Cancer Research

Cancer Affects microRNA Expression, Release, and Function in Cardiac and Skeletal Muscle

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Abstract

Circulating microRNAs (miRNA) are emerging as important biomarkers of various diseases, including cancer. Intriguingly, circulating levels of several miRNAs are lower in patients with cancer compared with healthy individuals. In this study, we tested the hypothesis that a circulating miRNA might serve as a surrogate of the effects of cancer on miRNA expression or release in distant organs. Here we report that circulating levels of the muscle-enriched miR486 is lower in patients with breast cancer compared with healthy individuals and that this difference is replicated faithfully in MMTV-PyMT and MMTV-Her2 transgenic mouse models of breast cancer. In tumor-bearing mice, levels of miR486 were relatively reduced in muscle, where there was elevated expression of the miR486 target genes PTEN and FOXO1A and dampened signaling through the PI3K/AKT pathway. Skeletal muscle expressed lower levels of the transcription factor MyoD, which controls miR486 expression. Conditioned media (CM) obtained from MMTV-PyMT and MMTV-Her2/Neu tumor cells cultured *in vitro* were sufficient to elicit reduced levels of miR486 and increased PTEN and FOXO1A expression in C2C12 murine myoblasts. Cytokine analysis implicated tumor necrosis factor α (TNFα) and four additional cytokines as mediators of miR486 expression in CM-treated cells. Because miR486 is a potent modulator of PI3K/AKT signaling and the muscle-enriched transcription factor network in cardiac/skeletal muscle, our findings implicated TNFα-dependent miRNA circuitry in muscle differentiation and survival pathways in cancer. *Cancer Res; 74(16); 4270–81. ©2014 AACR.*

Introduction

Extracellular/circulating microRNAs (miRNA) have emerged as minimally invasive biomarkers of cancer progression and therapeutic response (1–3). Imbalance in circulating miRNAs goes beyond cancer, as there is evidence for altered circulating miRNAs in Atherosclerosis and Alzheimer disease (4, 5). Because of relative stability of these circulating miRNAs, the sera miRNA profiling has been suggested to be highly sensitive screening assay for early detection of various diseases (6).

The source of circulating miRNAs, particularly in cancer, remains an enigma as levels of several of circulating miRNAs show opposing pattern in tumor and in circulation (7). Although tumor itself or circulating tumor cells are potential sources of miRNAs that are elevated in the sera/plasma of patients with cancer, consistent observation of lower circulating levels of specific miRNAs in patients with cancer compared with healthy controls suggest that systemic effects of cancer is causing overall changes in expression/release of miRNAs from distant organs (8–10). For example, a recent study evaluating sera miRNA as a potential risk biomarker of breast cancer using prospectively collected sera from Sister Study Cohort showed downregulation of 5 miRNAs in the sera of women who developed breast cancer (11). Another report using breast tumors and sera from Asian Chinese patients showed downregulation of miRNA in the sera of patients with cancer (7). Our recent study provided a hint to the contribution of secondary organs in cancer-associated circulating miRNA changes as we observed elevated U6 small RNA in the sera of patients with breast cancer who are clinically disease-free compared with healthy controls (12). We proposed that cancer-induced epigenomic changes in distant organs cause elevated expression and release of U6 from these organs. However, this possibility has not been experimentally verified and the underlying mechanisms are unknown.

The goals of this study were to identify miRNAs that are present at a lower level in circulation in breast cancer models and then to elucidate mechanisms responsible for reduced levels of specific circulating miRNAs. We used two transgenic mammary tumor models—one is an aggressive tumor model and the other with relatively longer latency—to ensure that the

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results obtained are not unique to a specific model. Our results reveal specific deregulation in the expression of cardiac/skeletal muscle–enriched miRNA miR486 in mammary tumor models. *In vitro* studies identified tumor necrosis factor α (TNFα) as a potential cancer-induced factor responsible for deregulation of miR486 expression.

Materials and Methods

Human serum sample processing and miRNA extraction

The Indiana University institutional review board approved the use of human sera samples. Susan G. Komen for the Cure Normal Breast Tissue Bank at the Indiana University Simon Cancer Center collected patient sera samples along with healthy volunteer controls after obtaining informed consent. All samples were collected in accordance with standard operating procedure described in the tissue bank website. MiRVana Kit was used to isolate miRNA from 250 μL of sera (Applied Biosystems). Sera were spiked with synthetic *C. elegans* miR39 mimic (Qiagen) before miRNA extraction and miR486 expression was normalized to spiked miR39 mimic levels. Characteristics of healthy controls and patients studied have been described in our previous publication (12).

Transgenic models of breast cancer

National Institutes of Health regulations about the use and care of experimental animals were followed while conducting animal studies and the study was approved by the Indiana University School of Medicine animal use committee. Male MMTV-PyMT or MMTV-Her2/Neu mice on a FVB/N background were randomly bred with normal FVB/N females to obtain female heterozygous for the PyMT and Her2/Neu oncogene. MMTV-PyMT and MMTV-Her2/Neu mice have been described previously (13, 14). Neu oncogene used in this transgenic model is an activated form with 16 amino acids in-frame deletion of the extracellular domain (14). Blood, heart, and muscle were collected for miRNA preparation at the age of 3 and 5 months from MMTV-PyMT and MMTV-Her2/Neu mice, respectively. As we have reported previously, MMTV-PyMT mice at this age have extensive tumor burden accompanied with metastasis to lungs (15). MMTV-Her2/Neu mice also develop lung metastasis by 5 months age, although their tumor burden and metastasis are not as extensive as in PyMT mice (data not shown, ref. 14). All animals had tumors at the time of tissue harvest. The age matched normal female mice were used as controls.

