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13. ABSTRACT (Maximum 200 Words)
Adenosine has angiogenic, growth-stimulatory and immunosuppressive properties that may be relevant for breast cancer progression. The second period of the funding term (12 months, Aug 1, 2002 to Jul 31, 2003) has been used to characterize in detail breast cancer cell lines developed in the first period. The in-depth characterization of eN-, GFP-, and ADA-overexpressing clones of MDA-MV-231 cells included their expression profiling and adhesion properties in in vitro assays. The expression profiling of the panel of breast cancer cells established that the high capacity to generate adenosine correlates with mesenchymal phenotype that is characteristic for invasive and metastatic breast cancer cells. Several membrane proteins that are characteristic for this phenotype, such as CD44, integrins α5 and β1 and EGFR were not altered in GFP and ADA overexpressing and in eN-suppressed MDA-MB-231 cells, suggesting that engineerd alterations were confined to the capacity to produce adenosine and express eN. Characterization of eN^- and ADA^+ cells led to discovery that eN is a receptor for Tenascin C and downregulation of eN caused decreased adhesion on this ECM. Finally, we have performed preliminary inoculations of developed cells in nude mice to test the relationship of growth of grafts to the expression of ADA and eN. Our results so far fully support the important role of adenosine and eN in breast cancer.

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1. Introduction

Overexpression of ecto-5'-nucleotidase (eN) in advanced breast carcinoma may lead to locally high concentration of signaling adenosine. Since adenosine has potent angiogenic, growth-stimulatory and immunosuppressive activities in other tissues, the question arises whether similar functions of this regulatory molecule would apply to breast cancer (reviewed in (1). This project has been designed to test this hypothesis and ask whether increased/decreased levels of adenosine in breast cancer may lead to acceleration/inhibition, respectively, of tumor progression in mice xenograft model.

The key enzymatic activity that produces pharmacologically active adenosine is ecto-5'-nucleotidase. We found recently that this protein, that is attached to the extracellular face of cell through the GPI anchor, is highly expressed in ER(-) breast cancer cells and clinical samples (2). We also found that expression of eN and another adenosine-regulating protein adenosine deaminase (ADA) is regulated by β-catenin, PKCα and estrogen receptor, further suggesting that increased expression of these proteins in breast cancer may lead to increased adenosine generation and breast cancer promotion.

Thus our previous data and the wealth of published observations on the physiological functions of adenosine in other tissues strongly suggest that eN and adenosine may constitute novel regulatory factors regulating breast cancer progression. If confirmed, both the eN directly and adenosine pharmacological activities indirectly may become targets for the development of new therapeutic agents.

2. Body of the Report

A. Maintaining and characterization of genetically modified breast cancer cell lines.

During the last 12 months (second year of the funding period) we have utilized tools developed in the initial funding period. These tools include breast cancer cell lines that have altered expression of eN and ADA. As proposed in the original application, we decreased the expression of eN by antisense cDNA and increased the expression of ADA by retroviral transfer of respective cDNA's in MDA-MB-231. After development of cells that stably downregulated eN (eN_{low}) and overexpressed EGFP (green fluorescent protein, clones H2 and F4), ADAw/t (clones G11 and H10) and ADAmut, we observed a significant loss of effectiveness of suppression of expression of eN but not ADA and EGFP. To solve this problem we performed two additional rounds of clonal selection of previously selected eN_{low} clones to select subclones that were more stable in long term culture. This strategy was successful and we have finally obtained three eN_{low} MDA-MB-231 clones that were further characterized (eN_{low} clones G12, G3 and H6).

We have started the characterization of cells to be used in this project by in-depth characterizing wild type cells. Since eN is a membrane protein attached to the extracellular face of the cell, we focused our expression profiling on other membrane and cytoskeletal proteins, especially those that, as eN, reside in membrane domains called lipid rafts. We used a broad panel of breast cancer cells that differ in their tumorigenic, invasive and metastatic capacities. In contrast to previous expression profiling, and in order to directly relate our results with specific cell phenotypes, we focused our analysis on proteins, rather than cDNA's, using specially optimized Western blot procedure. We were able to compare the expression of 56 membrane and cytoskeletal proteins in 15 cell lines. Among GPI proteins that reside in lipid rafts we found dramatic downregulation of CD24 and 45 kDa uPAR species and upregulation of eN and intestinal alkaline phosphatase (Fig. 1) in cells that previously were defined as more tumorigenic and invasive (3, 4). This surprisingly clear-cut expression profile prompted investigation of other membrane and cytoskeletal proteins in these cells. As shown in Fig. 2, the broader protein survey revealed a similar alteration in expression of specific proteins in ER(+) and ER(-) cell with somewhat variable expression in SK-Br-3 and MDA-MB-468 cells (Fig. 2). While E-cadherin was expressed mostly in ER(+) and control cells, other adhesion receptors such as integrins $\beta1$, $\alpha5$ and αV, CD44 and OB- and N-cadherins tended to co-express with eN (Fig. 2). Next, we investigated the expression of components of cytoskeleton and found that antibodies K8-12 and K8-13, that recognize cytokeratins 13, 15, 16 and 1, 5, 6, 7, 8, 10, 11 and 18, respectively, showed significant expression of these proteins in ER(+) and control cells. On the other hand, vimentin and smooth muscle actin was expressed in more metastatic ER(-)

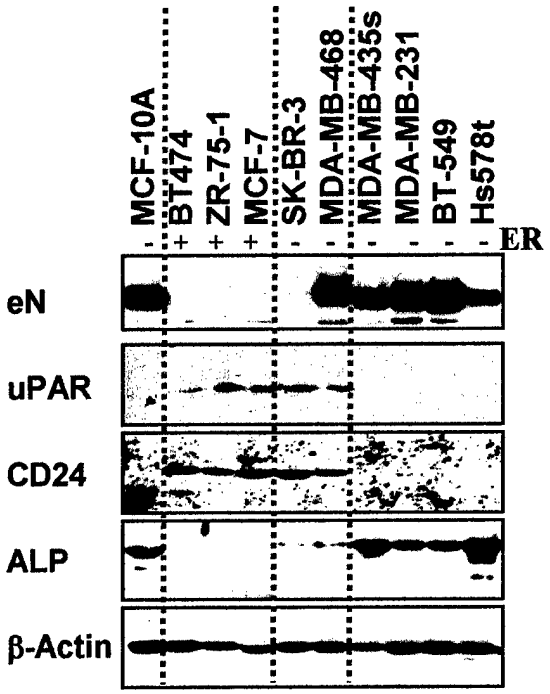


Fig. 1. Expression of GPI-anchored membrane proteins in breast cancer cell lines. 30 μg of cell lysate was taken for Western blot analysis.

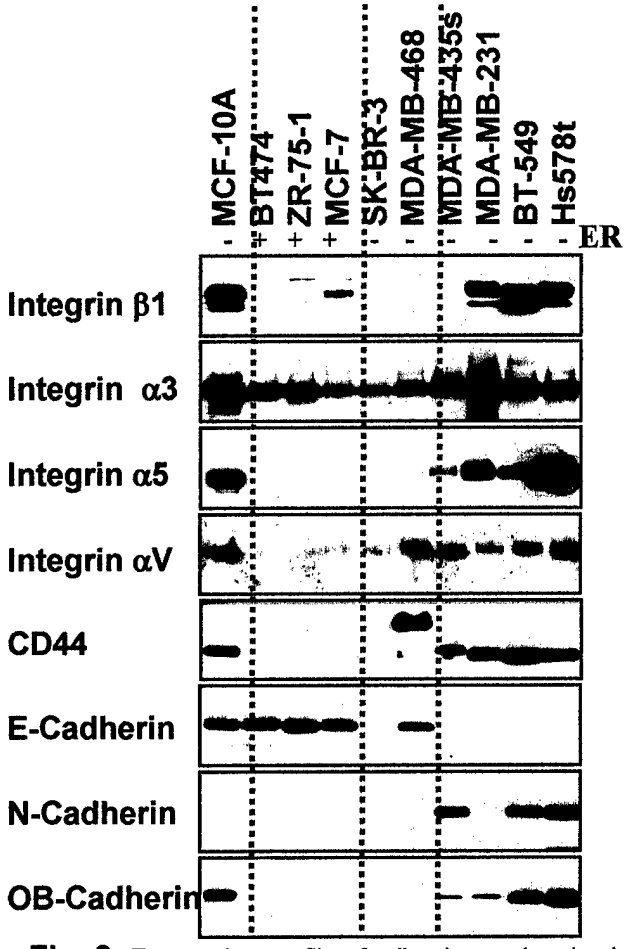
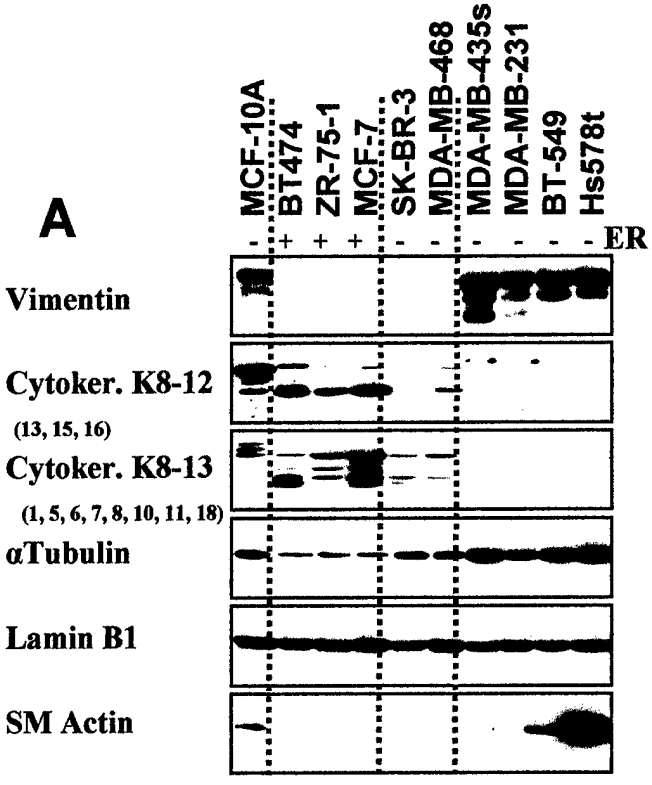


Fig. 2. Expression profile of adhesion molecules in a panel of breast cancer cell lines.



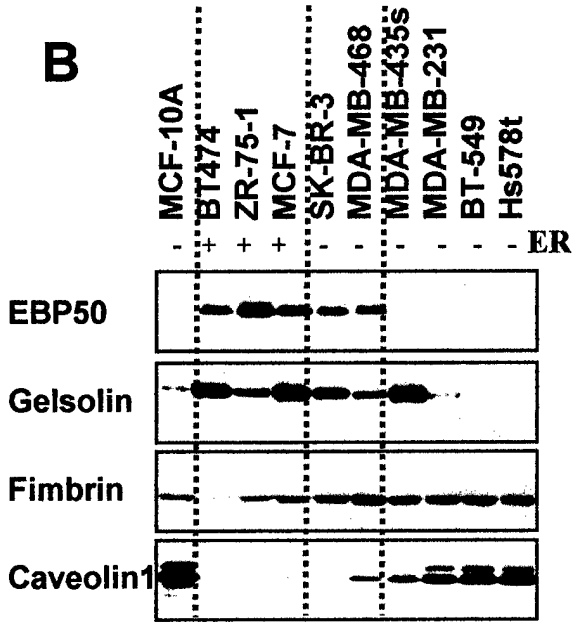


Fig. 3. A/B. The expression profile of cytoskeletal and cytoskelot-associated proteins in a panel of breast cancer cell lines.

cells with SM actin being less discriminatory than vimentin (Fig. 2C). Broader analysis of proteins associated with cytoskeleton showed that while EBP50 and gelsolin were associated with less invasive cells, fimbrin, talin, filamin and especially fascin and moesin tended to express at higher level in more invasive breast cancer cells (Figs. 2 and 3, other data in Appendices). Ankyrin B, on the other hand, was only expressed at high levels in c-Jun transformed cells and normal fibroblasts. Interestingly, caveolin-1 expression strongly coincided with eN further suggesting that, in addition to lipid rafts, also caveolae may have specific function in more invasive cells. This characterization revealed that eN expression and the high capacity to generate extracellular adenosine correlate with mesenchymal phenotype that is common for most aggressive breast cancer cell lines and is also typical for normal human fibroblast (Figures 1-3). Such shift in expression profile strongly suggest the epithelial to mesenchymal trandiferentiation (EMT) during breast cancer progression. Although in general this subcategorization was expected, the uniformity of expression profiles seems surprising and may suggest that significantly broader number of proteins may define and/or contribute to specific cell behavior. Significantly, cell lines SK-Br-3 and MDA-MB-468 fall in-between: while losing many epithelial markers such as E-cadherin and certain cytokeratins, they did not yet acquire full set of mesenchymal markers. Nevertheless, the expression of high EGFR and several key mesenchymal proteins in MDA-MB-468 cells was apparently sufficient to fully express metastatic behavior in mouse xenograft model (5).

In order to test whether genetically altered cells developed in the course of this work exhibited altered expression profile and/or cell behavior in adhesion assays, we performed several experiments to address this issues. To analyze the expression profile in altered MDA-MB-231 cells, we have tested the expression of several membrane proteins such as CD44, EGFR, Integrins $\alpha 5$ and $\beta 1$. Data shown in Fig. 4 indicate that there was no major shift of expression profile that would suggest altered phenotype inadvertently caused by genetic manipulations not related to the expression of ADA, EGFP and eN *per se*. Next we analyzed the adhesive properties of selected cells in *in vitro* cell adhesion assay. In this assay cells were plated on different ECM, incubated 30 min in incubator and nonadherent cells were removed with three rounds of washing with PBS. Remaining cells were quantitated and results are shown in Fig. 5 (representative of three independent experiments). These results suggest that while all cell clones have similar adhesive properties on collagen IV, Fibronectin, Laminin 1, Vitronectin and Restrictin (Tenascin R), eN_{low} cells (clone 6H) exhibit reduced adhesion on Tenascin C and on mixes of Tenascin C and fibronectin but not Tenascin C and Laminin 1. ADA-overexpressing clone were also somewhat less adherent on this ECM suggesting that adenosine removal may be important. Tenascin C is an important component of ECM in invasive breast carcinoma and interaction between this extracellular matrix and eN may have significant functional consequences. To further validate the specificity of interactions between Tenascin C and eN we tested the effect of Tenascin C on eN activity *in situ*. We incubated adherent w/t MDA-MB-231 cells with 10 μg/ml of all ECM

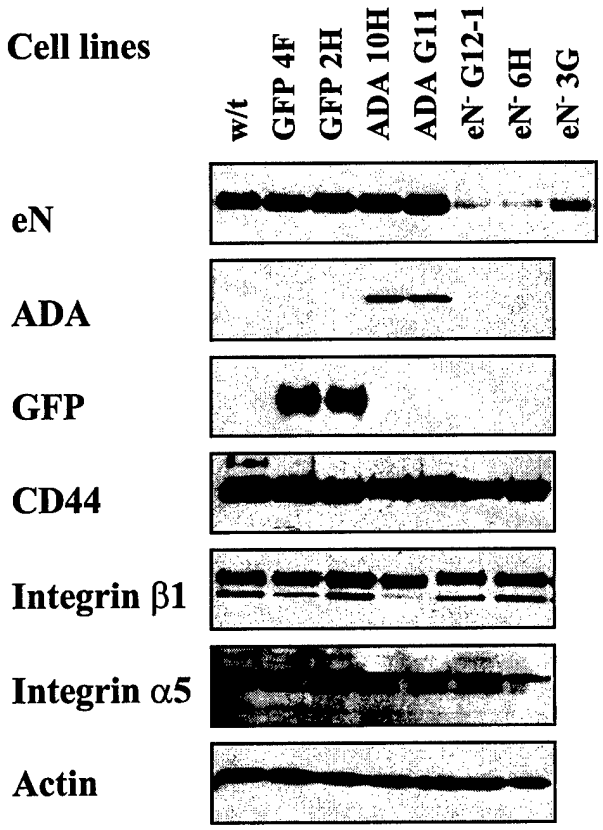


Fig. 4. Expression of eN, GFP and ADA in MDA-MB-231 clones developed for inoculation into nude mice. Comparison with the expression of other adhesion proteins surveyed in Fig. 1-3.

