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Epigenetic inhibition of the tumor suppressor ARHI by light at night-induced circadian melatonin disruption mediates STAT3-driven paclitaxel resistance in breast cancer

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors disclose no potential conflicts of interest

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Abstract

Disruption of circadian time structure and suppression of circadian nocturnal melatonin (MLT) production by exposure to dim light at night (dLAN), as occurs with night shift work and/or disturbed sleep-wake cycles, is associated with a significantly increased risk of breast cancer and resistance to tamoxifen and doxorubicin. Melatonin inhibition of human breast cancer chemo-resistance involves mechanisms including suppression of tumor metabolism and inhibition of kinases and transcription factors which are often activated in drug-resistant breast cancer. Signal Transducer and Activator of Transcription 3 (STAT3), frequently overexpressed and activated in Paclitaxel (PTX)-resistant breast cancer, promotes the expression of DNA methyltransferase one (DNMT1) to epigenetically suppresses the transcription of tumor suppressor Aplasia Ras homolog one (ARHI) which can sequester STAT3 in the cytoplasm to block PTX-resistance. We demonstrate that breast tumor xenografts in rats exposed to dLAN and circadian MLT disrupted express elevated levels of phosphorylated and acetylated STAT3, increased DNMT1, but reduced Sirtuin 1 (SIRT1) and ARHI. Furthermore, MLT and/or SIRT1 administration blocked/reversed Interleukin 6 (IL-6)-induced acetylation of STAT3 and its methylation of ARH1 to increase ARH1 mRNA expression in MCF-7 breast cancer cells. Finally, analyses of the I-SPY 1 trial demonstrates that elevated MT_1 receptor expression is significantly correlated with pathologic complete response following neo-adjuvant therapy in breast cancer patients. This is the first study to demonstrate circadian disruption of MLT by dLAN driving intrinsic resistance to PTX via epigenetic mechanisms increasing STAT3 expression and that MLT administration can reestablish sensitivity of breast tumors to PTX and drive tumor regression.

Keywords

Circadian disruption; Melatonin; STAT-3; Paclitaxel resistance

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer in women worldwide with an annual incidence of 1.4 million, and 450,000 deaths per year[1]. Approximately 30% of breast cancer cases present with locally advanced disease; another 30% will develop metastatic disease[2]. Although patients with distant metastases essentially remain incurable, the use of taxanes (paclitaxel) achieves response rates up to 48% in patients with metastatic breast cancer. Most of these patients will, however, eventually relapse after treatment[3–6].

Paclitaxel, an extract from the pacific yew Taxus tree[7], is a potent cytotoxic agent that stabilizes microtubules by binding β-tubulin to block cell division[8,9]. The cause of PTX-resistance in breast cancer is attributed to numerous mechanisms including constitutive activation of key oncogenic signaling pathways including phosphatidylinositol 3-kinase/protein B (PI3K/AKT), epidermal growth factor (EGFR) and human epidermal growth factor receptor (HER), downstream mitogen-activated protein kinases/extracellular signal-related kinases (MAPK/ERK1/2), STAT3, and c-SRC to name a few[10–18].

Resistance to PTX has also been linked to up-regulation and activation of STAT3[14,15] in response to extracellular stimuli such as cytokines including interleukin 6 (IL-6), growth factors, oncogenes (RAS), and kinases[19–26]. STAT3 is considered a potential oncogene, as its over-expression or gain-of-function mutations can transform cells to a tumorigenic phenotype[19–28]. However, STAT3 activity can be inhibited by Aplysia Ras homolog member 1 (ARHI), also known as DiRas3, a maternally imprinted tumor suppressor gene that encodes a 26-kDa GTP-binding protein with 60% homology to RAS[29], and whose expression is elevated in normal breast but diminished in breast cancer[30,31]. ARH1 is reported to bind and inhibit STAT3 activity by blocking its nuclear accumulation and, thus, transcriptional activity[32,33]. Furthermore, acetylation of STAT3 by IL-6 at Lysine 685 (k685) promotes STAT3's transcriptional activation of the DNMT1 gene that methylates the ARH1 promoter to block its transcription[34]. Finally, SIRT1, a class-III histone deacetylase involved in apoptosis regulation and tumorigenesis, deacetylates STAT3 at K685[35].