Quantitative reverse transcription PCR

Five microliters of miRNAs (for sera) or 100 ng (for tumor and normal mammary gland) was reverse transcribed into cDNAs in a final volume of 30 μL using a Taqman miRNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR (qPCR) was performed using Taqman universal PCR mix (Applied Biosystems) and specific primers. Primers for U6 (#001973), miR486 (#001278), miR202 (#001195), and miR30d (#000420) were purchased from Applied Biosystems. Each amplification reaction was performed in duplicate in a final volume of 20 μL with 2 μL of cDNA. qPCR reactions of sera from healthy subjects and patients with metastatic breast cancer for a particular probe were in the same plate to limit mechanical errors. The expression levels of miR486 were normalized to miR202 (mouse sera and mammary gland), U6 (cardiac and skeletal muscle), and miR30d (C2C12 cells) using $2^{-\Delta\Delta Ct}$ method. In case of C2C12 cells, 1 μg of miRNA preparation was used for cDNA synthesis. mRNAs from C2C12 cells were prepared using the RNAeasy Kit from Qiagen and subjected to qRT-PCR using the primers that amplify both Ank1 and sAnk1 (primer sequences; forward: 5′-GAC GCA TGA CCT ACA GTC TTC-3′ and reverse: 5′-GCT ATC CTC TCC CTT CTT CTC T-3′) and β-actin (forward: 5′-AAT GAG GCC GAG GAC TTT GAT TGC-3′ and reverse: 5′-AGG ATG GCA AGG GAC TTC CTG TAA-3′).

miRNA array

miRNA profiling was performed using the Taqman miRNA array A that included an assay plate containing 384 probes of rodent miRNAs (#4398979, Applied Biosystems). PCR was conducted as per instructions from the manufacturer using 9 μL of preamplified cDNAs. Preamplification reactions were done as per instructions from Applied Biosystems using the TaqMan MicroRNA RT Kit (part no. 4366596). The sera from four each of control, MMTV-PyMT, and MMTV-Her2/Neu were used for the array. Probes that showed undetectable signals were given a CT value of 40 for calculation. Supplementary Table S1 provides CT values for each of the probes in all 12 samples. Normalization using miR202 was done using the $2^{-\Delta\Delta Ct}$ method.

Cell culture and conditioned media

Mouse myoblast C2C12 cells were seeded in 6-well plates (5×10^5 cells per well) in DMEM plus 10% FBS overnight. Mammary tumor cells generated from MMTV-Her2/Neu (16) and MMTV-PyMT mice (15) were cultured overnight in the same media and changed to serum-free DMEM medium for 24 hours. C2C12 cells were treated with CM for 24 hours. For neutralizing antibody assay, conditioned media (CM) was preincubated with 1 μg/mL of anti-TNFα antibody (R&D systems) at room temperature for 1 hour before adding to C2C12 cells. To directly measure the effects of cytokines on miR486 expression, C2C12 cells were treated with 20 ng/mL of CCL2, IFNγ, IL1α, or TNFα (R&D Systems) overnight. For promoting differentiation to myotubes, 5,000 C2C12 cells were plated in 6-well plates and maintained in 2% of horse serum containing media for 7 days. Serum-free control or MMTV-PyMT or MMTV-Her2/Neu tumor cell line–derived CM were added 2 days after plating.

Western blotting

After indicated treatments, cells were washed in PBS and lysed in RIPA buffer with protease/phosphatase inhibitors (Sigma). Thirty micrograms of proteins were used for Western blotting. Antibodies against FOXO1A (Upstate), β-actin (Sigma), pAKT (Cell Signaling Technology), p27 (BD Biosciences), PTEN, and MyoD (Santa Cruz Biotechnology) were used for Western blot analyses as per instructions from manufacturers.

Cytokine array and ELISA

Serum-free CM derived from MMTV-Her2/Neu and MMTV-PyMT cultures were subjected to immunoblotting based

Table 1. Significantly altered circulating miRNAs in transgenic mice with mammary tumors

	MMTV-Her2/Neu vs. control		MMTV-PyMT vs. control	
miRNA	***P* value**	**Fold change**	***P* value**	**Fold change**
MMTV-Her2/Neu specific				
miR486	0.0001	−6.54	0.72	−1.11
miR7a	0.0025	58.47	0.69	−1.48
miR743a	0.007	2.03	1	−1
miR381	0.0079	2.66	1	−1
miR139-3p	0.015	88.62	0.03	46.2
miR129-3p	0.016	35.61	0.492	2.39
miR191	0.018	−4.45	0.21	2.00
miR34a	0.019	40.757	0.606	2.00
MMTV-PyMT specific				
miR136	0.978	1.01	2.19E-06	81.41
miR202-3p	0.537	−1.15	0.00046	3.35
miR574-3p	0.50	1.34	0.00094	7.92
miR146b	0.271	2.15	0.001	21.47
miR134	0.0248	6.4	0.0015	21.7
miR667	0.668	1.18	0.0017	5.28
miR685	0.633	1.43	0.002	21.7
miR24	0.03	4.2	0.003	8.9
miR132	0.019	49.99	0.0038	209.42
miR542-5p	0.45	2.6	0.005	97.17
miR324-3p	0.11	4.05	0.006	17.07
miR223	0.919	−1.06	0.0066	8.17
miR92a	0.718	−1.15	0.007	3.94
miR342-3p	0.08	2.73	0.007	5.83
miR410	0.857	1.31	0.0096	127.559
miR200a	0.038	7.07	0.01	13.62
miR484	0.639	−1.23	0.01	4.05
miR139-5p	0.715	1.255	0.01	6.96
miR429	0.035	53.58	0.01	173.95
miR187	0.94	1.07	0.01	19.58
miR30a	0.141	2.67	0.014	6.37
miR511	0.031	15.93	0.015	25.74
miR423-5p	0.51	−1.52	0.016	6.09
miR335-5p	0.364	5.14	0.016	155.23
miR146a	0.303	1.93	0.016	5.9
miR125a-3p	0.238	13.7	0.019	359.00
Commonly deregulated in both transgenic mice				
miR193b	0.009	6.7	0.0001	36.2
miR151-3p	0.0072	5.199	0.0003	13.73
miR27b	0.007	171.86	0.0004	3,013.24
miR671-3p	0.0095	47.41	0.0013	220.48
miR200b	0.014	9.82	0.018	8.7
miR183	0.016	23.44	0.004	56.17
miR210	0.02	6.53	0.00082	27.37
miR132	0.02	49.99	0.0038	209.4

NOTE: No normalization was used in this analysis.

cytokine profiling using the mouse cytokine array panel A (#ARY006, R&D Systems). ELISA was used to measure TNFα levels in mouse sera and CM (BioLegand, for mouse).

