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Dual, enzymatic and non-enzymatic, function of ecto-5′-nucleotidase (eN, CD73) in migration and invasion of A375 melanoma cells

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Ecto-5′-nucleotidase (eN, CD73) mediates extracellular adenosine production from 5′-AMP. Non-enzymatic functions of eN have also been reported. The aim of the study was to investigate the role of ecto-5′-nucleotidase in aggressive melanoma behaviour. Analysis of the involvement of eN in adhesion, migration and invasion revealed eN functions unknown to date. We found that following eN blockade by concanavalin A, the strength of adhesion to different ECM proteins was not altered, but at the same time the invasion ability of the cells was decreased. On the other hand, knocking down eN in melanoma cells did not influence cell invasion but abolished their migration on tenascin C (TnC). Ecto-5′-nucleotidase seems to fulfil a more distinct role as a receptor than as an enzyme in the cell interaction and mobility on TnC. Ecto-5′-nucleotidase activates also focal adhesion kinase and enhances the formation of complexes upon cell adhesion to TnC. All these observations prove that an eN-TnC complex is involved in cell migration and invasion and thus in the regulation of melanoma progression.

Key words: ecto-5′-nucleotidase; tenascin C; melanoma; migration; invasion

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INTRODUCTION

Melanoma accounts for less than 5% of skin cancer cases but is the cause of a large majority of skin cancer deaths. The American Cancer Society's most recent estimate for melanoma in the U.S.A. states that about 76 000 new cases will be diagnosed in 2012. Primary melanoma is largely surgically removable if diagnosed early, but the 5-year survival of patients with regional lymph node involvement decreases to about 50%. The prognosis is even poorer in patients with visceral metastasis, as the median survival rate is then only a few months (Balch *et al.*, 2001). Metastasis is then a leading cause of mortality and morbidity. The whole process of metastasis comprises successive or concomitant complex steps including cell adherence to and subsequent migration through extracellular matrix (ECM) and invasion of basement membranes, which ultimately allows the cells to spread throughout the body.

Ecto-5'-nucleotidase (eN, CD73) is a 70-kDa, GPI-anchored ectoenzyme localized in lipid rafts that catalyses the extracellular dephosphorylation of 5'-AMP (adenosine monophosphate) to adenosine. A growing body of evidence suggests CD73 implication in neoplastic progression. A highly active enzyme has been detected in breast carcinoma (Spychala *et al.*, 2004; Wang *et al.*, 2008), bladder (Stella *et al.*, 2010), ovarian (Cho *et al.*, 2006), thyroid (Kondo *et al.*, 2006), esophageal (Fukuda *et al.*, 2004) and prostate cancers (Hastie *et al.*, 2005), leukaemia (Mikhailov *et al.*, 2008), melanoma (Sadej *et al.*, 2006a), glioblastoma (Ludwig *et al.*, 1999) and glioma (Bavaresco *et al.*, 2008). Its gene is overexpressed also in estrogen receptor-negative breast cancer cell lines (Ostapkowicz *et al.*, 2006) and in highly invasive melanoma cell lines (Sadej *et al.*, 2006b). Using an orthotopic model of breast cancer MDA-MB-435 cells, CD73 was found to be upregulated in primary tumours and lymph node metastasis (Lee *et al.*, 2003). It was also expressed at a higher level in a metastatic compared with a non-metastatic breast cancer cell line. Additionally, analysis of clinical breast cancer biopsies has demonstrated a significant correlation between high CD73 and poor outcome, measured as tumour spread or distant recurrence within a 10-year follow-up period (Leth-Larsen *et al.*, 2009). However, there are also data presenting that elevated eN expression can predict a good prognosis for breast cancer patients (Supernat *et al.*, 2012).

