

Exercise-Induced Catecholamines Activate the Hippo Tumor Suppressor Pathway to Reduce Risks of Breast Cancer Development



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Abstract

Strong epidemiologic evidence documents the protective effect of physical activity on breast cancer risk, recurrence, and mortality, but the underlying mechanisms remain to be identified. Using human exercise-conditioned serum for breast cancer cell incubation studies and murine exercise interventions, we aimed to identify exercise factors and signaling pathways involved in the exercise-dependent suppression of breast cancer. Exercise-conditioned serum from both women with breast cancer ($n = 20$) and healthy women ($n = 7$) decreased MCF-7 (hormone-sensitive) and MDA-MB-231 (hormone-insensitive) breast cancer cell viability *in vitro* by 11% to 19% and reduced tumorigenesis by 50% when preincubated MCF-7 breast cancer cells were inoculated into NMRI-Foxn1^{nu} mice. This exercise-mediated suppression of cell viability and tumor formation was completely blunted by blockade of β -adrenergic signaling in MCF-7 cells,

indicating that catecholamines were the responsible exercise factors. Both epinephrine (EPI) and norepinephrine (NE) could directly inhibit breast cancer cell viability, as well as tumor growth *in vivo*. EPI and NE activate the tumor suppressor Hippo signaling pathway, and the suppressive effect of exercise-conditioned serum was found to be mediated through phosphorylation and cytoplasmic retention of YAP and reduced expression of downstream target genes, for example, ANKRD1 and CTGF. In parallel, tumor-bearing mice with access to running wheels showed reduced growth of MCF-7 (–36%, $P < 0.05$) and MDA-MB-231 (–66%, $P < 0.01$) tumors and, for the MCF-7 tumor, increased regulation of the Hippo signaling pathway. Taken together, our findings offer a mechanistic explanation for exercise-dependent suppression of breast cancer cell growth. *Cancer Res*; 77(18); 4894–904. ©2017 AACR.

Introduction

Persuasive epidemiologic evidence has emerged over the recent years outlining an inverse association between self-reported physical activity level and the risk of breast cancer (1–4). Moreover, physically active women diagnosed with breast cancer have lower risk of disease recurrence, as well as overall and cancer-specific mortality relative to their sedentary counterparts (5–9). This protection has been demonstrated for physical activity performed both before and after the patients' cancer diagnosis. As a consequence, this body of evidence has created the basis for cancer-specific exercise guidelines, recommending women to participate in exercise of moderate intensity for 150 minutes per week following a breast cancer diagnosis (10).

While observational data support a protective effect of physical exercise on breast cancer risk, recurrence, and mortality, limited mechanistic work has been published elucidating the potential biological pathways, driving this protection. Long-term reductions in various circulatory risk factors, for example, sex hormones, metabolic hormones, and low-grade inflammation, all of which are associated with weight loss, have gained considerable attention while linking the protective effect of exercise on cancer (11). However, our group recently highlighted that, in contrast to the long-term adaptations in breast cancer risk factors, acute changes in circulating exercise factors during a single exercise bout could reduce breast cancer cell viability by approximately 10% (12). This discovery has prompted us to conduct a comprehensive investigation into the antioncogenic potential of the acute systemic exercise response to identify exercise-induced cancer-controlling pathways and their exercise-dependent stimulants.

Against this background, we have performed the current study to explore the molecular mechanisms underlying the control of breast cancer viability and tumorigenesis through circulating factors induced during exercise. We have utilized *in vitro* models of the two breast cancer cell lines; MCF-7 [estrogen receptors (ER) and progesterone receptors (PR) positive and categorized within the largest subgroup of luminal breast cancer tumors], and MDA-MB-231 (triple negative for sex hormone receptors and representing a basal-like subgroup of breast cancer tumors; ref. 13). These two cell lines were incubated with human serum taken at rest or immediately after an acute exercise bout or with catecholamines; or used as inoculated tumors in wheel running interventions in

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-16-3125

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mice. Our primary objectives were to: (i) identify responsible intratumoral pathways involved in acute exercise-dependent inhibition of breast cancer cell growth; (ii) identify plausible exercise-dependent circulatory candidates stimulating these pathways; and (iii) investigate the potential differences between breast cancer cell subtypes, representing differences in sex hormone receptor status.

Patients and Methods

Human studies

Subjects. Human studies were performed at Copenhagen University in the period 2011–2014, according to the Declaration of Helsinki and after approval by the local ethical committee (H-3-2010-141). Written informed consent was obtained from all participants included in the studies. For the purpose of this study, we collected exercise-conditioned serum from healthy women and breast cancer patients receiving adjuvant chemotherapy, recruited in two different settings:

Two-hour acute exercise session in healthy women. Healthy women ($n = 7$) were recruited to participate in a one day acute exercise intervention combined with a nonexercise resting day trial. Inclusion criteria were: age between 18 and 30 years, no medication or oral contraceptives, nonsmokers, and normal weight. All participants completed the two test days and baseline characteristics were: age: 24.6 (1.0) years, BMI: 23.3 (2.7) kg/m², and VO_{2peak}: 44.5 (7.0) mL/kg/min [mean (SD)].

Two-hour acute exercise study in breast cancer patients receiving adjuvant chemotherapy. The participants of this study have previously been described (12). Briefly, women operated for stage I/II breast cancer and receiving adjuvant chemotherapy ($n = 20$) were recruited from the 6-week standard exercise rehabilitation program at the Copenhagen University Hospital (the Body and Cancer Program; ref. 14). Baseline characteristics of the subjects were: age: 49.5 (7.3) years, BMI: 26.0 (5.2) kg/m², and VO_{2peak}: 25.2 (8.3) mL/kg/min [mean (SD)]. The women were provided with oral and written information about the project, and had several days to decide on their participation. Once the patients had signed written consent, the experimental day was planned, aiming to take place halfway through the 6 weeks of training within the Body and Cancer program. In addition, it was ensured that the test day took place more than 7 days since last chemotherapy administration and with ≥ 2 days since last supervised exercise session.

Exercise interventions

Two-hour acute exercise session in healthy women. Participants came to the laboratory 3 times. On day 1, a medical examination and physiologic measures, including a VO_{2peak} test were performed. VO_{2peak} was measured directly by indirect calorimetry with a Cosmed Quark CPET system, after an incremental exercise test on a stationary cycle ergometer (Monark Ergonomic 839E). Following 5 minutes of warm-up at steady state, the incremental test was initiated at a baseline workload of Watts followed by 20 Watts increments every minute until volitional exhaustion, where Watt_{max}, maximum heart rate (HR_{max}) and VO_{2peak} were recorded. On day 2, the women rested throughout the stay and had blood samples drawn after 1 and 2 hours, which were immediately processed to serum and stored on -80°C until

further use. On day 3, they performed an acute bout of ergometer cycling at 55% of VO_{2peak} for 2 hours. Blood samples were drawn on the same time points as on day 2. Experimental day 2 and 3 were scheduled between day 7 and 12 of the women's menstrual cycle. Participants were fasting overnight for all experimental days and had refrained from physical exercise for 24 hours. Training intensity was documented by heart rate monitoring, showing heart rate levels above 70% of HR_{max} throughout the exercise bout (Supplementary Fig. S1A).

Two-hour acute exercise study in breast cancer patients. On the test day, the patients participated as normal in the Body and Cancer program, which consisted of 30-minute warm up, 1 hour resistance whole body training, and 30 minutes of high intensity spinning (pulse $> 80\%$ of HR_{max}) on stationary ergometer bicycles. The participants were asked to fast 2 hours prior to the exercise intervention. Blood samples were collected just before the program and immediately after the spinning program, and were processed to serum and stored on -80°C .

