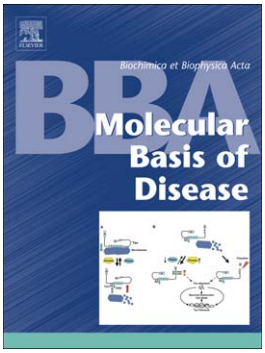

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TENASCIN C INTERACTS WITH ECTO-5'-NUCLEOTIDASE (eN) AND REGULATES ADENOSINE GENERATION IN CANCER CELLS

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Running Title: Imteraction of tenascin C with ecto-5'-nucleotidase

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Key words: Ecto-5'-nucleotidase, CD73, adenosine, breast cancer, extracellular matrix, tenascin C, cell adhesion, cell motility, invasion

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Abstract

Tenascin C is expressed in invasive human solid tumors; however its specific role in cancer biology remains obscure. Previously, we have found that ecto-5'-nucleotidase (eN) is a marker of ER (-) breast carcinoma and elevated expression correlates with invasive mesenchymal cell phenotype. To investigate for the potential relationship between eN and protein components of the extracellular matrix (ECM) we measured adenosine generation from AMP in cells incubated with soluble ECM proteins. We found that tenascin C was the only ECM component that strongly inhibited ecto-5'-nucleotidase (eN) activity *in situ* and adenosine generation from AMP (75% inhibition, $p<0.01$). The inhibition was comparable to that induced by concanavalin A, a well-defined and strong inhibitor of eN. Resin immobilized tenascin C, but not collagen, and only weakly fibronectin, specifically and quantitatively bound cell-extracted eN. We further developed breast cancer cell line with reduced eN expression and tested changes in cell adhesion on different ECM. Breast cancer cells expressing reduced eN attached 56% weaker ($p<0.05$) to immobilized tenascin C. This difference was not detected with other ECM proteins. Finally, control breast cancer cells migrated slower on tenascin C when compared with clone with reduced eN expression. These data suggest that eN is a novel and specific receptor for tenascin C and that the interaction between these proteins may influence cell adhesion and migration and also lead to decreased generation of local adenosine.

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Introduction

Extracellular matrix (ECM) has been established as a major factor regulating epithelial cell behavior and epithelial-mesenchymal interactions [1]. These regulatory functions may be critical in many physiological situations, for example during development and in pathological circumstances such as wound healing and cancer progression. Tenascin C belongs to non-structural ECM proteins that are believed to regulate cell adhesion and migration and to have signaling altering functions [2, 3]. For example tenascin C has shown strong anti-adhesive properties and counteracted cell adhesion to fibronectin by disrupting focal adhesion sites. Cells spread on tenascin C lack focal adhesions and actin stress fibers and instead form substratum contacts with radial actin microspikes [4, 5]. Tenascin C has been reported to bind (mostly through its FnIII domain) integrins, proteoglycans, cell adhesion molecules of immunoglobulin family and other ECM proteins and these complex interactions affected both Rho-mediated and Wnt signaling pathways [6].

Ecto-5'-nucleotidase (eN), an enzyme residing in lipid rafts, was previously proposed to directly interact with laminin and fibronectin in chicken gizzard [7, 8]. These studies suggested that eN, in addition to its adenosine generating functions, may participate in cell adhesion. We have demonstrated previously that eN and its enzymatic product adenosine are upregulated during breast cancer progression [9]. We have also shown that Wnt signaling pathway, which is frequently activated in epithelial tumors, activated eN expression and increased adenosine generation [10]. Given the known effect of adenosine in cell adhesion and motility [11-13] and previously described direct adhesive properties of eN, we set out to investigate whether eN may be involved in cell

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adhesion in metastatic breast cancer cells. In this communication we report that in human breast cancer cells eN strongly and specifically interacts with tenascin C and this interaction leads to (1) decreased generation of adenosine, (2) increased adhesion and, (3) decreased migration on tenascin C.