Several functions have been suggested for ecto-5'-nucleotidase during cancer progression. An enzymatic one, generating mostly locally adenosine subsequently activating adenosine receptors was proposed to be involved in the regulation of breast cancer cell invasion, migration and adhesion to ECM (Woodhouse *et al.*, 1998). The increased cancer cell mobility was diminished by AOPCP (α,β-methylene-adenosine diphosphate) — a specific inhibitor of eN activity (Wang *et al.*, 2008). Blocking the enzymatic activity significantly suppressed chemotaxis of breast cancer cells *in vitro* and lung metastasis in a mouse breast cancer model. On the other hand, anti-CD73 mAb significantly inhibited tumour growth as well as its spontaneous metastasis. The latter was essentially dependent on the induction of adaptive anti-tumour immune responses (Stagg *et al.*, 2010).

Independently of its enzymatic role, CD73 can mediate cell-cell adhesion being a co-receptor in T-cell activation, or regulate cell interaction with ECM components and migration on them (Airas *et al.*, 1995, 2000; Andrade *et al.*, 2011). CD73 has been considered also a motility factor in a regeneration and repair of the central nervous system, and development and progression of cancers.

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Abbreviations: AOPCP, α,β-methylene-adenosine diphosphate; AMP, adenosine monophosphate; ConA, concanavalin A; eN, ecto-5`-nucleotidase; ECM, extracellular matrix; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine; FAs, focal adhesions; FAK, focal adhesion kinase; TnC, tenascin C.

Earlier we found that it directly binds tenascin C (TnC), an ECM glycoprotein, and plays a role in the adhesion and migration of melanoma and breast cancer cells on TnC, revealing an agonist, receptor-like character of interaction (Sadej *et al*., 2006b, 2008). Acting as a docking molecule, CD73 mediates lymphocyte adhesion to endothelium (Airas *et al*., 1995) and by the same mechanism glioblastoma invasiveness (Fenoglio *et al*., 1997). siRNA-mediated knock-down of eN in MDA-MB-231 cells prevented their adhesion to ECM, suppressed their growth *in vivo* and *in vitro*, and finally inhibited invasion and migration. Abolishing eN activity by AOPCP also decreased cell adhesion, however, the effect was less significant than in the eN-knock-down cells, which suggested a distinct involvement of those two roles of ecto-5'-nucleotidase (Zhi *et al*., 2007).

Although it has earlier been speculated that eN can play such a bi-functional role — as a catalyst and a membrane receptor for extracellular matrix (Olmo *et al*., 1992) — the engagement of both these functions in cancer progression has never been directly proven or compared. The objective of our study was to verify the eN role in the key steps of melanoma metastasis — cell adhesion, migration and invasion. We did it by analysing the enzymatic versus non-enzymatic functions of ecto-5'-nucleotidase and concluded that both those faces of CD73 were involved in the aggressive behaviour of cancer cells. The enzymatic function seems to be primarily involved in invasion, whereas the non-enzymatic action of eN contributes to cell adhesion and migration on tenascin C through activation of focal adhesion kinase (FAK).

MATERIALS AND METHODS

Cells, antibodies and reagents. A375 cells (from ATCC) were grown in high-glucose DMEM supplemented with 10% FBS and penicillin/streptomycin (Sigma-Aldrich) in standard conditions (37ºC, 5% CO_2, humidified atmosphere). A375(eN$^-$) cells were generated using appropriate small interfering RNA (previously published sequence, GCCACTAGCATCTCAAATA (Zhi *et al*, 2007)) transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer protocol. Rabbit anti-eN polyclonal antibodies were described previously (Yegutkin *et al*., 2002) (a generous gift from Dr J. Spychala, University of North Carolina, USA): anti-FAK-Tyr397 antibodies were from Cell Signalling Technology; anti-actin antibodies were from Sigma-Aldrich. ECM proteins: human tenascin C was from Millipore and human fibronectin, rat collagen I and matrigel from BD Bioscience. Inhibitors: concanavalin A, EHNA (erythro-9-(2-hydroxy-3-nonyl)-adenine), dipyridamole, and AOPCP were purchased from Sigma-Aldrich.