Statistical analysis

Expression levels of sera miRNAs were compared using ANOVA. A P value of <0.02 was considered statistically

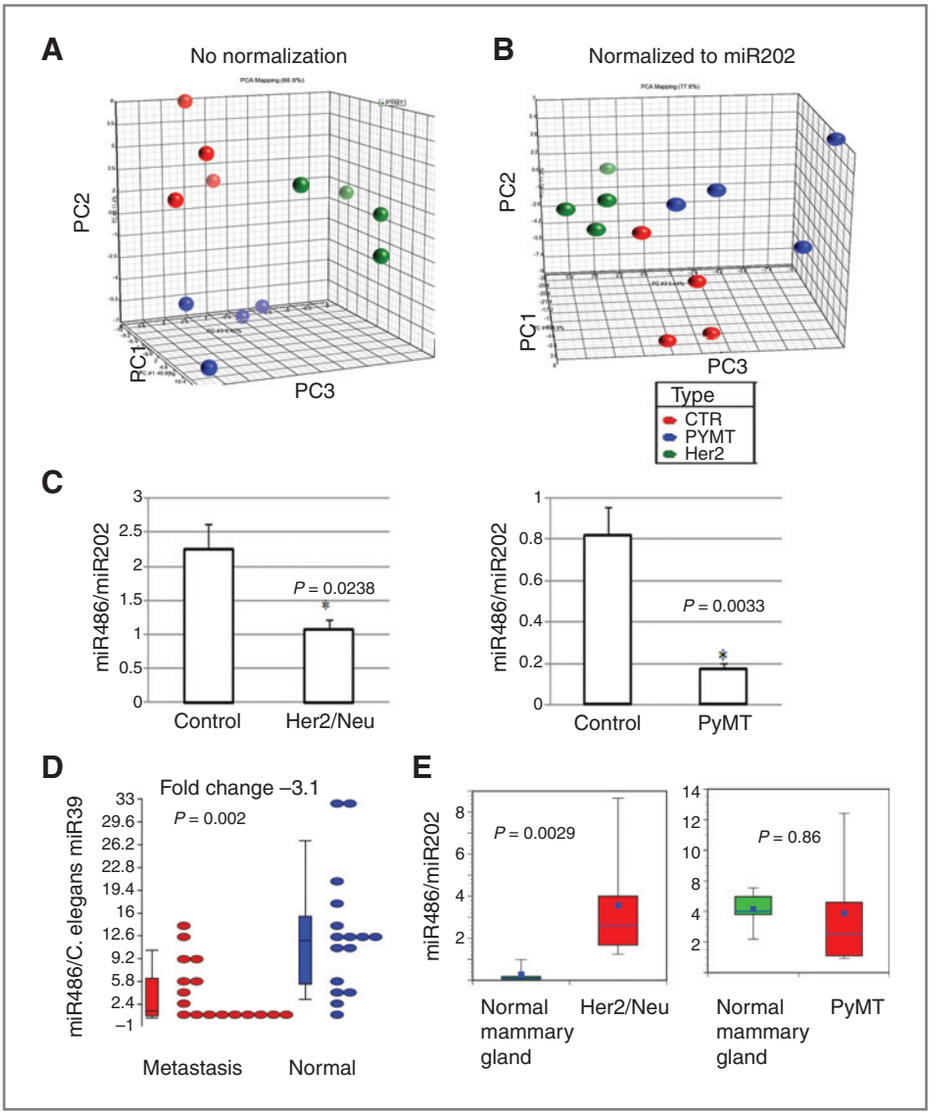

Figure 1. The sera of transgenic mice with mammary tumors display distinct miRNA profile. A, PCA of miRNAs in sera of control, MMTV-Her2/Neu, and MMTV-PyMT mice without normalization. B, PCA of miRNAs in sera of control, MMTV-Her2/Neu, and MMTV-PyMT mice after normalization with miR202. C, qRT-PCR analysis confirmed downregulation of miR486 in sera of transgenic animals with mammary tumors. D, analysis of circulating miR486 levels in healthy and metastatic patients using miRNA preparations after spiking sera with *C. elegans* miR39 to correct for technical variability. E, miR486 levels in the normal mammary gland (n, 7–8), MMTV-Her2/Neu ($n = 6$), and MMTV-PyMT–derived mammary tumors ($n = 10$).

significant in array experiments and <0.05 in validation experiments. miR486 in human sera was evaluated using ANOVA.

Results

Lower levels of circulating miR486 in transgenic mice with mammary tumors and in patients with breast cancer

To better understand the mechanism of lower circulating levels of specific miRNAs in patients with cancer compared with healthy controls, we profiled miRNAs in the sera of controls and transgenic mice with mammary tumors derived upon Her2/Neu (MMTV-Her2/Neu) or polyoma middle-T-antigen (MMTV-PyMT) oncogene overexpression (13, 14). Because of disagreements about normalization controls for such studies and each study claiming different miRNAs as better normalization controls (7, 17), data were analyzed without normalization first. In addition, analysis was conducted by selecting a miRNA, which showed highest stability value across samples, as a normalization control similar to the previously published Sister Study cohort report (11). Principle component analysis (PCA) showed a near perfect separation of three groups when data were analyzed without normalization compared with normalization using miR202 (Fig. 1A and B). Reason for the discrepancy in separation of samples to three groups with and without normalization is unclear. However, a recent study has shown PCA as a better method to identify circulating miRNAs (18). Samples utilized in this study were from inbred mice housed under similar condition, and collected and analyzed at the same time, which should limit inter-mice and technical variability. Therefore, extensive additional studies are required to find ideal normalization control. Alternatively, when another miRNA or small RNA is used as a normalization control, it may be ideal to present results as a ratio between test miRNA and specific normalization control RNA as we have presented below. Detailed miRNA profiles with CT values

Table 2. Significantly altered circulating miRNAs in transgenic mice with mammary tumors

	MMTV-Her2/Neu vs. control		MMTV-PyMT vs. control	
miRNA	***P* value**	**Fold change**	***P* value**	**Fold change**
MMTV-Her2/Neu specific				
miR204	0.009	8.9	0.507	2.66
miR375	0.017	2.82	0.712	−1.31
miR381	0.02	3.2	0.375	−3.63
MMTV-PyMT specific				
miR450b-5p	0.43	1.18	0.01	−3.63
miR142-3p	0.119	−2.02	0.01	−12.30
miR150	0.073	−1.89	0.014	−3.41
miR146b	0.397	2.59	0.018	6.16
Common to both				
miR486	0.0006	−5.69	0.001	−3.69

NOTE: Data were analyzed using miR202 as a normalization control. Although miR202 was present in all sera samples analyzed and showed least variability between samples, its levels were higher in the sera of MMTV-PyMT mice compared with control or MMTV-Her2/Neu mice.

and comparison between three groups with and without normalization are shown in Supplementary Table S1.