Methods

Reagents. Anti-eN rabbit polyclonal antibodies were described previously [14]. Anti-integrin ß1 I41720, integrin α5 I55220 antibodies were from Transduction Laboratories (Lexington, KY), anti-CD44s 13-5500 antibody was from Zymed (San Francisco, CA), anti-GFP antibodies were from Clontech BD Biosciences, (Mountain View, CA) and anti-β-actin antibodies were from Oncogene (Boston, MA). Human ECM proteins tenscin-c, fibronectin, vitronectin and laminin were from Chemicon (Temecula, CA), hyaluronic acid and ConA were from Sigma-Aldrich (St Louis, MO) and Matrigel and collagen IV were from BD Biosciences (Franklin Lakes, NJ). Cell culture media and FBS were from Gibco Invitrogen, Carlsbad, CA.

Measurements of extracellular adenosine generation from AMP (*in situ* assay). Breast cancer MDA-MB-231 cells and its derivatives (low eN and GFP-expressing clones) were plated at 750 to 1000 cells per well in 96 wells flat bottom plates. Twenty four hours after plating medium was changed to 80 µl of serum-free Opti-MEM (Gibco Invitrogen, Carlsbad, CA) with specific soluble ECM protein at 25 µg/ml (ConA at 5 mg/ml) and cells were preincubated for additional 30 min. Reaction was started by the addition of a mixture of {8-^{14}C} AMP (final concentration 100 µM, 3x10^5 cpm/well) and

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dipyridamole (inhibitor of adenosine uptake, final concentration 10 μM), in a final total volume of 200 μl per well. Samples (25 μl each) were withdrawn at 0, 30 and 60 min and analyzed for radiolabeled adenosine by TLC on a Kodachrome microcrystalline cellulose plates with fluorescent indicator. Adenosine and inosine spots were cut out and quantitated in scintillation counter as described before [10]. Results from 30 and 60 min time points were used to calculate average values. No radioactive IMP or other nucleoside degradation products were detected in significant amount (more than 3% of adenosine, as resolved on PEI-cellulose) in preliminary experiments. Rates of adenosine generation were linear within the times specified and the maximal amount of dephosphorylated AMP never exceeded 20%.

Cell adhesion assay. Cells were plated on 96-well plates (density 10^5 cells per well) pre-coated overnight (16 hrs) with 50 μg/ml (200 μl) of ECM protein in PBS at RT. Plates were transferred to 37^{o}C for 30 min pre-incubation and cells were allowed to adhere for 30 minutes in serum-free MEM medium at 37^{o}C. After incubation plates were washed 3 times with PBS to remove nonattached cells and adherent cells were quantitated with mitochondrial dye WST-1 supplied ready to use (1 mg/ml) from Roche Diagnostics (Indianapolis, IN). Absorbance was measured within 30 min at 450nm. Each experiment was done in duplicates and repeated 3 to 4 times.

Cell lines and development of eN-depleted and GFP-expressing MDA-MB-231 cell clones. Breast cancer ER negative MDA-MB-231 cells were obtained from Tissue Culture Facility at LCCC/UNC and originated from ATCC. Cells were maintained in

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MEM supplemented with Eagle salts, NaPyr, nonessential amino acids and 10% FBS as described before (Spychala, 2005)

To downregulate the expression of eN in MDA-MB-231 cells we have employed retrovirally mediated expression of anti-sense cDNA with pLNCX2 retroviral vector system from Clontech BD Biosciences, Mountain View, CA. Green Fluorescent Protein (EGFP) (Clontech) was used in control cells (pEGFP-N1 vector). We have generated the 1754 bp eN cDNA fragment using PCR with primers containing cloning sites SalI and XhoI. This fragment (1754 bp) started at 4^{th} base of the coding region and span over remaining of the eN coding and small part of the 5'-untranslated regions, and included added ATG site in reverse orientation at the 3' end. This cDNA was cloned into pLNCX2 vector in reverse orientation and used to transfect PT67 cells (supplied with the transfection kit from Clontech BD Biosciences) to produce viral particles. PT67 cell transfections were performed using CalPhos Mammalian Transfection Kit according to the manufacturer protocol. Supernatants from transfected PT67 cells 48 hours postransfection (containing viral particles) were used to transfect MDA-MB-231 grown at 15-20% confluency. Transfected MDA-MB-231 cells were selected with increasing concentrations of G418 (from 0.2 to 1.5 mg/ml) starting 24 hours post-transfection. Selected polyclonal cell population was further subcloned by limiting dilution and several clones for each transfection were expanded and tested for the expression of eN and GFP. Selected cell stocks were stored in liquid nitrogen and each sample was kept in culture for no more than 15 passages.