eN activity assay. Radioactive assaying of eN-catalysed reaction was performed as previously described (Sadej *et al*., 2006b; 2008). Briefly, cells were plated onto 48-well plates at 1×10^3 per well one day before experiment. Then the medium was exchanged for serum-free DMEM and 20 minutes before experiment supplemented with AMP (20 mM), adenosine (10 mM), 25 μM EHNA and 20 μM dipyridamole (to inhibit adenosine conversion and uptake). The reaction was initiated by the addition of 8-^{14}C-AMP (3×10^5 CPM/well) (Amersham) and 25 μl samples were withdrawn at 0, 30, 60 and 120 min. eN activity was measured as radiolabelled adenosine in samples separated overnight by thin-layer chromatography on Polygram SIL G/UV254 sheets (Macherey-Nagel, Duren, Germany) in chromatography buffer containing isobutyl alcohol/isoamyl alcohol/2-ethoxyethanol/ammonia/dH_2O (all from Sigma-Aldrich) at the ratio of 3:3:6:3:5. Identified at UV light spots were cut out and their radioactivity counted. When investigating the eN activity in the presence of concanavalin A (ConA), either TnC or matrigel (BD Bioscience) were used at 20 μg/ml. AOPCP at a final concentration of 10 μM was added 20 min before the initiation of reaction.

Adhesion assay. The standard static adhesion assay was carried out for 30 minutes. Cells were aliquoted (3×10^4) into 96-well plates pre-coated overnight with 200 μl of an ECM protein at 20 μg/ml concentration. After allowing adhesion for 30 minutes, the plates were gently washed 3 times with PBS and the adherent cells were quantified following alamarBlue dye assay (AbD Serotec).

Migration and invasion assay in Boyden chamber. The analyses were done using a standard Boyden chamber protocol. In brief, 1×10^5 cells were detached using enzyme-free cell dissociation solution (Millipore) and suspended in 500 μl of serum-free DMEM. Cells were subsequently added into the inner compartment of cell culture inserts (Falcon) with 8 μm pore size, the bottom side of each were coated with 10 μg/ml fibronectin, tenascin C or type-1 collagen (migration assay) or alternatively a high concentration of matrigel (invasion assay) diluted with DMEM at a 1:3 ratio and allowed to polymerise. Cells were allowed to migrate towards the complete medium for 8 h in migration assay or 24 h in invasion assay. Non-migrating cells were removed with a cotton swab and by PBS washing. AlamarBlue dye assay was used to quantify the number of migrating or invading cells. Experiments were done in duplicates, and three independent experiments were performed for each cell line.

Western blotting. Cells were grown to 70–80% confluency and then washed with cold PBS and disintegrated in lysis buffer; 50 mmol/L Tris/HCl (pH 7.5), 1% Triton X-100, 5 mmol/L EGTA, 1 mmol/L EDTA, 150 mmol/L NaCl, 10% glycerol, 1 mmol/L phenylmethylsulphonyl fluoride, 50 mmol/L sodium fluoride, complete protease inhibitor cocktail (Roche). For signalling analysis on ECM proteins, cells were starved overnight and then plated onto dishes covered with 20 μg/ml tenascin C or fibronectin for 10 or 30 minutes. Cell layers were scraped off and incubated on ice for 1 h. The lysates were cleared by centrifugation at 13 000 rpm for 10 minutes. Samples were separated by SDS/PAGE and transferred onto nitrocellulose membranes, which were immunoblotted with appropriate antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were developed by using BM chemiluminescence western blotting substrate (Roche).

Immunocytochemistry. Cells were plated on glass coverslips covered with 20 μg/ml tenascin C or fibronectin. One hour later spread cells were fixed with 2% paraformaldehyde/PBS for 15 minutes, permeabilized in 0.1% Triton-X100 and blocked with BSA. The staining with primary and fluorochrome-conjugated secondary antibodies was carried out and cells were analysed using an Axiovert Zeiss 200 microscope. Images were acquired using a digital camera and subsequently processed using the Axiovision image-processing programme (Zeiss).