Biochemical profile. Serum concentrations of IL6 were measured by ELISA (Meso Scale Discovery), lactate by ABL 800 flex blood gas analyzer (Radiometer), and EPI/NE by 2-CAT RIA kit (LDN), all according to the manufacturer's protocol.

Cell culture studies

Cancer cell lines. The human breast adenocarcinoma cell lines MCF-7 (ER⁺, PR⁺, HER2⁻) and MDA-MB-231 (ER⁻, PR⁻, HER2⁺) were cultured in DMEM, 1 g/L glucose (Invitrogen), 10% FBS (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). The *in vitro* studies were performed from 2014 to 2017. The two cell lines (MCF-7 and MDA-MB-231) were obtained from ATCC, which authenticates via short tandem repeat profiling. Upon receipt, cells were expanded and frozen in multiple vials and subsequently used for experiments within the same six passages (P14–P19). Both cell lines were periodically tested for mycoplasma by the MycoProbe kit (R&D Systems), and found negative. The cell lines were authenticated by routinely visual inspection of the morphologic phenotype. For all cell stimulation experiments, MCF-7 and MDA-MB-231 cells were plated in 96-well plates at concentrations 1×10^3 and 2×10^3 cells/well, respectively, in their normal growth media. After attachment to bottom of plate (6 hours), cells were serum starved overnight (18 hours).

Viability assays. To study the effect on cancer cells of human exercise-conditioned serum, viability of MCF-7 and MDA-MB-231 were measured by quantification of live proteases by CellTiter-Fluor cell viability assay (Promega). Quality control of the assay was performed before the actual experiments by plotting fluorescence signal against serial dilutions of cell counts (data not shown). Starvation media were replaced by growth media containing 10% human serum obtained at rest or after exercise in healthy women or breast cancer patients instead of FBS. All stimulations were performed in triplets. For the propranolol incubation study, 10% human serum obtained from breast cancer patients pre/post exercise \pm propranolol (Sigma Aldrich; 10 $\mu\text{mol/L}$) was added to the medium. Stimulated cells were incubated for 48 hours. Cells were added to 100 μL viability reagents, mixed by orbital shaking, and incubated for 1 hour at 37°C . For viability evaluation, fluorescence was measured at 400_{Ex}/505_{Em}.

For evaluation of the growth-inhibitory effect of EPI and NE, viability of MCF-7 and MDA-MB-231 cells were measured by both MTT assay (Promega) and by confluence (% fraction of area), quantified as a mean of three images/well in 3 wells by the image processing program ImageJ. Starvation media were replaced by growth media containing 0, 0.1, 1, or 10 $\mu\text{mol/L}$ EPI or NE, and media were changed daily throughout the experiments. MTT assays were performed according to manufacturer's protocol after 24, 48, and 72 hours of stimulation. Measure of confluence was performed after 48 hours of EPI/NE (10 $\mu\text{mol/L}$) stimulation.

Cellular localization by fluorescence microscopy. For visualization of the cellular localization of YAP, MCF-7 cells were stimulated with medium containing 10% rest or exercise-conditioned serum from breast cancer patients for 1 hour. Medium was removed and the cells were fixed with 4% formaldehyde fixative solution (Sigma Aldrich) and incubated for 15 minutes. The cells were washed, added 0.5% Triton X-100 solution (Sigma Aldrich) and incubated for another 15 minutes before blocking in 3% BSA for 60 minutes. After washing, the cells were stained with YAP antibody (63.7; Santa Cruz Biotechnology, # sc-101199) for 1 hour and AlexaFluor secondary antibody (Molecular Probes), before costaining with DAPI and Actin (Molecular Probes). Fluorescence was visualized by EVOS FL microscope.

Animal models

All mice studies were approved by the Danish Research Animal Inspectorate and conducted in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimentation. Female NMRI-Foxn1^{nu} mice (own breed) were housed in standard housing cages and were between 12 and 22 weeks old when the studies were initiated. The mice were maintained in a thermo-stated environment under a 12-hour light/dark cycle with free access to food and drinking water.

Tumor challenge. For cancer cell inoculation, MCF-7 and MDA-MB-231 cancer cells were grown until 70% confluent in growth media. In the preincubation studies, the standard 10% FBS was replaced by 10% human rest- or exercise-conditioned serum \pm propranolol (10 $\mu\text{mol/L}$) from healthy women or breast cancer patients for the last 48 hours prior to tumor inoculation. Five million MCF-7 or MDA-MB-231 cells were inoculated subcutaneously on the flank of the mice, and tumor growth was followed by caliper measurements and allowed to reach a maximum volume of 1,000 mm^3 . Mice with MCF-7 tumors had their drinking water supplemented with 10 $\mu\text{g/mL}$ water-soluble β -Estradiol (Sigma).

Voluntary wheel running and catecholamine treatment. At the day of tumor inoculations, mice were randomized to cages with/without a running wheel of 12 cm in diameter [MCF-7: $n = 14/10$; MDA-MB-231: $n = 13/12$ (control/exercise)], or to daily injections of EPI (0.05 mg/mL , 200 μL i.p.; MCF-7: $n = 10$; MDA-MB-231: $n = 10$) or NE (0.25 mg/mL , 200 μL i.p.; MCF-7: $n = 10$; MDA-MB-231: $n = 11$). Mice were housed in pairs to prevent isolation-induced stress; however, the running wheel allowed for both mice to run simultaneously. Tumor volume was followed using caliper readings and tumor volume was calculated by the formula: tumor volume = $\pi \times (\text{longest diameter} \times \text{diameter perpendicular to aforementioned} \times \text{depth})/6$. At termination, blood sugar was measured by Abbott Precision Xtra Glucose monitoring system and blood was collected, separated into serum

by centrifugation, and stored at -80°C . Tumors were excised and immediately frozen in liquid nitrogen. Animals showing any sign of illness during the intervention periods were euthanized and excluded from the studies.

Molecular methods

Detailed methodologic description of PCR and Western blotting analyses are presented in Supplementary Information.

Statistical analyses

Two-way ANOVA with subsequent Tukey posttest was used for multiple comparison of exercise with additional interventions. When the effects of interventions/stimulations were measured at different time points or at different concentrations, a two-way ANOVA with repeated measures was used, and for comparison of 2 groups, a two-tailed t test was used. Comparison of survival curves in the murine preincubation study was performed by a Log-rank (Mantel-Cox) test. Results are expressed as means \pm SEM unless otherwise described. A P value of less than 0.05 was considered to be statistically significant. Data analyses were performed using GraphPad Prism 7.02. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results

Effect of exercise-conditioned human serum on breast cancer cell viability and tumorigenic potential

First, we stimulated the breast cancer cell lines, MCF-7 and MDA-MB-231, with serum obtained from 20 breast cancer patients, participating in a 2-hour exercise session as part of a clinical rehabilitation program. After 48 hours of incubation with exercise-conditioned serum, breast cancer cell viability was reduced with 11% in the MCF-7 cells ($n = 19$, $P < 0.05$, Fig. 1A) and 9% in MDA-MB-231 cells ($n = 20$, $P < 0.05$, Fig. 1B) compared with breast cancer cells incubated with serum obtained prior to the exercise session. These data are in accordance with our recent findings (12). In parallel, we performed a controlled laboratory-based 2-hour moderate-intensity cycling intervention at 55% of $\text{VO}_{2\text{peak}}$ in 7 healthy young women (Supplementary Fig. S1A). As for the breast cancer patients, the viability of both breast cancer cell lines were significantly reduced by the exercise-conditioned serum (MCF-7; 1 hour: -10% , $P < 0.01$, 2 hours: -19% , $P < 0.001$, Fig. 1C, MDA-MB-231; 1 hour: -14% , $P < 0.05$, 2 hours: -13% , $P < 0.05$, Fig. 1D).