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Resin immobilization of ECM proteins and column chromatography of cell extracts. One milliliter of packed activated resin for each ECM protein from Aminolink Kit (Pierce, Rockford, Il) was prepared as described in manufacturers protocol and coupled with 1 mg each of tenascin C, fibronectin and collagen IV (each pre-dialyzed against PBS over night at 4^{o}C). Less then 10% of protein was left uncoupled. After coupling resins were placed in small columns (3 ml volume) and washed with PBS supplemented with 1 mM $ZnCl_2$ and $MgCl_2$. Cells extracts for chromatography were prepared using the following procedure. Harvested MDA-MB-231 cell pellets (collected from 10 225cm^2 culture dishes at 60-75% confluency, total 7.5x10^8 cells) were extracted with 25 mM Tris, pH 7.5 and 1% Triton X-100 in Dounce homogenizer, diluted 4 times with PBS supplemented with 1 mM $ZnCl_2$ and $MgCl_2$ and incubated 1 hr at room temperature with 500 units of phosphatidylinositol-specific phospholipase C (Sigma, St Louis, MO). Following incubation crude extracts were centrifuged at 10,000g for 10 minutes and clear supernatants were divided into 3 equal portions and applied on each column at 25 mg of total extract protein in 10 ml volume per each column at RT. Columns were washed with PBS supplemented with 1 mM $ZnCl_2$ and $MgCl_2$, and step-wise elution was performed with increasing concentrations of NaCl supplemented with 1 mM $ZnCl_2$ and $MgCl_2$. Ten 1 ml fractions were collected per each elution condition. Protein amount was determined in each fraction using Bradford reagent and peak fractions (in each case there were only 2 peak fractions representing total protein eluted under each condition) were combined. Twenty five μl samples were withdrawn for eN protein detection by Western blot. Thus, the procedure described above allows for direct comparison of immunoblot band intensities between samples from different columns.

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Western blot analysis. Harvested cells were extracted with 20 mM Tris, pH 7.4 and 0.2 mM PMSF by triple thaw/freeze (37^{o}C/-70^{o}C) and protein concentration in whole homogenates was assayed by Bradford method. Samples of 20 ug were loaded into each lane and separated in 10% polyacrylamide gel according to Laemmli procedure (SDS-PAGE). Resolved proteins were subsequently transferred onto Immobilon-P membrane (Millipore, Bedford, MA). Membranes were blocked either in PBS (pH 7.4) or in TBS (pH 8.4), both supplemented with 5% Carnation nonfat dry milk and 0.1 % Tween-20. Secondary antibody was conjugated with horse radish peroxidase and chemiluminescence developed with BM chemiluminescence western blotting kit (Roche, Indianapolis, IN) and detected on Kodak (Rochester, NY) X-Mat Blue XB-1 film.

Real time video tracking of cell movements. MDA-MB-231 control (GFP clone H2) and eN-depleted (eN$^{(-)}$ clone 12-1) cells were plated 2.5 hour before experiment in suspension cell culture dishes pre-coated with Tenascin or no coating (plastic). After 2.5 hour they were transferred to a semi-closed perfusion micro-incubator (Harvard Apparatus) placed on stage of the Olympus IX81 inverted microscope. The temperature of the chamber was kept at 37 ^{0}C and CO_2 flow was maintained over the cell culture dish. Microscope was equipped with Hamamatsu 4880 cooled CCD camera. Sequential time lapse images were taken every 5 minutes with 20x Ph1 objective for 3 hours. Time lapse sequences were then stored and analyzed off-line using Metamorph software package (Universal Imaging). Motility of every cell was determined by manual tracking of the cell position through time lapse sequence and automatic calculation of the traveled distance

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was performed by standard Metamorph routines. Tracking data were then transferred to Excel spreadsheet where the final numerical analysis was performed.

