Nischarin suppresses breast cancer progression through integrin alpha5-FAK-~~CyclinD1~~ mediated signaling events

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Background

Nischarin, a α5 integrin binding protein, has established role in cancer cell invasion. Based on its genomic locus, we hypothesized that it might be a tumor suppressor for breast cancer. α5 integrin has been identified as a prometastatic gene for breast cancer and thus identification of its regulator may be critical for further therapeutic interests.

Methods

Nischarin expression was examined in approximately 300 human breast cancer and normal tissues using quantitative PCR and immunohistochemistry. Loss of heterozygosity analysis was performed using three microsatellite markers located on Nischarin locus using normal and tumor tissues. Human metastatic MDA-MB231 breast cancer cells over expressing Nischarin clones were generated and tumor growth and metastasis were measured. To further evaluate the tumor suppressor function of Nischarin, we generated knockdown clones for Nischarin in MCF7 cells and measured tumor growth in mouse xenograft models. All statistical tests were two tailed analysis, and *P* value < 0.05 was considered statistically significant.

Results

Normal human breast tissue samples (~~60~~24) have significant higher expression of Nischarin compared to tumor tissue samples (~~60~~24) (*P*<0.01) with loss of heterozygosity correlating with Nischarin expression loss. Nischarin over expression in MDA-MB231 cells significantly reduced the tumor growth and metastasis compared to MDA-MB231 cells (*P*<0.01). Moreover, Nischarin knockdown in MCF7 significantly (*P*<0.01) promotes tumor growth in a mouse model. Mechanistically, Nischarin modulates α5 integrin expression and subsequent α5 integrin-FAK-Rac~~-CyclinD1~~ mediated signaling.

Conclusion

Nischarin may be a novel tumor suppressor and regulator of breast cancer progression that regulates α5 integrin and its downstream signaling pathway.

Introduction

We have previously identified a novel protein, Nischarin that selectively binds to the proximal transmembrane (IYILYKLGFFKR) region of the integrin α5 subunit cytoplasmic tail (1, 2)~~(1, 2)~~. Nischarin blocks Rac- induced cell migration and invasion in breast and colon epithelial cells, interacts with PAK1 to block PAK activation and influences actin filament organization (1)~~(1)~~. Nischarin also blocks PAK-independent Rac signaling (3, 4)~~(3, 4)~~, and interacts with LIMK to inhibit LIM kinase (LIMK) activation and LIMK-driven cell invasion (5)~~(5)~~. A human ortholog of Nischarin, IRAS, has also been shown to bind to the adaptor protein IRS4 to mediate translocation of α5 integrin from the cell membrane to endosomes (6)~~(6)~~.

Here we investigate whether Nischarin functions as a potential tumor suppressor in breast cancer. Several studies, including cytogenetic mapping and homozygosity mapping, have indicated that distinct regions of chromosome arm 3p are important for development of cancers including those of lung, breast, kidney, ovary, and cervix (7)~~(7)~~. As the NISCHARIN locus is located on chromosome 3p, these studies along with our loss of heterozygosity studies indicate that the NISCHARIN locus is lost in breast cancer patients. Our *in vitro* and *in vivo* data indicate that Nischarin acts to suppress breast cancer progression by regulating the α5 integrin-FAK-Rac~~-Cyclin D1~~ signaling cascade.

Materials and Methods

Cell culture

MDA-MB231, MCF7 and COS7 cells were obtained from ATCC, and maintained in high-glucose DMEM with 2 mM L-glutamine, 110 mg/mL sodium pyruvate, and 10% FBS.

Antibodies Used and their Source

Nischarin (BD Technologies), FAK (BD Technologies), Vinculin (Sigma), α5 integrin (Millipore), Ki67 (Novocastra labs), Phospho-FAK (Invitrogen).

~~, Cyclin D1 (Cell signaling technology), p19 ink (Abcam), Retinoblastoma (Rb) and Phospho RB (BD Biosciences), CDK4, CDK2, ERK, Phospho ERK (Santa Cruz Biotechnology).~~

Tissues used in the study

Human breast cancers (n=120) together with noncancerous tissues were obtained as surgical specimens from the U.S. patients with primary breast carcinoma. Frozen tissue sections were procured from the Southern Division (UAB, Birmingham, AL), Eastern Division (Philadelphia, PA), Mid-Atlantic Division (Charlottesville, VA), Mid-Western Division (Columbus, OH), and Western Division (Nashville, TN) of the Cooperative Human Tissue Network. Among 120 samples, 24 cancerous and 24 adjacent noncancerous tissues were from the same patients. Therefore, 120 samples were obtained from 96 patients (age range, 13 to 87 years). Sixty tumor samples represented 6 different histological types: 22, invasive ductal carcinoma (IDC); 4, ductal carcinoma in situ (DCIS); 17, mixtures of IDC and DCIS; 5, invasive lobular carcinoma (ILC); 6, mixtures of ILC and lobular carcinoma in situ (LCIS); and a miscellaneous group of 6 include 2 adenocarcinoma, 1 LCIS, 1 IDC+ILC mixture, 1 IDC+ILC+DCIS mixture, and 1 IDC+ILC+DCIS+LCIS mixture.

RNA isolation and real-time QPCR

Total RNA was isolated from tissues with an mRNeasy Kit (Qiagen). Quality of RNA was determined using Agilent 2100 bioanalyzer and an RNA Nano 6000 Lab chip kit (Agilent Technologies, Palo Alto, CA); the concentration was determined with a Nanodrop apparatus (Nanodrop Technologies, Wilmington, DE). Expression of

Nischarin was determined using TaqMan real-time assays (Applied Biosystem) in triplicate. Reverse transcription (RT) was performed using 500 ng of total RNA in a 20-µL reaction volume. Real-time PCR was performed using the standard TaqMan assay protocol using the ABI7900 Real-Time PCR Detection System (Applied Biosystems). The 20-µL PCR volume included 2 µL of RT product, 10 µL of TaqMan Universal PCR Master Mix, No AmpErase UNG (product number 4324018; Applied Biosystems), and 1 µL of primer and probe mix. The reactions were incubated in a 96-well plate at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Expression of mRNA was measured using threshold cycle values (Ct). The ΔCt was calculated by subtracting the Ct of GAPDH mRNA from the Ct of Nischarin mRNA.

~~**Primers used for Nischarin Real-time Q-PCR (from ABI)**~~

~~hNscex1617-ex16 Forward primer 5' CCCCAGGGCTCCTTTGC 3'~~

~~hNscex1617-ex16 Reverse primer 5' CTCTGCTGGGACCTCCTG 3'~~

~~hNscex1617-ex16 M2 (probe) 5' CGAGCGCAGGGCCAG 3'~~

~~**Primers used in RT-PCR assays:**~~

~~Human α5 integrin:~~

~~Sense primer 5' CGGGAGCACCAGCCATTTA 3'~~

~~Antisense primer 5' AGCAGGAGGCCAAACAGGA 3'~~

~~Human Nischarin:~~

~~Sense primer 5' CGGGAGCACCAGCCATTTA 3'~~

~~Antisense primer 5' AGCAGGAGGCCAAACAGGA 3'~~

~~β-actin:~~

~~Sense primer 5'AGCCATGTACGTTGCTATCCA 3'~~

~~Antisense primer 5' TCCTTAATGTCACGCACGATTTCC 3'~~

Tissue array analyses and Immunohistochemistry

TMAs were purchased from CYBRDI. TMAs were rehydrated and processed in citrate buffer (pH, 6) for 4 antigen retrieval cycles of 5 minutes each in a microwave oven (800 W). After blocking for 1 hour at room temperature with 0.1% Triton-X 100 and 10% normal goat serum in PBS, sections were incubated with primary antibodies at a dilution of 1:300 in the same solution at room temperature overnight. The sections were further processed with biotinylated secondary antibodies (1:300) and avidin-biotin-peroxidase complex (ABC) and finally visualized with 3' diaminobenzidine (Roche). All slides were scored for average staining intensity by 2 pathologists blinded to patient information. Staining intensity was graded as follows: 0, no expression; 1, weak expression; 2, moderate expression; and 3, strong expression. Average staining intensity was compared with the pathologic information provided by Cybrdi. An IgG control was used to estimate background staining.