To explore whether exercise-conditioned serum regulated the tumorigenic potential of the cancer cells, we inoculated female NMRI-Foxn1^{nu} mice with 5×10^6 MCF-7 or MDA-MB-231 breast cancer cells, which had been preincubated for 48 hours with resting or exercise-conditioned serum from the healthy women. Tumor incidence and growth were then followed without any further intervention. MCF-7 tumor incidence was markedly reduced for the cells preincubated with exercise-conditioned serum, as only 45% of mice inoculated with cells preincubated with exercise-conditioned serum formed tumors compared with 90% of the mice inoculated the MCF-7 cells preincubated with serum obtained at rest ($P < 0.05$, Fig. 1E). In continuation, end tumor volume was significantly reduced in the group of mice with tumors preincubated with exercise-conditioned serum (-76% , $P < 0.05$, Fig. 1F). Tumor incidence in mice inoculated with MDA-MB-231 cells preincubated with exercise-conditioned serum, tended to be lower than in mice inoculated with MDA-MB-231 cells preincubated with rest serum ($P = 0.09$, Fig. 1G). Yet, no

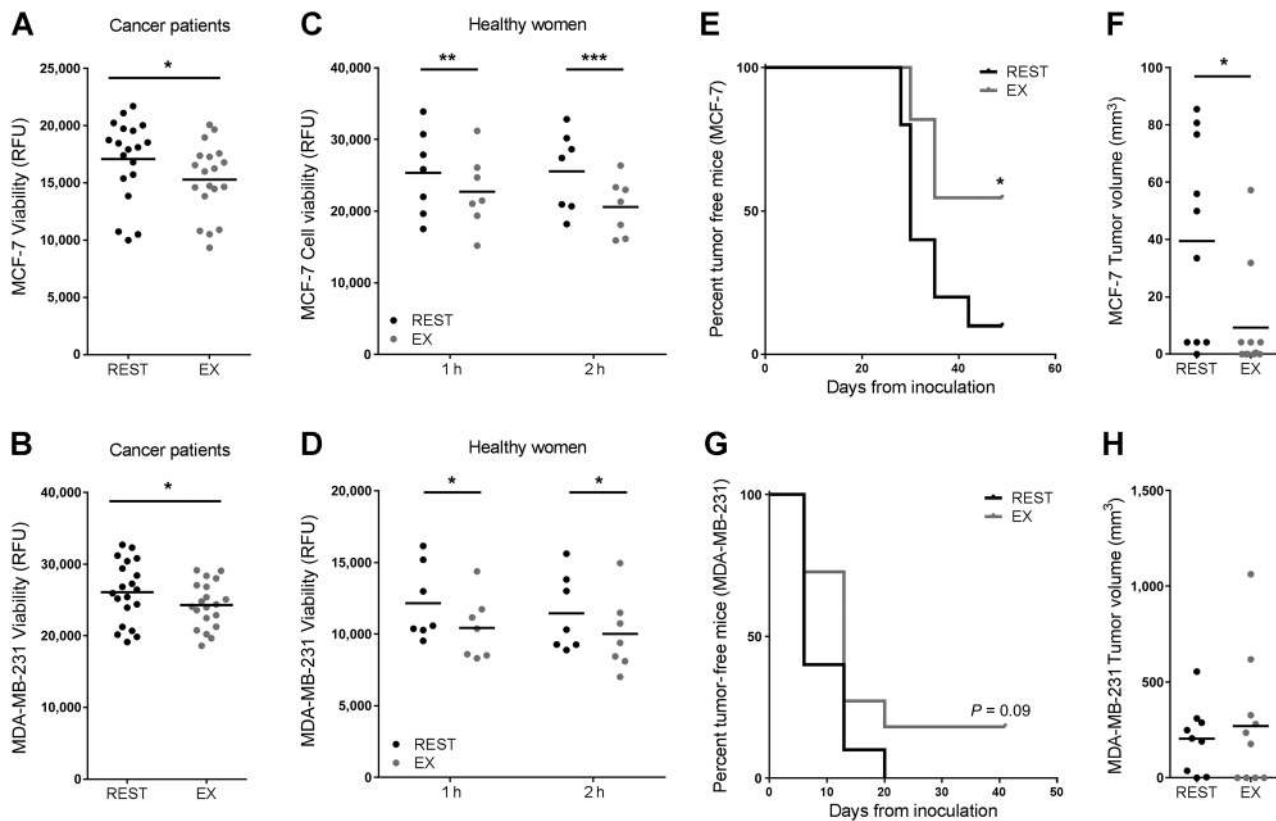


Figure 1.

Breast cancer cell viability and tumorigenesis after stimulation with human exercise-conditioned serum. Viability of MCF-7 (**A**) and MDA-MB-231 (**B**) cells after 48 hours of incubation with 10% human serum collected at rest or after 2 hours of combined resistance and endurance exercise in breast cancer patients ($n = 19$). Viability of MCF-7 (**C**) and MDA-MB-231 (**D**) cells after 48 hours of incubation with 10% human serum collected at rest or after 1 or 2 hours of cycling in healthy women ($n = 7$). Breast cancer cells were preincubated for 48 hours with 10% human serum collected from healthy women at rest or after exercise (EX) before inoculation into sedentary mice [$n = 10$ (REST), $n = 11$ (EX)]. Percent tumor-free mice in days after inoculation (**E**) and end tumor volume for MCF-7 cells (**F**). Percent tumor-free mice in days after inoculation (**G**) and end tumor volume for MDA-MB-231 cells (**H**). Statistical significance of the serum incubation studies was tested by a paired t test (breast cancer patients) and two-way ANOVA with repeated measures (healthy women). Tumor incidence was tested as a survival curve comparison by a Log-rank (Mantel-Cox) test and tumor volume as an unpaired t test. *, $P < 0.05$; **, $P < 0.01$.

difference in end tumor volume was observed between the two groups (Fig. 1H). These results demonstrate that exercise-stimulated changes suppress breast cancer cell viability, but even more strikingly reduce the tumorigenic potential of particular MCF-7 cells.

Exercise-induced epinephrine and norepinephrine mediate the cancer suppressive effect