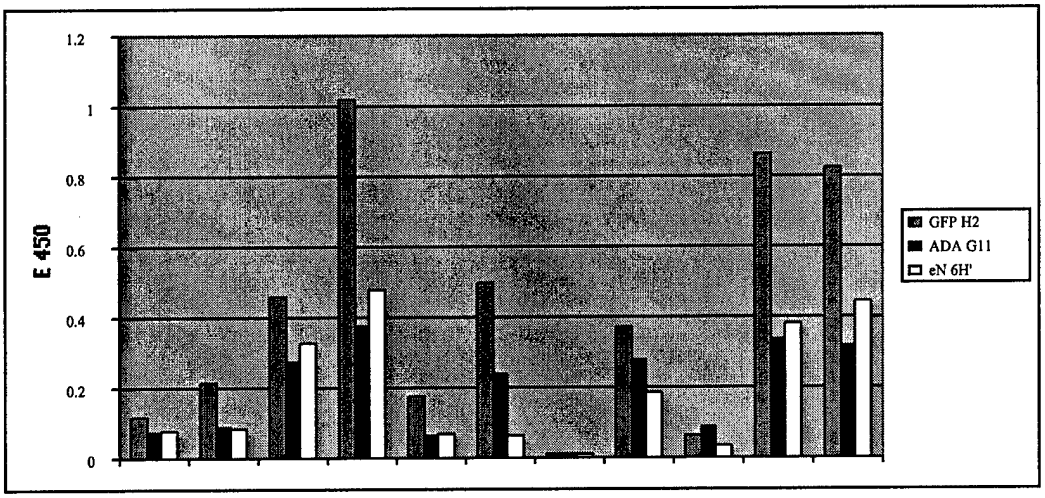


Fig. 5. Adhesion of MDA-MB-231 clones developed for inoculation to nude mice. Cells (10^5) were plated on different ECM for 30 min and after washing quantitated. Combined Fibronectin (F) and Tenascin C (T) and Laminin (L) and T were also tested.

tested above and assayed for the rate of adenosine generation from added radiolabeled AMP. Results shown in Fig. 6 demonstrate that only Tenascin C dramatically inhibited eN activity in live cells. Thus these experiments demonstrated for the first time a specific interaction between eN and Tenascin C and identified two-way functional relationship between these proteins: eN as a receptor for Tenascin C and Tenascin C as an inhibitor of eN and adenosine generation. This discovery has been an exciting new development that further validate our hypothesis on the important functions of eN and adenosine in breast cancer progression.

During last year we have also continued to develop MCF-7 non-tumorigenic cell line overexpressing eN and were unable to obtain positive clones. In contrast to the development of EGFP and ADA clones that posed no technical difficulties, the overexpression of eN in these epithelial cells, despite intensive efforts, has not been achieved. We have also tried alternative cell lines BT474 and ZR-75-1, that have similar expression profiles (Figs 1-3) to MCF-7 cells, with similar negative result. We do not have explanation for this outcome other than there may be a specific cellular requirement for the expression of this highly glycosylated GPI-anchored protein and expression "out of cellular context" may not be possible. Other reason may be that the eN which is highly expressed in mesenchymal cells represents and altered isoform (possibly by alternative splicing) and we are currently cloning eN from MDA-MB-231 cells to test this possibility.

B. Initial inoculations of modified MDA-MB-231 cell in nude mice.

We have utilized 9 mice to test if inoculation of 2.5 $x10^6$ cells per site will be sufficient to develop grafts of sufficient size for further analysis. In this experiment we used $EGFP_{high}$-1 (clone H2)cells as controls and ADA_{high}-1 (clone G11) and eN_{low}-1 (clone 6H) cells to manipulate tumor adenosine concentrations. Results shown in Fig. 7 demonstrate that while grafts ADA_{high}-1 grew as controls, eN_{low}-1 cells developed similarly sized tumors with significant delay. This result provides the first strong argument supporting the role of adenosine and/or eN in breast cancer growth. Currently, we are in a process of setting up a major experiment that will test two cell lines of each type and analyze the angiogenic and endothelial parameters by immunohistological and biochemical means.

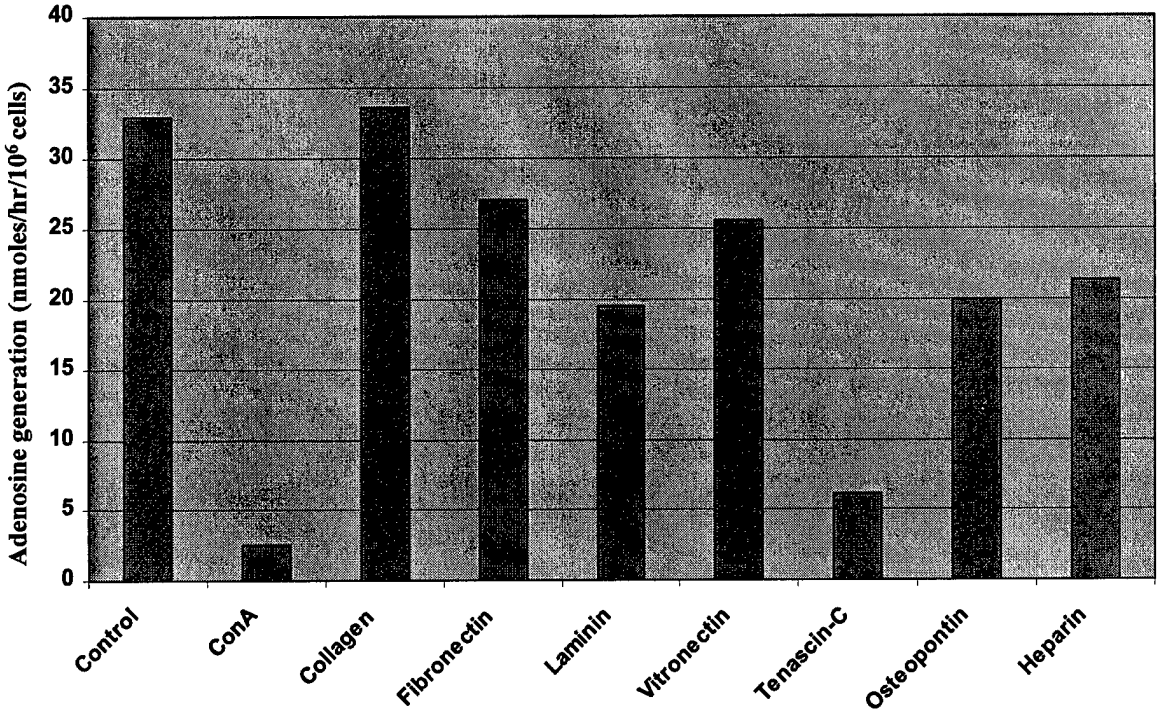


Fig. 6. The effect of extracellular matrix proteins at 10 μg/ml on adenosine generation from added radiolabeled AMP in MDA-MB-231 cells. Comparison with ConA, a known inhibitor of eN. Cells were preincubated for 30 minwith ECM proteins at wgich point AMP was added and adenosine was measured after 60 min by TLC. Representative of two experiments is shown.

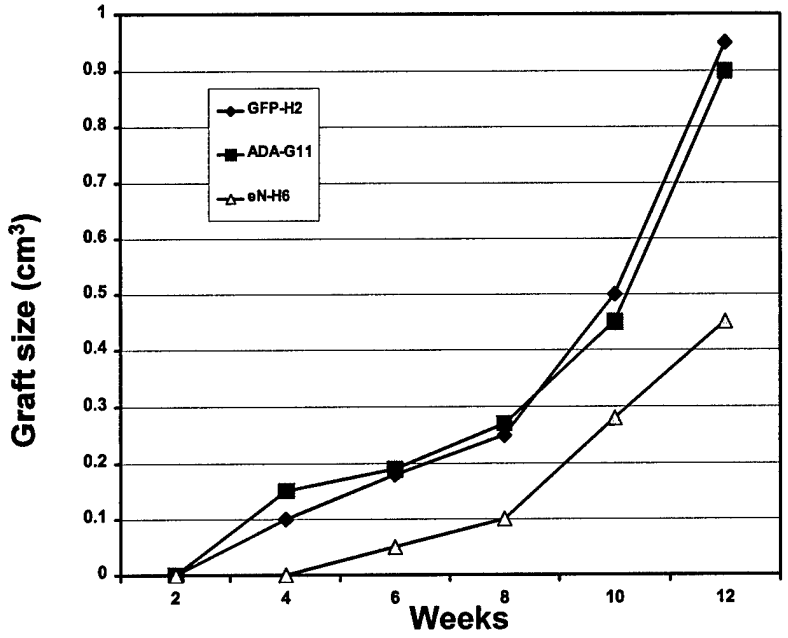


Fig. 7. The growth of orthotopically grafted cells GFP-H2, ADA-G11 and eN-H6 (eN_{low}) in nude mice. Graft sizes were measured weekly or more often and two-weeks measurements are shown in cm^3. Each point represents three independent grafts.

3. Key Research Accomplishments

- We have defined mesenchymal cell phenotype that is typical for advanced breast carcinoma as a site of increased eN expression and adenosine generation. Manuscript on this topic has been submitted to Cancer Research (2003). In addition, in the course of this work, a review on 5'-nucleotidases has been written and accepted in J. Biol. Chem. (Appendices)
- We have discovered that eN is a specific receptor for Tenascin C and characterized the relationship between these two proteins in terms of adhesion and generation of adenosine.
- In initial inoculations of nude mice with eN_{low}, ADA and GFP MDA-MB-231 cells we found that grafts expressing low eN grew slower than GFP and ADA expressing cells.

4. Reportable Outcomes

Our in-depth characterization of w/t and modified breast cabcer MDA-MB-231 cells revealed that the high potential to generate adenosine is typical for mesenchymal cells. The characteristic feature of these cells is that they are more motile and invasive and in breast cancer are more common for the advanced stage of the disease. This clear link between aggressive cell behavior and breast cancer progression on one hand and the capacity to generate adenosine further supports for the role of eN and adenosine in breast cancer. The specific interaction between eN and Tenascin C is a novel observation and may provide a basis for the mechanism of specific interactions between invasive cells and the substratum at the invasive end of breast carcinoma. Finally, preliminary testing showed that cells expressing low eN have lower tumorigenic potential in nude mice than control cells. Further testing is necessary to define the role of adenosine and eN in the mechanism of growth retardation of tumor grafts.

5. Conclusions

During last 12 months period we have accomplished several goals set for this stage of our research. We have performed in-depth characterization of genetically modified cell lines and tested them in preliminary inoculations. The results obtained so far clearly support our initial hypothesis on the role of adenosine in breast cancer progression. We are very excited to continue this project and are confident to fully define the role of both adenosine and eN in breast carcinoma by the end of the third and final year. As an unexpected bonus, the discovery of interactions of eN with Tenascin C, will strenghten the proposed novel functions of eN and adenosine in breast cancer. As proposed in the original proposal, our efforts now will concentrate on further analysis of xenograft growth in the context of eN and ADA expression and on defining the angiogenic activities of adenosine in tumor grafts by immunohistological and biochemical means.

Personnel involved in this project:

Jozef Spychala, Ph.D. P.I.
Anna Ostapkowicz, Tech II

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7. Appendices

3 Items:

A. Abstract from the 9th Purine and Pyrimidine Metabolism in Man Meeting in Amsterdam June 9th-13 2003.

B. Manuscript entitled "Lipid rafts remodeling in ER negative breast cancer" by Ostapkowicz Anna, Inai Kunihiro, Smith Leia, Kreda Silvia and Spychala Jozef submitted to Cancer Research (2003).

C. Manuscript entitled "Mammalian 5'-nucleotidases" by Vera Bianchi and Jozef Spychala accepted in J. Biol. Chem. (minireview series, 2003)

Appendice A **Jozef Spychala, Ph.D.**

The association of ecto-5'-nucleotidase (eN), integrin β1, EGFR and vimentin with invasive breast carcinoma: The role of eN in invasive phenotype.

Spychala Jozef, Ostapkowicz Anna, Naiki Hironobu, Noriki Sakon, Fukuda Masaru and Inai Kunihiro

Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, USA and Fukui Medical University, Fukui, Japan

Breast carcinoma is a heterogeneous disease and expression profiling has identified several subtypes that may be useful in determining more effective individualized treatment regimens in the clinic. Previously we have found ecto-5'-nucleotidase overexpression in ER negative breast cancer. In order to associate the expression of eN with specific cell phenotype, we have performed expression profiling at the level of protein in several breast cancer cell lines. Our focused analysis of membrane and cytoskeletal proteins revealed that eN is specifically co-expressed with a number of membrane proteins, such as EGFR, CD44, N-cadherin, OB-cadherin, caveolin, integrin β1 and integrin α5. Among cytoskeletal proteins eN co-expresses with vimentin, merlin, fascin, moesin and spectrin. Also several signaling molecules, such as tyrosine kinase Lyn, trimeric Gαi and PKC tightly co-express with eN. This expression profile is characteristic for normal fibroblasts and was also found in more aggressive and tumorigenic breast cancer cell lines that have undergone Epithelial to Mesenchymal Transdifferentiation (EMT). Since eN and several indentified membrane proteins, that either co-express or have exclusive expression pattern, are components of lipid rafts, these results suggests that there is a significant remodeling of this membrane microdomain in EMT. The co-expression of eN with vimentin also correlated with the responsiveness to ConcanavalinA in more aggressive breast cancer cell lines suggesting functional association. Preliminary survey of clinical samples from breast cancer patients show coexpression of eN with Integrin β1 and vimentin at the invasive edge of the tumor. Thus, these data suggest that elevated expression of eN and increased potential to generate extracellular adenosine may have specific functions related to cell migration and strengthen the significance of this protein as a novel marker for invasive and metastatic breast carcinoma.

The U.S. Army Medical Research Materiel Command under DAMD17-01-1-0351 supported this work.

Lipid rafts remodeling in ER negative breast cancer[1]

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[1] This work was supported by grants RO1-CA34085 and DOD grant DAMD17-01-1-0351

Abstract

Expression profiling of breast cancer cell lines and clinical samples revealed a significant shift in gene expression in ER negative breast cancer. Since we have found dramatic overexpression of eN (ecto-5'-nucleotidase or CD73), a GPI-anchored component of lipid rafts, in ER(-) breast cancer cell lines and in clinical samples, we undertook an investigation on the expression of other membrane and cytoskeletal protein elements in more aggressive and metastatic breast cancer cells. In addition, as an *in vitro* model of transition to ER-negative breast cancer we used the MCF-7 cell line and its two unrelated derivatives: drug resistant Adr2 and c-Jun transformed clone. Our directed protein expression profiling revealed a remarkably uniform shift in expression of a broad range of membrane, cytoskeletal and signaling proteins. These results suggest that there is a major remodeling of lipid rafts and underlying cytoskeletal network during transition to ER negative breast cancer. Interestingly, similar expression pattern was observed in normal fibroblasts, suggesting the common membrane determinants with motile mesenchymal cells. To investigate whether the observed changes in lipid raft composition would have functional significance, we clustered lipids rafts with lectin Concanavalin A and observed dose- and time-dependent association of lipid raft components with cytoskeleton, which was completely reversed by Latrunculin A, an inhibitor of actin polymerization. Furthermore, to test whether the ER(-) phenotype could be reversed by treatment with differentiationg agent, we treated ER(-) negative cells with TSA, an inhibitor of histone deacetylase, and observed broad reversal of mesenchymal and re-appearance of epithelial markers. Thus, our results suggest that during transition to more invasive breast cancer there is a significant structural and functional re-organization of lipid rafts and underlying cytoskeleton protein components that is reversed upon histone deacetylase inhibition.