Loss of heterozygosity analysis

Eighteen breast cancer specimens as well as the nearest normal tissue samples were screened using three polymorphic microsatellite markers spanning chromosome band 3p21.1 containing the *Nischarin* gene. Assays were performed by PCR amplification using fluorescent dye-labeled forward primer and unlabeled reverse primer. PCR was performed in 25-μL reaction volume containing 20 ng of genomic DNA, using Amplitag gold polymerase (Applied Biosystems) using the following conditions: Initial denaturation at 95°C for 2 min; 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and a final extension at 72°C for 10 min. 6 μl sample was loaded onto 7.5% polyacrylamide gel and allelic loss was determined upon ethidium bromide staining and the assay was repeated at least 3 times. Also PCR products were

visualized using an ABI genetic analyzer and the data were analyzed using GeneScan software. Microsatellite markers D3S3688, D35361 and D3S3026 were used in PCR reactions. The primers used in this study ~~were as follows~~were given ~~s~~in the supplementary methods section~~.~~ :

~~D3S3688~~

~~Forward primer:~~

~~5'CACCACTGCACTCCAG 3';~~

~~Reverse primer:~~

~~5'TGATTTGTTATTATCTCTTATGGG 3'~~

~~D3S3561~~

~~Forward primer: 5' TCCTGGGGACTGTGATG 3';~~

~~Reverse primer: 5' GGTGACTGGAGGTTCAAG 3'~~

~~D3S3026~~

~~Forward primer: 5' GCATCTTTGGTCCCAGCTAC 3';~~

~~Reverse primer: 5' TAAATGGAACACCCTGTGGT 3'~~

QPCR for micro-deletion analysis of Breast tissue-

Applied Biosystems SYBR Green Mix was used for genomic quantitative PCR (qPCR). Primers were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/). Briefly, 20 ng genomic DNA was used in a 25-µl reaction with 300 nM of primers. Reactions were performed in triplicate using ABI 7900 HT Real time PCR machine using conditions of 95°C for 10 min, then 40 cycles at 95°C for 15 s and 60°C for 1 min. Standard curves for Nischarin and GAPDH primers were generated using 10-fold dilution series ranging from 0.1 ng to 100 ng and slope value was determined. The C_t values for each primer pair were corrected/normalized using the C_t value of the GAPDH products for the same sample.

Corrected Ct (KCt) value was determined using the element of absolute standard curve, comparative C_T and relative standard methods as described (8)~~(8)~~. If the difference (ΔKCt) in corrected Ct (KCt) value in Normal and tumor sample is less than -0.35 is considered as micro-deletion, and if the difference is -0.35 to +0.35 is considered as no loss.

~~**Primers used for QPCR for micro-deletion analysis**~~

~~Human NIS QPCR 1 sense 5'TGGACACTTATACGGTGTGTTGGG3'~~

~~Human NIS QPCR 1 antisense 5'TCCTCGCAATCGCAGACCCTTCTA3'~~

~~Human NIS QPCR 2 sense 5'TACAGCGACTTCCATGACCTGCAT~~

~~Human NIS QPCR 2 antisense 5'ACAATGGAAAGTTGGCCTACGGGT3'~~

~~Human NIS QPCR 3 sense 5'TGGCAAAGAGCTTTGAAGATGCCC3'~~

~~Human NIS QPCR 3 antisense 5'TTGGCATCGCAAACCAAAGAGTCC3'~~

~~Human GAPDH QPCR sense 5'TTAGGAAAGCCTGCCGGTGACTAA3'~~

~~Human GAPDH QPCR antisense 5'AAAGCATCACCCGGAGGAGAAATC3'~~

~~**Cloning, RNAi constructs and stable cell line generation**~~

~~We employed lentiviral approaches to generate Nischarin over expressing clone in MDA-MB231 cells and shRNA clones for Nischarin knockdown in MCF7 cells. Full length human Nischarin amplified by PCR using the following sense primer 5'CTAGGAATTCGCCACCATGGAGCAGAAACTGATCTC3' and antisense primer 5'CTAGGGATCCCTAGCCGGGCCACCTGGCACC3'. For efficient translation, a kozak consensus sequence is engineered into the sense primer. The resulting 4545 base pair PCR product was cloned into the EcoRI and BamHI site of the pCDH-CMV-MCS-EF1-copGFP vector (System Biosciences, CA). Orientation and reading frame of insert was confirmed by DNA sequencing. Pseudo-viral particles were generated in HEK 293T cells after co-transfecting pCDH-Nischarin plasmid~~

~~with pCD/NL-BH*ΔAA and VSVG encoding pLTR-G (9) (Second generation lentiviral packaging and envelope particles kindly provided by Dr. Jakob Reiser, Gene Therapy, LSUHSC, New Orleans) using $CaCl_2$ method. Supernatant containing lentiviral particles were collected after forty eight hours and concentrated by ultracentrifugation for 25,000 rpm for 2 hour. Virus particles were reconstituted in 200 µl of serum free media. Lentiviral titer was measured by FACS analysis. MDA-MB231 and MDA-MB231 luciferase (received from Dr. J.W. Shay, University of *Texas* Southwestern Medical Centre, Dallas, USA) plasmids were transduced with Nischarin virus particles to get stable clones expressing Nischarin. GFP positive cells were sorted twice in FACS Aria sorter (Immunology core of LSUHSC, New Orleans). Western blotting and RT-PCR was performed to confirm the over expression of Nischarin in MDA-MB231 cells.~~

~~To generate stable clones of Nischarin knockdown in MCF7 cells, we obtained one shRNA miR construct for human Nischarin in lentiviral vector pGIPz, and a control plasmid which expresses turbo GFP tagged shRNAs from Open Biosystem (10).~~

~~The sequences shRNA were as follows:~~

~~Construct 1:~~

~~5'TGCTGTTGACAGTGAGCGCGCCTGTATTCTCTATTCCAATTAGTGAAGCCACAGATGTAATTGGAATAGAGAATACAGGCATGCCTACTGCCTCGGA3'~~

~~(V2LHS_254031)~~

~~Control Construct:~~

~~5'TGCTGTTGACAGTGAGCGCCCTGGACCTGTCCTACAACAATAGTGAAGCCACAGATGTATTGTTGTAGGACAGGTCCAGGTTGCCTACTGCCTCGGA3'~~

~~(V2LHS_98703)~~

~~Virus particles were generated as described above using the same envelope and packaging plasmids in HEK 293T cells. MCF7 cells were transduced with virus particles to generate stable clones of Nischarin knockdown cells. MCF7 transduced with virus particles for GFP were used as control for our experiments. We performed western blotting and RT-PCR to confirm the knockdown of Nischarin in MCF7 cells.~~