With a P value cutoff of <0.02 and no normalization, we observed 16 miRNAs being present differentially in the sera of MMTV-Her2/Neu mice compared with control mice, with two of them being downregulated (Table 1). In contrast, the sera of MMTV-PyMT mice contained elevated levels of 34 miRNAs (Table 1). Difference in number of circulating miRNAs between MMTV-PyMT and MMTV-Her2/Neu is statistically significant ($P = 0.01$, Fisher exact test, 2-tailed). Despite shorter latency, tumors and lung metastasis were more advanced in MMTV-PyMT transgenic mice compared with MMTV-Her2/Neu mice, which may be a reason for significantly higher number of circulating miRNA changes in MMTV-PyMT mice compared with MMTV-Her/Neu mice. Expectedly, unique changes were more common in the MMTV-PyMT mice (26 of 34) compared with the MMTV-Her2/Neu mice (8 of 16).

When miRNA profiles were normalized using miR202, only four miRNAs were present differentially in the MMTV-Her2/Neu sera compared with the sera from nontransgenic mice with one of them being lower, whereas five miRNAs were present differentially in the sera of MMTV-PyMT, with four of them being downregulated (Table 2). However, number of circulating miRNAs differentially present in the MMTV-PyMT may be an underestimation in this analysis because of overall increase in miR202 levels in MMTV-PyMT sera but not in MMTV-Her2/Neu sera compared with controls (Table 1). miR146b levels were elevated, whereas miR486 levels were lower in 1 or both transgenic mice models giving us the confidence that these two miRNAs are the major differentially expressed/secreted miRNAs in cancer.

We selected miR486 for further study because its expression is enriched in cardiac and skeletal muscle, which represents ∼40% of body mass (19). In addition, CT values ranged from 15 to 20, suggesting that its levels can be reliably measured. We verified the microarray results by qRT-PCR to confirm cancer-specific downregulation of circulating miR486 in both MMTV-Her/Neu and MMTV-PyMT mice using miR202 as a normalization control (Fig. 1C). As with microarray, in these validation experiments, miR486 levels were lower in sera of MMTV-Her2/Neu mice (3.78 ± 0.44 in control versus 1.55 ± 0.44 in MMTV-Her2/Neu, $P = 0.0004$) but not in sera of MMTV-PyMT mice when data were analyzed without normalization, further confirming reproducibility (data not shown).

To confirm the relevance of data obtained in animal models to human, we measured miR486-5P levels (equivalent of mmu-miR486) levels in the sera of patients with breast cancer with metastasis ($N = 17$) and healthy women ($N = 16$). Circulating miR486 levels were lower in patients with breast cancer metastasis compared with healthy (fold change $= -3.14$; $P = 0.002$; ANOVA, metastasis vs. normal; Fig. 1D).

To investigate whether there is any relationship between miR486 in tumor and in circulation, we measured miR486 levels in normal mammary gland and tumors from both transgenic mice. Although PyMT tumors and normal mammary gland expressed similar levels of miR486, Her2 tumors showed elevated miR486 expression compared with normal (Fig. 1E). Therefore, differential levels of circulating miR486 in three groups are less likely because of altered expression in tumors compared with normal mammary gland. We do acknowledge limitations of this analysis because normal mammary gland and tumors differ in their epithelial content. Similar analysis of public databases for miR486 in breast cancer and normal breast gave ambiguous results. Although The Cancer Genome Atlas (TCGA) dataset (20) showed lower miR486 in breast tumors compared with normal breast, three other datasets (GSE32922, GSE44124, and GSE53179; refs. 21 and 22) failed to demonstrate reduced miR486 in tumors (Supplementary Fig. S1A). Moreover, in the TCGA dataset, higher miR486 expression was associated with worst outcome, which is not compatible

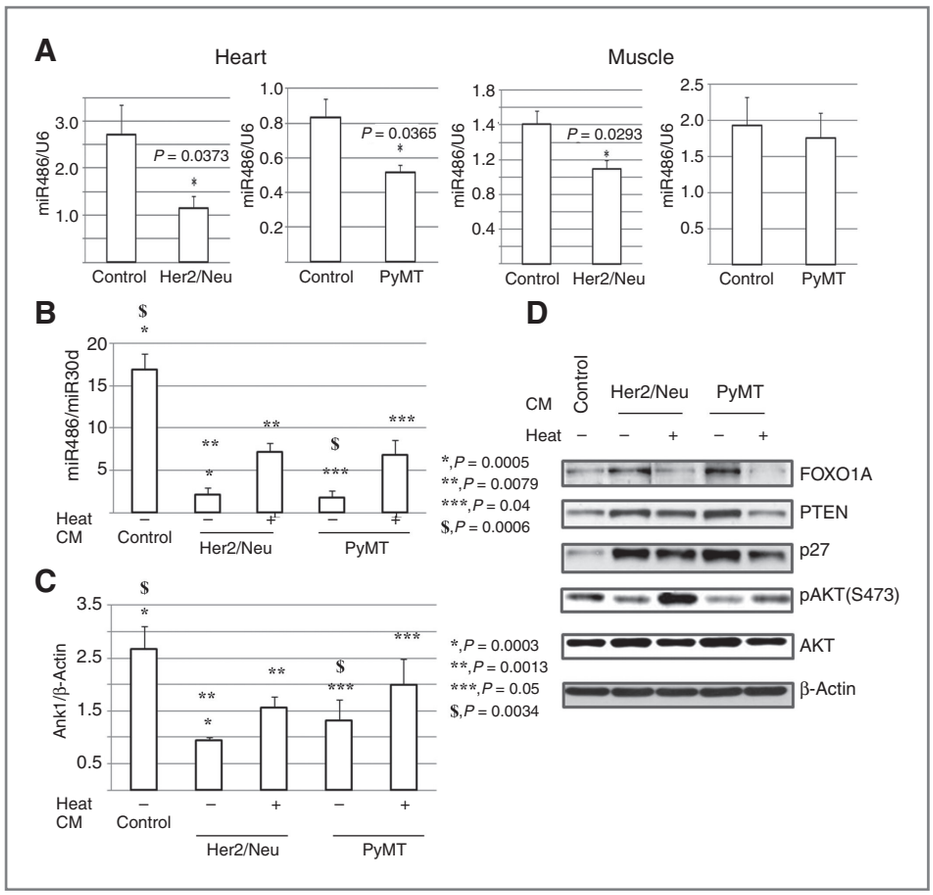

Figure 2. Cancer cell–derived soluble factors reduce miR486 expression in muscle and increase miR486 target proteins. A, heart of both MMTV-Her2/Neu and MMTV-PyMT and skeletal muscle of MMTV-Her2/Neu mice with mammary tumors expressed lower levels of miR486 compared with healthy controls ($N = 6$). B, CM from MMTV-Her2/Neu and MMTV-PyMT tumor-derived cell lines reduced miR486 expression in C2C12 cells. CM preheated at 80°C partially lost the ability to reduce miR486 expression. Asterisks and dollar sign indicate statistically significant differences. C, CM from MMTV-Her2/Neu and MMTV-PyMT tumor-derived cell lines reduced Ank1 mRNA. D, CM from MMTV-Her2/Neu and MMTV-PyMT tumor-derived cell lines upregulated miR486 target proteins in C2C12 cells.

with the observation of lower tumor-specific expression (Supplementary Fig. S1B). Although reduced expression of miR486 in breast tumors leading to lower circulating miR486 cannot be completely ruled out, based on the results in murine models and ambiguity of results obtained with breast tumors and normal breast tissue, we favor the alternative possibility of systemic effects of cancer causing lower circulating miR486 levels.