Introduction

Establishment of tumor markers that describe invasive and metastatic breast carcinoma is critical for more individualized therapeutic strategies. Since breast cancer is becoming an increasingly heterogeneous disease, the task of defining specific cancer cell phenotypes is especially challenging. Several individual breast cancer markers have been established and proven useful for target-specific pharmacological intervention. The clinically proven include estrogen and progesteron receptors and more recently Erb2 and EGFR. Estrogen receptor status in breast cancer is one of the most important discriminating factors that determine subsequent therapeutic strategy. Major differences in expression profiles of wide number of genes has been documented in ER(-) and ER(+) carcinomas (1), and both in *in vitro* studies and in the clinic, these differences were associated with either more motile and invasive phenotype or more aggressive course of the disease in the case of ER(-) breast carcinoma (2). Membrane proteins are means to interact with the extracellular milieu and the composition of membrane proteins is critical for cell behavior in general and invasive and metastatic properties in particular. However, the composition of membrane proteins during breast cancer progression has not been investigated in detail and this directly contributed to significant gaps in understanding of structure-function relationships at the cell surface during cell movement. Significantly, many important membrane proteins that were shown to contribute to cell behavior *in vitro*, often were not identified in subsequent genome-wide expression screens in clinical context.

Among different membrane microdomains, lipid rafts are one of the least understood elements. Although their lipid composition has been addressed in several studies, protein components were not systematically compared between invasive and noninvasive cells. Since several *in vitro* models of breast cancer exemplify transition to more invasive and metastatic state, we have chosen this model to study changes in lipid rafts composition after loss of ER expression. Our recent finding that ER(-) breast cancer cells express high level of eN (ecto-5'-nucleotidase) a GPI-anchored protein and a marker of lipid rafts provided an early argument for the lipid raft remodeling during transition to more aggressive breast carcinoma (3). In this study, we aimed to analyze whether there is a consistent alteration in expression of cytoskeletal membrane and lipid raft protein components across a wider population of ER positive and negative cells that would suggest a coordinate expression consistent with the motile and invasive phenotype. Although limited by the availability of suitable antibodies, the focus on expressed proteins rather than mRNAs allowed us to directly relate protein expression with the specific cell phenotype.

Materials and Methods

Cells. Breast cancer ER positive cells lines MDA-MB-474, ZR-75-1, MCF-7 and negative SK-BR-3, MDA-MB-468, MDA-MB-435s, MDA-MB-231, BT-549, Hs578t, nontrasformed MCF-10A, drug resistant MCF-7/Adr-2, c-Jun transformed MCF-7/c-Jun clone 2-33 and control MCF-7/neo clone 7-1 and human fibroblasts WI-38 were obtained from either Tissue Culture Facility at LCCC/UNC, ATCC or developed as described before (4). Cells were maintained in MEM supplemented with Eagle salts, NaPyr, nonessential amino acids and 10% FBS (most cell lines), in McCoy's supplemented with 15% FBS (SK-BR-3 cells), in Leibowitz L-15 supplemented with 10% FBS (MDA-MB-468 cells) and in MEGM medium supplemented with BPE, hEGF, Insulin, Hydrocortisone and 10% FBS (BioWhittaker medium for MCF-10A cells) in CO_2/O_2 atmosphere at 37° C, except for MDA-MB-468 cells which were grown at ambient atmosphere at 37° C. All media contained penicillin and streptomycin.

Reagents. All reagents were ACS or the highest purity commercially available. The following antibodies were used. Rabbit polyclonal anti-eN antibodies (for Western) were generated as described before (5). Anti-Gαs/olf sc-383, Gαi-2 sc-7276, Thy-1 sc-9163, CD24 sc-11406, Gβ sc-378, Gβ2 sc-380, cyclin D1 (sc-8396), Integrin β1 sc-8978, Integrin β2 sc-6624, Integrin β3 sc-6627, ERα sc-8005, Fyn sc-434, Lyn sc-15, Lck sc-433, c-Fgr sc-130, Hck sc-72, MDR1 sc-8313, N-Cadherin sc-8424, OB-Cadherin sc-9997, Caveolin-1 sc-894, PKCα sc 8393 and secondary HRP conjugated against goat and rat IgG were from Santa Cruz (Santa Cruz, CA). E-Cadherin C20820, FAK F15020, Integrin β1 I41720, Moesin M36820, EBP50 E83020 were from Transduction Laboratories (Lexington, KY). Anti-CD44s 13-5500 antibody was from Zymed (San Francisco, CA). Anti-c-Yes antibody 06-514 and c-Src GD11 were from Upstate (Lake Placid, NY), rabbit anti-uPAR (399R) were from American Diagnostica Inc. (Greenwich, CT), anti-β-actin were from Oncogene (Boston, MA) and anti-intestinal alkaline phosphatase antibodies were from

Lipid rafts isolation. A modified procedure for density gradient centrifugation using Nycodenz {5'-(N-2,3-Dihydroxypropylacetamido)-2,4,6-Triiodo-N,N-bis(2,3-Dihydroxypropyl) -isophtalamide}from Sigma-Aldrich (St. Louis, MO) was used to fractionate Triton X-100 soluble and insoluble membrane and cytoskeletal subdomains and components [Hostager, 2000 #12454]. For the purpose of centrifugation cell lysates were diluted 2-fold with 35% nycodenz and contained 3 to 4 mg of total protein per 0.5 ml. Density step-gradient was prepared by careful applying of 0.5 ml aliquots of increasing concentration of Nycodenz (35%, 25%, 22.5%, 20%, lysate in 17.5%, 15%, 12%, 8% and 4%) sequentially into Beckman (Palo Alto, CA) 13 x 51 mm polyallomer tubes. Note that lysate was placed in the middle of Nycodenz gradient. Tubes were centrifuged at 46K for 4 hrs in a Beckman 55Ti rotor at 4°C. Following centrifugation 0.5 ml fractions were carefully withdrawn and small pellet was resuspended in PBS containing 0.5% SDS and 1% Triton X-100 (fraction 10). Total of 10 fractions and control input lysate were analyzed for the distribution of proteins by Western blot. Typically, components of light lipid rafts and caveolae distributed into first 4 fractions, soluble cell components including cytosolic proteins were found in fractions 5 and 6 and cytoskeletal-associated heavy fractions were distributed in fractions 7 to 9.

Western blotting. Cell extracts, obtained by scraping cells in PBS in the presence of protein phosphatase and protease inhibitors and lysing with 1% Triton X-100/PBS, were loaded on the SDS-PAGE at 30 μg per lane. Separated proteins were transferred onto Immobilon-P 0.45 μM (Millipore, Bedford MA) PVDF membrane and used for probing with specific antibodies. Two buffer systems were used during incubations with antibodies: PBS supplemented with 5% Carnation fat-free dry milk and 0.2% Tween 20 or 25 mM Tris, pH 8.4 supplemented with 130 mM NaCl, 5 mM potassium phosphate, 5% fat-free dry milk and 0.2% Tween 20. Blots were re-used several times after air drying o/n at RT each time. Secondary antibodies conjugated to HRP and BM Chemiluminescence Western Blotting Kit (Roche, Indianapolis IN) were used to develop images on Kodak (Rochester, NY) X-Mat Blue XB-1 film. Fractions from Nycodenz gradient were loaded on SDS-PAGE at 5 μg/lane, except for fractions 1 to 3, where due to low protein content 0.25 μg/lane, 0.5 μg/lane and μg/lane was used, respectively.

Results

We used a broad panel of breast cancer cell lines, which ER status as well as invasive and metastatic potential has been well defined, thus enabling to correlate protein expression with cell phenotype (6). In this cell panel MCF-10A serve as nontumorigenic control cell line and SK-

Br-3 and MDA-MB-468 represent ER(-) cells that are much less tumorigenic than MDA-MB-435s, MDA-MB-231, BT-549 and Hs578t breast cancer cells (6). Previously we have reported dramatic upregulation of eN, a GPI-anchored membrane ecto-protein, in ER(-) breast cancer cells (3). Here we confirmed this observation and further compared the expression of eN with other lipid raft components such as uPAR, CD24, Thy-1 and alkaline phosphatase. As shown in Fig. 1, the expression of two established breast cancer markers, CD24 and uPAR (the 45 kDa species detected by 399R antibody), concurred with the ER receptor status, and were also found in SK-Br-3 and MDA-MB-468 cells. A reciprocal pattern was detected for intestinal alkaline phosphatase 64 kDa form and eN, two GPI proteins highly expressed in cells that previously were defined as more invasive (6, 7). The expression of Thy-1, a protein highly expressed in neuronal tissue, was not significantly altered in breast cancer cells. This surprisingly clear-cut expression profile prompted investigation of other membrane and cytoskeletal proteins in these cells. As shown in Fig. 2, the broader protein survey revealed a similar alteration in expression of specific proteins in ER+ and ER- cell lines with somewhat variable expression in SK-Br-3 and MDA-MB-468 cells (Fig. 2). While E-cadherin was expressed mostly in ER(+) and control cells, other adhesion receptors such as integrins $\beta 1$, $\alpha 5$ and αV, CD44 and OB and N cadherins tended to co-express with eN (Fig. 2A). Other membrane or membrane-associated signaling molecules such as EGFR, Lyn, PKCα also showed similar expression pattern (Fig. 2B). Next, we investigated the expression of components of cytoskeleton and found that antibodies K8-12 and K8-13, that recognize cytokeratins 13, 15, 16 and 1, 5, 6, 7, 8, 10, 11 and 18, respectively, showed significant expression of these proteins in ER(+) and control cells. On the other hand, vimentin and smooth muscle actin was expressed in more metastatic ER(-) cells with SM actin being less discriminatory than vimentin (Fig. 2C). Broader analysis of proteins associated with cytoskeleton show that while EBP50 and gelsolin were associated with less invasive cells, fimbrin, talin, filamin and especially fascin and moesin tended to express at higher level in more invasive breast cancer cells (Fig. 2D and E). Ankyrin B, on the other hand, was only expressed at high levels in c-Jun transformed cells and normal fibroblasts. Interestingly, caveolin-1 expression strongly coincided with eN further suggesting that, in addition to lipid rafts, also caveolae may have specific function in more invasive cells. Thus, this comprehensive expression pattern strongly subcategorized breast cancer cells into three distinct profiles: BT474, ZR-75-1 and MCF-7 cells having epithelial and MDA-MB-435s, MDA-MB-231, BT-549 and Hs578t having mesenchymal features. Although in general this subcategorization was expected, the uniformity of expression profiles seems surprising and may suggest that significantly broader number of proteins may define and/or contribute to specific cell behavior. Significantly, cell lines SK-Br-3 and MDA-MB-468 fall in-between: while losing many epithelial markers such as E-cadherin and certain cytokeratins, they did not yet acquire full set of mesenchymal markers. Nevertheless, the expression of high EGFR and several key mesenchymal proteins in MDA-MB-468 cells was apparently sufficient to fully express metastatic behavior in mouse xenograft model (8).

To further test whether transition to ER(-) status and more invasive phenotype will show similar shift in expression profile, we used two independent *in vitro* models of breast cancer. Development of drug resistance and overexpression of c-Jun in MCF-7 cells was shown to correlate with loss of ER expression and transition to more invasive phenotype (4, 9). In these cells we focused the expression survey on those membrane and cytoskeletal proteins that showed differential expression (Figs 1 and 2). Additionally, to ask whether the profile of membrane or cytoskeletal proteins may differentiate between "cancer metastatic" and normal motile

phenotypes, we included normal human fibroblasts WI-38. Data in Fig. 3 show that transition to more aggressive cell phenotype in both breast cancer cell models is accompanied by strikingly uniform downregulation of epithelial markers. Furthermore, the similarly uniform upregulation of mesenchymal markers in these cells showed an expression profile that is remarkably similar to normal human fibroblasts. Thus, the fact that drug resistant, c-Jun-transformed and all other invasive cells shown in Figs 1 and 2 exhibit the expression pattern that is similar to normal fibroblasts strongly suggest that progression to aggressive cell behavior is associated with the aquisition of normal motile "machinery" exemplified here by fibroblasts.

In addition to GPI-linked proteins that are components of lipid rafts at the outer lipid leaflet, members of the src-family of tyrosine kinases and G-trimeric proteins also may reside in lipid rafts by attachment of acyl groups to inner leaflet of lipid rafts. We compared the expression of these proteins in selected breast cancer cells as well as in fibroblasts. Data in Fig. 4A show that there was a significant upregulation of Lyn and less pronounced upregulation of Src, accompanied by downregulation of Lck. These observations were confirmed using a broader panel of breast cancer cell lines (data not shown). Among G-trimeric protein family members we observed significant upregulation of Gαi-2 and less pronounced upregulation of Gβ (using pan Gβ antibody) in ER(-) cells and in fibroblasts (Fig. 4B) that has also been confirmed using a broader panel of breast cancer cells (data not shown). The extensive similarities between ER(-) cell lines, including MCF-7 derived drug-resistant and c-Jun-transformed cells prompted us to test whether drug resistant phenotype, as exemplified by the expression of MDR1 and MRP1, has been expressed across the broader population of ER(-) cells. Indeed, MDR1 expression was dramatically induced in c-Jun-transformed MCF-7 cells and MRP1 expression was demonstrated in MDA-MB-231 cells (Fig. 4C). None of these proteins was detectable by Western blot in normal human fibroblasts.

Previous studies demonstrated that histone deacetylase inhibitors cause shift in expression pattern of selective groups of proteins. In breast cancer cells, TSA has been shown to induce the expression of ERα and E-cadherin in MDA-MB-231 cells. Based on these data we asked whether TSA may reverse expression of mesenchymal proteins that define the invasive and metastatic phenotype. Treatment of MDA-MB-231 cells with 1 μM TSA for 48 hours caused dramatic downregulation of eN mRNA (Fig. 5A) and protein (Fig. 5B). Other proteins that have been shown to contribute to invasive cell behavior, such as CD44, integrins β1 and α5, EGFR, OB-cadherin, moesin, PKCα and caveolin-1 and that were correlated with mesenchymal phenotype, were all strongly downregulated by TSA. On the other hand, epithelial markers such as E-cadherin, EBP50 and gelsolin were upregulated. The expression of several other proteins, such as Lyn, vimentin was only slightly downregulated, however, at 48 hours of drug treatment, there was increased incidence of apoptosis that may have masked further changes in gene expression. We conclude that in breast cancer model TSA induces changes in expression pattern that closely parallel cell differentiation, which in the context of mesenchymal cells, may be termed mesenchymal to epithelial transdiferentiation (MET), a process reciprocal to EMT.

Upon clustering, lipid raft may become associated with cytoskeleton and thereby trigger downstream signaling pathways [Villalba, 2001 #12474]. Preliminary results have shown that small but significant portion of lipid raft components was associated with cytokeletal elements, defined as pellets after 1 minute Triton X-100 lysis and centrifugation at 10K [Oda, 1992 #12455]. We analyzed lipid rafts and cytoskeletal distribution of these proteins in MCF-7 and MDA-MB-231 cells using density gradient centrifugation. Here, we used a modified Nycodenz density gradient centrifugation [Hostager, 2000 #12454] that combines the isolation of lipid rafts

and cytoskeletal elements using density gradient. Fractionation of Triton X-100 cell lysates on Nycodenz gradient caused predominant distribution of GPI proteins eN and CD24 to light lipid raft fractions (fractions 1 to 4) with little presence in heavier fractions 7 and 8 (Fig. 6A). In contrast, significant portion of uPAR in MCF-7 cells also distributed to soluble compartment (fractions 5 and 6). Surprisingly, Thy-1 was found exclusively in heavier, cytoskeleton-bound fractions (fractions 7 to 9), suggesting that this lipid raft component is constitutively anchored to the cytoskeleton in both cell types. Signaling proteins attached to the inner leaflet of plasma membrane, Lyn and Gαs, were also mostly associated with light lipid rafts fractions. To normalize and validate the separate distribution of lipid rafts, cytosolic and cytoskeletal complexes we pre-fractionated cells lysed in Triton X-100 treatment by centrifugation at 10K for 1 minute and separately run pellet and supernatant on Nycodenz density gradients. Results showing density gradient fractions probed for cytoskeletal proteins are shown in Fig. 6B. The results indicate that while α-tubulin was almost completely, and β-actin partially solubilized, vimentin and lamin B1 were mostly in pelleted cytoskeletal fractions. Interestingly, a portion of vimentin and β-actin were present in light lipid raft fractions, suggesting association of these cytoskeletal proteins with lipid rafts microdomains.