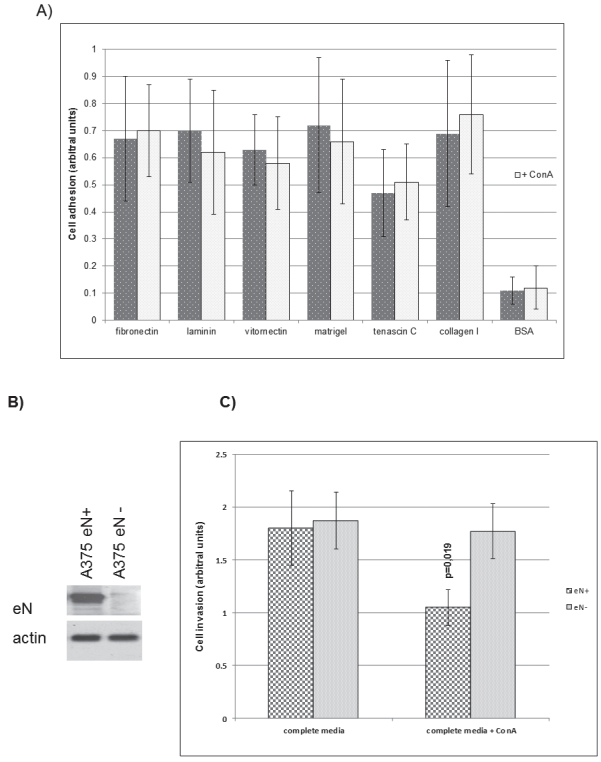

Figure 1. Ecto-5'-nucleotidase activity takes part in cell invasion but not in adhesion.
(A) Number of A375 cells adhering to different ECM proteins 30 minutes after plating; values are means±SD (n=4). **(B)** Western blotting detection of ecto-5′-nucleotidase in A375 melanoma cells. Specific siRNA was transfected into A375 cells with Lipofectamine 2000 to generate eN⁻ cells. eN⁺ cells — mock transfected cells, incubated only with Lipofectamine 2000. **(C)** Inhibition of ecto-5′-nucleotidase activity by ConA results in decreased invasion of A375 melanoma cells. Mock transfected eN⁺ and eN-knockdown A375 cells migrated in Boyden chamber through membrane covered with matrigel towards complete media (DMEM, 10% FBS) or media containing 20 µg/ml ConA. After 24 hours cells were counted following alamarBlue dye assay. The values presented are means ±S.D. (n=4), *p* value indicated on the graphs.

Statistical analysis. Data are expressed as means±SD of at least three independent experiments. Comparative data were analyzed with the unpaired Student's *t*-test using the Statistica 7.1 software. Differences for which $p < 0.05$ are considered as statistically significant.

RESULTS

Enzymatic activity of eN contributes to cancer cell invasion but not adhesion to ECM proteins

The processes of adhesion, migration and invasion are prerequisites of aggressive behaviour of a cancer cell. To investigate the role of eN in melanoma we studied its involvement in adhesion of A375 cells to different substrata. Earlier we found that eN works as a receptor for tenascin C by forming a complex with this protein (Sadej *et al.*, 2006b). Here, we checked whether the enzymatic activity of eN influences such formation. A375 melanoma cells were preincubated or not with concanavalin A, a well-defined inhibitor of eN (Navarro *et al.*, 1998; Stefanovic *et al.*, 1975), and then plated on different ECM proteins. The pretreatment did not significantly influence the strength of attachment in any case (Fig. 1A), suggesting that the eN-catalysed reaction *per se* is not necessary for adhesion. Then we knocked down the eN gene expression using previously characterized siRNA (Zhi *et al.*, 2007) (Fig. 1B). All subsequent experiments were done up to 96 h post transfection, the period when the transient knock-down of eN was sustained. Invasive potential of mock-transfected positive (eN⁺) *versus* siRNA-transfected negative (eN⁻) cells was tested on matrigel and no statistically significant differences between those cell lines were found without ConA. ConA addition into the medium, and thus effective blocking of the eN active site, significantly ($p < 0.05$) inhibited invasion of eN⁺ but not eN⁻ cells (Fig. 1C). This suggests an involvement of the enzymatic but not non-enzymatic functions of ecto-5'-nucleotidase in the cell invasion.