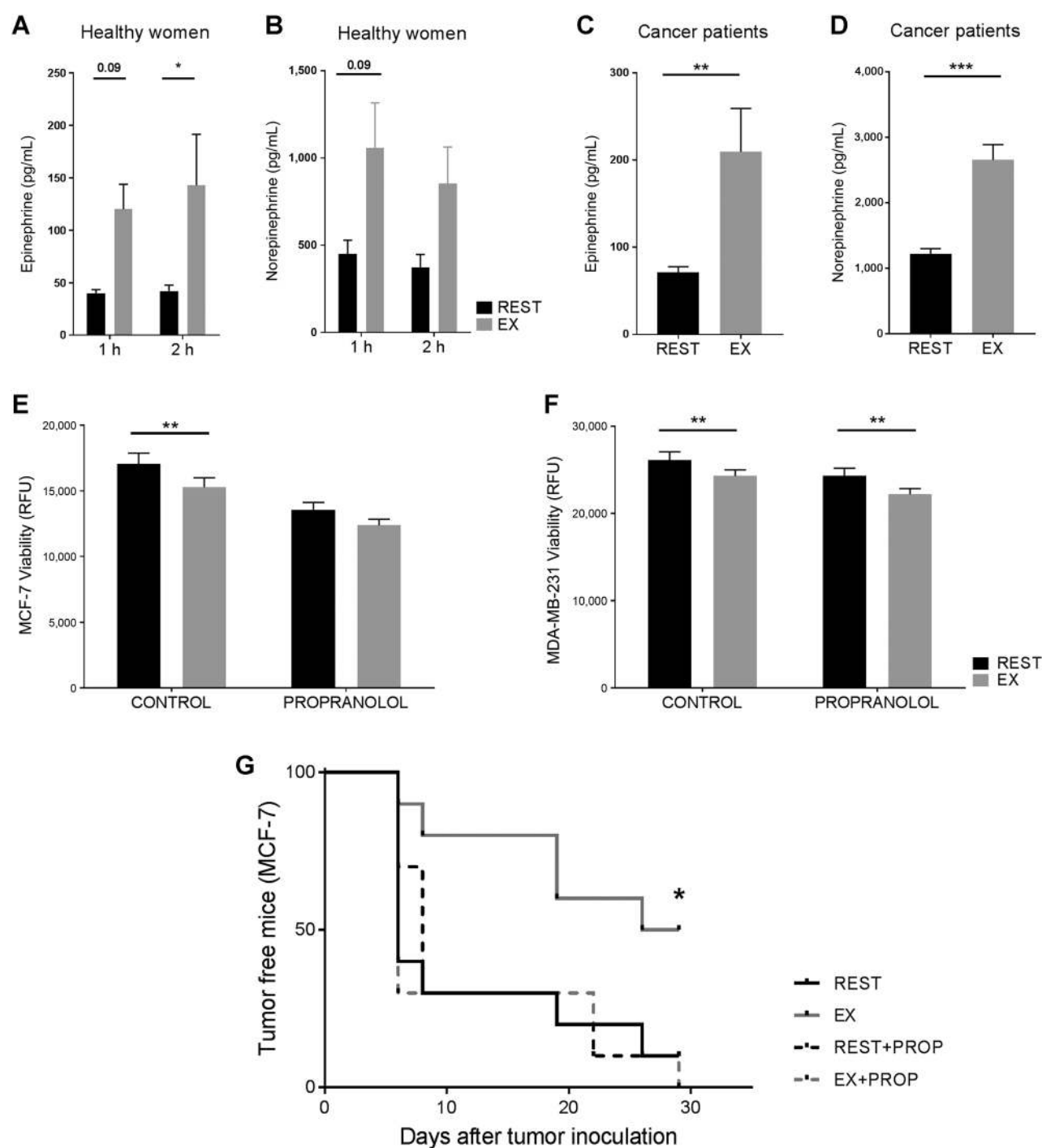
We went on to explore which exercise factors were responsible for the exercise-dependent suppression of the two breast cancer cell lines. Noticeable candidates were the catecholamines, that is, EPI and NE. These fight-or-flight hormones are markedly upregulated during high-intensity exercise (15, 16), as also observed in both human exercise intervention studies (Fig. 2A–D; ref. 12). In addition, we found elevated levels of other known exercise-related factors, that is, serum lactate was increased by 1.5-fold ($P < 0.01$, Supplementary Fig. S1B), and IL6 by 2.4-fold ($P < 0.05$, Supplementary Fig. S1C).

Next, we blocked β -adrenergic signaling by propranolol during the serum incubation studies to explore whether catecholamine-dependent signaling were driving the exercise-mediated

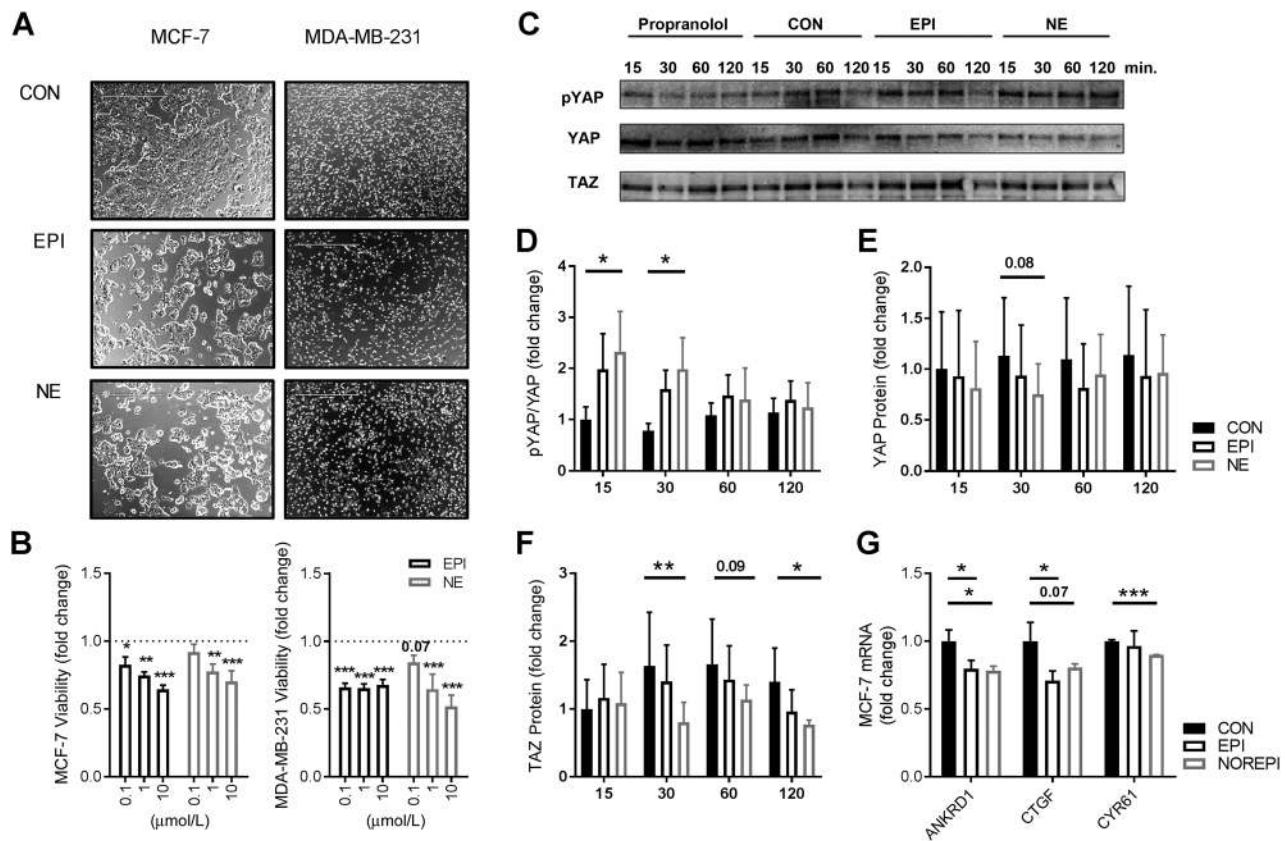
suppression on cell viability and tumorigenic potential. Addition of propranolol blunted the suppressive effect of the exercise-conditioned serum on cell viability in MCF-7 cells (Fig. 2E), while there was no attenuating effect in the MDA-MB-231 cells ($P < 0.01$, Fig. 2F). In continuation, we inoculated mice with MCF-7 cells preincubated with exercise-conditioned serum and propranolol treatment, and found that β -adrenergic blockade during the preincubation completely blunted exercise-mediated suppression on tumor formation (Fig. 2G). These results highlight the role of adrenergic signaling in the exercise-dependent control of breast cancer tumorigenesis.