The disruption of circadian time structure by LAN encountered during night shift work and/or disturbed sleep-wake cycles is reported to increase the risk of an array of diseases, including breast cancer[36]. We have demonstrated that the circadian hormone MLT, produced by the pineal gland at night, inhibits the growth of breast cancer through activation of its MT_1 G protein coupled MLT receptor[37]. We reported that disruption of the circadian MLT signal by exposure of nude rats to dLAN promotes the growth of ERα+ human breast tumor xenografts and drives intrinsic resistance to tamoxifen (TAM) and doxorubicin (Dox)[38,39].

In the present study, utilizing the circadian-complete (MLT producing), tissue-isolated breast tumor xenograft nude rat model, we tested the hypothesis that disruption of the circadian MLT signal, by exposure of tumor-bearing animals to dLAN, induces rapid breast tumor

growth and intrinsic resistance to PTX. In this study, we determined that administration of MLT during dLAN attenuates the expression, phosphorylation, and/or acetylation of the oncogene STAT3 to block dLAN-induced intrinsic resistance to PTX by inhibiting IL-6 expression, while increasing SIRT1 and ARH1 expression.

MATERIALS AND METHODS

Chemicals, reagents, cell lines and cell culture

The MCF-7 human breast cancer cells used in these studies were obtained from American Tissue Culture Collection (Manassas, VA), authenticated by ATCC, and, upon receipt, immediately expanded and frozen-down as stock for future studies. Low passage stocks (passage numbers 18–20) were used within one year of purchase. MCF-7 cells were maintained as previously described[37,38]. MCF-7 cells transfected with a constitutively active c-SRC construct (SRC-D) were finger printed (Sigma Aldrich, Bioreliance DNA barcode assay).

Animals, housing conditions, and diet

Ovariectomized female athymic, inbred nude rats (Crl:NIH- *Foxn1*^{rnu}), 1–2 weeks of age were purchased from Charles River Laboratories (Wilmington, MA) and were maintained in environmentally controlled rooms (25° C; 50–55% humidity) with controlled diurnal lighting schedule of 12h light:12h dark (LD12:12, 300 lux; 123 mW/Cm2; lights on 06:00 hours, and off at 18:00 hours). All rats were supplemented with estrogen pellets (0.72 mg of 17b-estradiol 60-day release form Innovative Research of America). One week prior to tumor implantation, the animals were switched to 12-hour light:12-hour dim light at night (dLAN) cycle (0.2 lux, with lighs on at 06:00 hours and off at 18:00 hours, a dLAN on at 18:00 hours and off at 06:00 hours), as previously described[37]. All procedures employed for animal studies were approved by the Tulane University Institutional Animal Care and Use Committee.

Arterial blood collection

Diurnal plasma MLT levels (pg/mL; mean ±1 SD) of naïve, female nude rats (n=12) maintained initially in the control LD12:12 cycle or in the dLAN photoperiod were measured along with total fatty acids, glucose, lactic acid, pO_2, and pCO_2, as previously described[38]. Briefly, blood collections (0.5 ml) were performed at designated 4-hour intervals at six circadian time points (04:00, 08:00, 12:00, 16:00, 20:00, and 24:00 hours) beginning at ZT2 (0800 hours) with each animal being subjected to cardiocentesis only once every 5 days during the 30-day period prior to tumor implantation.

