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## Nicotinamide phosphoribosyltransferase can affect metastatic activity and cell adhesive functions by regulating integrins in breast cancer

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### Abstract

NAD<sup>+</sup> metabolism is an essential regulator of cellular redox reactions, energy pathways, and a substrate provider for NAD<sup>+</sup> consuming enzymes. We recently demonstrated that enhancement of NAD<sup>+</sup>/NADH levels in breast cancer cells with impaired mitochondrial NADH dehydrogenase activity, through augmentation of complex I or by supplementing tumor cell nutrients with NAD<sup>+</sup> precursors, inhibits tumorigenicity and metastasis. To more fully understand how aberrantly low NAD<sup>+</sup> levels promote tumor cell dissemination, we here asked whether inhibition of NAD<sup>+</sup> salvage pathway activity by reduction in nicotinamide phosphoribosyltransferase (NAMPT) expression can impact metastasis and tumor cell adhesive functions. We show that knockdown of NAMPT, the enzyme catalyzing the rate-limiting step of the NAD<sup>+</sup> salvage pathway, enhances metastatic aggressiveness in human breast cancer cells and involves modulation of integrin expression and function. Reduction in NAMPT expression is associated with upregulation of select adhesion receptors, particularly  $\alpha v \beta 3$  and  $\beta 1$  integrins, and results in increased breast cancer cell attachment to extracellular matrix proteins, a key function in tumor cell dissemination. Interestingly, NAMPT downregulation prompts expression of integrin  $\alpha v \beta 3$  in a high affinity conformation, known to promote tumor cell adhesive interactions during hematogenous metastasis. NAMPT has been selected as a therapeutic target for cancer therapy based on the essential functions of this enzyme in NAD<sup>+</sup> metabolism, cellular redox, DNA repair and energy pathways. Notably, our results indicate that incomplete inhibition of NAMPT, which impedes NAD<sup>+</sup> metabolism but does not kill a tumor cell can alter its phenotype to be more aggressive and metastatic. This phenomenon could promote cancer recurrence, even if NAMPT inhibition initially reduces tumor growth.

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## Keywords

NAMPT; NAD<sup>+</sup> salvage pathway; integrins;  $\alpha\text{v}\beta\text{3}$ ; breast cancer; metastasis

## 1. Introduction

Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) metabolism is an essential regulator of cellular redox reactions, energy pathways, and a substrate provider for NAD<sup>+</sup> consuming enzymes<sup>1-3</sup>. These include ADP-ribose transferases, poly(ADP-ribose) polymerases (PARPs), and NADases such as CD38 and sirtuins. While NAD<sup>+</sup> consuming enzymes affect NAD<sup>+</sup> availability and typically degrade it to nicotinamide, redox reactions involve reduction of NAD<sup>+</sup> to NADH, which can be converted back to NAD<sup>+</sup> by NADH dehydrogenase activity of mitochondrial complex I<sup>1-9</sup>.

NAD<sup>+</sup> can be synthesized *de novo* from tryptophan, nicotinamide, nicotinic acid or nicotinamide riboside, or be derived via the salvage pathway. The initial and rate-limiting step within the NAD<sup>+</sup> salvage pathway is mediated by nicotinamide phosphoribosyltransferase (NAMPT), the enzyme that catalyzes conversion of nicotinamide to nicotinamide mononucleotide (NMN<sup>+</sup>) using phosphoribosylpyrophosphate as a co-substrate. NMN<sup>+</sup> is then converted to NAD<sup>+</sup> by nicotinamide nucleotide adenylyltransferases (NMNAT)<sup>1,2,10-12</sup>.

Cancer cells, especially highly proliferative cells in fast growing tumors such as triple negative breast cancers, generally accumulate high levels of DNA damage and genomic instability<sup>13-16</sup>. These cells can have increased NAD<sup>+</sup> degrading PARP activity for DNA damage repair, and thus a high need for NAD<sup>+</sup> to maintain cell viability. Therefore, fast growing cells often have low NAD<sup>+</sup> levels which sensitize them to further NAD<sup>+</sup> reduction<sup>1,17,18</sup>. Thus, it has been suggested that high NAMPT expression should enhance tumor cell survival by elevating NAD<sup>+</sup> levels<sup>19-21</sup>, while chemical inhibition of NAMPT to reduce cellular NAD<sup>+</sup> levels should inhibit tumor cell viability, especially when used in combination with PARP inhibitors. This strategy has been proposed as a therapeutic approach against breast cancer<sup>11,20,22,23</sup>.

In contrast to the concept that inhibition of NAMPT activity might eliminate tumor cells, we showed earlier that impaired NAD<sup>+</sup> metabolism activity and resulting decrease in NAD<sup>+</sup>/NADH redox levels in human breast cancer cells can actually significantly stimulate their metastatic properties<sup>7</sup>. Mechanisms underlying the enhanced metastatic aggressiveness were found associated with aberrant mitochondrial complex I and poor NADH dehydrogenase activity. Metastatic aggressiveness could be suppressed by enhancing complex I function through expression of yeast NADH dehydrogenase Ndi1 in the tumor cells. Augmentation of complex I activity enhanced the cellular NAD<sup>+</sup>/NADH balance and supported autophagy, while suppressing the mTORC1 pathway<sup>7</sup>. To more fully understand how reduced NAD<sup>+</sup> levels promote tumor cell dissemination, we here asked whether inhibition of NAD<sup>+</sup> salvage pathway activity by reduction in NAMPT expression can impact tumor cell adhesive properties. Aberrant cell adhesion supports the metastatic process by mediating tumor cell interaction with vascular cells or the lymphatic system, as well as with tissues and matrices

in target organs of metastasis<sup>24,25</sup>. As major adhesive, migratory and invasive tumor cell functions are mediated by adhesion receptors, we here asked whether interference with NAMPT affects adhesion receptor expression and function.

## 2. Materials and methods

### 2.1 Cell culture

MDA-MB-231 human breast cancer cells and their variants were stably transduced with Firefly luciferase (F-luc) using lentiviral expression vector pTacoma (CMV promoter) (BE Torbett, TSRI) to analyze metastasis by non-invasive bioluminescence imaging (55). Cells were grown in EMEM supplemented with nonessential amino acids, vitamins, 2 mM L-glutamine, 1 mM pyruvate, and 10% FBS.

### 2.2 NAMPT expression and knockdown

Lentiviral vector containing small hairpin RNA (shRNA) against NAMPT (shNAMPT) (TRCN0000116180) or non-mammalian targeting control shRNA (SHC0002) (shCT) were from Sigma-Aldrich, MO. Knockdown efficiency was quantified by real time PCR using FastStart Universal SYBR Green Master (Rox) (Roche) and the following primers: human NAMPT-F (GCCAGCAGGGAATTTTGTTA), human NAMPT-R (TGATGTGCTGCTTCCAGTTC), human GAPDH-F (GGGAAGGTGAAGGTCGGAGT), and human GAPDH-R (TCCACTTTACCAGAGTTAAAAGCAG). The same primers were used to analyze NAMPT gene expression in lung metastases developing in SCID mice after i.v. injecting shNAMPT versus shRNA control cells. Data were recorded and analyzed using an ABI-PRISM 7700 Sequence Detection System (Applied Biosystems) and Sequence Detector Software (SDS v2.0). Reduction of NAMPT protein expression in shNAMPT knockdown cells was confirmed by Western blot using cells lysed in Laemmli's buffer and analyzing 20 µg of total protein, probed with anti-NAMPT (ab45890, abcam, MA) or anti β-tubulin (Sigma-Aldrich, MO), and followed with secondary antibodies conjugated to IRDye 800 (NAMPT) or IRDye 680 (β-tubulin) using an Odyssey infrared imaging system (LI-COR Biosciences). Data were analyzed and quantified using Odyssey infrared imaging system application software v3.0.