~~**MTT cell proliferation assay**~~

~~Stably over-expressing Nischarin and knockdown cells were plated in 96-well flat-bottomed micro-plates at a density of 5000 cells per well in triplicate. After twenty-four hours the medium was replaced with MTT (Sigma-Aldrich) dissolved at a final concentration of 1 mg/mL in serum-free, phenol red-free RPMI 1640 (Life Technologies). The cells were incubated for 3 h to develop purple formazan precipitate. The MTT-formazan was then solubilized in DMSO, and the absorbance was measured at a wavelength of 570 nm.~~

Anchorage independent growth using soft agar assay

To assess anchorage independent growth, soft agar assays were performed. 50,000 cells were plated in one ml of 0.35% agarose/DMEM/10%FBS on top of 0.5% of agarose/DMEM/10%FBS coated 6-well tissue culture plates. After solidification of agarose, 1.5 ml of DMEM/10%FBS was added. Media was changed once in three days. After three weeks, colonies were stained with 0.1% crystal violet for one hour and destained with several washes of PBS. Number of colonies in soft agar was determined and digital images were captured using Epson Perfection V700 Photo Scanner. Adobe photoshop and Adobe illustrator were used to generate figures.

Tumor growth assays

Animal experiments were done in accordance with protocols approved by the Institutional Animal Care and Use Committees of Rockefeller University and

LSUHSC. We used 4- to 6-week-old female balb/c nude mice in xenograft studies. MDA-MB231 Nischarin and control cells or MCF7 and Nischarin knockdown cells were trypsinized, washed 3 times in PBS, and counted. Mice were anesthetized with xylazine-ketamine, and a small incision was made to visualize the mammary fat pads. Cells (5×10^6) were suspended in 100 μL of Matrigel diluted 1:1 in PBS and injected into 2 fat pads per mouse. We also placed tablet of 17β-estradiol 60 day release (Innovative Research of America, FL) into animals when performing experiments with MCF7 cells. Tumor growth was measured twice a week using calipers, and volume was calculated using the formula $\pi \times \text{length} \times \text{width}^2/6$ (9)~~(11)~~

Experimental metastasis followed by bioluminescent imaging

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For lung metastasis experiments, $1x10^6$ luciferase expressing MDA-MB231 cells with or without Nischarin in 100μl of PBS were injected into the tail veins of six week old Balb/c nude mice. On days 5, 10, 20, and 40 after injection of cells, animals were analyzed for metastatic disease by bioluminescence imaging using an IVIS-200 camera system for detection of luciferase expression (Xenogen) (10)~~(12)~~. Fifteen minutes prior to *in vivo* imaging, animals were anesthetized with 3% isofurane and injected intraperitoneally with D-luciferin (150 mg kg^{-1} in PBS) and animals were kept inside the warm chamber of Xenogen with 1.5% continuous flow of Isofurane along with oxygen to keep them anesthetic. For ex-vivo lung imaging, 300 μl of luciferin was injected intraperitoneally right before sacrificing the animals, and lungs were extracted and imaged using IVIS-200. The experiments were performed using 5 or 6 mice per condition and repeated 3 times. Also intact lungs were extracted and

subsequently fixed in 4% formalin (Sigma) and prepared for standard histological examination. The imaging results were analyzed using Living Image, version 3.0; Xenogen software. A region of interest (ROI) was manually selected over relevant regions of signal intensity and the intensity was recorded as the maximum number of photon counts within an ROI. Luminescent intensity obtained from each mouse is plotted using Graph Pad Prism software version 5.0 (San Diego, CA).

Truncation constructs of α5 integrin

Full-length α5 integrin and its truncations were described previously (11)~~(13)~~. The α5 c-10 construct expressing α5 integrin had only 10 amino acids instead of 27 in the cytoplasmic region, while α5 c-1 had only 1 amino acid; the entire cytoplasmic domain of 27 C-terminal amino acids including KLGFFKR residues was deleted, except for lysine in the proximal the trans-membrane region. The latter construct often is referred to as a tailless mutant.

~~**In vitro invasion assays**~~

~~Transwell inserts 8 μm pore size and a 24 well plate (6.5 mm wells; Corning) were used. Undersurfaces were pre-coated with poly L-lysine (0.01%; Sigma, St. Louis, MO), and rat tail type I collagen (10 μg/μL), or human fibronectin (10 μg/μL). MDA-MB231 cells transiently transfected with β-gal together with other selected plasmids for 24 hours, trypsinized, and re-suspended in serum-free DMEM. 400 μL serum-free DMEM or DMEM containing 10% FBS was added to the bottom chambers. The porous membrane in the top chamber was coated with Matrigel. A 100 μL volume containing 10^5 cells was added to the upper chambers to perform assays in triplicate. A separate well coated in respective substratum was seeded with the same quantity and volume of cells in DMEM containing 10% FBS for normalization with the total~~

~~number of cells transfected. Cells were allowed to invade through the ECM matrix for 48 hours at 37°C. The upper surface of the membrane was cleared of non-migrating cells with a cotton-tipped applicator. Transwells were fixed in a 2% paraformaldehyde solution and stained with X-gal as previously described (5). Stained cells were counted in five fields visually using a Zeiss light microscope (100 X magnification).~~

Luciferase Reporter Transfection and Dual Luciferase Assay

$5x10^4$ stably transduced MDA-MB231 Nischarin over expressing and MCF7 Nischarin knockdown cells were transfected with 400 ng either 923 bp or 26 bp of α_5 promoter construct of pGL3 vector (12)~~(14)~~ (generous gift from Dr. Jeffrey Ritzenthaler, Emory University School of Medicine, Atlanta, Georgia**),** along with Renilla vector. Lysates were prepared 48 hr after transfection, and reporter activity was measured with the Dual Luciferase Assay as suggested by manufacturer protocol (Promega). Data represent the average of three independent experiments and $p<0.05$ is considered as significant.

Cell cycle analysis using propidium iodide

MDA-MB231 cells expressing Nischarin (or control) were analyzed for cell cycle using propidium iodide staining. Briefly, cells were serum starved for 24 hours for cell synchronization, and cell cycle was performed after 72 hours. Cells were harvested and fixed in 70% ethanol for at least 30 minutes on ice. After washing with PBS, cells were labeled with propidium iodide (0.05 mg/ml) in the presence of RNase A (0.5 mg/mL) and incubated at room temperature in dark for 30 minutes. DNA content was analyzed using a BD Aria flow cytometer. Cell cycle peaks were obtained using auto curve fitting analysis with the Modfit program (Verity Software, Inc., Topsham, ME).

Rac GTPase assay

Rho/Rac/Cdc42 Activation Assay Combo Kit was used to perform Rac activation assay as described (Cell Biolabs). Briefly, MDA-MB231 GFP and MDA-MB231 GFP Nischarin cells were washed two times in cold PBS and then lysed in buffer B [50 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, 1% Triton X-100, 0.5 mmol/L $MgCl_2$, supplemented with 1 mmol/L PMSF, 1 µg/mL aprotinin, and 1 µg/mL leupeptin]. One milligram of total protein was then incubated with 30 µg glutathione *S*-transferase (GST)-PAK (PBD) beads for 30 min. The complexes were then washed, followed by SDS-PAGE, and the amount of active-Rac was determined by Western blot using Rac1-specific antibodies. Total cellular lysates were also separated by SDS-PAGE, and Western blot analysis with anti-Rac1 antibodies was done as a control for protein loading.