Although there is still little known on the function of lipid rafts in cancer cells, clustering of lipid rafts has been shown to trigger signaling pathways and association with cytoskeleton in lymphocytes. To test if altered composition of lipid rafts has functional consequences in breast cancer cells we used Concanavalin A (ConA) to induce lipid rafts clustering and see if this treatment leads to cytoskeletal association of lipid rafts components. This lectin was selected for three following reasons: first, ConA was demonstrated to cluster lipid rafts components in lymphocytes and cause cell activation, second, ConA was shown previously to functionally differentiate between ER(+) and ER(-) breast cancer cells (10), and third, this lectin has high affinity to eN and EGFR, two proteins that are highly expressed in most ER(-) cells and reside in lipid rafts (11, 12). Additionally, this lectin was shown to induce EGFR activation both *in vitro* and *in vivo* (13, 14) We incubated MCF-7 and MDA-MB-231 cells with ConA at 20 μg/ml for 25 minutes in TC incubator, scraped cells in ice-cold PBS containing protease and protein phosphatase inhibitors and lysed cell pellet in 1% Triton X-100 in PBS as described in Materials and Methods. Approximately 3 mg of total protein in Triton X-100 lysates was loaded and fractionated on Nycodenz density gradient. Results presented in Fig. 7A and 7B show that in contrast to MCF-7 cells, treatment of MDA-MB-231 cells with ConA caused dramatic re-distribution of several lipid raft components such as CD44, EGFR, Integrin β1, Lyn, Fyn, Gαi-2 and flotillin-1 and to lesser extent Gαs and src. In contrast, no significant re-distribution was seen in case of OB-cadherin in MDA-MB-231 cells. This re-distribution was transient and not detectable after 90 minutes of ConA stimulation (Fig. 7C) and completely reversed by preincubation with 0.5 μM Latrunculin, an inhibitor of actin polymerization (Fig. 7D). Similar results were obtained with BT474, ZR-75-1 ER(+) and BT549 and Hs578t ER(-) cells (data not shown). Importantly, despite the low residual presence of both eN and EGFR, no eN redistribution was observed in MCF-7 cells. The cell-specificity, time-dependence and the involvement of actin cytoskeleton indicate that ConA-induced lipid rafts clustering in breast cancer cells may have functional significance, as has been shown in lymphocytes, and not be a consequence of mere membrane protein aggregation by this polyvalent lectin. In future studies it will be important to define critical ConA receptors, in addition to eN and EGFR, as well as downstream signaling events initiated by lipid raft clustering in ER(-) cells.

Discussion

The analysis of expression of membrane, cytoskeletal and associated proteins in 12 breast cancer cell lines show that there is consistent shift in expression pattern between non-invasive and invasive cell phenotypes. Our focus on specific sets of membrane and cytoskeletal proteins allows us to directly correlate the expression pattern with well-defined specific cellular phenotypes. Although such comprehensive analysis of membrane and cytoskeletal proteins in a broad cell panel has not been performed in the past, few membrane and cytoskeletal proteins, that have been reported to express at variable levels between ER (+) and (-) cells, strongly validate our approach. Among these proteins vimentin, EGFR, CD44, fascin, E-cadherin were shown to be highly correlated with ER status both in breast cancer cell lines and in clinical samples [Wilson, 2002 #12473]. Our survey has largely extended this correlation and established a broader set of membrane and cytoskeletal proteins that define the motile and invasive phenotype. Although it may not be surprising that this invasive phenotype, common to all 6 more aggressive cell lines (MDA-MB-435s, MDA-MB-231, BT549, Hs578t, MCF-7/Adr2 and MCF-7/c-Jun), is largely recapitulated in normal fibroblasts, therefore providing a direct evidence for epithelial to mesenchymal transdiferentiation (EMT) in breast cancer cells, the extent of similarities, including increased expression of signaling molecules such as EGFR, PKCα, Lyn, Gαi-2, suggests that both structural proteins and regulatory circuitry has been adopted from normal mesenchymal cells. Therefore, the realization that a number of signaling molecules that are overexpressed in invasive and metastatic breast carcinoma, when compared with normal epithelium, actually belong to normal cell phenotype, may bring about better understanding of what is intrinsically normal and abnormal in cancer cells. As a result, a clearer distinction between proteins that are inducing oncogenic changes and proteins that are part of adopted phenotype may help in better selection of clinically useful drug targets. One example may be elevated PKCα, considered an important inducer of tumorigenesis, that is clearly elevated in normal human fibroblasts and in that sense seems to be a consequence rather than cause of progression to invasive breast carcinoma. Interestingly, in the context of MCF-10A nonmetastatic cells which express a number of mesenchymal markers, overexpression of PKCα was sufficient to induce changes in morphology, proliferation and motility [Sun, 1999 #12471]. However, given the expression of smooth muscle actin and both E-cadherin and vimentin, this cell line is likely of myoepithelial/basal cell origin that is characterized by a high invasive potential in nontransformed state (7). Accordingly, cancers derived from myoepithelial/basal cells represent a distinct and intrinsically aggressive subset of breast carcinoma (15). On the other hand, the expression of either MDR1 or MRP1 was specific for cancer cells only, suggesting that this protein may better represent transformed phenotype and consequently be more suitable target for pharmacological intervention.

Several of membrane proteins residing in lipids rafts are differentially expressed in invasive breast cancer cells, suggesting that there is a major remodeling of this membrane microdomain during breast cancer progression. GPI-linked proteins are typically considered markers of lipid rafts and in our study they show dramatic shift in expression profile. The downregulation of the 45 kDa species of uPAR and CD24 in ER(-) cells and the emergence of eN and IAP most likely have physiological consequences. Among five GPI proteins studied in this work three have known enzymatic activities. uPAR has intrinsic serine protease activity and participates in uPA activation and extracellular matrix degradation and thereby was postulated to perform important functions in cell invasion. This protein was shown to exist as 55 and 45 kDa species in cancer cells. uPAR was also shown to function as a signaling molecule that upon

ligand engagement (uPA) is able to initiate signaling cascade through association with integrins. Previous studies utilizing labelled uPA or anti-uPAR antibodies have demonstrated somewhat higher expression of uPAR in MDA-MB-231 cells (16), although it was detectable on Western blot utilizing 399R antibody only after induction with uPA (17). However, despite intensive efforts, using 399R antibody we were only able to detect relatively low 45 kDa uPAR levels in this cell line after longer blot exposures (data not shown). CD24 is a new marker for breast and ovarian carcinoma [Fogel, 1999 #9649; Kristiansen, 2002 #12472] with positive estrogen receptor status (18). This mucin-like heavily glycosylated protein was shown to be a ligand for P-selectin and proposed to mediate rolling in endothelium (19). On the other hand, eN and IAP that seem to replace uPAR and CD24 during breast cancer progression, both have phosphohydrolase activity and participate in dephosphorylation of extracellular nucleotides (mostly adenosine phosphates) and generation of signaling adenosine (20). It is important to note that the apparent loss uPAR proteolytic and pro-invasive activity during breast cancer progression may well be compensated by the increase in MMP-2 and MMP-9 metaloproteinases in ER(-) cells reported previously [Tester, 2001 #10949]. However, the specific roles of eN and IAP in invasive breast carcinoma remain to be defined. The strong association of eN with ER(-) breast carcinoma reported before (3), and with mesenchymal phenotype demonstrated in this work, support the view that this protein or its catalytic product adenosine may have specific function in invasive cell physiology. Adenosine, the product of both eN and IAP, acting through a family of receptors, has well established roles in adhesion, growth regulation, vasodilation and angiogenesis that may be important for breast cancer progression (reviewed in (20)).

In addition to more obvious changes in expression of cadherins, cytokeratins and vimentin during progression to metastatic breast carcinoma, there are more subtle, but nevertheless consistent, changes in expression of underlying cytoskeleton-associated proteins. The down-regulation of EBP50 and gelsolin, two proteins independently associating with actin cytoskeleton, correlate with upregulation of moesin, fascin and to lesser extent talin and filamin. EBP50 may associate with ERM proteins (Ezrin, Radixin, Moesin) and thereby regulate anchoring of lipid rafts to cytoskeleton [Itoh, 2002 #12475]. A number of functional associations between proteins that overexpress in mesenchymal cells have been established. CD44/moesin and CD44/Lyn interactions collaborate in triggering invasiveness and chemoresistance (21, 22). Vimentin and fimbrin form functional complexes in macrophages (23). Thus, all these proteins seem to have distinct roles in aiding assembly of links between actin or vimentin cytoskeleton and membrane proteins. In that capacity, they are well suited to regulate adhesion/deadhesion and membrane fluidity that is critical for efficient cell motility. Although it is well accepted that metastasis requires cells to become motile and invasive, the molecular basis of cell migration, and underlying genetic changes that induce the motility, are not well defined. Our analysis identified a broad group of membrane and cytoskeletal proteins that seem to change as a "package" representing epithelial and mesenchymal phenotypes. Although many other proteins will be likely identified, those defined in this work may be a starting point for use in functional *in vitro* studies and, as a markers, in the clinic.

It is not yet clear whether expression profile exemplified here by ER(-) cells with high eN and alkaline phosphatase and low uPAR and CD24 *in vitro* represents a distinct clinical subset of advanced breast carcinoma. A number of membrane and cytoskeleton-associated proteins shown to be elevated in this survey were also shown to co-express in clinical setting, thus suggesting that specific protein groups may functionally co-express during cancer progression *in vivo*.

EGFR and vimentin were shown to co-express in specific subset of advanced breast carcinoma distinct from both erbB2-positive and ER-positive cases [Wilson, 2002 #12473]. CD44, moesin, and fascin were also found to express at higher level in advanced breast cancer (24-26). Recent study found a striking correlation of CD44-positive and CD24-negative cells derived from breast carcinoma with their tumorigenic potential (27).

Histone deacetylase inhibitors were shown to be potentially effective anticancer agents (28). In breast cancer cells they inhibited cell proliferation *in vitro* (29) and decreased carcinogen-induced mammary tumorigenesis and tumor growth *in vivo* (30, 31). Interestingly, trichostatin A, a widely used HDAC inhibitor, activated the expression of ERα in MDA-MB-231 cells suggesting that there may be potential reversal of ER(-) phenotype in these cells. We have utilized this model to show that, indeed, upon TSA treatment there was systematic reversal of mesenchymal expression profile accompanied by the emergence of such epithelial marker as E-cadherin and increased expression of uPAR, CD24, gelsolin and EBP50. Thus, this altered expression profile strongly suggests that many membrane and cytoskeletal proteins investigated in this work represent a protein set that is typical for motile phenotype and that this set of proteins is utilized by cancer for invasion and metastasis.

Furthermore, these experiments also suggest that breast cancer has a high potential for plasticity that could enable transition from ER(+) to ER(-) state. Although there seem to be a controversy whether loss of ER expression is a common clinical stage of the disease, the transition to ER(-) has been frequently recapitulated *in vitro* with ER(+) breast cancer cell lines (4) and was associated with the development of drug-resistant, invasive and metastatic phenotype. In MCF-10A cells, that are frequently used as a model of pre-malignant phenotype, a spontaneous shift between ER negativity and positivity has been reported in long term culture (32) and a derivative MCF-10TA generated heterogeneous malignant variants that were both ER(+) and ER(-) (33), suggesting that such shift is possible. A strong argument for the transition from ER positivity to negativity was obtained in animal model of breast cancer where the large T-antigen transgenic mouse tumor progression was accompanied by the loss of ERα expression (34). However, it is still unclear whether decrease in ER positivity after anti-estrogen receptor therapy in the clinic represents a transition to ER(-) carcinoma or is just a temporary decrease in ER expression and a definitive evidence for such transition in the clinic is still lacking (35). Since our data and previously reported results demonstrated that breast cancer cells have remarkable intrinsic plasticity, we suggest that this feature may be the reason that during transition to metastatic phase of the disease cells may temporarily acquire motile and mesenchymal phenotype that would allow them to invade and colonize distant sites where, under the influence of local conditions, cells would revert to epithelial growth. Such scenario, if conformed, would make the specific pharmacological targeting of metastatic cells much more difficult.

Figures and Legends

Fig. 1.

Differential expression of GPI proteins in. Thirty ug of cell lysate was loaded onto each lane and separated on SDS-PAGE as described in Materials and Methods. Each experiment was repeated at least twice.

Fig. 2.

Differential expression of membrane and cytoskeletal proteins in hyperplastic MCF-10A and a panel of breast cancer cell lines. A) adhesion proteins, B) Signaling and regulatory proteins, C) Cytoskeletal proteins, D) and E) Cytoskeleton associated proteins. Protein loading and other conditions as described in Materials and Methods.

Fig. 3.

Differential expression of selected membrane, adhesion and cytoskeletal (A) and regulatory and cytoskeleton-associated (B) proteins in *in vitro* model of breast cancer progression, drug resistant and c-Jun-transformed MCF-7 cells. Comparison of expression profile with human normal fibroblasts WI38.

Fig. 4.

Differential expression of lipid rafts associated Src trimeric G-protein family members in (A). Jurkat T-cell extract shown as a control for the expression of Lck. (B) Differential expression of MDR1 and MRP1 in selected breast cancer cell lines and human normal fibroblasts WI38.

Fig. 5.

Effect of TSA on the expression of selected membrane, cytoskeletal and associated proteins in MDA-MB-231 cells. Experimental conditions described in Materials and Methods.

Fig. 6

Fractionation of Triton X-100 lysates on Nycodenz density gradient. (A) Scheme of Nycodenz gradient procedure. (B) Distribution of eN, Thy-1, uPAR, CD24, Lyn and Gαs in MCF-7 and MDA-MB-231 cells. (C) Normalization of Nycodenz density gradient centrifugation with cytoskeletal proteins.

Fig. 7

Clustering of membrane proteins with Concanavalin A and induction of re-distribution of lipid raft components into high density fractions in MDA-MB-231 (A) but not in MCF-7 (B) cells. The re-distribution of lipid raft components in MDA-MB-231 is reversed by prolonged treatment and in the presence of actin polimerization inhibitor Latrunculin B (C).