Ecto-5′-nucleotidase modulates cancer cell migration on tenascin C

Our recent studies on WM9 melanoma and MDA-MB-231 breast cancer cells suggested that in both those lines ecto-5'-nucleotidase is directly engaged in interaction with tenascin C (Sadej *et al.*, 2006b, 2008). Yet we needed to check if the enzyme was similarly engaged in the cell migration on the same substratum. It appeared that depletion of eN attenuated the rate of migration on tenascin C in approximately 50% of cells. No such phenomenon was observed with fibronectin or collagen type I (Fig. 2A). Similarly to the earlier studied cells (Sadej *et al.*, 2006b, 2008), tenascin C inhibited the enzymatic activity of eN in A375 melanoma cells (Fig. 2B). Then we asked about the nature of the eN involvement in cell

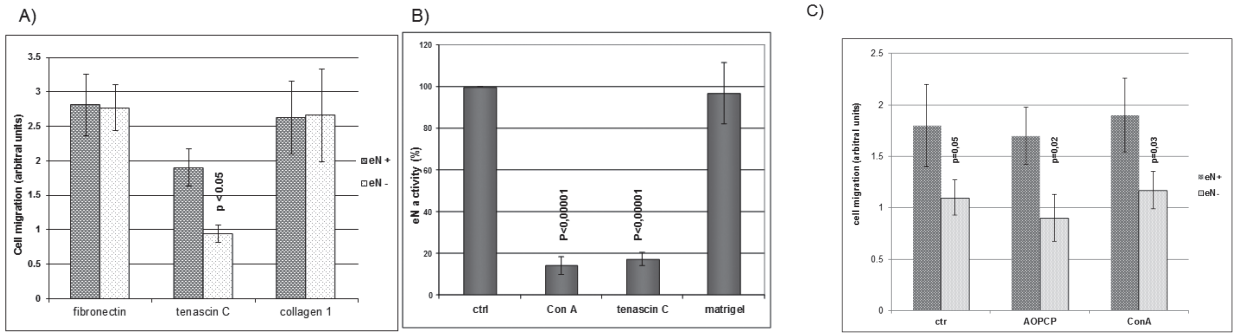

Figure 2. Ecto-5′-nucleotidase takes part in cell migration on tenascin C independently of eN activity.
(A) Boyden chamber migration assay. Insert membrane was coated with 20 µg/ml of tenascin C; assay was carried out for 8 h. Values are means ±S.D. (n=3). **(B)** Measurement of eN activity as described in Materials and Methods. Concanavalin A was used as a control. Values are means ±S.D. (n=3). **(C)** A375 migration on tenascin C in the presence of eN inhibitors. Values are means ±S.D. (n=3), *p* value indicated on the graphs.