Regulation of the Hippo tumor suppressor pathway by epinephrine and norepinephrine

We proceeded to explore whether EPI and NE directly could regulate breast cancer cell viability in cell culture studies. Both cancer cell lines displayed visible decreases in viability by microscopy (Fig. 3A), and when quantified as percent cell confluence after 48 hours of stimulation with EPI (MCF-7: -25% , $P < 0.01$; MDA-MB-231: -18% , $P < 0.01$) or NE (MCF-7: -41% , $P < 0.01$; MDA-MB-231: -6% , $P < 0.001$; Supplementary Fig. S2A and S2B).

**Figure 2.**

Effect of β -adrenergic receptor signaling in breast cancer growth response to exercise stimulus. Serum levels of epinephrine (**A** and **C**) and norepinephrine (**B** and **D**) at rest and after 1 and 2 hours of exercise in healthy women ($n = 7$) and after 2 hours of exercise in breast cancer patients ($n = 20$). Viability of MCF-7 ($n = 19$; **E**) and MDA-MB-231 ($n = 20$; **F**) cells stimulated with 10% serum obtained from breast cancer patients at rest (REST) or after 2 hours of exercise (EX) \pm propranolol (control/propranolol). Breast cancer cells were preincubated for 48 hours with 10% human serum collected from breast cancer patients at rest or after exercise (EX) and with the same serum plus 10 μ mol/L propranolol (REST+PROP and EX+PROP) before inoculation into sedentary mice ($n = 10$ in each group). **G**, Percent tumor-free mice in days after inoculation. Statistical significance of the catecholamine concentrations was tested by two-way ANOVA, with repeated measures (healthy women) and a paired t test (breast cancer patients). MCF-7 viability was tested by two-way ANOVA, with repeated measures and tumor incidence as a survival curve comparison by a Log-rank (Mantel-Cox) test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

**Figure 3.**

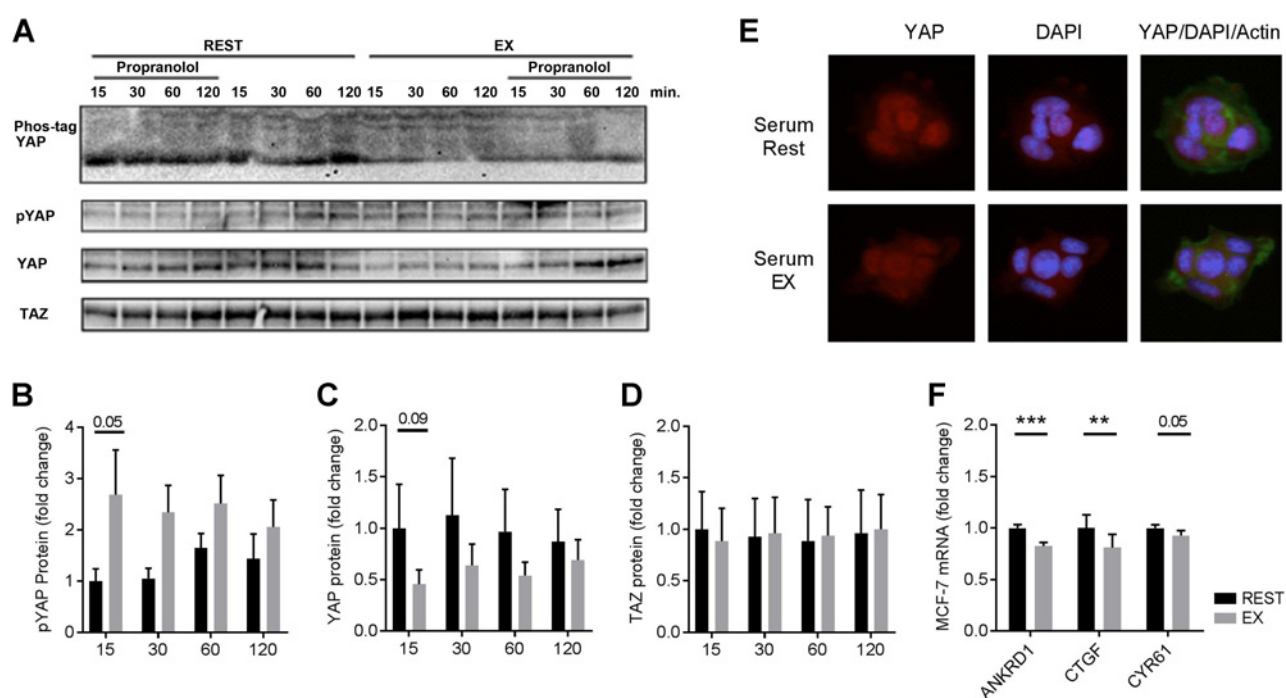
Direct growth-inhibitory effects of epinephrine and norepinephrine on breast cancer cells. **A**, Microscope images of MCF-7 and MDA-MB-231 cells stimulated with 10 $\mu\text{mol/L}$ EPI, 10 $\mu\text{mol/L}$ NE, or vehicle (CON) for 48 hours. **B**, Viability of the cancer cells after stimulation with EPI and NE at concentrations of 0, 0.1, 1, and 10 $\mu\text{mol/L}$ for 72 hours ($n = 3$). Results are shown as fold change from nonstimulated cells (dotted line). **C**, Representative blots of MCF-7 cells stimulated with growth medium (CON) +10 $\mu\text{mol/L}$ propranolol, EPI, or NE for 15–120 minutes. Quantification of pYAP (Ser127)/YAP (**D**), YAP (**E**), and TAZ protein expression in MCF-7 cells ($n = 3$; **F**) related to total protein (reactive brown). **G**, mRNA expression of genes downstream of YAP/TAZ in MCF-7 cells after 48 hours of stimulation with CON/EPI/NE ($n = 3$). Data are presented as means \pm SEM. Statistical significance was tested by two-way ANOVA with repeated measures. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

In continuation, we demonstrated a dose-dependent growth-inhibitory effect of both catecholamines for both breast cancer cell lines at 24, 48, and 72 hours by MTT assays [10 $\mu\text{mol/L}$ at 72 hours: MCF-7: EPI: -36% ($P < 0.001$), NE: -30% ($P < 0.001$); MDA-MB-231: EPI: -32% ($P < 0.001$), NE: -48% ($P < 0.001$); Fig. 3B, Supplementary Fig. S2C and S2D].

EPI and NE have been shown to regulate the Hippo tumor suppressor signaling pathway (17, 18). This developmental signaling pathway is dysregulated in several types of cancer, including breast cancer, where activation of the oncoproteins, YAP/TAZ, are associated with tumor growth and progression, tumor formation, metastasis, and drug resistance (19). When the Hippo pathway is induced, YAP and TAZ are phosphorylated and thus inactivated. In line with the original studies, we found that EPI and NE directly regulated the Hippo tumor suppressor pathway in the two breast cancer cell lines (Fig. 3C–F; Supplementary Fig. S3A–S3D). Both EPI and NE induced a rapid phosphorylation of YAP Ser127 at 15 and 30 minutes ($P < 0.05$; Fig. 3C and D), and a reduction in total TAZ protein at 30–120 minutes ($P < 0.01$; Fig. 3F) in MCF-7 cells. In continuation, expression levels of genes downstream of YAP/TAZ were significantly downregulated (Fig. 3G).