MCF-7 tumor xenografts development in nude mice and transplantation into female athymic nude rats

MCF-7 breast tumor xenograft development in athymic nude female mice for tumor xenograft transplantation as tissue-isolated tumors into athymic nude female rats was conducted as previously described[38,39]. When tumor weights reached approximately 2.5g estimated weight, one-half of the animals (n=3/group) maintained in LD12:12, dLAN, or dLAN supplemented with MLT (dLAN + MLT) were treated daily at 1600 hr with either

PTX (4 mg/kg/day) or diluent by i.p. injection. This study consisted of 6 groups: Group I (dLAN), Group II (dLAN + PTX), Group III (dLAN + MLT), Group IV (dLAN + MLT + PTX), Group V (LD12:12, endogenous nighttime MLT), and Group VI (LD12:12 + PTX). All animals receiving exogenous MLT received it as a supplement in their nighttime drinking water (0.1 mg/mL) such that they received approximately 2.5 mg MLT daily intake, based on a daily water intake of about 25 mL, which simulates the high normal nighttime physiologic levels of MLT.

A-V Tumor Measurements

Analyses of A-V differences between the different treatment groups in tumor MLT, glucose, lactate, fatty acids, and tumor production of 13-HODE and levels of cAMP, [^{3}H]-thymidine incorporation into DNA and DNA content, were conducted as previously described[38,39].

Tumor lysate extraction and Western blot analysis

Total cellular protein was extracted from each tumor xenograft and Western blot analysis was performed as described previously[38,39]. Blots were probed with various antibodies including (t) total and phospho (p)-HER2, HER3, ERK1/2 Thr202/Tyr204, ERK1/2, AKT Ser473, AKT, SRC Ser536, SRC, CREB Ser133, CREB, RAS, STAT3 Tyr707, Acetyl-STAT3 (K685) and STAT3, SURVIVIN, and FASCIN from Cell Signaling (Danvers, MA).

Immunohistochemistry (IHC) of cytoplasmic and nuclear expression of Tyr(p)-705 STAT3 in MCF-7 breast tumor xenografts

Sections of tumor xenografts from the dLAN, dLAN + PTX, dLAN + MLT, and dLAN + MLT + PTX treatment groups were Formalin-fixed and paraffin-embedded into tissue blocks. Tissue sections were cut to 4-μM thickness, mounted on polylysine-coated slides, de-waxed, rehydrated in ethanol, and rinsed in phosphate-buffered saline. Antigen retrieval was performed by pretreating slides with citrate at pH 6 with microwaving for 10 minutes, and endogenous peroxidase activity was blocked using 0.3% H_2O_2. The slides were incubated with anti-pSTAT3 antibody (15μg/ml) and biotin-labeled secondary antibody, streptavidin/peroxidase, diminobenzidine, and counter stained with Hematoxylin/Eosin. Sections were imaged at a magnification of X 100, using an Olympus 1X70 microscope with a digital camera (Olympus, Tokyo, Japan).

IL-6 induction of STAT3 acetylation and MLT-mediated SRIT1 de-acetylation of STAT3

To investigate the capacity of IL-6 to repress and MLT to inhibit STAT3 acetylation and to regulate ARHI mRNA expression, MCF-7 cells in 5% FBS were treated with IL-6 (50 ng/ml) in the presence or absence of MLT (10^{-8} M or 10^{-9} M), or the SIRT1-inhibitor EX-527 (0.5 μM). Cells were harvested after 12 h frozen in liquid nitrogen and stored at −80° C until total cellular protein was extracted. Western blot analyses were conducted as described above using an acetylation specific antibody for K685 of STAT3.

Real-Time RT-PCR analysis of IL-6 and ARHI

Following in vitro treatment of MCF-7 cells with IL-6 (50 ng/ml), IL-6 + MLT (10^{-9} M), and IL-6 + MLT+ the SIRT1-inhibitor EX-527 for 48 hr, total RNA was extracted by using

the TRIzol Reagent (Thermo Fisher Scientific). Total RNA was also extracted from tumor xenografts samples for dLAN, dLAN +PTX, dLAN + MLT, and dLAN + MLT + PTX group tumor xenografts for analysis of IL-6 mRNA. First strand cDNA was synthesized by using SuperScript II Reverse Transcriptase (Thermo Fisher Scientific). Real Time PCR (qPCR) was performed using QuantStudio3 apparatus (Applied Biosystem, Foster City, CA), and PowerUp SYBR Green Master Mix kit (Life Technologies, Carlsbad, CA). The DNA denaturation step at 95° C for 10 minutes was followed by 40 cycles of 95° C for 15 seconds, 60° C for 1 minute. The primers for ARHI were: forward 5'-TCTGCCCGCCCTGCTTAT-3' and reverse: 5'-TTGCCGTCGCCACTCTTG-3'. The primers for IL-6 were: 5'-GGTACATCCTCGACGGCA