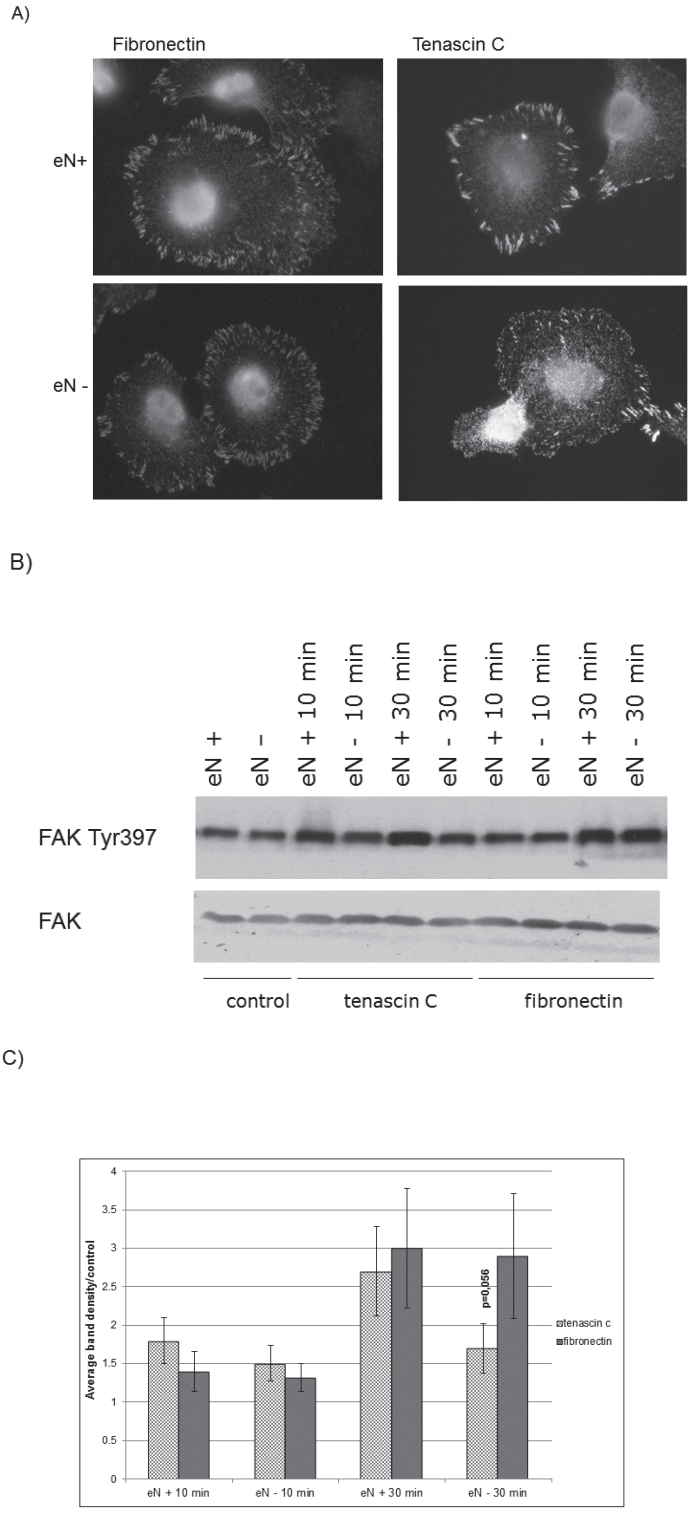

Figure 3. Adhesion of A375 melanoma cells to tenascin C results in activation of focal adhesion kinase (FAK) in eN-dependent manner.
(A) Focal adhesion kinase (FAK) distribution in mock transfected eN+ and eN- A375 cells adhering for 1 h to fibronectin or tenascin C. **(B)** Phosphorylation of FAK on Tyr397 in A375 melanoma after adhesion on tenascin C or fibronectin. Overnight-starved cells were seeded on dishes coated with 20 µg/ml tenascin C or fibronectin. Activation of FAK was analysed after 10 and 30 minutes of adhesion **(C)** Densitometric analysis of FAK-Tyr397 phosphorylation in response to tenascin C or fibronectin adhesion, ratio to control — non activated FAK (average band density, ImageJ software, three independent experiments). Values are means ±S.D., *p* value indicated on the graphs.

motility on tenascin C. Analysis of eN+ *vs* eN– cells migration in the presence of AOPCP or ConA showed no influence of both inhibitors on the cell migratory potential (Fig. 2C). These data indicate that a non-enzymatic but not the enzymatic function of cell surface eN is important for cell migration on tenascin C.