Results

Ecto-5'-nucleotidase is the main source of adenosine in epithelial cells [15] and we have tested the effect of soluble tenascin C, laminin, collagen IV, vitronectin, hyaluronic acid and matrigel (all at 25 μg/ml) on the adenosine generation from AMP by attached MDA-MB-231 breast cancer cells. Data shown in Fig. 1 demonstrate that soluble tenascin C inhibited the adenosine generation from AMP by ecto-5'-nucleotidase by 75.8 % ($p<0.01$, $n=3$). This enzyme is the major adenosine producing activity in breast cancer cells and, accordingly, Concanavalin A (ConA), a specific eN inhibitor decreased adenosine production to a similar extent. Other ECM proteins either did not significantly affect eN activity, or as in the case of vitronectin and fibronectin, inhibited adenosine generation only weakly, although in the case of vitronectin the inhibition did reach the significance of $p<0.05$ ($n=3$, ANOVA t-test).

To further evaluate whether there is a direct and specific interaction between eN and tenascin C and possibly other ECM proteins, we have immobilized tenascin C, fibronectin and collagen IV on resin to investigate whether solubilized eN will be retained in the column in the ECM protein-specific manner. For this purpose we used eN solubilized with phosphatidylinositol-specific phospholipase C (PLC) from MDA-MB-231 cell extracts. As shown in Fig. 2, when each column was loaded with equal amount of eN, only the column with immobilized tenascin C retained most of applied eN (as determined by Western blot). Much smaller amount of eN was retained with immobilized

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fibronectin and no retention was observed with collagen IV. Furthermore, increasing concentrations of NaCl eluted column-bound eN in higher amounts and at higher NaCl concentrations from tenascin C column than from fibronectin column.

To investigate the interaction of eN with tenascin C and its potential significance in cell adhesion and migration we have developed a derivative of MDA-MB-231 cells with decreased expression of eN, achieved with stable expression of anti-sense eN cDNA. As shown in Fig. 3 two clones with anti-sense cDNA and GFP sense (control cells) each were selected and tested for expression of several proteins. Both eN anti-sense clones had eN expression reduced by over 90% as determined by enzymatic activity assay (see legend for Fig. 3). Representative membrane proteins that are important in cell adhesion, such as CD44, integrins $\beta 1$ and $\alpha 5$ were not significantly affected. These cells were subsequently used to compare the adhesion of cells to different substrata. Data shown in Fig. 4 demonstrate that while the low eN expression did not have significant effect on adhesion to plastic, collagen IV, laminin, fibronectin and vitronectin, the adhesion on tenascin C was reduced by approximately 50% ($p<0.05$, $n=4$) in cells with low eN expression.

MDA-MB-231 cells were previously shown to migrate on several substrata and we hypothesize that altered adhesion on tenascin C may influence cell motility of $eN^{(-)}$ clone. We have used video tracking technique to test the migration velocity. Data shown in Fig. 5 demonstrate that downregulation of eN caused increase in cell mobility on tenascin C. However, the cell mobility increase did not reach the significance of $p<0.05$.

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Discussion

Tenascin C is highly expressed during development and in tumor stroma and its expression is negatively correlated with the survival prognosis of patients with glioma, breast and gastric tumors [6, 16]. It has been detected in many other solid tumors and in sarcomas, however it's relation to tumor invasiveness is not fully understood. Although initial findings suggested that tenascin C had anti-adhesive properties in the context of other ECM proteins such as fibronectin, it is now recognized that these interactions may be cell-type specific. An example that illustrate this cell-specific behavior has been presented [17] and shows that when plated on patterned ECM substrates, sensory neuritis easily crossed borders between laminin and tenascin C, however satellite cells stopped migrating and lined up at borders to tenascin C. Other reports also demonstrate that tenascin C may have both pro-migratory and anti-migratory properties, depending on cell type and ECM composition. Such complex behavior suggests that there are other tenascin C receptors and/or interacting proteins expressed on cell surface that may regulate migration and invasiveness in a cell-specific manner.