Moreover, NAMPT Knockdown was confirmed by western blot. Cells were lysed with Laemmli's buffer and 20µg of total protein was loaded in a SDS-page gel. Western blots were incubated with antibodies against: NAMPT (ab45890, abcam, MA) and β-tubulin (Sigma-Aldrich, MO). Antibody binding was detected following incubation with secondary antibodies conjugated to IRDye 680 (β-tubulin) 800 (NAMPT), using an Odyssey infrared imaging system (LI-COR Biosciences). Data were analyzed and quantified using Odyssey infrared imaging system application software v3.0.

### 2.3 NAD<sup>+</sup>/NADH measurement

NAD<sup>+</sup> and NADH were analyzed independently in extracts of whole cells ( $1 \times 10^6$ ) prepared as previously described<sup>7</sup>. Concentrations were determined using a fluorescence NAD<sup>+</sup>/NADH detection kit according to the manufacturer's protocol (Cell Technology, Inc).

## 2.4 Metastatic activity

For experimental metastasis, female 6–8 week-old C.B-17/SCID mice were injected intravenously (i.v.) with F-luc-tagged cancer cells:  $2.5 \times 10^5$  MDA-MB-231 shCT or MDA-MB-231 shNAMPT cells. Mice were imaged weekly (IVIS 200, Xenogen) 10 min after i.p. injection of D-luciferin (100 mg/kg). Bioluminescence was quantified as photons/second/cm<sup>2</sup> in defined regions of interest using Living Image software. Animal work complied with National Institutes of Health and institutional guidelines (TSRI is AAALAC accredited).

## 2.5 Cell-adhesion assay

Cell-adhesion assays were performed in 96-well plates coated with 50  $\mu$ l of one of the following matrix proteins: human vitronectin (10  $\mu$ g/ml), fibrinogen (20  $\mu$ g/ml), fibronectin (10  $\mu$ g/ml) or collagen I (10  $\mu$ g/ml), in PBS overnight at 4 °C. The plates were washed twice with TBS and blocked with 2% BSA in TBS overnight at 4 °C. Cells were harvested with versene and washed with adhesion buffer (serum free EMEM medium containing 0.5% BSA) and then pre-incubated with or without 10  $\mu$ g/ml function blocking monoclonal anti-integrin antibodies:  $\beta$ 3 (M21.3),  $\alpha$ v (HB8448),  $\alpha$ v $\beta$ 5 (PIF6),  $\beta$ 1 (P5D2) and  $\alpha$ 5 (P1D6)<sup>26–29</sup> for 15 min at RT. Cells were then seeded at  $4 \times 10^4$ /well in the presence of the antibodies. Adhesion time was 35 min at 37°C. Non-adhered cells were removed by adding floatation medium (0.9% NaCl, 80% Percoll) as described previously<sup>30</sup>. Attached cells were fixed with 3.3% glutaraldehyde and 20% Percoll. The attached cells were stained with crystal violet, lysed with 0.5% Triton X-100 in water, and quantified by measuring optical absorbance at 585 nm.

## 2.6 Integrin expression

Surface integrin expression was analyzed by flow cytometry as follows: cells were harvested with versene, washed with PBS, and resuspended in ice cold binding buffer (1% BSA in TBS pH 7.4). 50  $\mu$ l containing  $10^5$  cells were then incubated with 30  $\mu$ g/ml of anti-integrin antibodies:  $\alpha$ v $\beta$ 3 (VNR1),  $\alpha$ v $\beta$ 5 (PIF6),  $\alpha$ 5 (P1D6) and  $\beta$ 1 (P5D2)<sup>26–29</sup>, in binding buffer for 45 min on ice. Cells were washed with cold PBS and incubated with 50  $\mu$ l of 1:100 APC-conjugated goat anti-mouse polyclonal antibody in binding buffer for 45 min on ice. After washing, cells were analyzed by flow cytometry using a FACS-Calibur 2 in FL-4 (Becton-Dickinson).

## 2.7 Integrin affinity based on binding of ligand mimetic antibody

The affinity state of integrin  $\alpha$ v $\beta$ 3 was assessed based on binding of soluble ligand mimetic RGD-containing human antibody ScFv1<sup>26</sup> which was here re-engineered into an intact IgG (EW84-IgG). Tumor cells were harvested with versene, washed first in ice cold cation free TBS and then in TBS with 2mM Ca<sup>2+</sup>, before incubation with EW84-IgG15 at  $\mu$ g/ml in TBS/Ca<sup>2+</sup> on ice for 45 min. After washing in TBS/Ca<sup>2+</sup>, cells were incubated with APC-conjugated anti-human Fc in TBS/Ca<sup>2+</sup> for 45 min on ice, then washed as above and read in FL-4 on a FACS-Calibur 2.  $\beta$ 3 integrin expression in these experiments was verified with non-cation dependent and non-ligand mimetic mAb AV-10 detected with goat-anti mouse APC conjugate<sup>31</sup>.

### 3. Results

#### 3.1 NAMPT expression affects metastatic activity of human breast cancer cells

To further understand how changes in cellular NAD<sup>+</sup> levels and NAD<sup>+</sup>/NADH ratios affect the ability of breast cancer cells to metastasize, we knocked-down expression of NAMPT, the rate-limiting enzyme in NAD<sup>+</sup> synthesis. NAMPT converts NAD<sup>+</sup> precursor nicotinamide to nicotinamide mononucleotide (NMN<sup>+</sup>) which is then coupled with the AMP moiety of ATP to form NAD<sup>+</sup> <sup>1,8,10</sup>. Using an shRNA approach, NAMPT gene and protein expression was significantly reduced in MDA-MB-231 human breast cancer cells (Fig. 1A, B). This resulted in reduced NAD<sup>+</sup> and slightly reduced NADH concentrations in these cells, and significantly lowered cellular NAD<sup>+</sup>/NADH ratio (Fig. 1C). Upon injection of the tumor cells into the venous circulation of SCID mice, NAMPT knock-down cells showed significantly enhanced metastatic activity compared to control shRNA transduced cells (Fig. 1D,E). Reduced levels of NAMPT expression in the highly metastatic knockdown cells were confirmed to be stable even in the metastatic niche (Fig. 1F). NAMPT Knockdown was confirmed to be stable even in the metastatic niche. Thus, reduction in NAD<sup>+</sup> synthesis caused enhanced aggressiveness in the already highly metastatic MDA-MB-231 breast cancer cells.