Ecto-5′-nucleotidase regulates function of focal adhesion kinase (FAK) in A375 migrating on tenascin C

Formation and turnover of focal adhesions (FAs) are critical for efficient cell migration. One of the main regulators of the process — focal adhesion kinase (FAK) — is recruited at an early stage to the sites of adhesion and mediates many of the downstream responses crucial for actin contraction and subsequent motility. It has been reported that tenascin C can activate FAK (Midwood & Schwarzbauer, 2002; Nagaharu *et al.*, 2011). Therefore we addressed the question whether the observed eN-dependent differences in cell migration on tenascin C are due to different FAK activity in eN+ *vs* eN– cells. Immunostaining of FAK in A375 cells revealed that eN does regulate FAK function after adherence to tenascin C. eN+ cells formed classical FAs on either tenascin C or fibronectin (Fig. 3A). eN depletion abolished the engagement of FAK in adhesions but only in cells attached to tenascin C. In those cells the distribution of FAK in eN– cells was sparse comparing to fibronectin. This suggests a specific regulation of FAK in response to the signal from tenascin C. FAK also showed a consistent difference in the kinetics of its phosphorylation (Fig. 3B) dependent on the type of substratum. In eN+ cells, a sharp increase of FAK-Tyr397 phosphorylation was observed whereas in eN- cells the degree of phosphorylation induced by tenascin C was significantly lower (Fig. 3C). These results show that deficiency of eN brings about specific alterations of FAK signalling pathways but only in cells adhering to tenascin C.

DISCUSSION

The major finding of this study is that eN has two individual roles in melanoma cells: (i) the non-enzymatic, involved in cell adhesion and migration, (ii) the enzymatic activity playing a role in invasion. Moreover, the formation of focal adhesions and activation of FAK upon placement of cells on tenascin C were found to be dependent on the presence of eN.

Ecto-5'-nucleotidase has been proven to contribute to cancer progression at multiple levels. Targeting CD73 (eN) is involved in treatment of cancer. Accordingly, the enzymatic activity of eN was shown to increase the resistance of breast cancer

cells to doxorubicin (Ujházy *et al*., 1996). The overexpression of CD73 gene facilitated invasion, migration and adhesion to ECM of breast cancer cell lines due to its enzymatic ability to generate adenosine. The increased cell mobility was blocked by an eN inhibitor — AOPCP (Wang *et al*., 2008). Adenosine generated by CD73 enhanced tumour cell chemotaxis. Consistent with the role of adenosine in promoting metastasis, the administration of an adenosine analogue to 4T1.2 tumour-bearing mice significantly increased the number of spontaneous lung metastasis without any effect on primary tumour growth. The 4T1.2 cells were found to express A_1 and A_{2B} adenosine receptors. Treatment with A_{2B} adenosine receptor agonist decreased *in vitro* chemotaxis and *in vivo* lung metastasis, whereas A_1 agonist had no effect. Then it was suggested that generation of extracellular adenosine by tumour cells' CD73 promotes breast cancer metastasis to the lungs through the activation of A_{2B} adenosine receptor (Woodhouse *et al*., 1998). However, the effect of CD73 on tumour metastasis *in vivo* may extend beyond its enzymatic activity. For instance, CD73 may directly enhance adhesion of tumour cells to the endothelial layer, thereby promoting their migration across the vascular endothelium. Earlier work showed that CD73 can promote the binding of human lymphocytes to endothelial cells (Airas *et al*., 1995). Small interfering RNA-mediated knock-down of eN in MDA-MB-231 cells prevented their adhesion to ECM, suppressed their growth *in vivo* and *in vitro* and inhibited invasion and migration. AOPCP pretreatment also significantly decreased cell adhesion, although its influence was not as spectacular as with CD73 RNAi, suggesting independent or complementary roles of different eN functions. These properties of ecto-5'-nucleotidase correlated with inhibition of matrix metalloproteinases (MMP)-2 and MMP-9 expression and activity (Zhi *et al*., 2007). MMPs are a family of zinc-dependent endopeptidases with the primary function of degrading ECM proteins. Expression of MMPs and their activation in tumour cells, as well as tumour-surrounding stromal cells, have been implicated in tumour cell invasion and metastasis (Hua *et al*., 2011). We found earlier that eN is coexpressed with MMP-2 in melanoma cells (Sadej *et al*., 2006b). One can suggest that regulation of MMPs function by eN could be one of the mechanisms underlying its contribution in cancer progression.