Exercise-conditioned serum is controlling breast cancer cells through the Hippo signaling pathway

Next, we investigated whether the Hippo tumor suppressor pathway was regulated by exercise-conditioned serum (Fig. 4). Incubation with exercise-conditioned serum induced phosphorylation of YAP in MCF-7 cells in a time-dependent manner, peaking already at 15 minutes, which was evident by YAP phos-tag staining (Fig. 4A), as well as phosphorylation on YAP Ser127 ($P = 0.05$, $n = 3$, Fig. 4B). In parallel, total protein levels of YAP tended to decrease ($P = 0.09$, Fig. 4C), while TAZ protein remained unchanged (Fig. 4D). In MDA-MB-231 cells, there were no significant differences in pYAP ($P = 0.13$), YAP, or TAZ protein expression after 1 hour of incubation with exercise-conditioned serum (Supplementary Fig. S4A–S4D). Phosphorylation of YAP leads to its cytosolic sequestration, and subsequent proteolytic breakdown. Accordingly, we depicted the cellular localization of YAP after incubation with exercise-conditioned serum, and found that YAP was indeed sequestered in the cytosol following serum incubation for 1 hour (Fig. 4E). Correspondingly, this deactivation and cytosolic sequestration of YAP, resulted in decreased expression of YAP target genes in MCF-7 cells [MCF-7: ANKRD1:

**Figure 4.**

Effect of exercise-conditioned serum on Hippo signaling in MCF-7 cells. **A**, Representative blots of phos-tag YAP, pYAP (ser127), YAP, and TAZ in MCF-7 cells after stimulation with medium containing 10% serum from breast cancer patients at rest or after 2 hours of exercise (EX) \pm propranolol for 15–120 minutes. Quantification of pYAP/YAP (**B**), YAP (**C**), and TAZ (**D**) protein in MCF-7 cells ($n = 3$). **E**, Fluorescence images of MCF-7 cells stained for YAP, DAPI, or YAP/DAPI/Actin after incubation with REST or EX serum for 1 hour. **F**, mRNA expression of genes downstream of YAP/TAZ in MCF-7 cells after 48 hours of stimulation with REST/EX serum from breast cancer patients ($n = 13$). Data are depicted as mean \pm SEM and statistical significance was tested by two-way ANOVA with repeated measures. **, $P < 0.01$; ***, $P < 0.001$.

–17% ($P < 0.001$); CTGF: –19% ($P < 0.01$); CYR61: –7% ($P = 0.05$); Fig. 4F]. In the MDA-MB-231 cells, exercise-conditioned serum had less effect on the expression levels of these genes [MDA-MB-231; ANKRD1: –6% ($P = 0.06$), CTGF: –4% ($P < 0.05$), CYR61: –2% ($P > 0.99$; Supplementary Fig. S4E)]. By stimulating MCF-7 cells with serum in combination with propranolol, we found that β -adrenergic blockade reduced the serum-mediated phosphorylation of YAP (Fig. 4A and Supplementary Fig. S4F).

Voluntary wheel running regulates tumor growth in mice

To evaluate the effect of exercise on breast cancer tumor growth *in vivo*, female NMRI-Foxn1^{nu} mice were inoculated with MCF-7 or MDA-MB-231 tumor cells, and randomized to home cages with or without running wheels. Mice with access to running wheels ran voluntarily approximately 4 km per night/cage (Supplementary Fig. S5A). For MCF-7 tumor-bearing mice, all mice from the exercise group completed the study while 4 mice from the control group were euthanized due to signs of illness. For MDA-MB-231 tumor-bearing mice, 2 mice from the exercise group and 4 mice from the control group did not complete the study. Growth of both MCF-7 (–36%, $P < 0.05$) and MDA-MB-231 (–66%, $P < 0.01$) tumors were reduced by voluntary wheel running compared with control mice (Fig. 5A and B). We have previously seen that propranolol treatment can blunt the exercise-dependent suppression of tumor growth in B16 melanoma-bearing wild-type mice

(20). Here, we also attempted to block β -adrenergic signaling during the exercise intervention, but the NMRI-Foxn1^{nu} mice did not tolerate the propranolol treatment. In tumors from the running mice, we found no difference in protein expression of pYAP (Fig. 5C–D), which may simply reflect that these tumors were excised at rest. We did however find an overall down-regulation of downstream genes in the MCF-7 tumors from mice with access to running wheels ($P < 0.05$, two-way ANOVA, Fig. 5E), while the expression of these genes were not affected in MDA-MB-231 tumors (Fig. 5F).

Exercise-dependent control of breast cancer progression has been suggested to relate to weight control (21); however, in these lean chow-fed mice, we did not observe any differences in body weight or blood glucose between the control and wheel running groups at the end of the study (Supplementary Fig. S5B), and neither body weight nor blood glucose levels correlated with tumor volume in either of the two groups (Supplementary Fig. S5C). Moreover, we have previously shown that wheel running controls growth of melanoma tumors through an exercise-dependent mobilization of immune cells (20); however, in our breast cancer models, wheel running only tended to increase immune cell infiltration as reflected by the expression of immune cell markers in MDA-MB-231 tumors (Fig. 5G and H). Thus, we did not find evidence to suggest that neither weight control nor immune cell infiltration were the primary exercise-induced mechanisms responsible for suppression of tumor growth in these models.

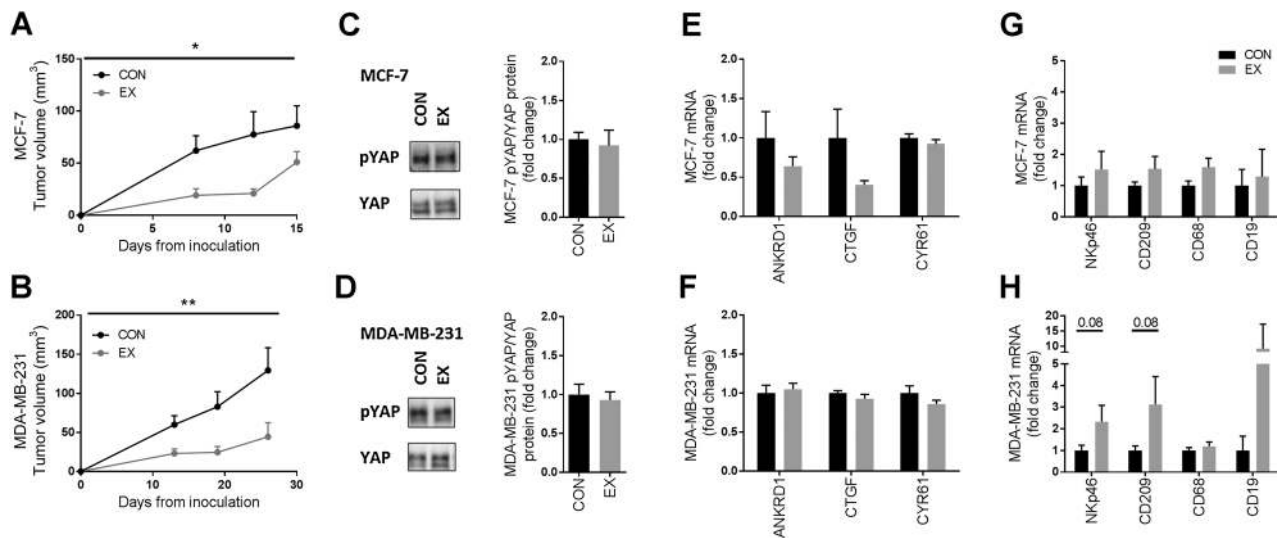


Figure 5.