We propose that ecto-5'-nucleotidase (eN) is a novel receptor for tenascin C. Data presented in Figures 1 and 2 demonstrate that there is highly specific interaction of eN with tenascin C and this interaction influences cell adhesion, migration (Figures 4 and 5) and adenosine generation. Previously, chicken gizzard ecto-5'-nucleotidase was shown to interact with laminin and fibronectin [7, 8], however our data show that in the case of human eN there was relatively weak interaction with fibronectin and no interaction with laminin. The interaction of avian eN with tenascin C has not been investigated. Among 7 ECM proteins tested in this work, only tenascin C potently inhibited eN enzymatic

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activity. The strong interaction of eN with tenascin C, as determined by column chromatography, and the inhibition of eN enzymatic activity by soluble tenascin C, both support the physiological significance of this interaction. In addition, altered generation of adenosine, a known regulator of adhesion and migration, further implicate adenosine as an additional effector of this interaction. Adenosine was shown, depending on the adenosine receptor subtype, to exhibit anti-adhesive properties [11-13]. One may thus propose that upon eN – tenascin C interaction, decreased local generation of adenosine may further enhance the adhesive properties of tenascin C toward cells with high eN expression. In simple terms, such interaction would actually decrease metastatic behavior of cells expressing high eN, however this notion will require further investigation. However, our results may potentially help reconcile previous conflicting findings showing that in human cancer tenascin C correlated with both poor and favorable clinical prognosis, depending on the tumor type [16]. One may speculate that metastatic cells that do not express specific tenascin C receptors (such as eN) may have enhanced motility due to it's mostly anti-adhesive properties. Conversely, cells with high eN or other tenascin C receptors would have impaired motility in the presence of extracellular tenascin C. The *in vivo* scenario is likely to be much more complex due to the unknown source of extracellular tenascin C in each particular setting. Is tenascin C produced by the same cells as is eN or it may be discharged by other type of cells and in this way impair the migration of eN expressing cells? Or is there a potential for self regulation of eN by cells producing their own tenascin C?

It is not yet clear if tenascin C and eN expression correlate during development and in tumorigenesis. Our previous studies demonstrate that Wnt signaling, which has a

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role in inducing the expression of tenascin C [18], may also control eN expression [10], suggesting that there may be a particular circumstance for the co-expression of these two proteins. Furthermore, in a broad panel of breast cancer cell lines eN expression correlated with a mesenchymal expression profile that was coordinately altered by inhibition of histone deacetylases [19]. These data suggest that eN, along with vimentin and other mesenchymal proteins, potentially including tenascin c, may be coordinately regulated during development and in tumorigenesis. However, the co-expression of these proteins in tumor samples and any functional links between them *in vivo* have yet to be established.

eN may be directly involved in tumor cell invasion through production of adenosine from extracellular AMP or through protein-protein interactions with other cell surface or ECM proteins. Very recent reports on overexpression of eN in T-47D breast cancer cells indicate that adenosine generated from eN may be a positive factor in motility and invasion [20]. This interesting observation could be collaborated by earlier findings on the role of adenosine receptors in adhesion (reviewed in [21]). However, the eN inhibitor AMPCP used in this study appears to have additional effects on purinergic receptors and on nucleotide metabolism [15], that may independently affect cell behavior and, thus, the enzymatic role of eN in breast cancer invasion will have to be independently confirmed. On the other hand, direct engagement of eN using either specific monoclonal antibodies or ConA, a specific eN inhibitor, have been shown to exert profound effects on T-cell activation [22]. These observations opened up the possibility that a specific eN interacting protein(s) may trigger a intracellular signaling cascade. However, such interacting protein has not yet been identified. Although our

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present study suggests that tenascin C may be such eN interacting partner, the signaling properties of such interaction, if any, will need to be addressed in future studies.

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Figure legends

Figure 1. Effect of soluble extracellular matrix proteins on adenosine generation from AMP in MDA-MB-231 cells. The amounts of generated adenosine and inosine from AMP were representative of eN *in situ* activity and compared between different ECM proteins added. Control cells did not contain any added protein. Conversion rates of AMP to adenosine/inosine were calculated and compared with the rate in control wells (1195±322 nmoles /hr/10^6 cells, relative 100%). Data represent mean, ± SD from 3-4 experiments. Statistical analysis was performed using ANOVA t-test.