Cardiac and skeletal muscle of transgenic mice with mammary tumors express lower miR486 compared with controls

To determine whether lower circulating miR486 in transgenic mice with cancer can be attributed to its lower expression in muscle and heart, we measured miR486 in miRNA preparation from these organs. Animals used in these experiments are different from those used for sera miRNA analysis. Indeed, miR486 levels were lower in the heart and muscle of MMTV-Her2/Neu transgenic mice ($n = 6$) and in the heart of MMTV-PyMT mice compared with control mice ($n = 6$ for control, $n = 5$ for muscle, $n = 4$ for heart; Fig. 2A). U6 is an appropriate control for heart and muscle tissue based on a previous study and our finding that it was not significantly different between PyMT, Her2, and control mice (19).

Conditioned media from MMTV-Her2/Neu and MMTV-PyMT mammary tumor cell lines reduce miR486 in C2C12 cells

To delineate the mechanism involved in cancer-induced changes in miR486 expression in muscle, we utilized undifferentiated murine myoblast C2C12 cell line as a model system. The effects of CM from tumor cell lines derived from MMTV-Her2/Neu and MMTV-PyMT mice on miR486 expression in these cells were measured. The CM from both MMTV-Her2/Neu and MMTV-PyMT tumor-derived cell lines reduced miR486 levels in this myoblast cell line (Fig. 2B). The ability to inhibit miR486 expression was reduced partially when CM was pretreated for 20 minutes at 80°C, indicating that a protein factor(s) in the CM is repressing miR486 expression. miR30d expression was used as a normalization control. However, results were similar when data were analyzed without normalization in majority of experiments (data not shown).

miR486 is transcribed from the intron 40 of the *Ankyrin-1 (Ank1)* gene, which encodes for an ankyrin repeat protein and an erythroid-specific enhancer–promoter controls its expression in erythroid cells (19). However, muscle cells express a smaller Ank1 (sAnk1) transcript containing exon 39a and exons 40 to 42 utilizing an alternative promoter immediately upstream of exon 39a. sAnk1 and miR486 are expressed

coordinately in muscle cells and muscle-enriched transcription factors MyoD and myocardin-related transcription factor-A (MRTF-A) control their expression (19). Because CM from tumor-derived cell lines reduced miR486 in C2C12 cells, we examined the effects of CM on Ank1 expression. Similar to miR486, the expression of Ank1 was reduced in CM-treated cells and preheat-treated CM was less efficient in reducing Ank1 expression (Fig. 2C). Please note that RNAs for these experiments were prepared from different batch of cells using independent methods and β-actin was used as a control for normalization in Ank1 expression analysis. Thus, two independent assays demonstrate an effect of cancer cell–derived factors on the expression of miR486.

FOXO1A and PTEN are the well-established targets of miR486. Therefore, by downregulating PTEN, miR486 activates the PI3K/AKT survival pathway in cardiac and skeletal muscle (19). In addition, miR486 has been shown to suppress muscle wasting by targeting FOXO1 (23). Because tumor cell line–derived CM reduced miR486 levels, we determined the expression levels of its target proteins in C2C12 cells with and without CM treatment. CM pretreated at 80°C for 20 minutes was used a control. CM from both MMTV-Her2/Neu and MMTV-PyMT cell lines increased the levels of FOXO1A and PTEN with concomitant decline in pAKT levels (Fig. 2D). The levels of FOXO1A target protein p27 were elevated in cells treated with CM from MMTV-Her2/Neu and MMTV-PyMT tumor cell lines (24). None of these changes were observed when cells were incubated with heat-treated MMTV-PyMT cell line–derived CM. For unknown reason, heat-treated MMTV-Her2/Neu tumor-derived cell line CM was still able to increase PTEN and p27 but not FOXO1A. Nonetheless, these results provide evidence for a factor(s) derived from cancer cells in reducing miR486 levels and as a consequence, increasing miR486 targets in the myogenic cell line.

TNFα, secreted by cancer cells, alters miR486 expression in C2C12 cells

We used cytokine arrays to identify cytokines present in the CM from both cell lines. This array measures 40 different cytokines. Although each CM contained unique cytokines (TIMP1 in case of PyMT and G-CSF, CCL-1, CCL-5, CXCL1, CXCL2, CXCL10, and IL1ra in case of Her2/Neu), GM-CSF, TNFα, CCL-2, IFNγ, and IL1α were the common cytokines secreted by both tumor lines (Fig. 3A). The ability of some of these cytokines to alter miR486 and Ank1 expression was examined. Although all four cytokines tested reduced miR486 expression, only IL1α and TNFα reduced both miR486 and Ank1 expression (Fig. 3B and C). These results suggest that although TNFα and IL1α reduce miR486 by targeting Ank1 regulatory regions, CCL-2 and IFNγ regulate miR486 expression and/or maturation independent of Ank1 gene transcription.

We focused on TNFα because of its previously described role in reducing MyoD expression in muscle, an important transcription factor required for miR486 expression, and in muscle dysfunction (19, 25). Toward this end, we pretreated CM with either control IgG or neutralizing antibody against TNFα and then applied to C2C12 cells. TNFα neutralizing antibody significantly prevented CM-mediated suppression of miR486 and Ank1 expression (Fig. 3D and E). Using ELISA assay, we confirmed the presence of TNFα in CM from MMTV-Her2/Neu and MMTV-PyMT cell lines and in sera of transgenic mice with tumors (Fig. 3F and G). Similar analysis of sera from patients with metastatic breast cancer and healthy donors showed a trend of elevated circulating TNFα in patients with cancer compared with healthy (Supplementary Fig. S1C).