Effects of wheel running on tumor growth and Hippo signaling. Tumor volume of MCF-7 tumors ($n = 10$; **A**) and MDA-MB-231 tumors cells ($n = 9-10$; **B**) in mice with access to running wheels (EX) or no running wheels (CON). pYAP (Ser127) and YAP protein in MCF-7 (CON = 6, EX = 5; **C**) and MDA-MB-231 ($n = 8$; **D**) tumors related to total protein (reactive brown). Gene expression levels of genes downstream of YAP/TAZ in MCF-7 tumors (CON = 6, EX = 7; **E**) and MDA-MB-231 tumors (CON = 6, EX = 4; **F**). Gene expression levels of immune markers in MCF-7 (CON = 6, EX = 7; **G**) or MDA-MB-231 (CON = 6, EX = 4; **H**) tumors from mice with access to running wheels. All mRNA levels are related to β -actin. Data are depicted as mean \pm SEM. Statistical significance of tumor volume was tested by two-way ANOVA with repeated measures, and protein and mRNA expression was tested by unpaired t tests. *, $P < 0.05$; **, $P < 0.01$.

Control of tumor growth in mice by daily injections of epinephrine and norepinephrine

Finally, we investigated if EPI and NE had direct effects on tumor growth *in vivo*, by inoculating MCF-7 and MDA-MB-231 cells in NMRI-Foxn1^{nu} mice, and subsequently treated with daily physiologic doses of EPI (0.05 mg/mL, 200 μ L, i.p.) or NE (0.25 mg/mL, 200 μ L, i.p.), mimicking the increases occurring during acute exercise. Concordant with the tumor suppression observed with wheel running (Fig. 5A and B), we found that both EPI and NE reduced tumor growth of MCF-7 tumors [EPI: -85% ($n = 9$, $P < 0.001$), NE: -53% ($n = 6$, $P < 0.05$), Fig. 6A] and MDA-MB-231 tumors [EPI: -59% ($n = 9$, $P < 0.01$), NE: -58% ($n = 10$, $P < 0.01$), Fig. 6B]. As for the wheel running intervention, daily injections of EPI or NE had no effect on body weight (Fig. 6C and D), and end tumor volume did not correlate with body weight (Fig. 6E and F).

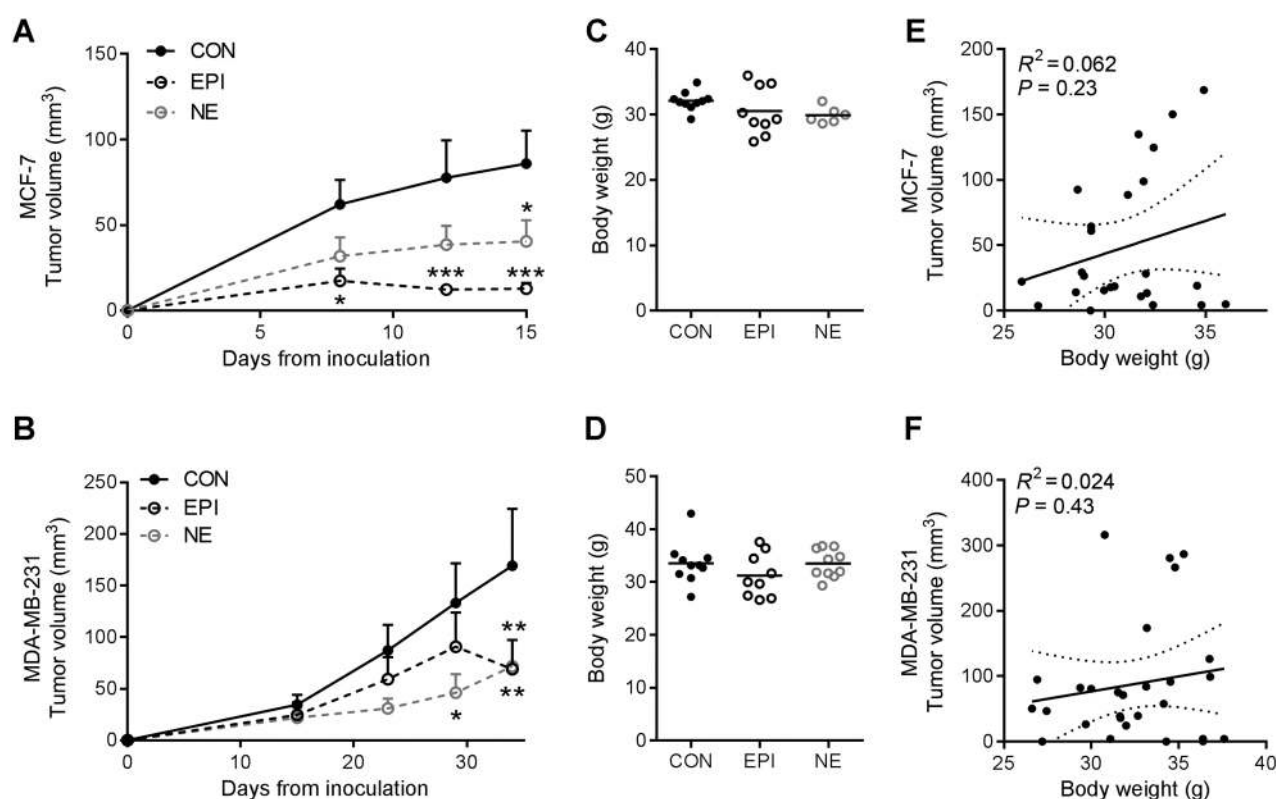
Discussion

Despite a large number of published training intervention studies in women with breast cancer, relative little is known about the molecular mechanisms whereby exercise and exercise-induced factors influence breast cancer biology and progression. The current study is the first to demonstrate that systemic changes occurring during exercise can reduce breast cancer cell viability and decrease tumorigenesis through regulation of the Hippo signaling pathway. This exercise-dependent control of breast cancer was driven by induction of catecholamines, that is, EPI and NE, both of which directly regulated the Hippo signaling pathway, suppressed breast cancer cell viability and reduced tumor formation *in vivo*. We have used two distinct breast cancer cell lines, and while both cell lines were regulated by exercise and catecholamine stimulation, the less aggressive hormone-sensitive

cell line, MCF-7, displayed greater sensitivity to exercise-mediated activation of Hippo signaling.

Solid epidemiologic data show that regular exercise is associated with lower risk of developing breast cancer, as well as decreased risk of disease recurrence (5, 8, 9). Recently, excitement about the identification of exercise-dependent immune cell regulation and myokine secretion in cancer control have highlighted the clinical potential of utilizing exercise in cancer therapy (22). Yet, to fully explore the potential of exercise in control of breast cancer, a detailed molecular understanding of the effect of exercise and exercise factors in breast cancer biology is warranted. To this end, we have recently shown that systemic changes occurring during acute exercise can directly inhibit breast cancer cell viability (12). In line with these results, we here demonstrate that serum taken during or immediately after an exercise session from both breast cancer patients and healthy women could reduce cancer cell viability *in vitro* by approximately 10%. Yet, the effect of exercise-conditioned serum on the tumorigenic potential was even more strikingly. Here, preincubation with exercise-conditioned serum reduced MCF-7 tumor formation with 50%.