#### 3.2 NAMPT expression modulates breast cancer cell adhesive properties

Important cellular functions that help mediate tumor cell dissemination during metastasis are adhesive properties that support cell interaction with matrix proteins during cell adhesion, migration, and invasion. To investigate whether breast cancer cell binding to the extracellular matrix is altered when NAD<sup>+</sup> metabolism is changed, we analyzed MDA-MB-231 cell attachment to major matrix proteins, comparing NAMPT knock-down cells versus controls. We found that cell adhesion to vitronectin, fibrinogen, and type I collagen were clearly enhanced when NAMPT expression was reduced, while attachment to fibronectin was not significantly altered (Fig. 2A). Cell attachment to these extracellular matrix proteins is primarily mediated by adhesion receptors of the integrin family<sup>25</sup>. We therefore analyzed if down-regulation of NAD<sup>+</sup> metabolism through NAMPT knock-down affected expression of integrins known to support cell interaction with the tested matrix proteins. Two major changes were observed. NAMPT knock-down was associated with expression of integrin  $\alpha v \beta 3$  which is largely missing MDA-MB-231 parental or knock-down control cells, and an upregulation of  $\beta 1$  integrins (Fig. 2B, C). Attachment of MDA-MB-231 parental cells to vitronectin is known to be mediated primarily by integrin  $\alpha v \beta 5$ <sup>32</sup>. Expression of this integrin was not changed in response to NAMPT knock-down. The upregulation of  $\alpha v \beta 3$  expression upon NAMPT knock-down is therefore likely to contribute to the enhanced vitronectin adhesion of these cells (Fig. 2A). Attachment of MDA-MB-231 parental cells to fibronectin is mediated predominantly by integrin  $\alpha 5 \beta 1$ <sup>32</sup>. We found no change in  $\alpha 5$  integrin subunit expression in NAMPT knock-down cells, and this correlated with their unchanged adhesion to fibronectin (Fig. 2A,B). In contrast, attachment to collagen I was upregulated in NAMPT knock-down cells, consistent with increased expression of  $\beta 1$  integrins as seen by flow cytometry (Fig. 2B). The predominant collagen receptor of MDA-MB-231 cells is integrin  $\alpha 2 \beta 1$ <sup>33</sup>, and this receptor may be affected by NAMPT knock-down induced alteration in NAD<sup>+</sup> metabolism.

Together, our results indicate that interference with NAD<sup>+</sup> metabolism by reduction of NAMPT expression in breast cancer cells is associated with upregulation of specific integrins, and results in alterations in cell adhesion to major extracellular matrix proteins.

### 3.3 NAMPT expression specifically modifies $\beta 3$ and $\beta 1$ integrin adhesion receptors in breast cancer cells

Inhibition of NAD<sup>+</sup> generation through NAMPT mediated NAD<sup>+</sup> synthesis from NAD<sup>+</sup> precursor nicotinamide most prominently impacts expression of  $\alpha v\beta 3$  and  $\beta 1$  integrins in the examined breast cancer cells. To analyze the contribution of upregulated  $\alpha v\beta 3$  and  $\beta 1$  integrin expression to tumor cell adhesion, we used function blocking anti-integrin antibodies. NAMPT knock-down cells were compared to shRNA control treated cells for adhesion to key extracellular matrix proteins in the presence or absence of antibodies directed to  $\beta 3$ ,  $\alpha v\beta 5$ ,  $\alpha v$ ,  $\alpha 5$  or  $\beta 1$ , alone or in combination (Fig. 3).

Increased MDA-MB-231 cell adhesion to vitronectin upon NAMPT knock-down was confirmed, and a clear reduction of this enhanced adhesion was seen in the presence of blocking anti- $\alpha v\beta 5$  and anti- $\alpha v$  antibodies (Fig. 3A, E). These results identify  $\alpha v\beta 5$  as the predominant receptor for vitronectin in control as well as in NAMPT knock-down cells where  $\alpha v\beta 3$  is upregulated in the entire cell population (Fig. 2C). Failure of the anti- $\beta 3$  antibody to markedly reduce the enhanced vitronectin adhesion of NAMPT knock-down cells, and the strong inhibition by anti- $\alpha v\beta 5$  and anti- $\alpha v$  could indicate a cooperative effect of  $\alpha v\beta 3$  and  $\alpha v\beta 5$  in these cells, which strengthens vitronectin adhesion that cannot be abolished by the anti- $\beta 3$  antibody alone. Furthermore, NAD<sup>+</sup> metabolism may modulate  $\alpha v\beta 5$  activity rather than expression.  $\beta 1$  integrin targeting had no effect, confirming that no  $\beta 1$  integrin such as  $\alpha 8\beta 1$ , a known vitronectin receptor<sup>34</sup>, is involved (Fig. 3A, E).

To directly address a functional role of  $\alpha v\beta 3$  in the adhesion of NAMPT knock-down cells, fibrinogen was included as a substrate. Parental MDA-MB-231 cells almost completely lack  $\alpha v\beta 3$  and cannot attach to fibrinogen, as this protein is not recognized by any other adhesion receptor expressed by these cells. The functional contribution of integrin  $\alpha v\beta 3$ , upregulated in NAMPT knock-down cells, was evident in the fibrinogen adhesion analysis. The role of this receptor in supporting fibrinogen attachment was confirmed, as  $\beta 3$ - and  $\alpha v$ -blocking antibodies completely inhibited adhesion to this substrate, while anti- $\alpha v\beta 5$  had no effect (Fig. 3B,E).

Supporting the results on fibronectin adhesion in Fig. 2, use of function blocking anti-integrin antibodies demonstrated that  $\alpha 5\beta 1$  is the predominant adhesion receptor on MDA-MB-231 cells for this substrate, regardless of the NAMPT knock-down effect on NAD<sup>+</sup> metabolism (Fig. 3C,E). Blocking  $\beta 1$  integrin function reduced adhesion to fibronectin to the same level in control and NAMPT knock-down cells. This finding, and the strong inhibition of adhesion in both cell types by combining antibodies against  $\alpha v$  and  $\beta 1$ , could indicate that MDA-MB-231 cells express and utilize integrin  $\alpha v\beta 1$  for adhesion to fibronectin. This receptor has not been previously reported as mediating fibronectin adhesion in these breast cancer cells. There are no function blocking antibodies to  $\alpha v\beta 1$ , and it is therefore difficult to definitively demonstrate its role here. It is possible that  $\alpha v\beta 1$  is slightly upregulated in



NAMPT knock-down cell and contributes to the observed slight enhancement of fibronectin adhesion in these cells.

Tumor cell attachment to collagen I was found enhanced when NAMPT expression was reduced (Fig. 2A). Treatment with neutralizing anti- $\beta 1$  antibody blocked collagen I adhesion nearly completely in control and NAMPT knock-down cells (Fig. 3D,E). This indicates that the observed upregulation of  $\beta 1$  integrin expression upon NAMPT knock-down (Fig. 2B) is responsible for the increased ability of the cells to interact with collagen I, a major substrate found in tissues and the vessel wall.