Figure 2. Western blots of affinity column eluates. Wash represents volume collected during extract application and subsequent wash with PBS supplemented with 1 mM $ZnCl_2$ and $MgCl_2$, and concentrated to 10 ml for direct comparison with extract before application. For other samples protein containing fractions were combined and used directly for SDS-PAGE.

Figure 3. The expression of eN and GFP and three representative adhesion proteins in wild type and stably selected MDA-MB-231 cell clones after transfection with sense GFP and anti-sense eN cDNA and. The adenosine generation rates from AMP in a *in situ* assay in obtained clones were: 1) w/t 1302+409; 2) GFP 4F 1049+389; 3) GFP 2H 1245+420; 4) $eN^{(-)}$ 12-1 92+44; 5) $eN^{(-)}$ 6H 128+58 nmoles/hr/10^6 cells.

Figure 4. Adhesion assay of control (GFP-expressing) and eN-depleted ($eN^{(-)}$ 6H) MDA-MB-231 clones on 96 well plates pre-coated with ECM proteins. Readings of absorbance at 450 nm were taken to calculate the mean absorbance values. Data represent mean, ± SD from 3-4 experiments. Statistical analysis was performed using ANOVA t-test.

Figure 5. The migration of control (GFP-2H) and eN-depleted MDA-MB-231 clones on plates pre-coated with tenascin C. Data represent mean, ± SD from 3 experiments.

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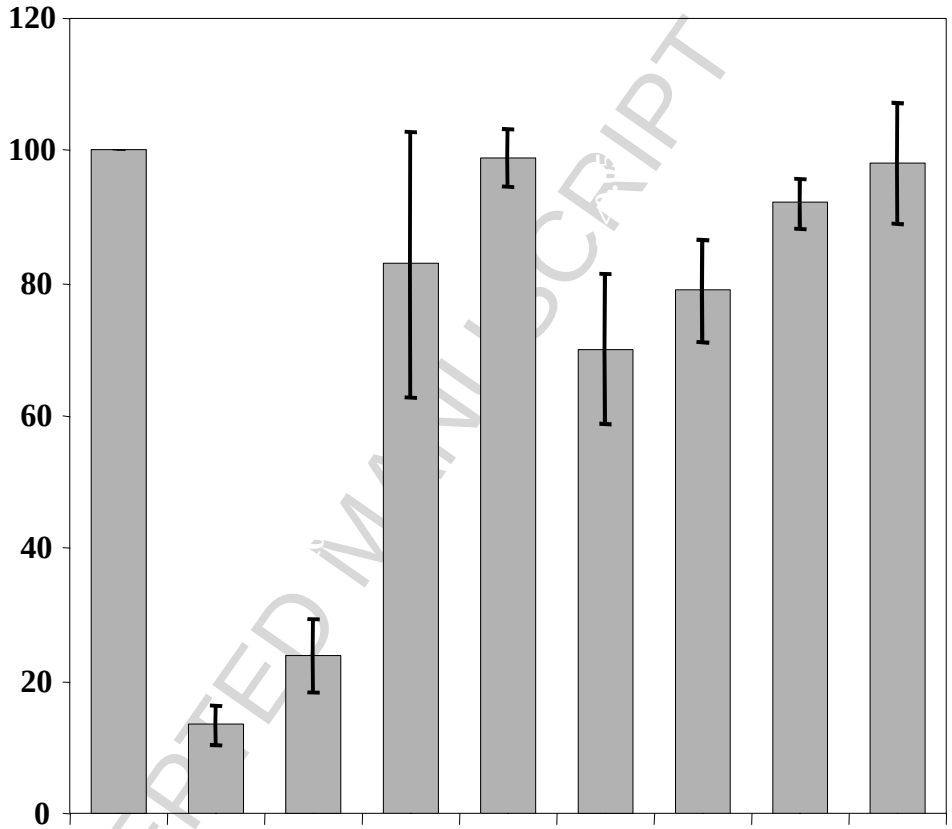

Fig. 1. Sadej et al.