CM from tumor cell lines reduce miR486 expression in myotubes

The above studies were performed in undifferentiated C2C12 cells. To determine whether CM from tumor cell lines reduce miR486 in myoblasts undergoing differentiation to myotubes, we grew C2C12 cells in media containing 2% horse serum (26). CM from both cell lines reduced the levels of miR486 in the differentiated cells (Fig. 4). We also noted lower cell density when cells were treated with tumor cell–derived CM compared with control CM, possibly indicating an effect of CM on the miR486-mediated cell survival pathway (Fig. 4).

Deregulation of the PI3K–PTEN–AKT survival network in cardiac and skeletal muscle of transgenic mice

Although *in vitro* studies indicated a clear effect of factors secreted by cancer cells on miR486 expression and survival signaling network in myoblasts, we wanted to confirm similar scenario in intact animals. Toward this end, we measured the levels of miR486 target proteins in extracts from heart and muscle. Heart from transgenic animals contained lower levels of pAKT compared with control animals despite insignificant difference in PTEN levels between groups (Fig. 5A). PTEN is highly abundant protein in heart, which may be a reason for not detecting differences in its levels between groups. In contrast, muscle of transgenic mice contained elevated PTEN and lower pAKT (Fig. 5B). Because PI3K/pAKT has a cardioprotective function and prevents muscle atrophy by inhibiting FOXO transcription factors (27), whereas elevated levels of phosphorylated/activated p38 kinase is associated with apoptosis and is often increased during ischemic heart disease (28), we next measured the levels of phospho-p38 (T180/Y182). Indeed, extracts of heart from transgenic mice contained elevated phospho-p38 compared with extracts from control mice (Fig. 5A). These results suggest that tumor-induced factors such as TNFα reduce the expression of miR486 in heart, leading to impairment in the PI3K/AKT-dependent survival pathway and the elevated p38 kinase pathway.

Because sera of transgenic animals contained elevated levels of TNFα, which can affect miR486 expression by targeting MyoD (19), we next examined MyoD protein levels in heart and muscle of control and transgenic animals. MyoD levels were lower in skeletal muscle of tumor-bearing animals (Fig. 5B). Similar trend was observed in heart, although differences did not reach statistical significance. In summation, our results suggest the effect of cancer on miRNA expression in distant organs with an impact on the survival-signaling network in these organs.

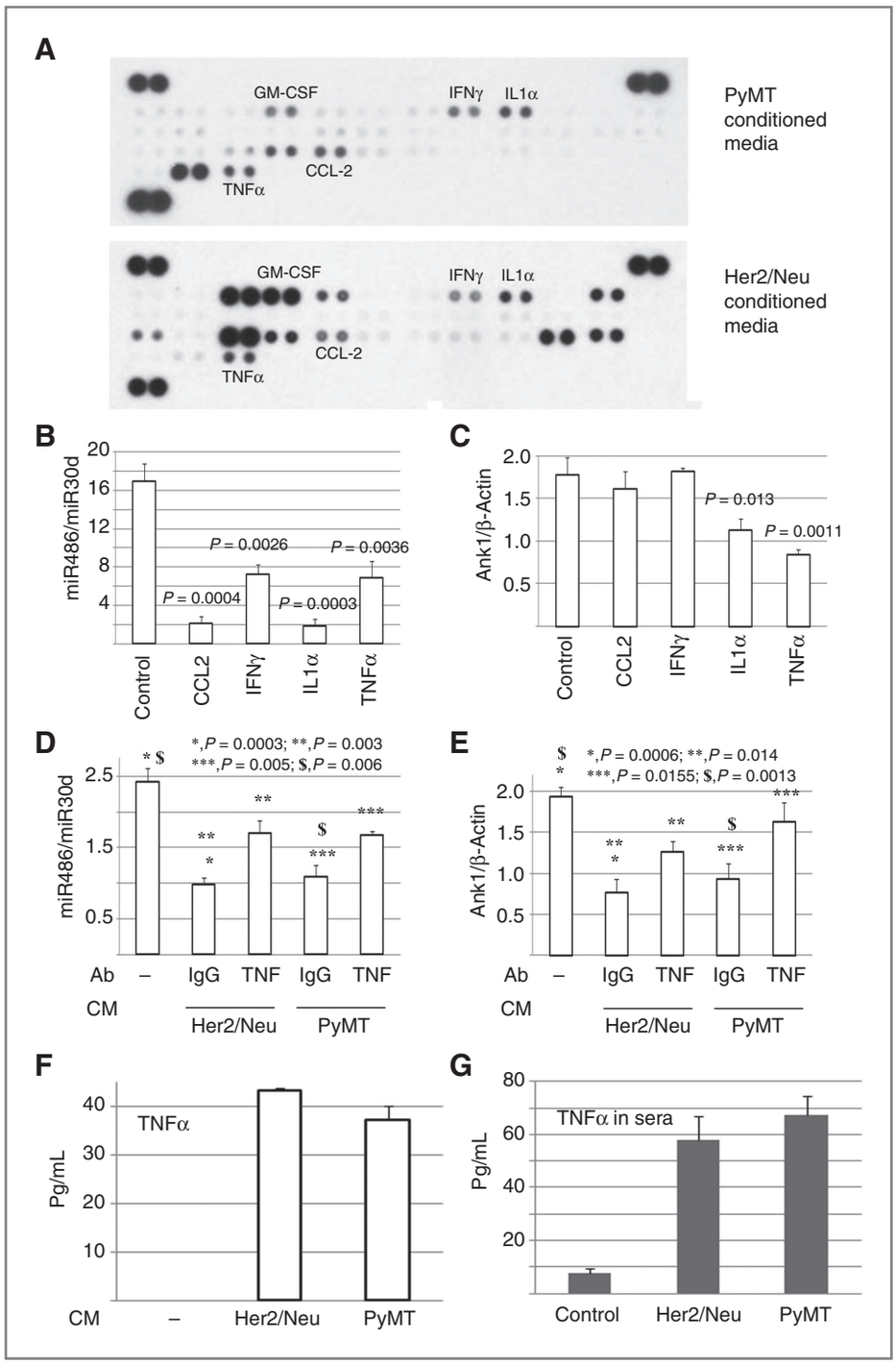

Figure 3. TNFα mediates miR486 repression. A, cytokine array identified GM-CSF, CCL2, IFNγ, IL1α, and TNFα as common cytokines secreted by both MMTV-Her2/Neu and MMTV-PyMT tumor-derived cell lines. B, the effects of CCL2, IFNγ, IL1α, and TNFα on miR486 expression in C2C12 cells. C, the effects of CCL2, IFNγ, IL1α, and TNFα on Ank1 expression in C2C12 cells. D, neutralizing antibody against TNFα partially reversed the effects of CM from MMTV-Her2/Neu and MMTV-PyMT tumor-derived cell lines on miR486 expression in C2C12 cells. E, neutralizing antibody against TNFα reduced the effects of CM from MMTV-Her2/Neu and MMTV-PyMT tumor-derived cell lines on Ank1 expression in C2C12 cells. F, CM from MMTV-Her2/Neu and MMTV-PyMT tumor-derived cell lines contained TNFα. G, sera of MMTV-Her2/Neu and MMTV-PyMT mice contained higher levels of TNFα compared with control mice.