### 3.4 NAMPT expression influences the $\beta 3$ integrin affinity state in breast cancer cells

It is well known that the affinity state of integrins expressed at the cell surface influences ligand recognition and the ability of the receptors to bind soluble ligand<sup>35</sup>. Classical examples are platelet and leukocyte integrins that can be activated and require activation to support platelet cohesion during thrombus formation<sup>36</sup>, and to mediate leukocyte arrest within the vasculature at sites of inflammation<sup>37</sup>. We showed earlier that integrin  $\alpha v\beta 3$  can also exist in distinct states of activation in tumor cells, and that expression of the high affinity receptor in breast cancer cells supports their metastatic activity<sup>38,39</sup>. To analyze the affinity state of integrin  $\alpha v\beta 3$ , which we found upregulated in MDA-MB-231 breast cancer cells upon NAMPT knock-down (Fig. 2B), we studied the ability of the cells to bind a ligand mimetic antibody that we developed earlier<sup>26</sup>. This antibody mimics ligands of  $\alpha v\beta 3$  that express the RGD integrin-binding motif by displaying an RGD sequence within CDR3 of the heavy chain<sup>26</sup>. Even though multiple integrins are known to recognize the RGD motif in multiple natural ligands, our ligand mimetic antibody is specific for integrin  $\alpha v\beta 3$  only<sup>26</sup>. Originally isolated from a phage displayed human cancer patient scFv antibody library, we now re-engineered the scFv into an intact IgG and analyzed soluble IgG binding to the NAMPT knock-down tumor cells as a measure of integrin  $\alpha v\beta 3$  affinity in these cells. We found that NAMPT knock-down cells, but not their control cells, bound the ligand mimetic IgG in a cation dependent manner (Fig. 4A). In the presence of  $\text{Ca}^{2+}$ , antibody binding was observed (Fig. 4A) proportionate to binding of non-cation and non-activation dependent anti- $\beta 3$  mAb AV10 (Fig. 4B). This finding - first - confirms upregulated expression of  $\alpha v\beta 3$  integrin upon NAMPT knock-down in the tumor cells, and - second - indicates that most of the expressed  $\alpha v\beta 3$  is present on the tumor cells in a high affinity state. This notion is supported by our result that binding of the ligand mimetic antibody seen with  $\text{Ca}^{2+}$  did not increase in the presence of  $\text{Mn}^{2+}$ , a cation known to induce a high affinity state in most integrins (not shown)<sup>26</sup>.

Together, our results indicate that inhibition of  $\text{NAD}^+$  synthesis from  $\text{NAD}^+$  precursor nicotinamide by interference with NAMPT expression affects the expression of certain integrins on tumor cells and thereby influences their adhesive and ligand binding properties. Most notably, integrin  $\alpha v\beta 3$  is upregulated in association with NAMPT knock-down from a nearly undetectable level in the parental and control tumor cells. Importantly, not only the expression of the integrin is induced but also the presentation of the receptor at the cell surface in a conformation that is compatible with a high affinity state, as evidenced by the ability of the receptor to bind ligand from solution. This result is supported by our finding

that the induced expression of  $\alpha v\beta 3$  in NAMPT knock-down cells supports adhesion of the tumor cells to immobilized fibrinogen, a protein that requires  $\alpha v\beta 3$  activation for support of cell adhesion<sup>31</sup>.

## 4. Discussion

This study demonstrates that NAMPT, the enzyme catalyzing the initial and rate-limiting step in the NAD<sup>+</sup> salvage pathway, can regulate metastatic activity in tumor cells, and that this can involve modulation of integrin expression and function which alter tumor cell adhesive functions associated with tumor cell dissemination.

### 4.1 Involvement of NAD<sup>+</sup> metabolism in regulating metastatic activity

We showed earlier that impaired NAD<sup>+</sup> metabolism activity and resulting decrease in NAD<sup>+</sup>/NADH redox levels in human breast cancer cells can significantly stimulate their metastatic properties<sup>7</sup>. Mechanisms underlying enhanced metastatic aggressiveness were found associated with aberrant mitochondrial complex I and poor NADH dehydrogenase activity, resulting in altered autophagy functions and enhanced mTORC1 activity<sup>7</sup>. Furthermore, we showed that therapeutic enhancement of the NAD<sup>+</sup>/NADH ratio through treatment with NAD<sup>+</sup> precursors can inhibit breast cancer metastasis and interfere with oncogene driven breast cancer progression in the MMTV-PyMT mouse model<sup>7</sup>. Detailed mechanisms through which enhancement of NAD<sup>+</sup> metabolism may impact tumor progression can be complex. NAD<sup>+</sup> and its reduced form NADH are cofactors of more than 200 metabolic reactions in mammalian cells, including regulation of NAD<sup>+</sup> degrading enzymes, such as PARPs, Sirtuins, and CD38. These enzymes and NAD<sup>+</sup>/NADH levels contribute to tumor suppressor functions of p53, BRCA1, and BRCA2, and can affect chromatin structure and genomic stability<sup>2,5,9,40–43,44,45,46,47</sup>. Our current results indicate a role of NAD<sup>+</sup> metabolism in regulating tumor cell adhesive functions that may have direct impact on metastatic behavior.

### 4.2 Regulation of NAD<sup>+</sup> metabolism through NAMPT in cancer cells

Within the NAD<sup>+</sup> salvage pathway, catalytic activity of NAMPT is significantly lower than NMN adenylyl transferase (NMNAT) activity which converts NMN<sup>+</sup> to NAD<sup>+</sup>, and therefore NAMPT limits the rate of this pathway. In human cancer, intracellular as well as extracellular NAMPT (iNAMPT and eNAMPT) can be upregulated<sup>12,23,48,49</sup> and associate with a more aggressive phenotype and poor prognosis<sup>50</sup>. While iNAMPT is the primary regulator of NAD<sup>+</sup> salvage from NAD<sup>+</sup> precursor NAM, eNAMPT is secreted by specific cell types including adipocytes, macrophages and hepatocytes, and apparently has very low catalytic activity in NAD<sup>+</sup> synthesis<sup>12,49</sup>. However eNAMPT may regulate intracellular pathways and cell survival by affecting cellular glucose metabolism through insulin-mimetic activity. Rapid tumor cell growth further demands increased functionality of NAD<sup>+</sup> degrading enzymes that support high transcriptional activity. Compensatory upregulation of NAMPT expression in these cells could therefore support sustained growth and cell viability. Thus, NAMPT has been suggested as a target for anti-cancer therapy<sup>1,10–12,20,23</sup>. Surprisingly, our results demonstrate that inhibition of NAMPT expression and function, at levels that do not kill tumor cells can lead to a significantly more aggressive phenotype<sup>7</sup>.



Integrin alterations, such as upregulation of  $\alpha v \beta 3$  and  $\beta 1$  integrin expression and modulation of functional activity as demonstrated in this study, likely contribute to the enhanced metastatic activity which we found associated with NAMPT downregulation in breast cancer cells.