ACCEPTED MANUSCRIPT

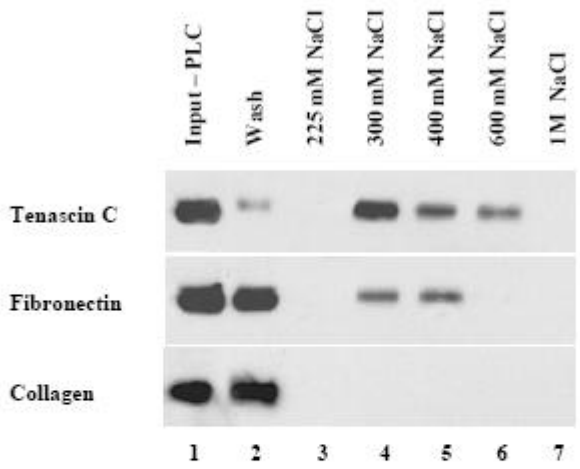

Fig. 2. Sadej et al.

ACCEPTED MANUSCRIPT

Figure 3

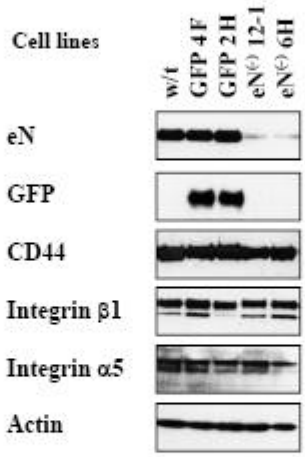

Fig. 3. Sadej et al.

ACCEPTED MANUSCRIPT

Figure 4

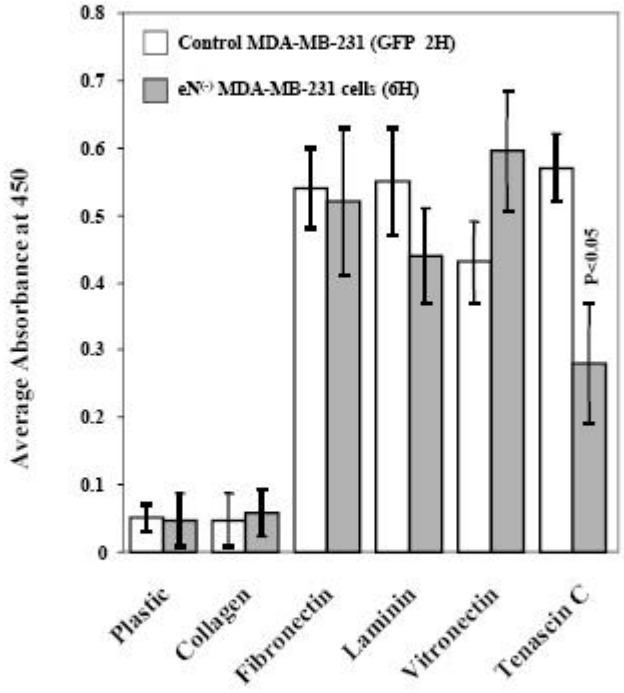

Fig. 4. Sadej et al.

ACCEPTED MANUSCRIPT

Figure 5

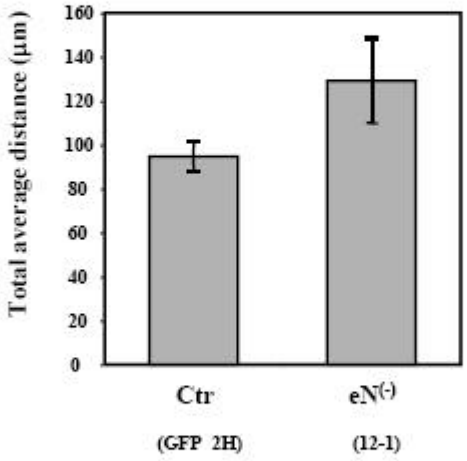

Fig. 5. Sadej et al.