Lung Histology

Mice were sacrificed and lungs were inflated and fixed with one ml of Z-fix solution overnight followed by washing with PBS and dehydration in 70% ethanol. Tissue paraffin-embedding, sectioning and H&E staining were performed by Morphology and Imaging Core facility at LSUHSC.

Microarray data retrieval and bioinformatics analysis

Public-domain expression microarray data was obtained from web-sites and further analyzed using Web-based microarray analysis software (13)~~(15)~~.

Statistical analyses

Statistically significant differences between samples were determined by a paired, 2-tailed *t* test. Statistical significance of Nischarin expression differences samples were also determined by paired, 2-tailed *t* test. One-way analysis of variance (ANOVA) was used to assess differences in Nischarin expression between tumor types. Tukey's multiple comparisons test was used for all possible pair-wise comparisons.

Results

Decreased Nischarin expression during breast cancer progression

We investigated Nischarin expression in human breast cancer cell lines and specimens from human breast cancer patients. We initially examined Nischarin expression in breast cancer and breast epithelial cell lines. Nischarin mRNA expression was lowest in highly invasive cancer cell lines (MDA-MB231), lower in moderately invasive cells (MCF7, T47D, BT474) compared to non-tumorigenic MCF10A cells (Supplementary Figure 1). To evaluate Nischarin expression in human breast cancer specimens, we assessed Nischarin mRNA expression in 60 human breast cancer specimens relative to 60 normal tissues. Nischarin was strongly expressed in normal tissues, while there was decreased expression in the breast cancer specimens compared with the normal tissue specimens ($p < 0.01$; Figure 1 A). Among the 60 breast cancers, 22 were invasive ductal carcinoma (IDC); 4, ductal carcinoma in situ (DCIS); 17, a mixture of IDC and DCIS; 5, invasive lobular carcinoma (ILC); 6, a mixture of ILC and lobular carcinoma in situ (LCIS) and 6, a more complex mixture of these categories. Compared to the noncancerous tissues, all of the specimens with invasive components exhibited less expression than the normal tissues (Figure 1B). ~~In contrast, normal breast and DCIS appeared to express similar levels of Nischarin, suggesting that loss of Nischarin expression is associated with the invasive phenotype.~~

To explore whether there was any association of Nischarin expression with breast cancer stage, we quantified Nischarin mRNA expression in a panel of 45 cDNA (Origene) prepared from breast cancer tissue from different stages of breast cancer. Nischarin expression was high in Stage 0 specimens, while Stage I-IV specimens had lower expression (Supplementary Figure 2). To assess Nischarin

expression during breast cancer progression, we further quantified Nischarin mRNA in a panel of 24 primary breast tumor samples with matched normal breast tissue from the same patient. Nischarin expression was decreased from the normal to the breast cancer specimens in 71% (17/24) of these matched pairs, with the average Nischarin expression decreasing from adjacent normal tissue to breast cancer tissue ($p<0.01$, Figure 1C), supporting loss of Nischarin expression during breast cancer progression. To examine whether Nischarin expression was decreased in a broader spectrum of human breast cancers and further explore any associations with clinical and pathological data, we examined Nischarin expression in three publically available data sets of human breast cancer specimens analyzed by the Web-based microarray bioinformatics tool, Oncomine (14-16)~~(16-18)~~. Analysis of the publically available message expression databases supported decreased Nischarin expression in breast cancer compared with normal breast (Supplementary Figure 3A; $p< 0.05$). We also examined association of Nischarin mRNA expression with breast carcinoma tumor grade (grade 1, n=258; grade 2, n=536; grade 3, n=457) (Oncomine). Nischarin expression was significantly lower in high-grade tumors relative to low-grade tumors ($p< 0.05$) (16-22)~~(18-24)~~ (Supplementary Figure 3B). Taken together, these data support decreased Nischarin expression as a common event in human breast cancer, with loss of expression associated with invasive disease, higher tumor grade and disease progression. We next examined Nischarin protein expression in breast cancer cell lines and tumor tissues. Consistent with Nischarin message expression data, Western blot analysis detected little Nischarin in highly invasive MDA-MB231 cells or in invasive ductal and invasive lobular breast carcinoma tissues, while Nischarin protein expression could be detected in MCF7 cells, normal breast tissue and in ductal carcinoma in situ (DCIS; Figure 1D). To examine Nischarin protein expression in a

broader spectrum of human breast cancers, we performed immunohistochemical analysis on 3 tissue microarrays (TMA) obtained from CYBRDI consisting of 236 breast cancer and normal mammary tissue samples. These arrays were stained for Nischarin and then scored for staining intensity by two pathologists. Intensity of staining was scored as the average staining intensity of the epithelial cells in each sample (0, no staining; 1, light staining; 2, medium staining; and 3, intense staining). Representative staining intensities are shown in Figure 1E. We used three different arrays (CC08-01-005; CC08-00-001; CC08-21-002) and established that normal breast specimens had the highest Nischarin expression, lowest levels are for grade III with approximately equally lower levels in grades 1 and IIfollowed by grade I and grade III tumors (Figure 1F, $p<0.05$). Furthermore, we compared average staining intensity for Nischarin in different stages of breast cancer in all three microarrays (CC08-00-001, CC08-21-002, and CC08-01-005). Quantification of the average Nischarin staining intensity indicated that Nischarin expression was reduced in invasive carcinoma relative to normal breast tissue (Figure 1G). These data are consistent with Nischarin mRNA level studies, supporting loss of Nischarin expression in association with invasive disease and higher tumor grade.

Genomic Loss of the NISCHARIN locus in human breast cancer

Nischarin maps to 3p21, a cytogenetic region reported to exhibit loss of heterozygosity (LOH) in a variety of human cancers including breast cancer (23, 24)(25, 26). To further investigate the mechanism for loss of Nischarin expression during the breast cancer progression, we examined LOH at the NISCHARIN locus using microsatellite markers in DNA samples extracted from 18 human breast cancers and patient-matched normal tissue counterparts. With three microsatellite markers, we established that 50% of tumor samples (9 of 18) exhibited LOH at the Nischarin locus

(data shown for D3S3026 marker) (Figure 2A and B). Importantly, LOH at the NISCHARIN locus correlated with decreased Nischarin expression (Figure 2C).

In addition, we performed QPCR micro deletion analysis in twenty matched pair of breast tissue samples using three pairs of primers (see methods for details) designed in the intron 2, 3 and 6 of human Nischarin gene (data not shown for intron 2 and 3 primers). The QPCR analysis further validated the loss of Nischarin locus in 12 out of 20 matched tumor samples (data shown for intron 6 primers) (Figure 2D). These results support that Nischarin is down regulated in a large proportion of primary human breast cancer patients through LOH.

As decreased Nischarin expression is frequently observed in human breast cancers, we investigated whether Nischarin expression could be a prognostic marker for breast cancer patients. We analyzed multiple expression data sets for which both Nischarin expression and recurrence-free survival data were available (25)~~(27)~~. We stratified the patient population (n=286) into 2 groups based upon Nischarin expression, and found that patients with elevated Nischarin expression showed significantly increased recurrence-free survival (Figure 2E, $p<0.05$). Specifically, a 2-fold increase in Nischarin expression conferred a 2.8-fold decrease in risk of tumor recurrence. These data suggest that decreased Nischarin expression may predict decreased recurrence-free survival in breast cancer patients.