#### 4.3 NAD<sup>+</sup> metabolism induced modulation of cell adhesion, integrin expression and activity

Among the cell adhesion receptors found upregulated in response to NAMPT knock-down in this study, is integrin  $\alpha v \beta 3$  which is not significantly expressed by the parental tumor cell population. This integrin is known to be associated with a highly aggressive phenotype in breast cancer<sup>38</sup> where it critically supports tumor cell interaction with a number of Arg-Gly-Asp (RGD) containing extracellular matrix proteins in tissues, the vessel wall, and matrices of the lymphatic system. Furthermore, our results show that inhibition of NAD<sup>+</sup> synthesis through NAMPT knock-down is accompanied by increased tumor cell adhesion to collagen I, another prominent component of the vascular basement membrane and connective tissue. Enhanced collagen adhesion is primarily mediated by  $\beta 1$  integrins which we also found upregulated in response to NAMPT knock-down, but in part by de novo expressed  $\alpha v \beta 3$ . While  $\alpha v \beta 3$  does not support cell interaction with native collagen, RGD sequences within this matrix protein are exposed when it is degraded, and  $\alpha v \beta 3$  can now recognize collagen fragments<sup>51</sup>. Reported association of uPAR with  $\alpha v \beta 3$  in cell membrane lipid rafts can foster activation of metalloproteinases that efficiently degrade collagen<sup>52,53</sup> and may thereby contribute to  $\alpha v \beta 3$  mediated collagen adhesion.

It is still an open question why and through which mechanisms only select integrins are modified in the breast cancer cell model we studied, while other integrins are apparently not affected. In a glioma model, it has been reported that genetic and pharmacologic inhibition of NAMPT and resulting fluctuations in intracellular NAD<sup>+</sup>/NADH levels affect cell motility and invasion<sup>54</sup>. Another important consideration is that nicotinamide riboside kinases (Nrk) within the NAD<sup>+</sup> pathway may impact cell adhesion. In particular, Nrk2 which catalyzes phosphorylation of nicotinamide riboside (NR) and nicotinic acid riboside (NaR) to form NMN and nicotinic acid mononucleotide (NaMN), can regulate deposition of the extracellular matrix protein laminin and cell adhesion to this substrate<sup>55</sup>. This process apparently involves the ability of Nrk2 to directly interact with the  $\beta 1$  integrin subunit.

#### 4.4 Impact of integrin affinity state on tumor cell adhesive properties and metastatic activity

We discovered earlier that tumor cells can carry integrin adhesion receptors in distinct states of affinity, and that constitutive expression of the high affinity conformer of certain integrins strongly promotes an invasive, metastatic phenotype<sup>24</sup>. Our previous studies on mechanisms mediating blood-borne tumor cell interaction with vascular cells during metastasis specifically revealed high affinity tumor cell integrin  $\alpha v \beta 3$  as a critical receptor for disseminating cells<sup>56</sup>. We found that high affinity  $\alpha v \beta 3$  supports tumor cell interaction with platelets, transendothelial migration, and invasion of matrices found in target tissues<sup>57</sup>. Importantly, targeting of the high affinity conformer of  $\alpha v \beta 3$  with ligand-mimetic antibodies

that we derived from the cancer patient immune repertoire, prevented hematogenous metastasis<sup>26</sup> and also interfered with advanced metastatic lesions<sup>58</sup>.

In the present study, interference with tumor cell NAD<sup>+</sup> metabolism by knock-down of NAMPT expression was found associated with upregulation of integrin  $\alpha\beta3$  in MDA-MB-231 breast cancer cells and with expression of this receptor in its high affinity form. This result is remarkable in two major ways. First, MDA-MB-231 cells largely lack  $\alpha\beta3$  indicating that alterations in NAMPT levels can induce an adhesion receptor that is not otherwise expressed. Second, we determined the affinity state of NAMPT knock-down associated tumor cell  $\alpha\beta3$  based on binding of a soluble ligand-mimetic antibody which we isolated and identified as a metastasis blocking reagent<sup>59</sup>. Binding of this  $\alpha\beta3$ -specific soluble ligand to NAMPT knock-down cells indicates that the cells can likely interact with soluble ligands, such as plasma proteins, that circulating tumor cells encounter and use for retention within the target organ vasculature and extravasation from the blood stream. Our results show that alteration in NAD<sup>+</sup> levels, through genetic modification of NAMPT expression, modify integrin expression and function through conformational changes in the receptor at the tumor cell surface. This finding conceptually agrees with a previous study showing that mono-ADP-ribosylation of an integrin subunit affected structure and function of the adhesion receptor<sup>60</sup>. This principle might also apply to integrin  $\alpha\beta5$ , as our current study shows that NAMPT knockdown increased  $\alpha\beta5$  dependent cell adhesion to vitronectin without modifying  $\alpha\beta5$  surface expression.

#### 4.5 Implications for clinical cancer progression and therapy

NAMPT inhibitors such as FK866/APO866 and CHS828/GMX1777 are being evaluated for anti-cancer effects in the clinic<sup>61</sup>, based on the rationale that NAMPT supports cell viability and growth by promoting DNA repair and pro-survival pathways such as ERK1/2 and p38 activity, expression of matrix degrading metalloproteinases such as MMP-2/9, and angiogenesis<sup>19-21,62-64</sup>. While some of these processes involve NAMPT mediated regulation of the NAD<sup>+</sup> salvage pathway, others apparently depend on the less well understood cytokine activity of the enzyme<sup>65</sup>. Notably, NAMPT was suggested to play a role in development of chronic inflammation which is known to promote tumor progression and chemoresistance<sup>62,66,67</sup>. However, only few studies correlated NAMPT expression in tumors with patient outcome<sup>48</sup>.

Clinically used NAMPT inhibitor FK866 is cytotoxic for a number of different tumor cells and noncompetitively inhibits NAMPT activity. The drug has been used in phase I and II clinical trials. CHS828, another NAMPT inhibitor originally identified as an antihypertensive agent, inhibits NAD<sup>+</sup> synthesis, can kill cancer cells, and has also been tested in phase I and II trials<sup>11</sup>. Despite the promise of *in vitro* and mouse model studies, no objective tumor remission could be documented in the clinic, even though transient reductions in VEGF levels associated with angiogenesis were observed with NAMPT inhibitors given as single agents<sup>11</sup>. Next generation NAMPT antagonists are being developed, mainly to improve selectivity and pharmacologic characteristics such as plasma half-life<sup>11</sup>. Targeting of NAMPT, a rate-limiting enzyme of the vital NAD<sup>+</sup> salvage pathway required for cellular energy metabolism, appears logical when the goal is to kill a cell.

However, there are two major implications that need to be considered and more fully investigated to unambiguously identify NAMPT as a target for effective cancer therapy. First, the specificity of NAMPT inhibitors for cancer cells has not been sufficiently addressed. Second, as documented in our present study and previous report<sup>7</sup>, incomplete inhibition of NAMPT which does not kill a tumor cell can alter its phenotype and make it more aggressive and metastatic. This phenomenon could associate with enhanced cancer recurrence, even if NAMPT inhibitor treatment reduces the size of an existing tumor or metastatic lesion.