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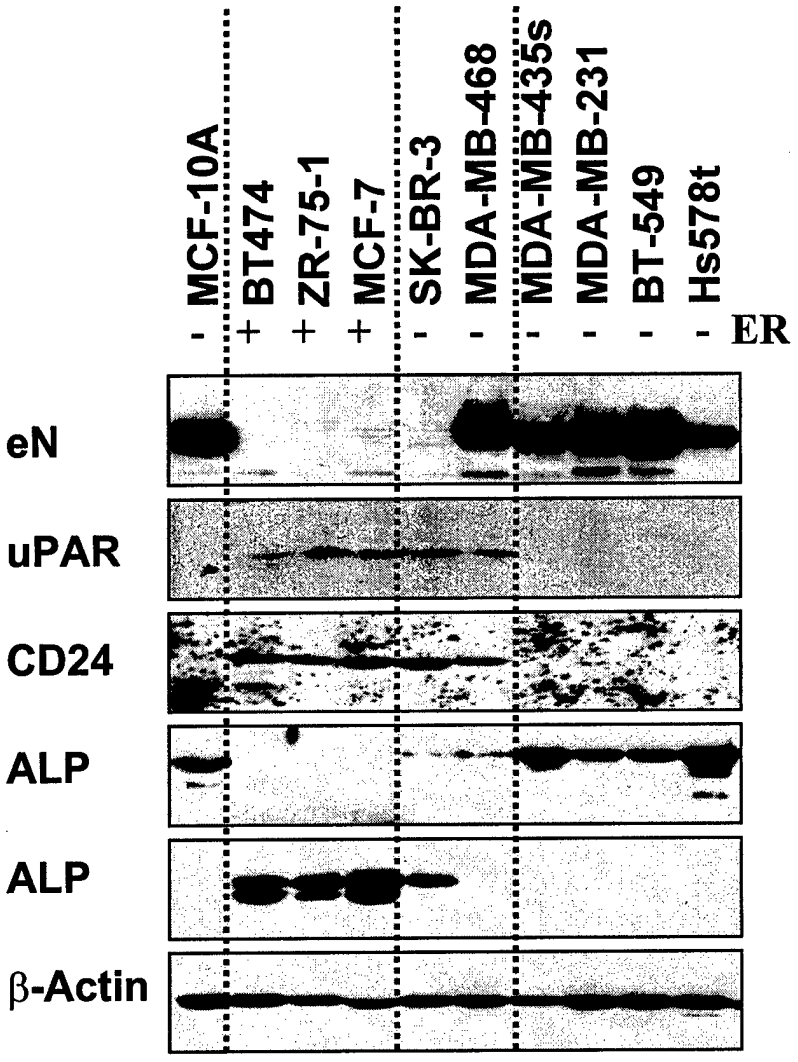
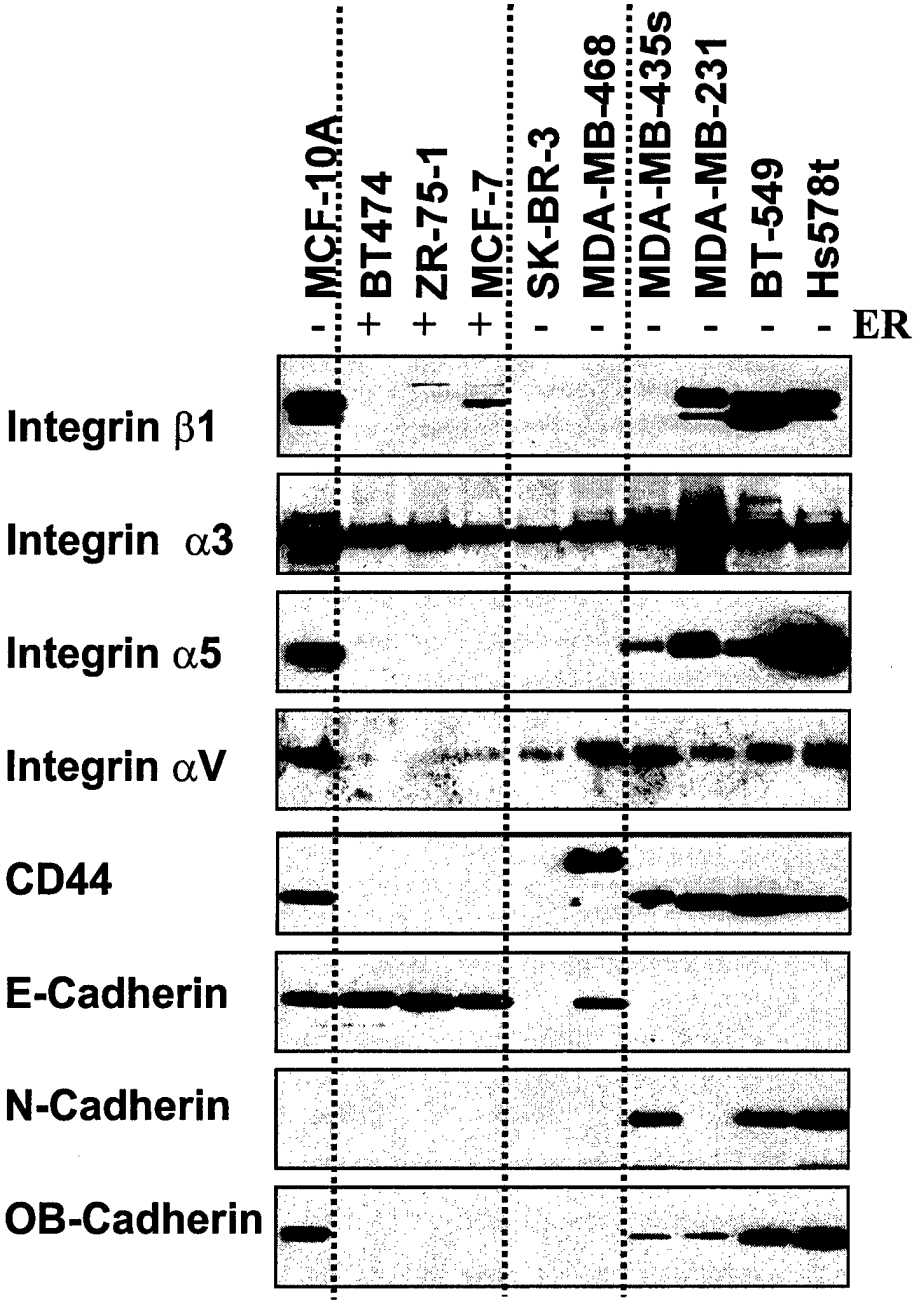


Spychala et al, Fig. 1.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- BT-549
- Hs578t
ER
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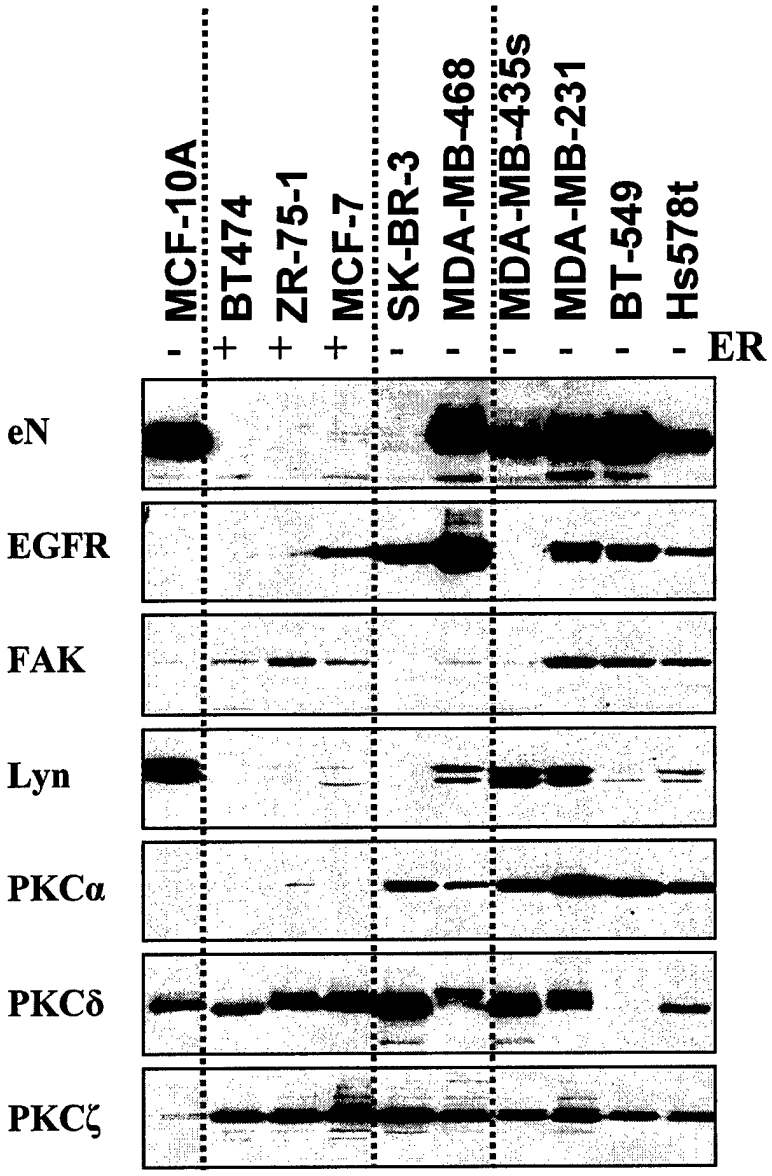
OB-Cadherin

Fig. 2A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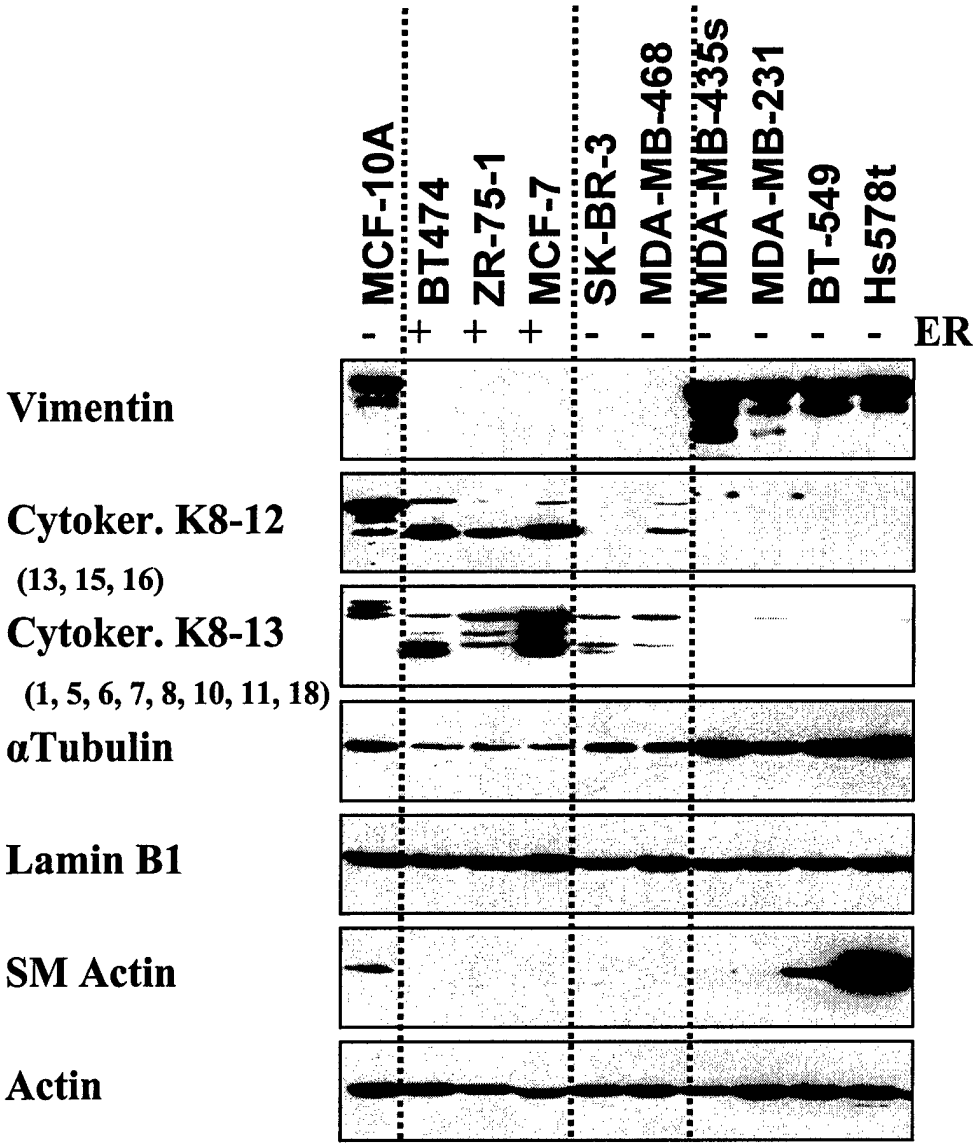
PKCζ

Fig. 2B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MCF-7
SK-BR-3
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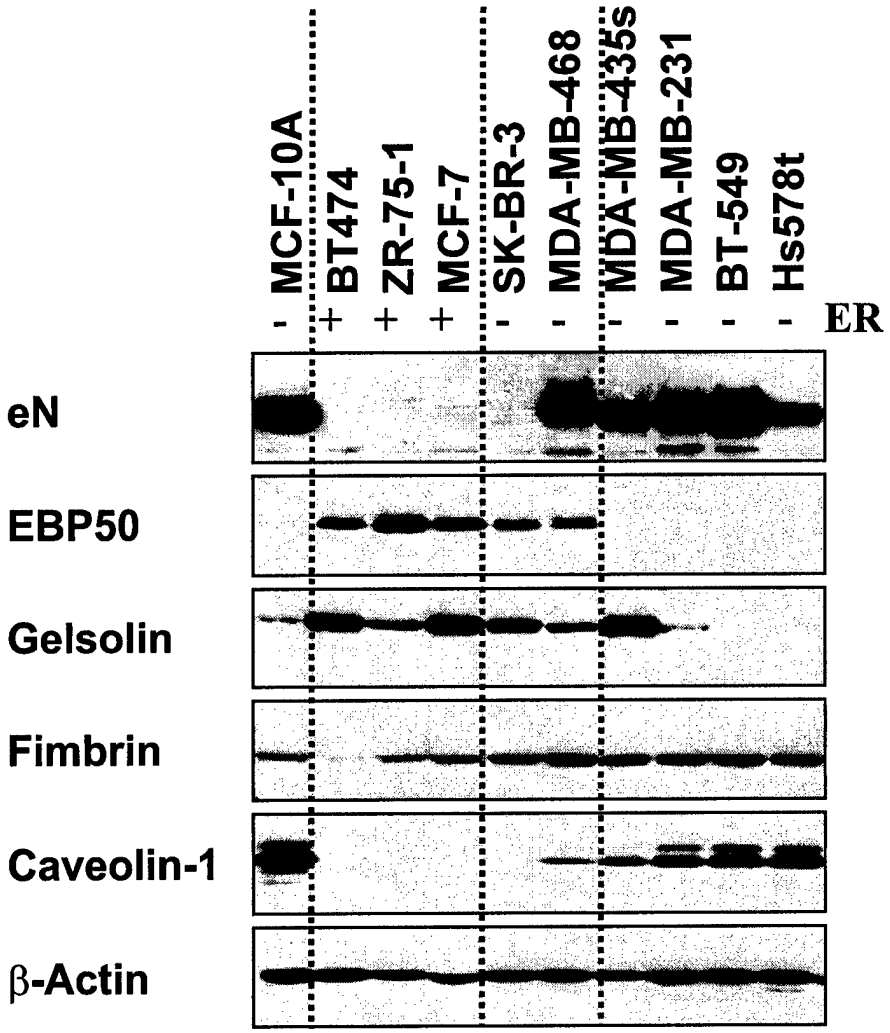
BT-549
Hs578t
ER
- + + + - - - - - -
Vimentin
Cytoker. K8-12
(13, 15, 16)
Cytoker. K8-13
(1, 5, 6, 7, 8, 10, 11, 18)
αTubulin
Lamin B1
SM Actin
Actin

Fig. 2C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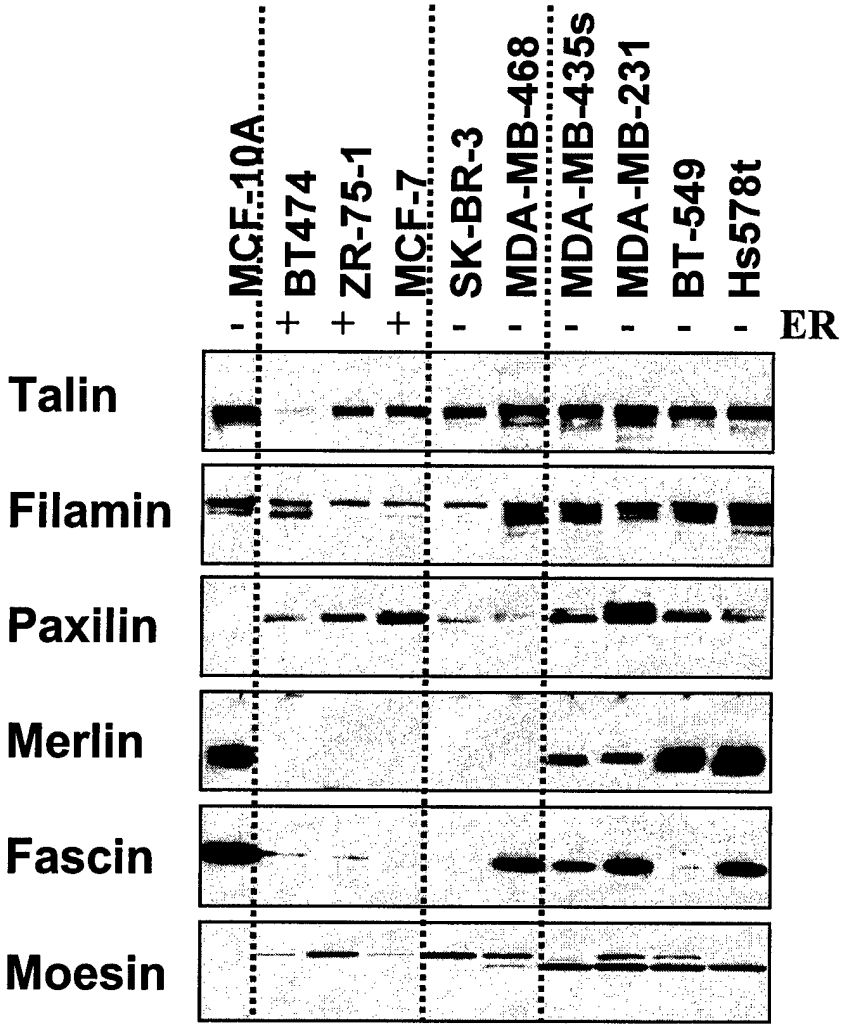
Gelsolin
Fimbrin
Caveolin-1
β-Actin