Nischarin suppresses cell growth, anchorage independent growth and tumor growth *in vivo*

Loss of Nischarin expression during breast cancer progression suggested that Nischarin might normally function to prevent cancer progression. We have previously demonstrated that Nischarin decreases the migratory potential of breast cancer cells

(14). Cell proliferation is an important determinant of tumor growth and metastasis. To examine whether Nischarin could function as a putative tumor suppressor, we initially assessed the effects of Nischarin expression on the growth of MDA-MB231 cells. While MDA-MB231 cells expressing vector control grew robustly, growth of Nischarin expressing cells was significantly inhibited ($p<0.05$) (Supplementary Figure 4), supporting an effect of Nischarin on breast cancer cell growth. As Nischarin inhibited MDA-MB231 cell growth, we investigated whether Nischarin could inhibit anchorage-independent growth. Parental vector-expressing MDA-MB231 cells exhibited high potential to form colonies in soft-agar. However, expression of Nischarin substantially reduced soft-agar colony formation (Figure 3A, B).

Our *in vitro* studies suggested that down-regulation of Nischarin expression in MDA-MB231 breast cancer cells may provide a growth advantage. To investigate this possibility, we stably expressed GFP-Nischarin or GFP control in MDA-MB231 cells using lentiviral expression vector. Cells were injected into mammary fat pads of Balbc nu/nu mice, and tumor volume was followed. Although tumors formed in both groups, tumors from cells expressing only GFP alone consistently grew more rapidly and attained greater volumes than tumors from Nischarin-expressing MDA-MB231 cells. Overall, tumors from control cells attained a mean calculated volume ten times greater than tumors from Nischarin-expressing cells (Figure 3C and 3D). Western blot analysis of tumors confirmed that Nischarin-expressing tumors continued to express Nischarin (Figure 5E), suggesting that inhibition of tumor growth is due to Nischarin expression. These results support a possible tumor suppressor function for Nischarin *in vivo*. As our *in vitro* studies suggested an effect of Nischarin on cell proliferation, to investigate this *in vivo*, we performed immunohistochemical analysis for Ki67, a marker for proliferation. Nischarin expressing tumors had significantly less Ki67

staining than GFP expressing controls (Figure 3E, F; $p<0.01$), supporting a role for Nischarin in regulating tumor growth in part through the effects on cell proliferation.

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Nischarin inhibits lung metastasis

We further examined the function of Nischarin *in vivo* using an experimental lung metastasis model. In this model, tumor cells are injected directly into the blood stream bypassing the step of invasion and intravasation. MDA-MB231 cells were genetically engineered to express the firefly luciferase gene (referred to as MDA-MB231-luc, generous gift from Dr. Jerry W Shay; University of Texas Southwestern). These cells were transduced with Nischarin or vector control lentivirus particles. Nischarin expressing MDA-MB231 luc or its vector control cells were injected into tail veins of nude mice. After two hours of injection, bioluminescent imaging was performed to establish whether the cells were homing to the lung and subsequent bioluminescent imaging was performed every week for eight weeks. The mice injected with Nischarin expressing MDA-MB231 demonstrated a significant reduction in bioluminescent signal compared to vector control MDA-MB231 (Figure 4A). Our data indicate that the bioluminescent signals from mice injected with Nischarin expressing MDA-MB231 cells were six orders of magnitude less than those of controls injected with vector-expressing MDA-MB231 cells (Figure 4C). *Ex vivo* bioluminescent imaging of lungs from these mice after eight weeks also revealed that Nischarin suppresses lung metastasis (Figure 4B).

To further confirm the effect on metastasis, we performed histological analysis of lung tissues from the mice that were injected with breast cancer cells through the tail veins. Lung tissues were extracted and sectioned, and Hematoxylin and Eosin (H&E) staining revealed that very few metastases were formed in the lungs of mice injected with Nischarin transfected cells (Figure 4D). In contrast, lungs from mice

injected with vector control cells were heavily infiltrated by metastases. Further pathologic examination of tumor metastasis to the lungs in MDA-MB231 Nischarin mice revealed that these metastases were small and isolated compared with the large, locally invasive metastases observed in MDA-MB231 control mice (data not shown). These results suggest that Nischarin is an important regulator of breast cancer cell invasion and metastasis *in vivo*.

Nischarin inhibits MDA-MB231 invasion through the α5 integrin

Cell invasion is an important event in tumor progression, with adhesion of tumor cells to the extracellular matrix (ECM) and migration of tumor cells both contributing to cancer cell invasion and metastasis (26)~~(28)~~. To define the mechanism by which Nischarin suppresses invasion, we assessed the effects on the extracellular matrix. While restoring Nischarin expression did not alter basal invasion or serum stimulated invasion in the presence of poly-lysine (Supplementary Figure 5A) or collagen (Supplementary Figure 5B), Nischarin inhibited the invasiveness of MDA-MB231 cells in the presence of fibronectin (Figure 5A), suggesting that Nischarin inhibits tumor cell invasion specifically in the context of fibronectin.

We have previously established a specific protein-protein interaction between Nischarin and α5 integrin (1)~~(1)~~. It is known that the α5 integrin complexes with β1 integrin to form the classic fibronectin receptor, which plays a vital role in both cell migration and cell adhesion. Since Nischarin specifically interacts with α5 integrin and specifically inhibited cancer cell invasion in the context of fibronectin, we investigated whether these effects were mediated through α5 integrin by expressing α5 integrin in the context of the different ECM proteins and assessing Nischarin's

effects on invasion. While α5 integrin had no effect on the cell invasion of MDA-MB231 cells to polylysine (Supplementary Figure 5A) or collagen (Supplementary Figure 5B), exogenous α5 integrin did abrogate Nischarin-mediated inhibition of invasion to fibronectin (Figure 5A, B).

We had previously demonstrated that the proximal trans-membrane region (KLGFFKR) of α5 integrin is important for interaction with Nischarin (2)~~(2)~~. To examine the specificity of α5 integrin's effects on invasion and whether the cytoplasmic region or interaction with Nischarin were necessary, we introduced α5 truncation constructs lacking either 16 amino acids in the cytoplasmic domain (contains the essential Nischarin binding region, and referred to as α5 c-10) or the entire cytoplasmic domain (does not contain the essential Nischarin binding region, and referred to as α5 c-1). While α5 c-10 was partially able to recapitulate the effects of α5, α5 c-1 was largely inactive (Figure 5B). These data suggest that Nischarin's interaction with α5 integrin is critical for Nischarin-mediated inhibition of invasion. As these data suggested that the effects of Nischarin were largely dependent on the interaction of Nischarin with α5 integrin, we investigated whether Nischarin regulated α5 integrin expression. To examine this possibility, we performed RT-PCR on Nischarin- expressing cells using α5 integrin-specific primers. In the MDA-MB231 model, Nischarin expression potently decreased α5 integrin expression both at the message level (Figure 5C) and the protein level (Figure 5D). These data suggest that a reduction of α5 integrin expression by Nischarin could be one of the mechanisms by which Nischarin functions as a tumor suppressor. To establish whether this mechanism was operating *in vivo*, we analyzed expression of Nischarin and α5 integrin in MDA-MB231 tumors formed in nude mice in the presence and absence of Nischarin. Similar to our studies *in vitro*, Nischarin expression potently decreased α5

integrin expression *in vivo* (Figure 5E), supporting Nischarin induced loss of α5 integrin expression as one mechanism for the tumor suppressing effects of Nischarin.