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## Abbreviations

<b>AMP</b>	Adenosine monophosphate
<b>ATP</b>	Adenosine triphosphate
<b>BRCA1</b>	Breast cancer 1
<b>BRCA2</b>	Breast cancer 2
<b>eNAMPT</b>	Extracellular nicotinamide phosphoribosyltransferase
<b>ERK</b>	Extracellular signal-regulated kinases
<b>iNAMPT</b>	Intracellular nicotinamide phosphoribosyltransferase
<b>MMTV-PyMT</b>	Mouse mammary tumor virus - polyoma middle T antigen
<b>mTORC1</b>	Mammalian target of rapamycin complex 1
<b>MMP</b>	Matrix metalloproteinases
<b>NAD+</b>	Nicotinamide adenine dinucleotide
<b>NADH</b>	Reduced nicotinamide adenine dinucleotide
<b>NaMN</b>	Nicotinic acid mononucleotide
<b>NAMPT</b>	Nicotinamide phosphoribosyltransferase
<b>NaR</b>	Nicotinic acid riboside
<b>Ndi1</b>	NADH-ubiquinone reductase (H(+)-translocating
<b>NMN+</b>	Nicotinamide mononucleotide
<b>NMNAT</b>	Nicotinamide nucleotide adenylyltransferases
<b>NR</b>	Nicotinamide riboside
<b>NRK</b>	Nicotinamide riboside kinases

<b>PARPs</b>	Poly (adenosine diphosphate-ribose) polymerases
<b>SCID</b>	Severe Combined Immunodeficiency
<b>shRNA</b>	Small hairpin RNA
<b>uPAR</b>	urokinase Plasminogen activator receptor
<b>VEGF</b>	Vascular endothelial growth factor

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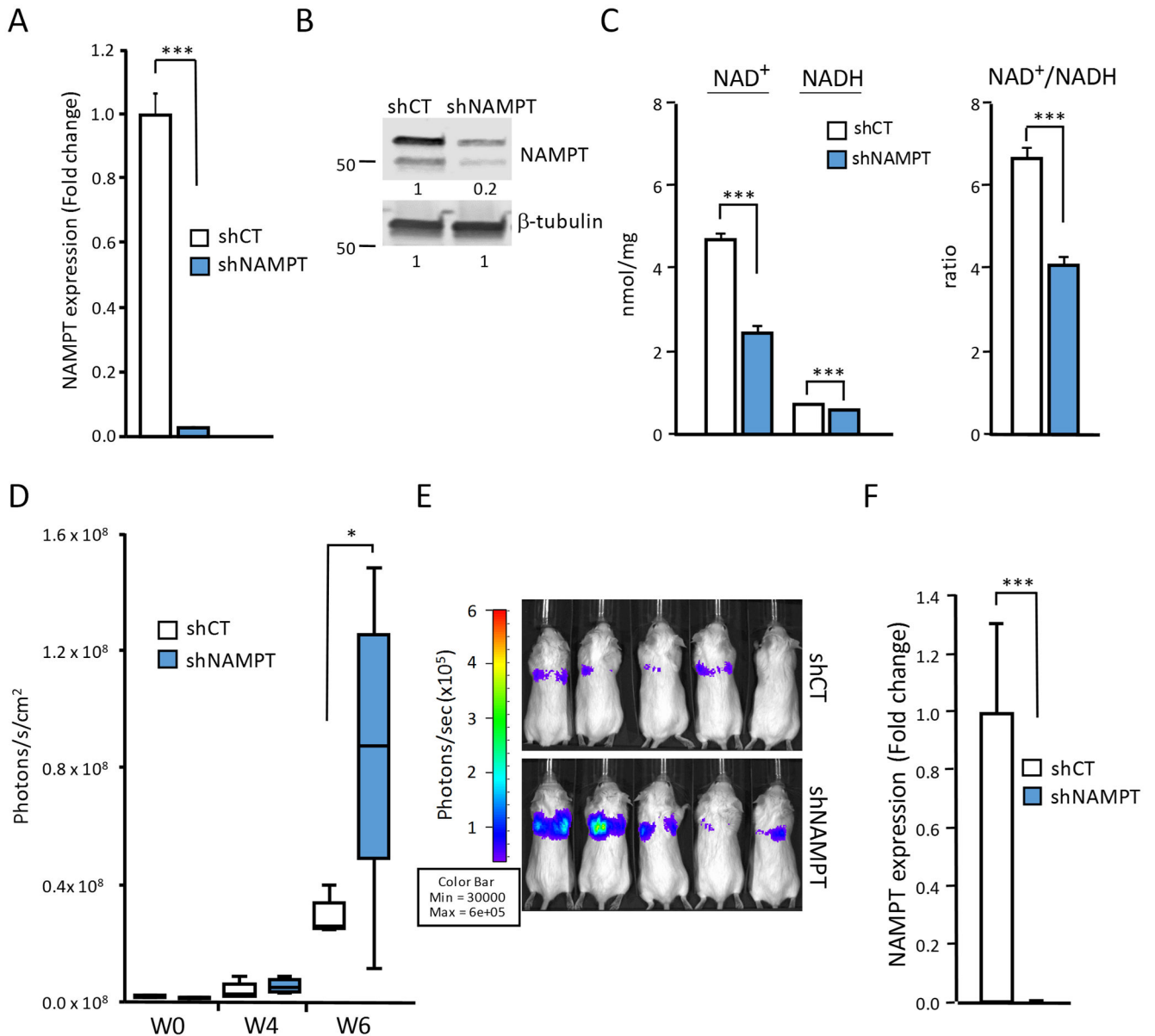
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### Highlights