Fig. 2D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Fascin
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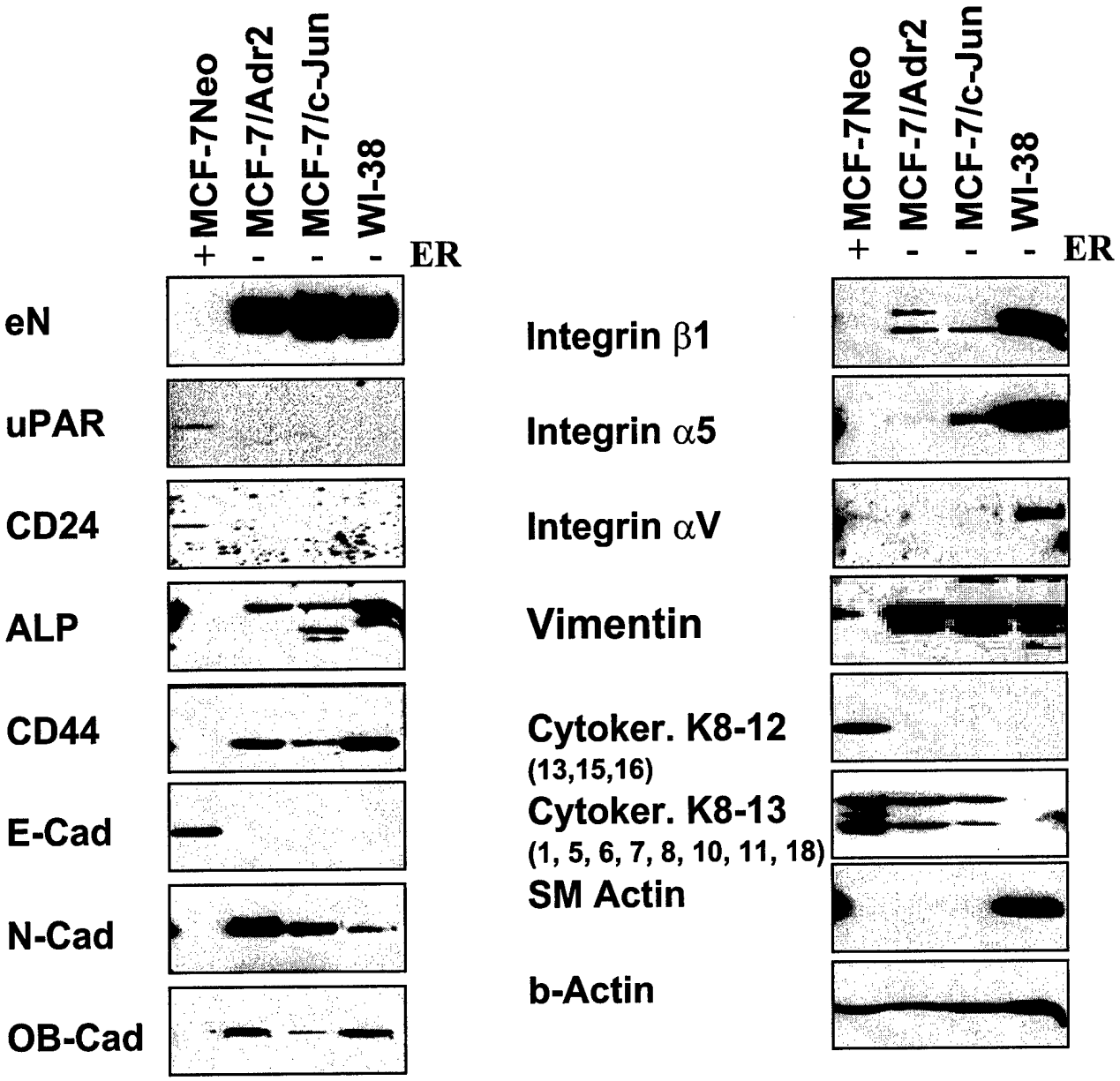
Fig. 2E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ER
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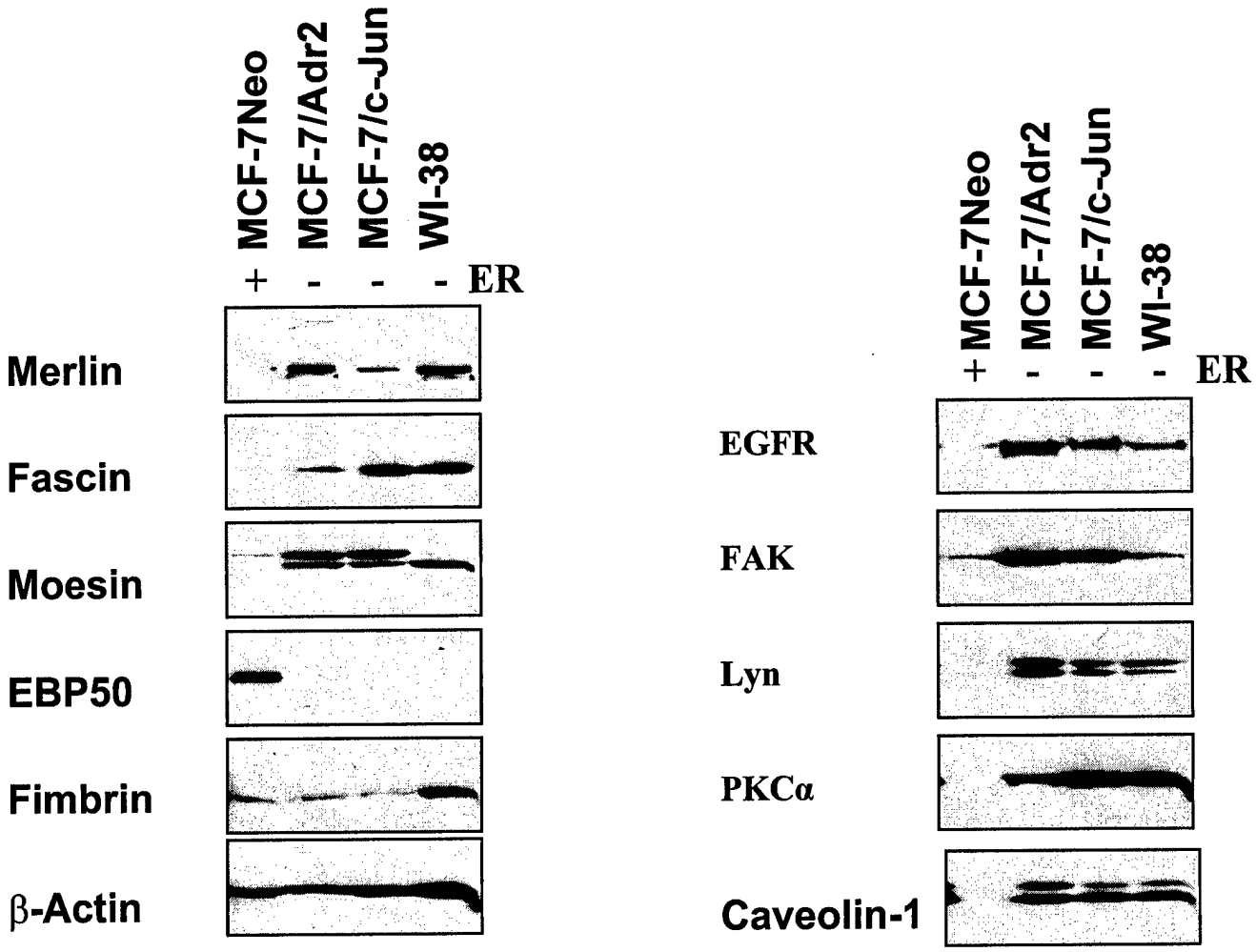
N-Cad
OB-Cad
+ MCF-7Neo
- MCF-7/Adr2
- MCF-7/c-Jun
- WI-38
ER
Integrin β1
Integrin α5
Integrin αV
Vimentin
Cytoker. K8-12
(13,15,16)
Cytoker. K8-13
(1, 5, 6, 7, 8, 10, 11, 18)
SM Actin
b-Actin

Fig. 3A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- MCF-7/Adr2
- MCF-7/c-Jun
- WI-38
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Merlin
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Moesin
EBP50
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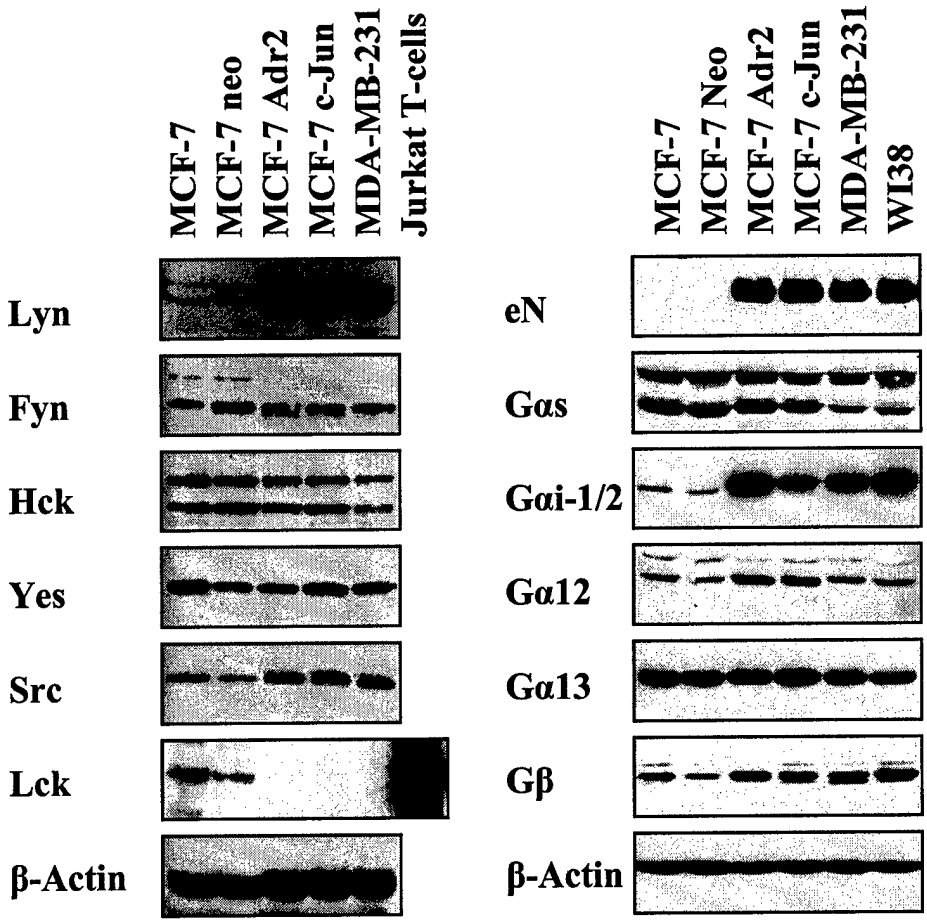
β-Actin
+ MCF-7Neo
- MCF-7/Adr2
- MCF-7/c-Jun
- WI-38
ER
EGFR
FAK
Lyn
PKCα
Caveolin-1

Fig. 3B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MCF-7 Adr2
MCF-7 c-Jun
MDA-MB-231
Jurkat T-cells
Lyn
Fyn
Hck
Yes
Src
Lck
β-Actin
MCF-7
MCF-7 Neo
MCF-7 Adr2
MCF-7 c-Jun
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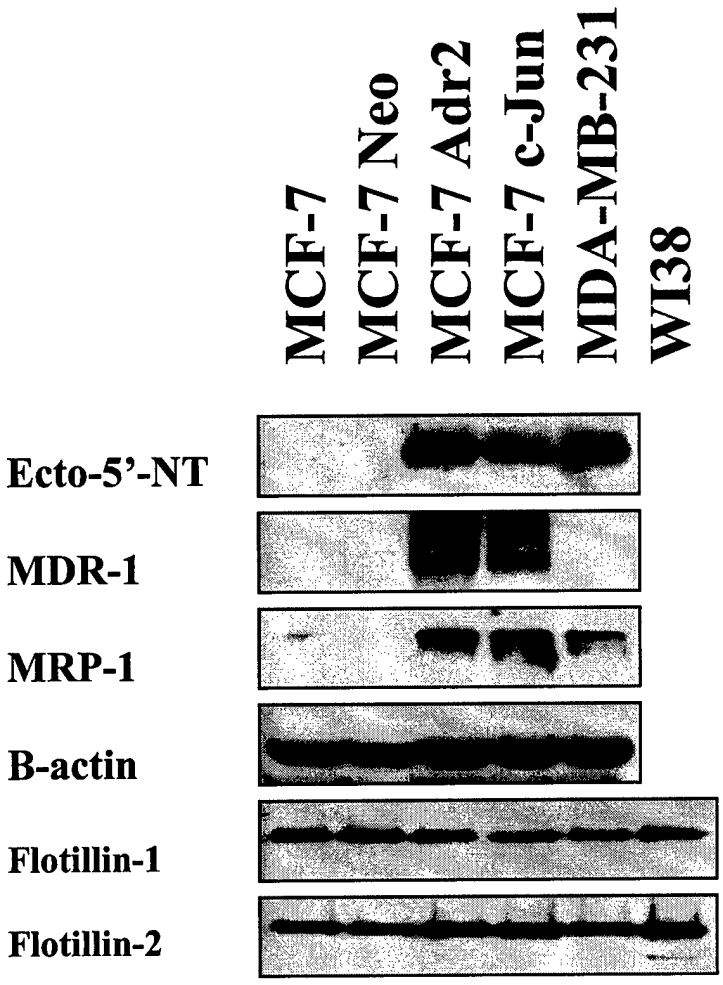
β-Actin

Fig. 4A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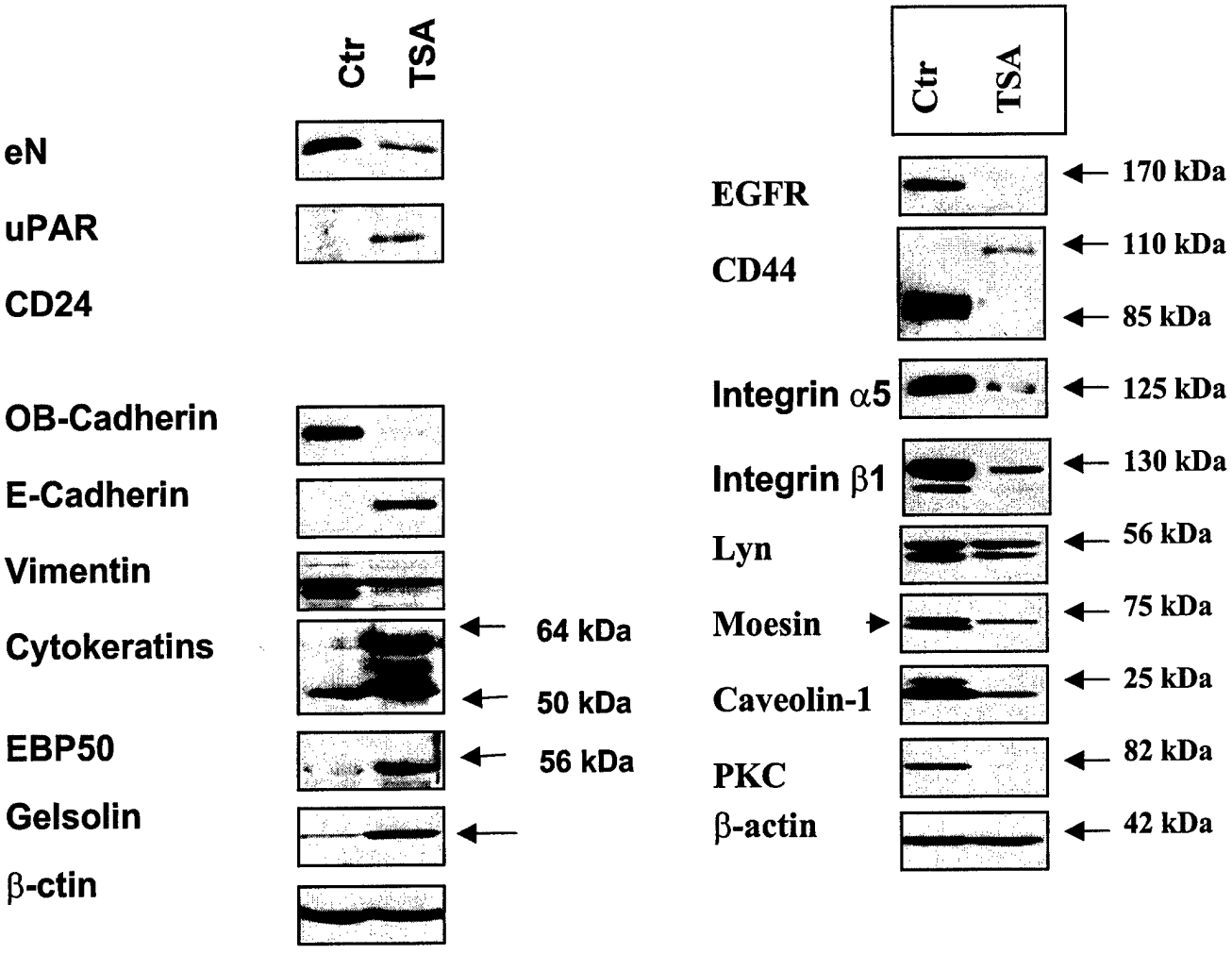
Flotillin-1
Flotillin-2