Nischarin modulates α5 integrin expression

To examine whether Nischarin expression modulates the transcriptional regulation of α_5-integrin, we performed dual luciferase assay on stable clones of MDA-MB231 over expressing Nischarin and transiently expressing either full length 923 bp α5 promoter or 26 bp of α5-integrin promoter construct fused with luciferase in a pGL3 vector (generous gift from Dr. Ritzenthaler from Emory University, GA); the 26 bp construct served as a negative control. Over expression of Nischarin significantly ($p<0.01$) decreased the full-length α5-integrin promoter activity (Figure 6A). These data suggested potential transcriptional regulation of α5 integrin by Nischarin. Our data demonstrated that Nischarin decreases α5 integrin expression. To further define the mechanism for Nischarin's tumor suppressor effects, we examined the effects of Nischarin on α5-mediated signaling events (27)~~(29)~~. Since α5 integrin stimulates FAK signaling, FAK has been shown to promote cell migration and cell invasion, and FAK is over expressed in human cancers, we examined whether Nischarin effects FAK phosphorylation as a surrogate for FAK activation. In the MDA-MB231 model, Nischarin expression potently decreased FAK phosphorylation (Figure 6B). To investigate whether the effects of Nischarin were due to altered α5 integrin expression, we rescued loss of α5 integrin expression with exogenous α5 integrin or the α5 integrin cytoplasmic domain mutants α5 c-1 and α5 c-10. While restoring α5 integrin expression reversed Nischarin mediated suppression of FAK phosphorylation, α5 c-1 and α5 c-10 were only partially able to do so (Figure 6C). To establish whether this mechanism was operating *in vivo*, we analyzed expression of

Nischarin and phosphorylated FAK in MDA-MB231 tumors formed in nude mice in the presence and absence of Nischarin. Similar to our *in vitro* studies, Nischarin expression potently decreased FAK phosphorylation *in vivo* (Figure 6D). Taken together these studies support a model in which Nischarin functions as a tumor suppressor by decreasing α5 integrin expression to decrease FAK signaling.

Nischarin knockdown promotes α5 integrin expression

As restoring Nischarin expression inhibits tumor growth and metastasis, we examined whether loss of Nischarin expression could promote these processes in weakly tumorigenic and invasive MCF7 cells. Nischarin shRNA effectively reduced Nischarin expression at the message (Figure 7A) and protein level (Figure 7B). While restoring Nischarin expression in MDA-MB231 cells reduced α5 integrin expression, reducing Nischarin expression induced α5 integrin expression in MCF7 cells (Figure 7A and 7B, middle panels). Consistent with the gain of function results in MDA-MB231 cells, Nischarin knockdown cells demonstrated significantly increased α5 integrin promoter activity (Figure 7C, $p<0.01$). These data suggest that a reduction of α5 integrin expression by Nischarin could be one of the mechanisms by which Nischarin functions as a tumor suppressor in breast cancer.

Increased integrin alpha5 (ITGA5) expression during breast cancer progression

Since Nischarin regulates alpha5 integrin expression, we examined the expression of alpha5 integrin in breast tumors. We assessed integrin alpha5 mRNA expression in 54 human breast specimens relative to 54 normal tissues. In contrast to Nischarin data (shown in Figure 1), alpha5 integrin was weakly expressed in normal tissues, while there was increased expression in the breast cancer specimens compared with the normal tissue specimens ($p<0.05$; Supplementary Figure 6~~7~~A). Compared to the noncancerous tissues, IDC and IDC+DCIS specimens exhibited

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more expression than the normal tissues (Supplementary Figure 7 6 B). To further assess integrin alpha5 expression, we quantified ITGA5 mRNA in a panel of 24 primary breast tumor samples with matched normal breast tissue from the same patient. ITGA5 expression was increased from the normal to breast cancer specimens in 91% (22/24) of these matched pairs, with the average ITGA5 expressing increasing from adjacent normal tissues to breast cancer tissue ($p<0.001$). (Supplementary Figure 6C) Furthermore, we compared the expression of Nischarin and ITGA5 in 54 tumor specimens (Supplementary Figure 6D). Our data clearly show that Nischarin and ITGA5 have inverse correlation with regard to their expression levels.

Nischarin knock down upregulates tumorigenic potential *in vitro* and *in vivo*

As restoring Nischarin expression inhibited cell growth, we investigated the effect of knockdown of Nischarin on anchorage independent growth. As expected down regulation of Nischarin enhanced anchorage independent growth relative to control shRNA expressing cells (Figure 7D and 7E), with an increase in the size and number of soft agar colonies. Consistent with this, Nischarin knockdown increased proliferative activity of MCF7 cells in culture (Supplementary Figure 7 6A). shRNA silencing of Nischarin also potently increased the invasiveness of MCF7 cells through Matrigel (Supplementary Figure 7 6B). These data indicate Nischarin knockdown enhances *in vitro* tumorigenic functions.

We next examined whether reducing endogenous levels of Nischarin would affect *in vivo* tumorigenic properties. MCF7 cells expressing GFP alone, control shRNA and Nischarin shRNA expressing cells were injected into mammary fat pads of Balbc nu/nu mice, and tumor volume was measured. Mice bearing Nischarin shRNA cells developed significantly large tumors compared with either mice bearing

vector alone and scrambled control shRNA cells (Figure 7F and 7G). Taken together, these results support Nischarin as a candidate tumor suppressor in breast cancer.

Nischarin ~~induces G1/S arrest and~~ inhibits breast cancer cell invasiveness through inhibition of FAK and Rac

~~Since cell growth is tightly regulated by a series of positive and negative regulators of the cell cycle, and both gain and loss of function studies demonstrated a role for Nischarin on regulating cell proliferation, we analyzed the effects of Nischarin on cell cycle progression and its associated proteins. Nischarin transfected cells demonstrated a G1/S arrest (65% compared with 49% in control), which resulted in a decrease in S phase population (Figure 8A). The cell cycle progression is tightly regulated by a complex network of positive and negative cell cycle regulatory molecules, such as cyclin-dependent kinases (CDK), cyclins, and CDK inhibitors. CDK4-CDK6 (cyclin D1) and CDK2 (cyclin E) complexes phosphorylate retinoblastoma protein (Rb) in the mid to late phase of the G1 phase. Therefore, the expression levels of CDK4, CDK6, CDK2, cyclin D1 and phosphorylated Rb were examined in tumors extracted from MDA-MB231 Nischarin or control xenografts. As shown in Figure 8B, Nischarin expressing tumors exhibited decreased expression of cyclin D1, CDK4 and phosphorylated Rb (Figure 8B). As expected there was no change in the level of CDK2 (data not shown). Furthermore, the expression levels of cyclin dependent kinase inhibitors in the G1/S phase were also examined. As shown in Fig 8B, the expression level of p19 was significantly increased.~~

Since it is known that α5 integrin activates Rac and Rac regulates ~~cyclin D1~~PAK, we hypothesized that Nischarin may regulate Rac activation. Nischarin expressing MDA-MB231 cells and GFP control cells were serum starved and

stimulated with serum for one hour and Rac GTPase assays were performed. In Nischarin expressing cells, Rac GTP loading was significantly reduced (Figure 8A~~C~~). These data suggest that Nischarin regulates α5 integrin expression which affects Rac mediated signaling to regulate tumorigenesis. ~~downstream cyclin and Rb phosphorylation events.~~ Moreover, ~~cyclin D1 is activated by~~PAK1 regulates ERK phosphorylation, and thus we examined whether Nischarin regulates ERK phosphorylation. As predicted, restoring Nischarin expression reduced ERK phosphorylation (Figure 8A~~B~~). In contrast, suppression of endogenous Nischarin in MCF-7 xenograft tumors stimulated ~~cyclin D1 and CDK4 expression, Rb,~~ FAK and ERK phosphorylation as well as Rac GTP loading (Figure 8B~~D~~). These data suggest an important role for Nischarin. As we examined these signaling mechanisms in tumor xenografts from two distinct breast cancer models, using both gain and loss of function approaches, these results strongly support a role for Nischarin in regulating the FAK-Rac-ERK-~~cyclin D1 Rb~~ signaling cascade to regulate breast cancer progression (Figure 8C~~E~~).