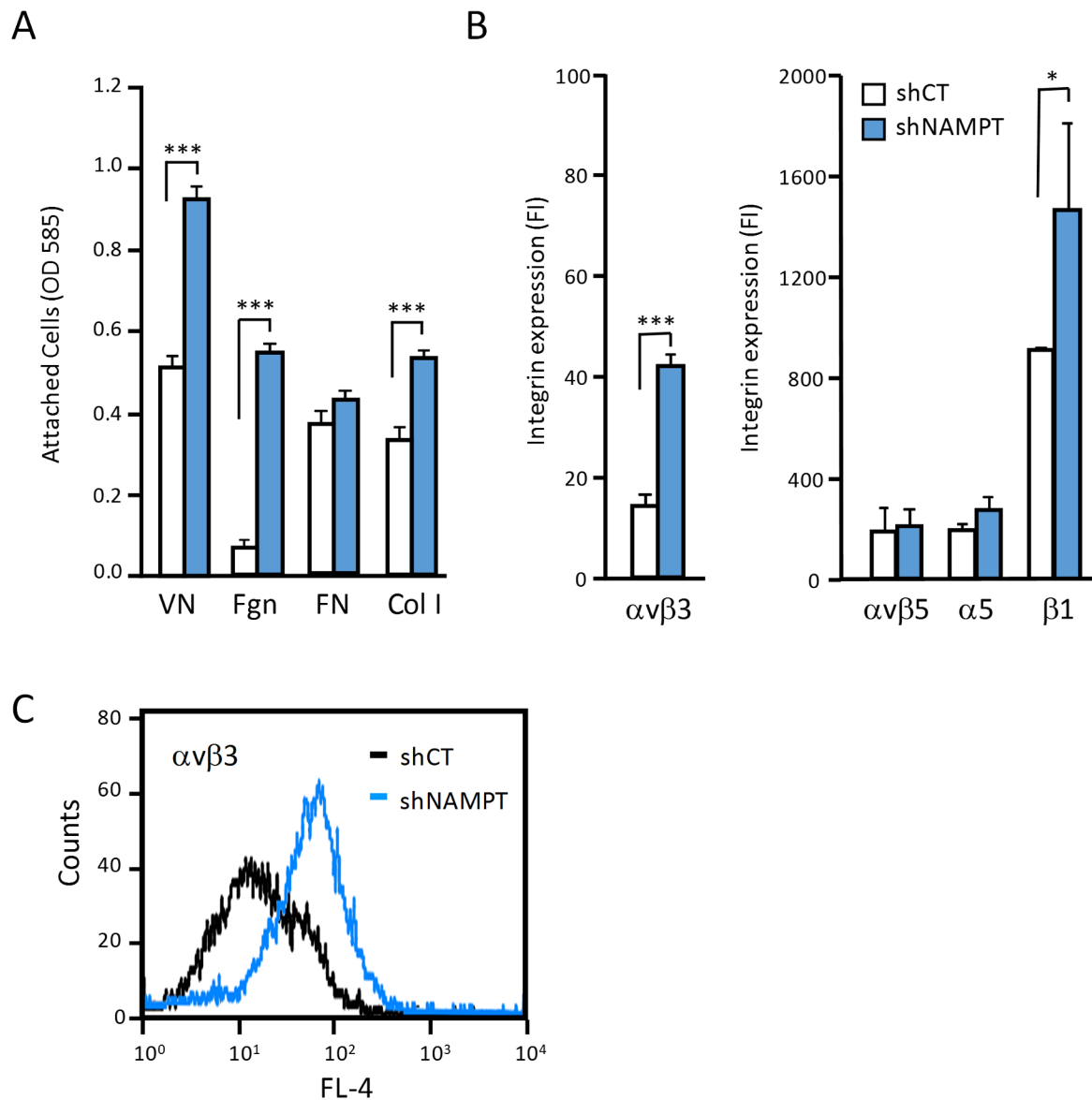
- Inhibition of NAD<sup>+</sup> salvage pathway activity by reduction in nicotinamide phosphoribosyltransferase (NAMPT) expression enhances metastatic aggressiveness in human breast cancer cells, and this involves modulation of integrin adhesion receptor expression and function.
- Reduced NAMPT is associated with upregulation of  $\beta$ 1 integrins and  $\alpha$ v $\beta$ 3 in tumor cells, and with receptor expression in a high affinity form that supports enhanced adhesion to extracellular matrix proteins.
- The results indicate that incomplete inhibition of NAMPT in tumor cells that fails to kill all cells, may render remaining cells more aggressive and thereby promote cancer recurrence.



**Figure 1. Down regulation of NAMPT expression reduces NAD<sup>+</sup>/NADH ratios and enhances metastatic activity in human breast cancer cells**

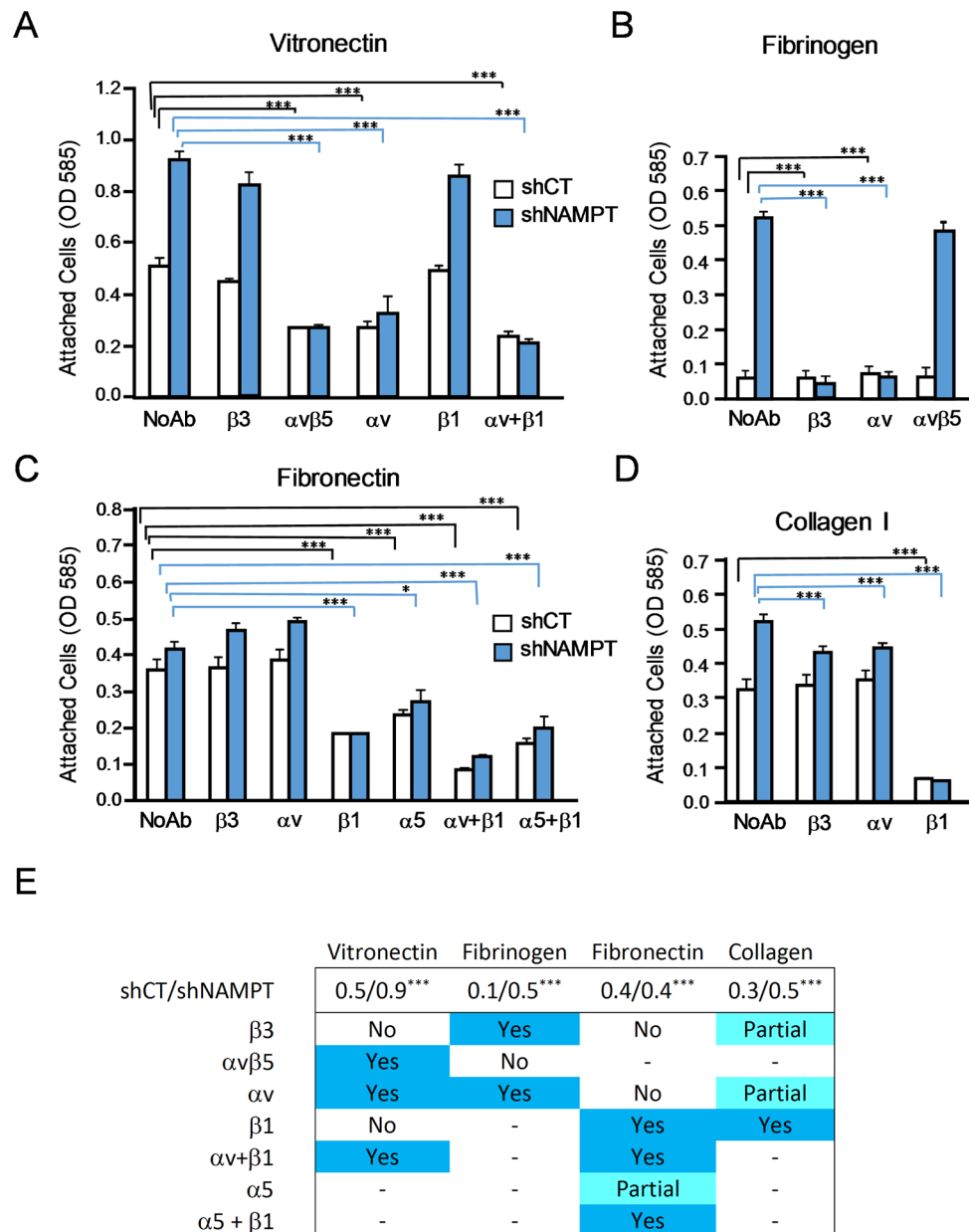
(A) NAMPT knockdown (shNAMPT) reduced NAMPT expression by 97 % in MDA-MB-231 cells, compared to controls transduced with scrambled shRNA (shCT). NAMPT mRNA levels were analyzed by real time PCR and are expressed relative to GAPDH (\*\*P<0.001) (n = 3). (B) NAMPT knockdown (shNAMPT) reduced NAMPT protein expression in MDA-MB-231 cells, shown by Western blot analysis. Quantification of NAMPT protein was related to  $\beta$ -tubulin expression. (C) Interference with NAD<sup>+</sup> synthesis and salvage pathways reduced absolute amounts of NAD<sup>+</sup> and NADH, and cellular NAD<sup>+</sup>/NADH ratios. NAD<sup>+</sup> and NADH were analyzed independently in whole cell extracts of  $1 \times 10^6$  cells. Metabolite concentrations were determined using a NAD<sup>+</sup>/NADH fluorescence detection kit (Cell Technology, Inc). Groups were compared by unpaired two-tailed