Fig. 4B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Gelsolin
β-ctin
Ctr
TSA
EGFR
170 kDa
CD44
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85 kDa
Integrin α5
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β-actin
42 kDa

Fig. 5

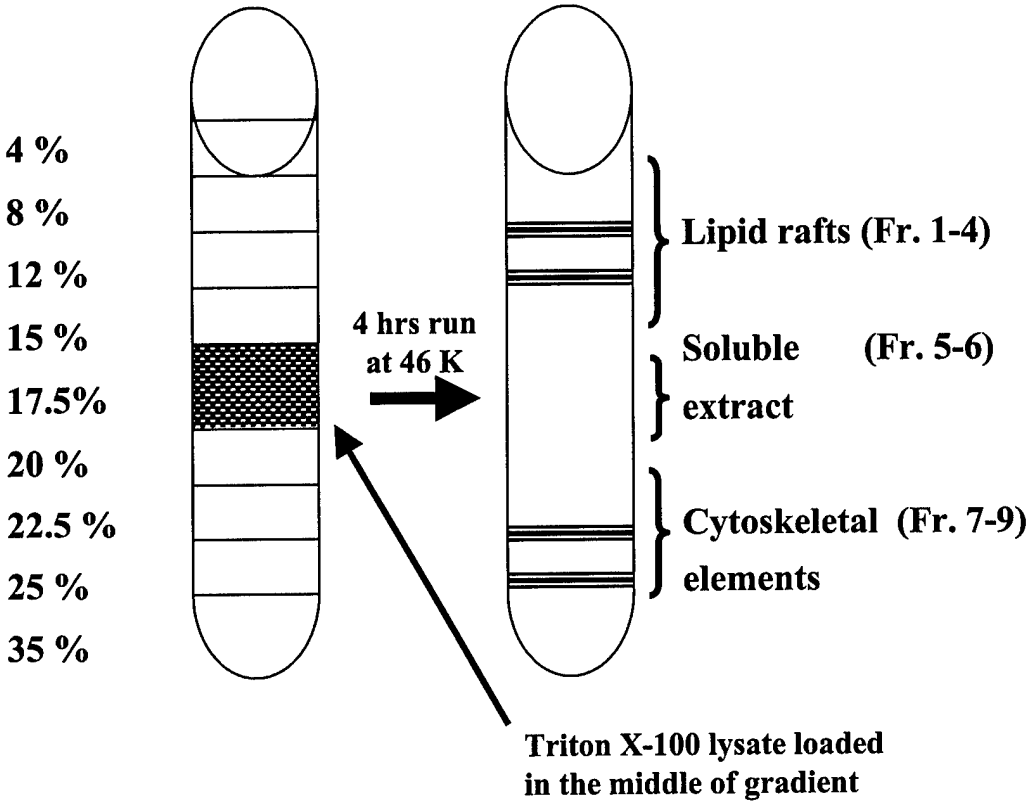
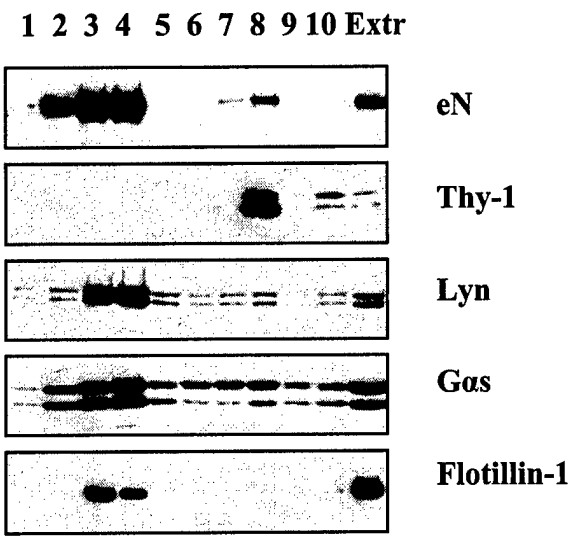

4 %
8 %
12 %
15 %
17.5%
20 %
22.5 %
25 %
35 %
4 hrs run
at 46 K
Lipid rafts (Fr. 1-4)
Soluble (Fr. 5-6)
extract
Cytoskeletal (Fr. 7-9)
elements
Triton X-100 lysate loaded
in the middle of gradient

Fig. 6A

MDA-MB-231 cells



MCF-7 cells

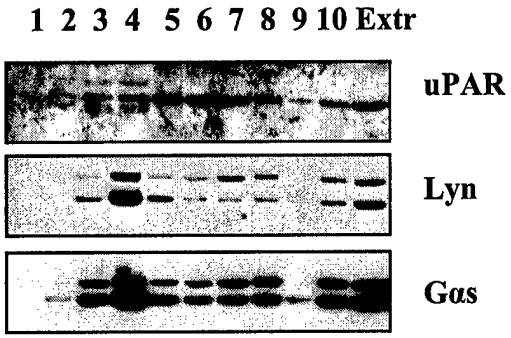


Fig. 6B

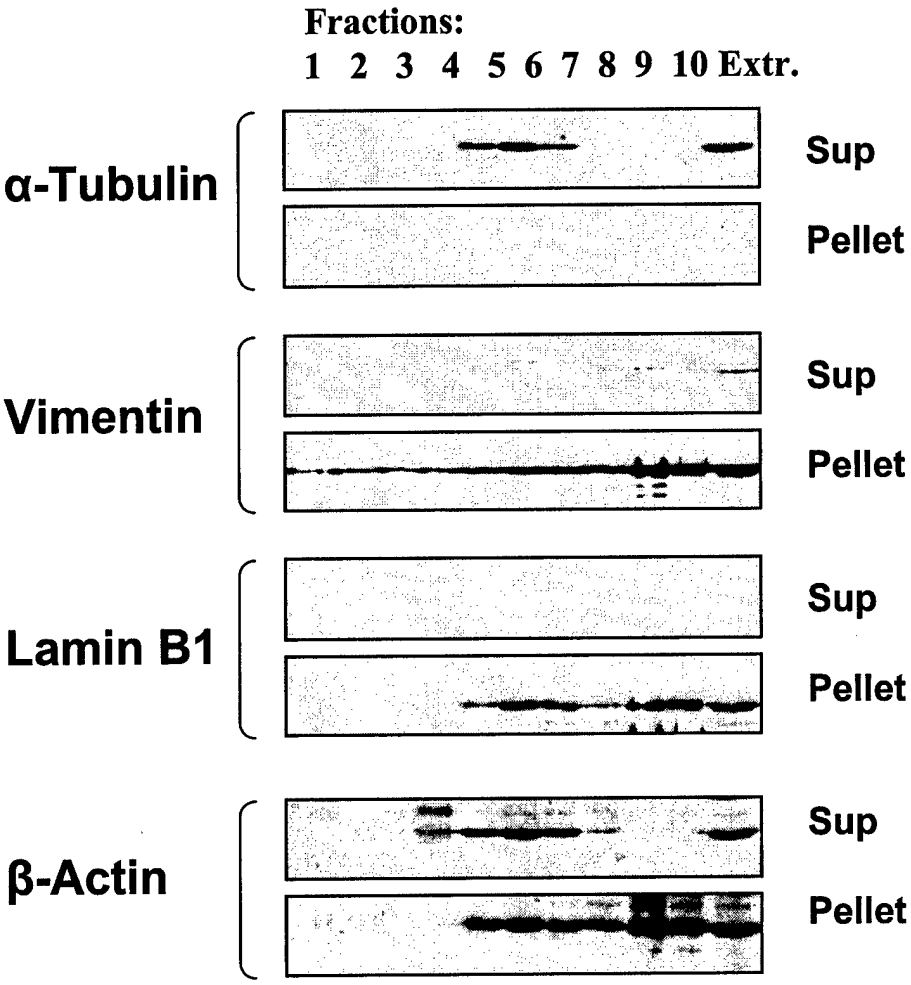

Fractions:
1 2 3 4 5 6 7 8 9 10 Extr.
α-Tubulin
Sup
Pellet
Vimentin
Sup
Pellet
Lamin B1
Sup
Pellet
β-Actin
Sup
Pellet

Fig. 6C

MDA-MB-231 cells

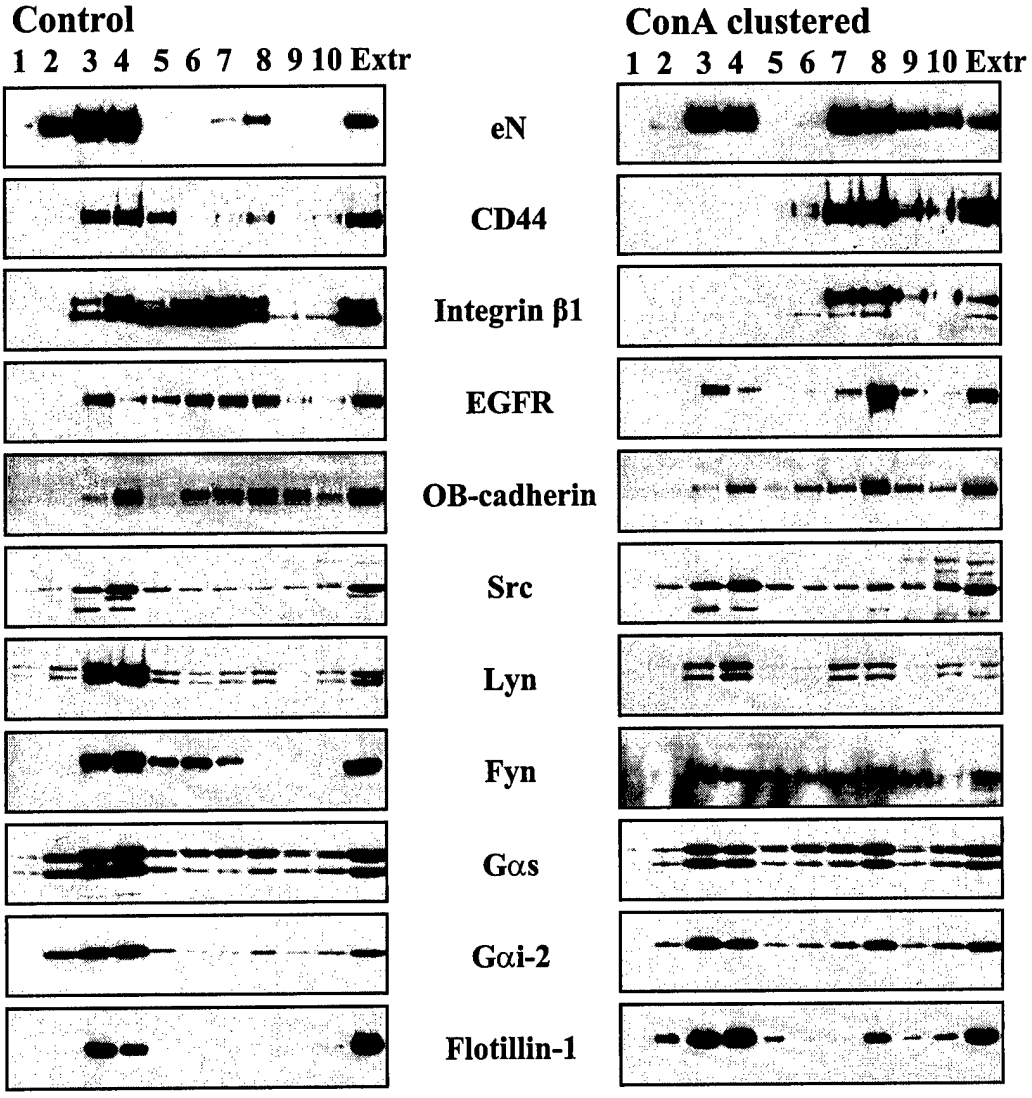


Fig. 7A

MCF-7 cells

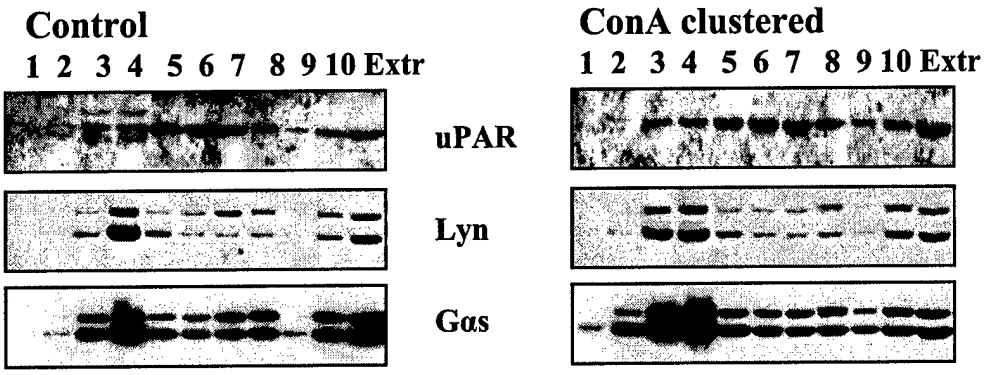
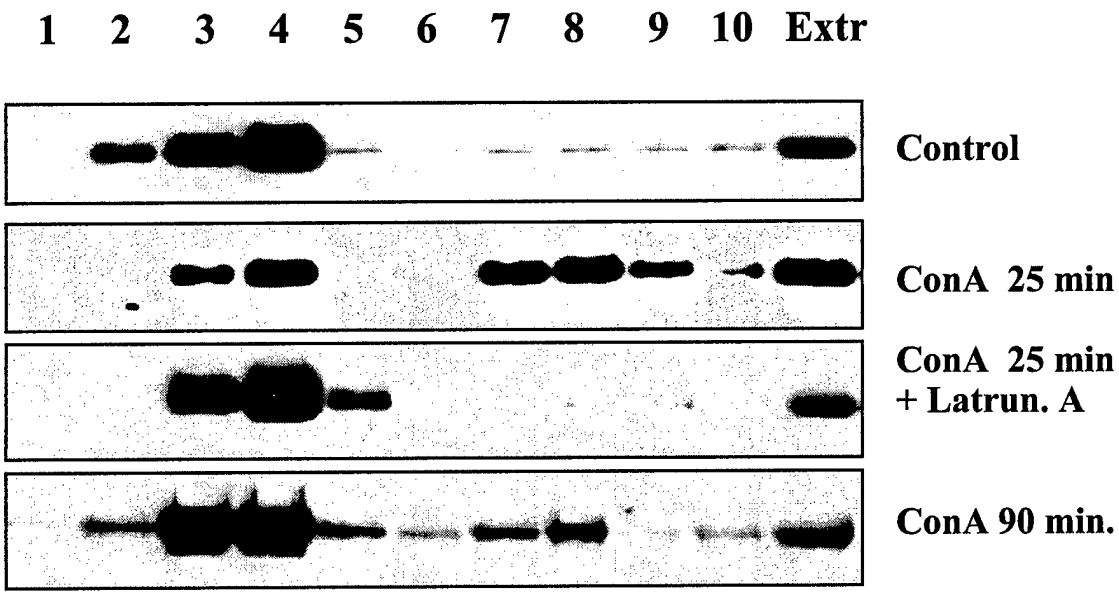


Fig. 7B


1 2 3 4 5 6 7 8 9 10 Extr
Control
ConA 25 min
ConA 25 min
+ Latrun. A
ConA 90 min.