Discussion

Based on our previous studies, which defined Nischarin as a regulator of cell migration and invasion (3-5)~~(3-5)~~, we hypothesized that Nischarin may function as a tumor suppressor in human breast cancer. Here we demonstrate that Nischarin expression is frequently decreased at the message and protein level in human breast cancers. Nischarin loss correlates with acquisition of the invasive phenotype, with loss correlating with tumor grade and corresponding to a decrease in patient survival. LOH at the NISCHARIN locus is one mechanism for loss of Nischarin expression, with 50% of breast cancer patients exhibiting LOH and LOH closely correlating with loss of Nischarin expression. Moreover, a recent study using genome wide promoter

methylation analysis identified Nischarin promoter methylation in 30% of breast cancers (28)~~(30)~~, supporting epigenetic regulation and LOH as mechanisms resulting in loss of Nischarin expression in breast cancer. Functionally, restoring Nischarin expression inhibits proliferation and soft-agar colony formation *in vitro* and tumor growth *in vivo*, while directly silencing Nischarin expression increases cell proliferation and soft-agar colony formation *in vitro* and tumor growth *in vivo*. Mechanistically restoring Nischarin expression decreased α5 integrin expression and reduced FAK phosphorylation, leading to down regulation of ERK~~, which in turn downregulated cyclin D1 and Rb phosphorylation~~. In a reciprocal manner, shRNA-mediated silencing of Nischarin expression increased α5 integrin expression, FAK phosphorylation and ERK phosphorylation~~, which in turn upregulated cyclin D1 expression and Rb phosphorylation~~. Furthermore, our data show an inverse relationship between ITGA5 and Nischarin expression. These results suggest that Nischarin is a potential tumor suppressor in breast cancer functioning to potently regulating α5 integrin, FAK and Rac and to regulate ~~Cyclin D1 mediated~~ tumorigenesis. Taken together these results strongly support Nischarin as a putative tumor suppressor in breast cancer.

Integrins have been shown to play a significant role in cancer progression, especially by promoting tumor cell survival, tumor angiogenesis, and metastasis; thus, agents targeting integrins have great therapeutic potential (29)~~(31)~~. Our data agree with several recent studies showing that α5β1 integrin is important to induction of invasion of breast carcinoma cells. Also in several cell types, regulation of α5β1 mediated action of ERK and FAK signaling has been demonstrated (30)~~(32)~~. Our data further corroborates that Nischarin down regulation of α5 integrin affects ERK and FAK phosphorylation. A recent study elegantly showed that FAK catalytic activity is

required for α5β1-stimulated Src activation through FAK phosphorylation of Src (27)~~(29)~~. It is known that E-cadherin functions as a tumor suppressor that regulates epithelial mesenchymal transition (EMT)(31)~~(33)~~. In several breast cancers, E-cadherin levels are significantly decreased, similar to Nischarin levels, further suggesting that Nischarin and E-cadherin reduce tumor growth through the modulation of α5-FAK signaling. Furthermore, down-regulation of E-cadherin up-regulates α5 integrin protein expression through activation of the EGFR/FAK/ERK1 signaling pathway (32)~~(34)~~ suggesting an inverse correlation between E-cadherin and α5 integrin expression levels. While α5β1 functions as a tumor promoter, an antagonist of this integrin was shown to block proliferation, adhesion, and anchorage-independent growth of human astrocytoma cells (33)~~(35)~~. Further support comes from a study demonstrating that α5 expression is elevated in metastatic B16F10 melanoma cells (34)~~(36)~~. The exact mechanism of Nischarin regulation of ITGA5 is currently unknown. One potential mechanism could be through the association of Nischarin leucine zipper domains (3)~~{Alahari, 2004 #14}~~ ~~(Alahari et al, 2004)~~ with other leucine zipper containing proteins (35)~~{Venugopal, 1998 #96}{Alahari et alVenugopal and Jaiswal, 1998)~~. I~~i~~t is possible that Nischarin leucine zipper domains may bind to c-fos/AP1 complex proteins, which are known to upregulate transcription of several genes involved in proliferation, and are shown to regulate the expression of ITGA5 (36,)~~{Gingras, 2009 #94}~~ ~~(~~37)~~{Larouche, 2000 #97}~~. ~~(Gingras et al, 2009ref).~~ Thus, Nischarin may regulate ITGA5 expression through AP1 complex transcription factors. However, Nisc~~a~~harin is primarily localized to the cytosol and ~~it is not clear~~ what stimulates its translocation to the nucleus remains to be determined. ~~, which needs to be further investigated.~~

Based on our data, we propose a model in which Nischarin reduces α5 expression leading to reduction of FAK phosphorylation and Rac GTP loading ~~that leads to regulation of cyclin D1~~, which in turn reduces tumor growth. In addition to effects on α5 integrin and FAK as shown above, Nischarin also regulates PAK, and LIMK signaling, all of which have defined roles in breast carcinogenesis (1, 3, 5)~~(1, 3, 5)~~. Whether Nischarin mediates some of its tumor suppressor roles in breast cancer through regulation of these pathways is an area of current investigation.

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Figure Legends

Figure 1: Loss of Nischarin expression during breast cancer progression

A: Nischarin mRNA expression by QPCR in sixty normal breast and sixty breast cancer tissues ($p<0.01$). **B:** Detailed analysis of normalized Nischarin mRNA expression by QPCR in sixty breast cancer specimens and sixty normal breast tissue specimens ($p<0.01$). n=number of tissues. **C:** Relative expression of Nischarin in 24 patient-matched breast cancer and adjacent noncancerous breast tissue ($p< 0.01$) **D:** Western blotting analysis of protein lysates prepared from MCF7 and MDA-MB231 cells and tissue lysates from normal breast, breast tumor (ductal carcinoma in situ), and invasive ductal and invasive lobular carcinoma tissues (U.S. Biologicals). **E:** Representative Immunohistochemical staining for breast cancer array core (original magnification, × 20) for Nischarin in normal breast (score 3), or grade I (score 2), grade II (score 1) and grade III (score 0) infiltrating ductal carcinoma (Scale bar = 500μM). **F:** ANOVA analysis of Nischarin expression ($p<0.01$) among the normal and grade I, grade II and grade III groups. **G:** Dunn's post hoc analysis of the normal, ductal and lymph node metastasis samples ($p< 0.05$).