Student's t-test (\* $P < 0.05$ ). Data are expressed as mean  $\pm$  SEM. **(D)** NAMPT knockdown increased lung colonization activity in MDA-MB-231 cells. Metastatic growth was measured by repeated non-invasive bioluminescence imaging (x-axes: time in weeks). Groups were compared by unpaired two-tailed Student's t-test (\* $P < 0.05$ ). (\* $P < 0.05$ , n = 6/group). **(E)** Representative bioluminescence images of mice (n=5), 6 weeks after i.v. injection of  $2.5 \times 10^5$  MDA-MB-231 control or shNAMPT expressing cells. **(F)** Down regulation of NAMPT expression in NAMPT knockdown cells (shNAMPT) was maintained in lung metastases. Lung lesions developing in SCID mice after injection of shNAMPT cells versus control cells (shCT) as shown in **(E)** were analyzed for NAMPT gene expression as detailed in **(A)**.



**Figure 2. NAMPT knockdown increases breast cancer cell adhesion to select substrates and upregulates  $\beta 3$  and  $\beta 1$  integrin expression**

(A) NAMPT knockdown (shNAMPT) enhanced MDA-MB-231 cell adhesion to vitronectin (VN), fibrinogen (Fgn), and collagen type I (Col I) compared to controls transduced with scrambled shRNA (shCT). (\*\*\*) $P < 0.001$  ( $n = 3$ ) (B) NAMPT knockdown (shNAMPT) enhanced expression of integrin  $\alpha v \beta 3$  and  $\beta 1$  integrins in MDA-MB-231 cells, measured by flow cytometry based on fluorescence intensity (FI) using mAbs directed against the  $\alpha v \beta 3$  complex,  $\alpha v \beta 5$  complex, or the  $\alpha 5$  and  $\beta 1$  integrin subunits (\* $P < 0.05$ ) ( $n = 3$ ). (C) Representative histogram of cell surface expression of integrin  $\alpha v \beta 3$  in NAMPT knockdown (shNAMPT) MDA-MB-231 cells or their controls transduced with scrambled shRNA (shCT).

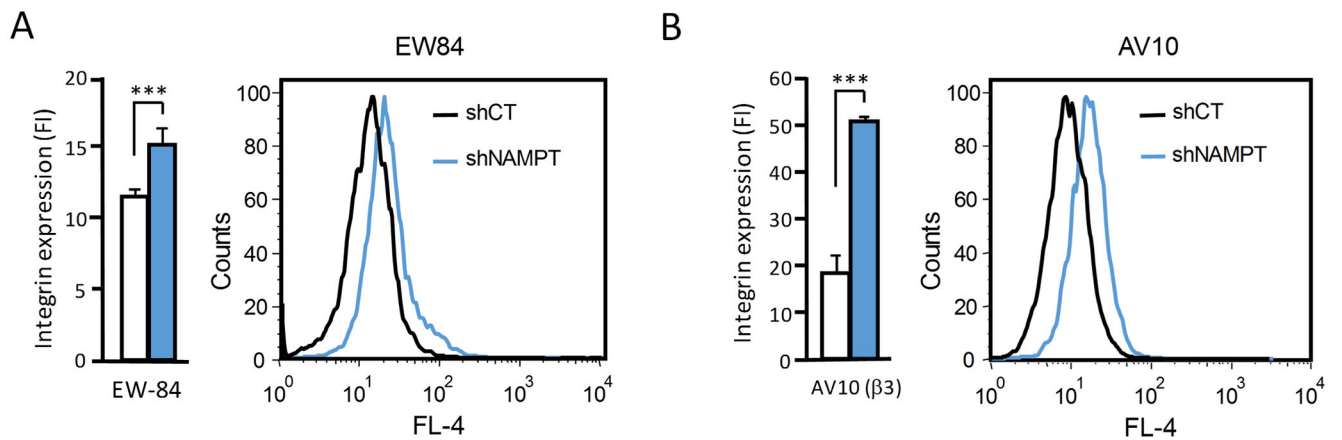


**Figure 3. NAMPT knockdown increases  $\alpha v\beta 3$  and  $\beta 1$  integrin function in mediating breast cancer cell adhesion**

Adhesion of MDA-MB-231 NAMPT knockdown cells (shNAMPT) or controls transduced with scrambled shRNA (shCT) to vitronectin (A), fibrinogen (B), fibronectin (C) and collagen type I (D) in the absence (no Ab) or presence of function blocking antibodies directed to the  $\alpha v\beta 3$  complex, the  $\alpha v\beta 5$  complex, or to integrin subunits  $\alpha v$ ,  $\alpha 5$ , or  $\beta 1$ , alone or in combination. (\* $P < 0.05$ , \*\*\* $P < 0.001$ ) (n = 3) (E) Overview of integrin contributions to MDA-MB-231 cell adhesion to the indicated substrates in response to NAMPT knockdown in the tumor cells. Values reflecting cell adhesion of control compared to NAMPT knockdown cells are listed in the top row of the table (shCT/shNAMPT) (\*\* $p < 0.001$ ) (n=3). Yes = this integrin was found to contribute to adhesion of both shNAMPT and



shCT MDA-MB-231 cells as a neutralizing antibody against this integrin significantly inhibited cell attachment (>50%); Partial = adhesion to this integrin was partially inhibited, No = no significant inhibition. - = no measured; (n=3).



**Figure 4. NAMPT knockdown leads to expression of integrin  $\alpha v\beta 3$  in its high affinity conformation on human breast cancer cells**

(A) Measurement of high affinity  $\alpha v\beta 3$  expression: binding average (left panel) and representative histogram (right panel) of ligand mimetic  $\alpha v\beta 3$ -specific human monoclonal antibody (IgG EW84) to MDA-MB-231 NAMPT knockdown cells (shNAMPT) or controls transduced with scrambled shRNA (shCT) in the presence of calcium, measured by flow cytometry. (B) Measurement of overall  $\beta 3$  integrin expression: binding average (left panel) and representative histogram (right panel) of integrin  $\alpha v\beta 3$  reported on MDA-MB-231 NAMPT knockdown cells (shNAMPT) or controls transduced with scrambled shRNA (shCT) by staining with non-activation dependent anti- $\beta 3$  mAb AV10, measured by flow cytometry.