Discussion

A number of recent reports have described circulating miRNAs as potential biomarkers of cancer (7, 11, 17). However, this field still suffers from lack of reproducibility, as there is a minimum overlap in cancer-specific circulating miRNAs identified in different studies. There are two possible explanations for this lack of reproducibility; first is the difficulty in finding a suitable normalization control and second is the recent realization of large scale transcriptome variation among healthy humans, which makes it difficult to assign "normal" value to circulating miRNAs (29). Nonetheless, at least few specific miRNAs have been detected in disease conditions in more than one study. For example, elevated circulating miR181a and miR222 have been observed in breast cancer in more than one

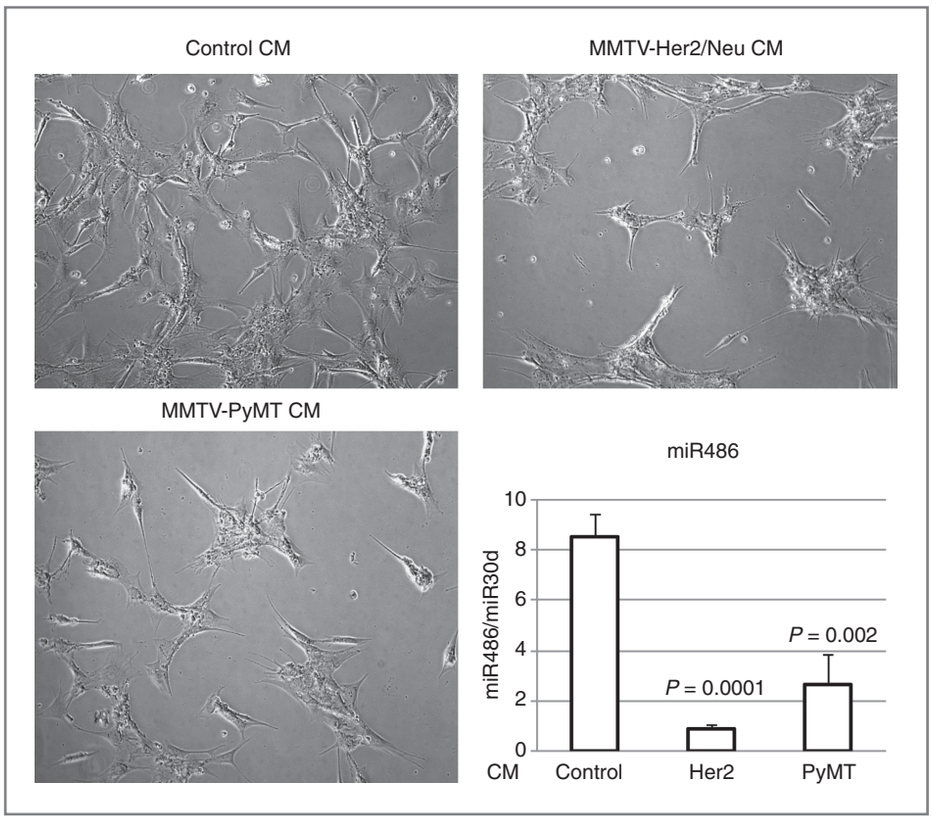

Figure 4. CM from tumor cell lines decrease miR486 expression in differentiated C2C12 cells. C2C12 cells were allowed to undergo differentiation by replacing FBS with horse serum. Phase-contrast images of myotubes under control or CM-treated conditions are shown. The levels of miR486 with or without CM treatment are also shown.

study (7, 11, 17). miR151-3p, miR134, and miR671-3p identified in our animal models have been shown to be elevated in the sera of patients with cancer (7, 11). Therefore, studies that combine analysis in transgenic animal models in a similar genetic background and in human samples, as done in this study, may identify a set of circulating miRNAs that can be used as biomarkers in a clinical setting.

Several miRNAs that are present at higher levels in the sera of transgenic mice have previously been shown to have oncogenic role. For example, miR27b, which is elevated in the sera of both transgenic mice (Table 1), is a context-specific oncogene and both Her2 and TNFα increase its expression in breast cancer cells (30). In fact, miR27b was not measurable in the sera of control animals but readily detectable in the sera of transgenic mice (Supplementary Table S1). Furthermore, combination of two circulating miRNAs, miR27b, and miR15b, has been shown to discriminate patients with non–small cell lung cancer from healthy controls (31). miR210 is a hypoxia-inducible oncogene, which predicts poor outcome in patients with breast cancer (32). Consistent with rapid tumor progression, which often leads to hypoxia, MMTV-PyMT tumor-bearing mice had much higher circulating miR210 than MMTV-Her2/Neu tumor-bearing mice (Table 1). Circulating levels of miR210 and miR200b correlate with the presence of circulating tumor cells in patients with breast cancer (10). miR146a and miR146b elevated in the sera of MMTV-PyMT mice have previously been shown to target BRCA1 and are expressed at a higher level in basal-like and triple-negative breast cancers (33). In addition, circulating miR146 may suggest overall inflammatory status as both miR146a and miR146b are NF-κB–inducible miRNAs (34, 35). miR183, which is elevated in the sera of both transgenic mice, is overexpressed in ductal carcinoma *in situ* compared with normal breast (36). Thus, deregulation of certain miRNAs in breast/mammary tumors is common across species and independent of specific oncogenic events.

There have been limited attempts to understand why circulating levels of certain miRNAs are lower in cancer despite consistent observation of such a phenomena. We selected miR486 as a model miRNA to study this aspect because it is a unique miRNA with no family members and is expressed predominantly in heart and muscle, which represent 40% of body mass (19). Unlike most other miRNAs with family members sharing targets and thus compensating for the loss of expression of a family member, loss of miR486 expression is likely to have consequences. Consistent with this possibility, disease phenotypes are associated with reduced miR486 expression as evident in the muscle of patients with Duchene muscular dystrophy (37). Physical exercise can have a negative influence on its release into circulation, suggesting a link between muscle biology and circulating levels of miR486 (38). Our results have demonstrated an effect of mammary tumors on its expression in skeletal and cardiac muscle. Similar scenario may exist in other cancers as lower circulating miR486-5p in relation to miR21 or miR126 is observed in patients with lung cancer with poor outcome (39).