Figure 2: Frequent loss of Nischarin gene locus in breast cancer correlates with loss of Nischarin expression. A: Loss of heterozygosity on Nischarin locus. Representative results showing allelic loss in tumors 2, 3, 4, 6 and 7 (shown by asterisks) after PCR products were separated on a 7.5% TBE polyacrylamide gel. (M, molecular weight marker; N, normal tissue; T, tumor tissue). **B:** LOH was confirmed using an ABI sequencer and quantified using Gene scan software. A representative sample with LOH is shown. **C:** Quantitative PCR analysis of Nischarin mRNA in breast cancer specimens was compared with LOH and without LOH. Red bars represent the tissue samples with loss of heterozygosity and black bars represent the samples that did not show any allelic loss. **D:** QPCR micro-deletion analysis of

Nischarin locus in matched breast tissue samples. Paired tissues in the left panel do not show any locus loss (ΔKCt values of 0 ± 0.35) whereas paired tissues in the right panel show micro-deletion of Nischarin locus (ΔKCt below -0.35) in tumor tissues. n= number of tissues. **E:** Low levels of Nischarin predict decreased recurrence-free survival in women with breast cancer (n=286) ($P<0.05$).

Figure 3: Restoring Nischarin expression inhibits anchorage independent growth and tumor growth A: MDA-MB231 cells were stably transfected with Nischarin or control plasmid and assayed for anchorage independent growth (Scale bar=100μM). **B:** Quantitative analysis of number of soft agar colonies in Nischarin expressing cells. **C:** Nischarin or GFP expressing MDA-MB231 cells were injected into the breast fat pads of female nu/nu mice (n=5). A representative picture of vector and Nischarin cells produced tumors in nude mice is shown. Tumors and site of tumor cells injected are shown with asterisks. **D:** Quantitative data showing primary tumor growth in Nischarin or GFP expressing MDA-MB231 tumors. **E:** Paraffin embedded tissue sections of primary tumors from mice with MDA-MB231 (Top panel) and MDA-MB231-Nisch (bottom panel) cells were immunostained with anti-Ki 67 antibody (right) and H&E (left). **F:** Number of Ki67 positive cells field per view in MDA-MB231 and MDA-MB-231 Nischarin expressing tumor ($P<0.01$).

Figure 4: Nischarin over expression inhibits lung metastasis in Nude mice model A: Representative bioluminescent image of the indicated mice 5, 10, 20 and 40 days after tail vein injection are shown. PTV; post tail vein injection; **B:** Ex-vivo bioluminescent imaging of mouse lungs from vector control and Nischarin expressing cells after eight weeks. **C:** Quantification of bioluminescence data. Data shown are as mean ± SEM (n=5). **D:** H&E analyses showing staining of lung tissue sections from mice injected with MDA-MB231 cells stably expressing vector control, mice injected

with MDA-MB231 cells stably expressing Nischarin. T indicates tumor metastases (Scale bar = 500μM).

Figure 5: Nischarin regulates α5-mediated invasion and down regulates α5 integrin expression. A: MDA-MB-231 cells were transiently transfected with Nischarin +vector, vector alone, or Nischarin + α5 integrin. β-gal was introduced in every transfection to visualize the cells. **B:** MDA-MB231 cells were transfected with Nischarin + α5 integrin, Nischarin + vector, Nischarin+ α5 integrin c-1, or Nischarin+ α5 integrin c-10 and serum induced invasion assay was performed (n=3). **C:** Nischarin, α5 integrin and β-actin mRNA expression were examined by RT-PCR in MDA-MB231 cells expressing GFP or Nischarin. **D:** Nischarin, α5 integrin and vinculin protein expression were examined by Western blot analysis in MDA-MB231 cells expressing GFP or Nischarin. **E:** Nischarin, α5 integrin and vinculin protein expression were examined by Western blot analysis in MDA-MB231 xenograft tumors expressing GFP or Nischarin.

Figure 6: Nischarin inhibits the α5 integrin mediated signaling pathway. A: Dual luciferase assays on the indicated portions of the α5 integrin promoter in the presence of Nischarin or GFP control. Y axis: arbitrary units of luciferase activity ($^{**}p < 0.01$; n = 3). **B:** Phosphorylation of FAK at tyrosine-397 (P-FAK), total FAK, and Nischarin and vinculin protein expression were examined by Western blot analysis in MDA-MB231 cells expressing GFP or Nischarin. **C:** MDA-MB231 cells were transiently transfected with 1ug of Nischarin, 2μg of α5+1 μg of Nischarin, 2μg of α5 c-1+1μg of Nischarin, 2μg of α5 c-10+1μg of Nischarin, and the lysate made from these cells were immunoblotted with respective antibodies. The α5 integrin cytoplasmic domain

specific antibody (AB1928 from Millipore) detected robust α5 integrin signal only in full length α5 integrin transfected cells. **D:** Phosphorylation of FAK at tyrosine-397 (P-FAK), total FAK and Nischarin protein expression were examined by Western blot analysis in MDA-MB231 xenograft tumors expressing GFP or Nischarin.

Figure 7: Nischarin silencing upregulates α5 integrin expression and promotes tumor growth A: Nischarin, α5 integrin and β-actin mRNA expression were examined by RT-PCR in control MCF-7 cells, MCF-7 cells expressing shRNA to Nischarin or scrambled shRNA. **B:** Nischarin, α5 integrin and vinculin protein expression were examined by Western blot analysis in control MCF-7 cells, MCF-7 cells expressing shRNA to Nischarin or scrambled shRNA. **C:** Dual luciferase assays on the indicated portions of the a5 integrin promoter in the presence of shRNA to Nischarin or scrambled shRNA(**$p < 0.01$; $n = 3$). Y axis: arbitrary units of luciferase activity. **D**. Anchorage-independent growth assay of MCF7 cells infected with lentivirus expressing GFP alone, scramble, or shRNA to Nischarin (scale bar =100μM). **E:** Quantification of anchorage-independent growth assay of MCF7 cells infected with lentivirus expressing GFP alone, scramble shRNA, or shRNA to Nischarin. ($^{**}p < 0.01$; $n = 3$) **F:** Representative images of the mice showing primary tumor growth of MCF-7 cells with GFP alone, scramble shRNA or shRNA to Nischarin (n=5) Tumors and their site of injections are shown with asterisks. **G:** Quantitative data showing tumor volumes of MCF-7 cells with GFP alone, scramble shRNA or shRNA to Nischarin. Data represent averages of three independent experiments. All data are shown as mean ± SD.

Figure 8: Nischarin regulates the cell cycle and cell cycle regulators. A: Rac1 GTPase assay in MDA-MB231 expressing GFP or Nischarin. Nischarin and ~~vector~~

GFP transduced MDA-MB231 cells were ~~synchronized for~~serum starved overnight ~~by serum starvation and cell cycle analysis was performed after staining with propidium Iodide after 72 hours (n=3~~and stimulated with serum for one hour~~). **B:** Expression of the indicated proteins by Western blot analysis in MDA MB231 GFP or MDA MB231 Nischarin xenograft tumors. **C:** Rac1 GTPase assay in MDA MB231 expressing GFP or Nischarin. **D**~~**B:**~~ Expression of the indicated proteins by Western blots in MCF-7-scramble shRNA or MCF-7-shRNA-Nischarin xenograft tumors. ~~**E**~~**C:** Model of the mechanism of Nischarin action during breast cancer progression.

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