In earlier studies we observed that eN interacts with tenascin C. This was demonstrated as the direct binding in both functional adhesion and migration assays. TnC was also shown to inhibit eN enzymatic activity (Sadej *et al*., 2006b; 2008). Tenascin C is a secreted hexameric glycoprotein which is a non-structural component of ECM. TnC is highly expressed in the microenvironment of many tumours and acts in diverse biological processes, including cell adhesion, proliferation, migration, differentiation, primary tumour growth, metastasis and survival with all effects being dependent on cell type and tissue context (Orend & Chiquet-Ehrismann, 2006). Although TnC is absent or expressed at a very low level in adult tissues, its expression can be induced during tissue remodelling, inflammation and tumourigenesis (Chiquet-Ehrismann & Chiquet, 2003; Orend & Chiquet-Ehrismann, 2006). The majority of human melanoma cell lines express and secrete tenascin C. In normal human skin, TnC levels are low, but they are significantly elevated in melanoma and correlate with its progression and metastasis. High tenascin C expression has been observed at the invasive front of tumours and the intensity of TnC staining correlated with cells spread to sentinel lymph nodes, better than tumour thickness (Breslow depth) (Ilmonen *et al*., 2004; Kääriäinen *et al*., 2006). Similarly, an analysis of tenascin C in primary melanoma has revealed that an absence of TnC in the stroma of the invasion fronts and within tumour cells correlates with benign disease behaviour and a lower risk of developing metastases (Fukunaga-Kalabis *et al*., 2010).

TnC is generally agreed to promote proliferation and migration of cancer cells (Yoshida *et al*., 1999). It co-localizes in tumours with fibronectin and procollagen-I and forms tubular mesh-works and channels ensheathing melanoma cells. These special channel-like structures facilitate spreading of tumour cells (Kääriäinen *et al*., 2006). Thus eN, as a potential receptor for tenascin C, can be directly involved in melanoma development. Our data suggest that eN primarily takes part in migration of A375 cells on tenascin C independently of its enzymatic function. Although TnC as well as AOPCP and ConA inhibit the eN enzymatic activity, they did not impair the cell migration on a layer of TnC. These results suggest that direct binding of tenascin C to ecto-5'-nucleotidase includes critical catalytic residues of eN (as inhibition abolishes the effect) but its activity *per se* is not necessary for the cell migration on TnC.

We found also that interaction of eN with TnC results in phosphorylation of FAK at Tyr397. Cellular adhesion and migration requires remodelling and reorganization of focal adhesions — large multi-protein assemblies at sites of interaction with underlying extracellular matrix. In FAs, aggregated receptors, e.g. integrins, through various signalling molecules mediate interaction of ECM components with the cytoskeleton. FAK is a crucial component of FA (Shen & Guan, 2001) and phosphorylation of FAK at tyrosine 397 is a key determinant of how FAK controls FA turnover (Hamadi *et al*., 2005). It is worth noting that FAK was previously identified as one of downstream targets in CD73-mediated cell motility (Ode *et al*., 2011). On the other hand, tenascin C was found to activate the FAK-induced epithelial-mesenchymal-like change in cancer cells (Midwood & Schwarzbauer, 2002; Nagaharu *et al*., 2011). Taking all together, we believe that activation of FAK is dependent on the presence of CD73 in cells interacting with tenascin C. This could be a key mechanism of the cell adhesion and migration in the presence of this ECM protein.

We conclude that the observations presented here prove that ecto-5'-nucleotidase acts at different stages of metastatic cascade. Its mechanism of action is complex and involves two independent roles: a non-enzymatic and the enzymatic one, both contributing to progression of the disease.

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