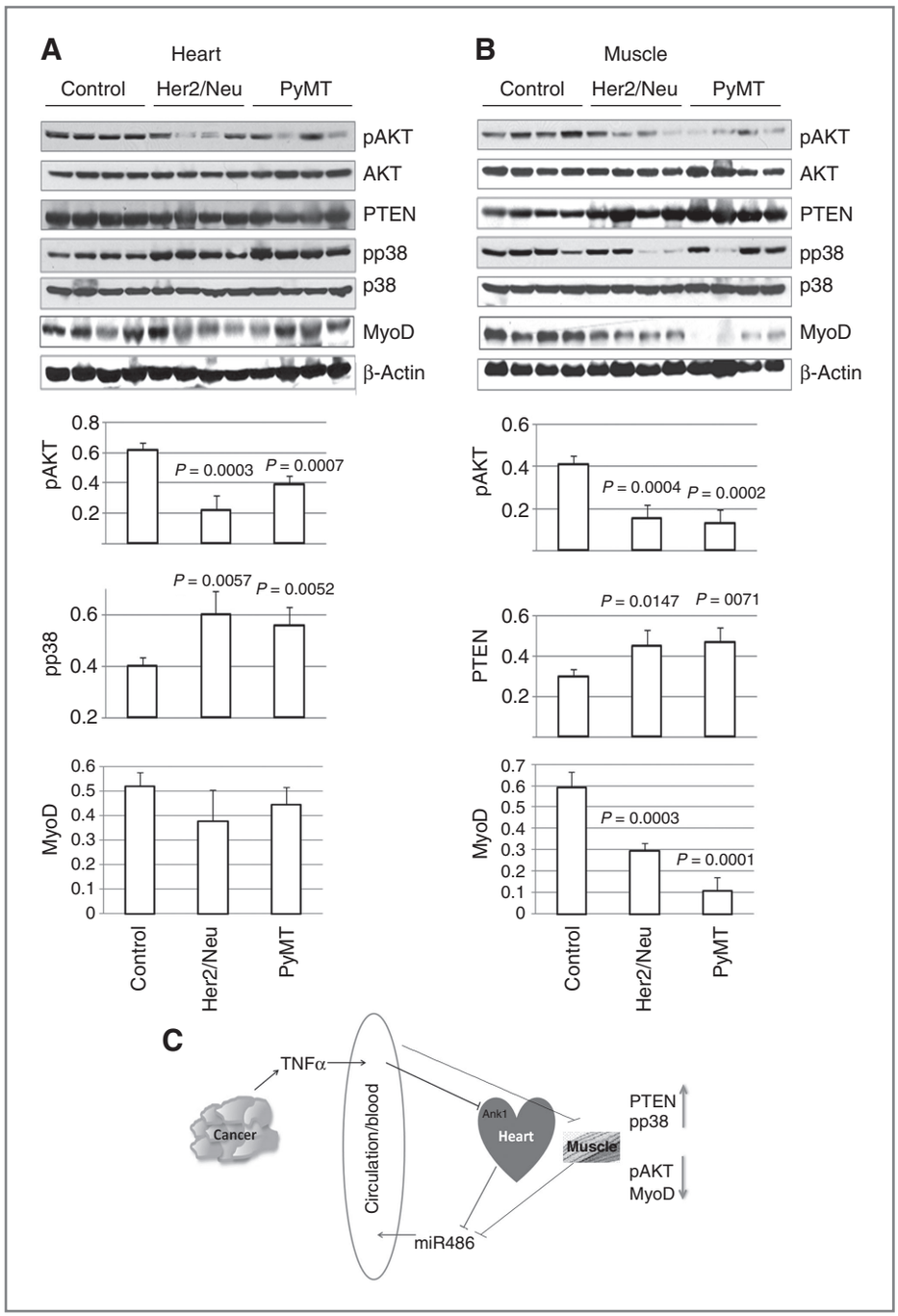

Figure 5. Heart and muscle of transgenic mice display a defective PI3K/AKT pathway compared with control mice. A, pAKT, PTEN, MyoD, and pp38 levels in the heart of mice. Quantitative differences are shown at the bottom. Extracts from four mice in each group were used. B, pAKT, PTEN, MyoD, and pp38 levels in the muscle of mice. C, model depicting the effects of cancer on signaling events in heart and muscle, leading to lower miR486 in circulation.

To date, most research on cardiac health of patients with cancer is focused on cardiac toxicity of cancer therapy (40). Our study raises the possibility that cardiac dysfunction occurs during cancer progression. As presented schematically in Fig. 5C, such a cancer-induced collateral damage to heart/muscle involves a miRNA network. Similarly, cancer-induced skeletal muscle dysfunction and cachexia is observed in at least 50% of patients although cachexia is rare in patients with breast cancer (41). Recently, cachexia has been defined as a syndrome that progresses through various stages; precachexia to cachexia to refractory cachexia (42). Because tumor-bearing transgenic mice did not show severe cachexia at the time of sacrifice but displayed some of the molecular defects associated with skeletal muscle dysfunction, including lower MyoD expression, drop in circulating miR486 levels may provide an indication to precachectic stage.

Neutralizing antibody against TNFα is already in clinical use for other diseases (43). At least in animal models, TNFα

neutralizing antibody reduced mammary tumor growth (44). It may be worth considering these treatments not only to inhibit tumor growth but also to reduce side effects of cancer. The efficacy of a treatment in patients with metastasis is often measured by the ability of the drug to shrink metastasis. However, few of these drugs, including anti-TNFα antibody, may not be effective in reducing metastasis but effective in reducing cancer-induced collateral damage. Such treatments may help to extend and/or improve quality of life. Because RNA-based therapies are increasingly being developed as treatment with improved delivery system (45, 46), replenishing miRNAs that are lower in circulation of patients with cancer may be an option to restore cardiac and muscle function.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: D. Chen, H. Nakshatri
Development of methodology: D. Chen, H. Nakshatri
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Chen, C.P. Goswami, R.M. Burnett, W. Muller
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Chen, H. Nakshatri
Writing, review, and/or revision of the manuscript: D. Chen, C.P. Goswami, P. Bhat-Nakshatri, H. Nakshatri
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Anjanappa, P. Bhat-Nakshatri, H. Nakshatri
Study supervision: H. Nakshatri

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