Mammalian 5'-nucleotidases

Vera Bianchi[1]* and Jozef Spychala[2]

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email: vbianchi@mail.bio.unipd.it;

Footnote: [1] Review.

Aknowledgments: We thank Agnes Rinaldo-Matthis for providing figure 1.

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Nucleoside monophosphate phosphohydrolases or 5'-nucleotidases (members of EC 3.1.3. and EC 3.1.3.6) dephosphorylate non-cyclic nucleoside monophosphates to nucleosides and inorganic phosphate. Seven human 5'-nucleotidases with different subcellular localization have been cloned (Table I). Sequence comparisons show high homology only between cN-IA and B and also cdN and mdN, however the existence of common motifs within the active site suggests similar catalytic mechanism for the intracellular 5'-nucleotidases. Several 5'-nucleotidases are more ubiquitously expressed (eN, cN-II and cdN), while other display tissue-specific expression (cN-I, cN-III and mdN).

Here we summarize recent advances on the structure and cellular functions of the cloned 5'-nucleotidases. We also propose a revised nomenclature, agreed upon with other colleagues active in the nucleotidase field.

Table 1. Classification of 5'-nucleotidases

Revised Protein Nomenclature	Full Name and Gene Symbol	UniGene Cluster No	Aliases	References
eN	Ecto-5'-nucleotidase, *NT5E*	Hs.153952	Ecto-5'-NT; Low Km 5'-NT; eNT; CD73	(1)
cN-IA	Cytosolic 5'-nucleotidase IA, *NT5C1A*	Hs.307006	AMP-specific 5'-NT; cN-I	(2, 3)
cN-IB	Cytosolic 5'-Nucleotidase IB, *NT5C1B*	Hs.120319	cN-IA homolog; AIRP	(4)
cN-II	Cytosolic 5'-nucleotidase II, *NT5C2*	Hs.138593	High Km 5'-NT, Purine 5' NT; GMP, IMP-specific 5'-NT	(5)
cN-III	Cytosolic 5'-nucleotidase III, *NT5C3*	Hs.55189	PN-I; P5'N-1; UMPH	(6)
cdN	Cytosolic 5'(3')-deoxynucleotidase, *NT5C*	Hs.67201	dNT-1; PN-II	(7, 8)
mdN	Mitochondrial 5'(3')-deoxynucleotidase, *NT5M*	Hs.16614	dNT-2	(9)

Catalytic mechanism. Crystal structures are known for human mdN (10) and cdN (Rinaldo-Matthis and Nordlund, in preparation) and for *E.coli* periplasmic 5'-nucleotidase (11), a homologue of eN. All intracellular nucleotidases share a DXDXV/T motif that is critical for catalysis. eN belongs to a separate family that includes also 2'-3'-cyclic phosphodiesterases and apyrases.

The crystal structure of mdN and work on the active site of cN-II revealed a great deal on reaction mechanism that likely apply to all other intracellular 5'-nucleotidases (10, 12). The key reaction intermediate is phosphoaspartate at position 41 in the DXDXV/T motif (12). The second aspartate in the above motif also has role in the reaction (Fig.1) (10). The pentacovalent phosphorus generated at phosphoaspartate 41 has the same organization as the intermediate detected in the structure of beta-phosphoglucomutase (13). The x-ray structure of cdN suggests a catalytic mechanism identical to that of mdN and differences within the active site account for differences in substrate specificity (Rinaldo-Matthis & Nordlund, in preparation). Using the structurally important residues the best alignment was between the two deoxynucleotidases and cN-III (10). Two 5'-nucleotidases, cN-II and cN-III, exibit reverse phosphotransferase activity (14^1, 15^1), possibly due to higher stability of the phosphoenzyme intermediate or faster exchange of the nucleoside product with the nucleoside acceptor.

The active site of *E. coli* 5'-nucleotidase, the paradigm for eN, contains two zinc ions and the catalytic dyad Asp-His (11). No phosphoenzyme intermediate is formed during catalysis but a water molecule performs the nucleophilic attack on the phosphate (16).

Properties of 5'-nucleotidases. All 5'-nucleotidases have relatively broad substrate specificity. In agreement with the structural information on the active sites (10, 11), all family members, except eN, are absolutely dependent on Mg for activity. Table 2 summarizes the major enzymatic features of 5'-nucleotidases and refers to known nucleotide analog inhibitors. The most active inhibitors described so far are pyrimidine nucleotide and nucleoside analogs inhibiting cN-I at nmolar or low micromolar concentrations with up to 1000-fold selectivity for cN-I relative to cN-II or eN (18). Two pyrimidine phosphonates inhibit cdN and mdN (8,20), with weaker inhibition of cN-I (20).

Specific properties of individual 5'-nucleotidases are discussed below.

Table 2. Distinctive features of 5'-nucleotidases.

Enzyme	Protein structure (monomer kDa^)	Substrate affinity (K_m)	Effect of ATP	pH optimum	Known inhibitors (ref)
eN	Dimer (63)	μM	_	7.5	(17)
cN-IA°	Tetramer (41)	μM-mM$^+$	+ (ADP ++)*	7	(18)
cN-II	Tetramer (65)	sub mM	++	6.5	(19)
cN-III	Monomer (33)	sub mM	none	7.5	-
cdN	Dimer (23)	mM	none	6-6.5	(20)
mdN	Dimer (26)	sub mM	none	5.5	(20)

^ Predicted from cDNA sequence and not including posttranslational modifications. °cN-IB not yet characterized. * ADP and dADP are best activators. $^+$μmolar K_m values for pyrimidine deoxynucleotides, mmolar or submillimolar for purine substrates (3,18).

a) Ecto-5'-nucleotidase (eN), also known as CD73, is heavily glycosylated protein bound to the outer surface of the plasma membrane by a GPI anchor (1). As other GPI-linked and membrane anchored proteins, eN co-localizes with detergent resistant and glycolipid-rich membrane subdomains called lipid rafts. Although it has broad substrate specificity, AMP is considered to be the major physiological substrate (22-24). Independently of the enzymatic function, the protein acts as co-receptor in T cell activation (23[1]) and as cell-adhesion molecule (24[1]). eN is variably expressed in a wide number of cell types in physiological and pathological conditions (23[1], 24[1]). The proximal promoter region of the gene contains a number of tissue-specific elements that may explain variable level of expression (25,26).

b) Cytosolic 5'-nucleotidase IA (cN-IA) was named AMP-specific 5'-nucleotidase for its high specific activity with AMP at millimolar concentrations, however subsequent detailed kinetic studies revealed high affinities toward deoxypyrimidine monophosphates (18). It is highly expressed in skeletal and heart muscle where it has a physiological function in the generation of signalling adenosine during ischemia (2, 27). However, the high affinity for deoxynucleotides suggests for the potential role role in their metabolism. A homologous sequence related to human autoimmune infertility gene (*AIRP*) and with highest expression in testis has been cloned and designated cN-IB (4).

c) **Cytosolic 5'-nucleotidase II (cN-II)** is a 6-hydroxypurine-specific nucleotidase, most active with (d)IMP (14^1) and positioned to regulate ATP and GTP pools. The tetrameric protein is stimulated by (d)ATP and GTP and regulated by substrate and phosphate in a complex manner (14^1, 27-29), possibly involving subunit association and dissociation (30). Under physiological conditions cN-II can catalyze phosphotranfer from a purine nucleotide donor to inosine or guanosine (14^1, 31). This reaction is responsible for the activation of several anti-viral and anti-cancer nucleoside analogs that are not substrates of cellular nucleoside kinases (14^1).

d) Cytosolic 5'-nucleotidase III (cN-III) is highly expressed in red blood cells where it participates in the degradation of RNA during erythrocyte maturation (15^1). It prefers pyrimidine ribo- over deoxy-nucleotides with CMP as best substrate. It is inactive with purine nucleotides. The enzyme has phosphotransferase activity (32), but less efficient than cN-II (31). The sequence of cN-III coincides with that of p36, an interferon alfa-induced protein of unknown function (6). Alternative splicing of exon 2 gives rise to two protein products 286 and 297 a.a. long (33), with the shorter form corresponding to cN-III.

e) Cytosolic 5'(3')-deoxynucleotidase (cdN) is an ubiquitous enzyme, first purified to homogeneity from human placenta (34). It is the major deoxynucleotidase activity in cultured human cells (20, 35). In contrast to cN-III, human cdN is not strictly pyrimidine-specific and also works efficiently with dIMP and dGMP. dAMP is a poor substrate and dCMP is inactive (8,34). The enzyme is very active on 2'- and 3'-phosphates (7, 34). Neither the highly purified human placental cdN nor the recombinant mouse and human enzymes showed phosphotransferase activity (7,34), in contrast to what was reported for cdN purified from human RBC (32).

f) Mitochondrial 5'(3')-deoxynucleotidase (mdN) is highly homologous to cytosolic cdN (52% amino acid identity). The two enzymes are coded by nuclear genes with identical structure, probably derived by a gene duplication event (9). With its high preference for dUMP and dTMP mdN shows remarkably narrow substrate specificity. Similarly to cdN, mdN prefers deoxy- over ribonucleotides and also accepts 3'- and 2'-nucleoside monophosphates (9,35). Its enzymatic features suggest that mdN regulates mitochondrial dTTP and prevents accumulation of mutagenic dUTP within mitochondria.

Detection and quantitation of 5'-nucleotidases . Detection of individual nucleotidases using enzymatic assay in cell lysates is problematic because different nucleotidases are co-expressed in the same tissue or cell type. The problem was earlier addressed by immunotitration (14^1), more recently by a strategy that exploits differences in optimal assay conditions between ubiquitous nucleotidases (20). Enzyme assays are performed either with radioactive substrate or non-labeled nucleotides measuring production of inorganic phosphate. Phosphotransferase activity is assayed by conversion of a radioactive nucleoside to nucleotide (12,31). Differential assays also take advantage of inhibitors of individual nucleotidases (8,17^1,20,35). Multiple-tissue Nothern blots reveal tissue-specific expression of nucleotidases (3,9) and by overexposure can detect wider low-level expression (3). Quantitative and semi-quantitiative RT-PCR is used to measure mRNAs (4,36) although the correlation between mRNA and protein levels may not be always straightforward (4). A significant limitation of tissue-wide surveys is the cell heterogeneity of tissues and one specific cell type overexpressing given nucleotidase (for example high level of eN in endothelial cells) may significantly blurr the expression profile. Polyclonal or monoclonal antibodies are potentially good tools for quantitation, however only eN has been reliably assessed in cells or cell extracts using either Flow Cytometry or Western blotting (22, 23). Immunofluorescence or immunocytochemistry were also employed to study tissue and cell distribution of cN-1A (2) and cN-II (14^1).

Physiological role of 5'-nucleotidases: insights from overexpressing cell lines. By opposing the phosphorylation of nucleosides by kinases, intracellular 5'-nucleotidases participate in substrate cycles that regulate the cellular levels of ribonucleoside and deoxyribonucleoside monophosphates and, by extension, ribonucleotide and deoxyribonucleotide pools (37, 38). Intracellular 5'-nucleotidases have relatively high K_ms and operate on substrates generally present at (very) low concentrations. Thus they are exquisitely sensitive to oscillations of substrate concentration. However, given their overlapping substrate specificities, it is difficult to tie a given enzyme to the maintenance of a specific nucleotide pool. Important information has been obtained with cell lines engineered to overexpress specific nucleotidases. The higher expression induces the excretion of nucleosides that reflect the *in vivo* substrate selectivity of the 5'-nucleotidase. By this strategy cN-IA was shown to operate on AMP (27,39), and cN-II to act on IMP and GMP (27,38). Which particular 5'-nucleotidase is involved in deoxynucleotide pool regulation has not been yet fully established. Murine cdN was shown to regulate all pyrimidine deoxyribonucleotide pools (38). However, in human cells dCMP is dephosphorylated by a different enzyme, as human cdN is inactive on dCMP. A potential candidate is cN-IA that has high affinity for all deoxyribonucleotides (3,18), although it is still not clear whether the expression of this enzyme outside of skeletal and heart muscle is sufficient to perform this function (3). Strategies such as knockout mice and siRNA may help solve these issues. Indeed, dowregulation of mdN in cultured human cells by siRNA showed that mdN participates in a mitochondrial substrate cycle with the mitochondrial thymidine kinase (C. Rampazzo & V. Bianchi, unpublished).

Clinical implications of 5'-nucleotidases. The only known genetic syndrome due to 5'-nucleotidase deficiency is the hereditary haemolytic anemia caused by mutation of cN-III (15^1). Accumulation of normally undetectable pyrimidine nucleotides in erythrocytes of affected subjects underlies the important role of cN-III during maturation of RBC.

Antiviral and antiblastic nucleoside analogs that are used in the clinic must be activated by phosphorylation to exert their therapeutic effect. Intracellular 5'-nucleotidases influence the metabolism of these analogs by reversing the activation step and thereby decreasing the pharmacological efficacy of a given compound. Several of the *in vitro* models of nucleoside analog resistence were linked to high expression of cN-II and cN-IA (3, 41, 43). Consistent with the role of 5'-nucleotidases and nucleoside kinases in substrate cycles described earlier, the relative ratio of nucleoside kinase to 5'-nucleotidase have been found to have predictive clinical value (43). Based on these new clinical implications of 5'-nucleotidase activity one may suggest that development of compounds that inhibit this enzyme may increase the efficacy of existing drugs. Alternatively, development of new nucleoside analogs that are poor substrates for 5'-nucleotidases may lead to more effective therapies in the future.

Phosphorylation by the phosphotransferase activity of cN-II is the only known mechanism for the activation of analogs such as 2'-3'-dideoxyinosine (14^1). The substrate specificity of 5'-nucleotidases towards individual analogs permits to taylor the therapy to the biochemical phenotype of the target cells. The level of expression of a relevant 5'-nucleotidase may be a prognostic factor for patients (43). In the development of resistance to nucleoside analogs in *in vitro* selected cell lines the relative ratio between a nucleoside kinase and a 5'-nucleotidase may be a determining factor (43). However in some cases it is difficult to reconcile the known substrate specificity of the nucleotidase with the resistant phenotype, e.g. in the case of araC resistance in cells with elevated cN-II activity. Analyses of the substrate specificity of recombinant 5'-nucleotidases for various analog monophosphates (3,8) and the pattern of

resistance to analogs in transfected cell lines overproducing a specific 5'-nucleotidase (3) become very important to understand *in vivo* resistance to nucleoside analogs and to design more efficient analogs. Several antiviral nucleoside analogs show striking mitochondrial toxicities (42). The crystal structure of mdN (10) may help design new antiviral compounds more susceptible to dephosphorylation by this enzyme to limit mitochondrial side-effects.

Conclusions. The presence in the human genome of at least seven genes for 5'-nucleotidases suggests that these enzymes perform important metabolic functions. With the enzymes available in recombinant form it will soon be possible to complete their biochemical characterization. Gene regulation remains instead an unchartered field. We do not know how strict is the tissue-specific expression of cN-I and cN-III, how the variable expression levels of the ubiquitous nucleotidases shown by multiple-tissue northern blots are obtained, if expression of individual enzymes can be induced or repressed in specific circumstances, if patterns of expression are altered in specific tumors.

The new RNA technologies and microarray analyses of overall expression profiles in cells will contribute to clarify the role of 5'-nucleotidases in the regulation of nucleotide pools. We are looking forward to the new information to come.

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Figure legend

Fig.1. Structure of the active site of mdN with the pentavalent phosphorous intermediate produced by nucleofilic attack of asp41 on the phosphate (10). Asp 41 and asp 43 are the two aspartates in the motif conserved in intracellular 5'-nucleotidases.