Endurance exercise has been suggested to alter Hippo signaling in skeletal muscles through AMPK-, EPI-, and IL6-driven regulations of YAP (23); however, the effect of exercise on Hippo signaling is undescribed in cancer. The Hippo tumor suppressor signaling pathway is involved in basal processes like cellular growth, differentiation, and apoptosis, and is particularly recognized for its role in tissue development (24). In many cell types, including myoblasts, cardiomyocytes, and several cancer cells YAP hyperactivity increases cell proliferation (25–29). An even more striking effect of activation of Hippo signaling and thus reduced YAP activation is a pronounced suppression of tumor formation (17, 30–32), corresponding precisely to the effect seen in our studies with preincubation with exercise-conditioned serum. The

**Figure 6.**

Effect of epinephrine and norepinephrine on tumor volume. MCF-7 (CON = 10, EPI = 9, NE = 6; **A**) and MDA-MB-231 (CON = 10, EPI = 9, NE = 10; **B**) tumor volume in mice receiving daily injections of EPI (0.05 mg/mL, 200 μ L i.p.) or NE (0.25 mg/mL, 200 μ L i.p.) compared with control mice (CON). Body weight (minus tumor) of the mice with MCF-7 (**C**) or MDA-MB-231 (**D**) tumor challenge. Correlation analyses of MCF-7 (**E**) and MDA-MB-231 (**F**) tumor volume relative to body weight. Data represent means \pm SEM. Statistical significance was tested by two-way ANOVA with repeated measures for tumor volume, by unpaired *t* tests for body weight, and as linear regression analysis for tumor volume/body weight correlations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Hippo signaling pathway has been shown to be dysregulated in several types of cancer, including breast cancer, where the activation of the oncoproteins YAP/TAZ have been associated with a poor prognosis (19), and the therapeutic role of YAP has been studied in gastric cancer and hepatocellular carcinoma, where molecules targeting YAP caused pronounced tumor regression in mice (33, 34).

Activation of Hippo signaling comprises of phosphorylation and thus inhibition of the two homologous transcription factors, YAP and TAZ. This phosphorylation leads to sequestration of YAP/TAZ in the cytoplasm, thereby preventing the induction of their target genes, that is, genes involved in cell proliferation and survival (35). The cytoplasmic sequestration also leads to ubiquitin-mediated degradation of YAP/TAZ, further inhibiting their function (36). Here, we demonstrate that exercise-conditioned serum has the potential to regulate all of these steps, including a 2-fold induction of YAP phosphorylation, retention of YAP to the cytoplasmic compartment, a 50% reduction in the amount of total YAP protein, and 17%–19 % decrease in the expression of YAP target genes, in particular in MCF-7 cells. Although the regulation at each step might not seem dramatic, the notion that acutely induced exercise factors can elicit these responses is highlighting that these physiologic changes may translate into a pronounced effect on tumor formation.

The Hippo pathway is regulated by numerous extracellular stimuli, among other factors signaling through G protein-coupled receptors (17, 24). While most G-coupled receptor ligands have shown to inhibit Hippo signaling, the catecholamines have shown to stimulate Hippo signaling through β -adrenergic receptors, resulting in inactivation of YAP/TAZ (24, 37). In line with this, we found that the exercise-dependent breast cancer suppression could be mimicked by EPI or NE. Both of these factors were markedly upregulated in our acute human exercise interventions, and blockade of their signaling through β -adrenergic receptors during the serum incubation studies, blunted the exercise-mediated growth suppression in the MCF-7 cells, as well as tumor formation. Moreover, direct stimulation of the breast cancer cell lines with either EPI or NE activated Hippo signaling, reduced breast cancer cell viability, and suppressed tumor growth when EPI and NE were injected daily in tumor-bearing mice. This control of breast cancer tumor growth by β -adrenergic stimulation is in accordance with studies from other groups (38, 39). We have recently shown that EPI could mimic the exercise-mediated control of melanoma tumor growth through mobilization and activation of NK cells, resulting in increased intratumoral immune cell infiltration (20). In the breast cancer tumors in the current study, we also evaluated the effect of wheel running on immune cell infiltration, and although wheel running tended to increase

the infiltration of some immune cell subsets, it could not explain the pronounced effect on tumor growth control.

Throughout this study, we have utilized MCF-7 and MDA-MB-231 breast cancer cell lines. These comprise markedly different phenotypic characteristics, that is, MCF-7 cells are sex hormone sensitive with a luminal A classification, representing the majority of breast cancer tumors in patients. In contrast, the MDA-MB-231 cells are triple negative with claudin-low classification, representing another subgroup of breast cancer tumors, typically with a worse prognosis (13). Despite these marked differences in the molecular profiles, the progression of both breast cancer cell lines was affected by wheel running, exercise-conditioned serum, as well as both catecholamines. However, the two cell lines displayed differential responses regarding regulation of the Hippo signaling pathway. The MCF-7 cells showed a rapid phosphorylation of YAP with both exercise-conditioned serum and EPI and NE, sequestration to the cytosol and suppression of downstream genes. In our hands, MDA-MB-231 cells showed a less pronounced induction in YAP phosphorylation by EPI and NE, while exercise-conditioned serum only regulated Hippo signaling to a minor degree. Yet, Yu and colleagues have comprehensively shown that EPI can phosphorylate and deactivate YAP in MDA-MB-231 cells in time- and dose-dependent manners (24). One marked difference between the two cell lines is the β -adrenergic receptor density, which is much higher in MDA-MB-231 cells compared with MCF-7 cells (40). Furthermore, as many signaling pathways converge at YAP, additional exercise-stimulated signaling pathways may cross-communicate to explain the differential response. Chen and colleagues have shown that LIF, a member of the IL6 superfamily, is an upstream tumor suppressor, which inhibited breast cancer metastasis through Hippo and YAP signaling (31). LIF is also recognized as an exercise-induced muscle-derived factor, that is, myokine (41), and in line with this, we have previously shown that such myokines can inhibit the proliferation of breast cancer cells (42). A recent study has also demonstrated that Hippo signaling interacts with ER α signaling, and loss of Hippo signaling conferred resistance to antihormonal therapy (43).

It is important to distinguish between the acute and rapid exercise/stress response, demonstrated to suppress breast cancer cell viability in this study, and chronically upregulated stress responses. During exercise, systemic levels of catecholamines increase several fold, but these levels return rapidly to baseline levels after exercise cessation, with the half-life of EPI being within minutes. In contrast, chronic stress is associated with persistent elevations of catecholamine levels, as well as other stress factors, for example, cortisol (44). In this situation, several studies have

documented that the chronic upregulation of stress factors, including the catecholamines is associated with increased tumor progression (45).

Taken together, we show an omnipresent exercise-dependent suppression of breast cancer cell viability and tumorigenic potential that depends on acutely exercise-induced catecholamines and suggest a role for regulation of the Hippo signaling pathway. The identification of EPI and NE as active oncostatic factors in the exercise-mediated control of breast cancer cell viability and tumor formation suggests that exercise of moderate to high intensity is the most optimal regimen for generation of an acute systemic response with potential to directly control breast cancer cell development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acknowledgments

The authors acknowledge Ruth Rousing, Hanne Villumsen, Anne-Kirstine Lundby, Lone Pejjs, and Noemi Galicki James for their technical assistance.

Grant Support

This study was supported by research grants from The Danish Cancer Society (to P. Hojman), the Danish Cancer Research Foundation (to C. Dethlefsen), and Dagmar Marshall Fund (to C. Dethlefsen). The Centre for Physical Activity Research (CFAS) is supported by a grant from TrygFonden (to B.K. Pedersen). During the study period, the Centre of Inflammation and Metabolism (CIM) was supported by a grant from the Danish National Research Foundation (DNRF55 to B.K. Pedersen). CIM/CFAS is a member of DD2 - the Danish Center for Strategic Research in Type 2 Diabetes (the Danish Council for Strategic Research, grant no. 09-067009 and 09-075724 to B.K. Pedersen).

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Received November 16, 2016; revised April 19, 2017; accepted June 27, 2017; published OnlineFirst September 8, 2017.

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