TCT-3' and reverse 5'-GTGCCTCTTTGCTGCTTTCAC-3' All PCR analyses were performed in triplicate in a volume of 20 μl, using 96-well optical-grade PCR plates and optical sealing tape (Bio-Rad). Differences in the expression of the ARHI and IL-6 transcripts were normalized with respect to GAPDH expression and expression was calculated with the formula 2−Δct.

Methylation-specific PCR analysis

The status of ARHI promoter methylation at CpG I and II was studied by combined bisulfite restriction analysis (COBRA) assay. Briefly, genomic DNA was isolated from MCF-7 cells treated with IL-6, IL-6 + MLT (10^{-8} M), and IL-6 + MLT + EX-527 (SIRT1-inhibitor) for 48hr, using Puregene Core Kit A (Qiagen, Hilden, Germany) and was bisulfite treated using the EZ DNA Methylation kit (Zymo Research, Irvine, CA). Aliquots of bisulfite converted DNA (2 μl) were amplified by PCR. Primers used for COBRA were as follows: CpG I Forward: 5′-GTAAGGGAGAAAGAAGTTAGA-3′, Reverse: 5′ TACTATCCTAACAAAACCCTC-3′; CpG II Forward: 5′-GTTGGGTTAGTTTTTTATAGTTGGTT-3′, Reverse: 5′-AACCAAACA ACCTAAAAAACAAATAC-3′. After amplification, PCR products were digested with the restriction enzyme *Taq*I (New England Biolabs, Ipswich, MA) for CpG I and III or *Bst*UI (New England Biolabs) for CpG II and subjected to electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

Impact of MT1 receptor expression and MLT on ARHI expression and PTX-resistance in breast cancer cells in vitro.

To determine if elevated expression of the MT1 MLT receptor could enhance the sensitivity of MCF-7 cells to MLT and enhance MLT mediated expression of ARH1, MCF-7 cells were treated with diluent (0.01% ethanolic cell culture media), MLT (10 nM), trichostatin A (TSA, 0.7 μM), or TSA + MLT for 24 h. Cells were then harvested in lysis buffer after and total cellular protein isolated and analyzed by Western blot analysis for the expression of MT1 (ab87639, Abcam, Cambridge, MA) and ARHI protein levels as described above.

Twenty four hours after treatment with TSA (TSA, 0.7 μM) or diluent (0.01% ethanolic media) paclitaxel-resistant MCF-7 cells (MCF-7/PAC, gift from Dr. Timothy Grant, University of Leicester, UK) were plated at a density of 20×10^5 cells per ml in six-well plates in IDMEM supplemented with 10% FBS[45]. Five hours after seeding, cells were

treated with PTX (uM),PTX, TCA (0.7 μM), MLT (10 nM), or TCA + MLT. On specific days (1, 3, 5, or 7), cells were trypsinized, mixed with 2% trypan blue, and total and viable cells counted on a haemocytometer.

Analysis of MT_1 receptor expression in breast tumors in I-SPY 1 Trial

Analyses of the association between MT_1 receptor expression levels and response to neo-adjuvant chemotherapy were based on array data from the publicly available caINTEGRATOR tool associated with the I-SPY 1 trial—CALGB 150007/150012, ACRIN 6657[46]. MT_1 expression was represented by probe id A_24_P266019, corresponding to RefSeq transcript NM_005958; official symbol: MTNR1A. The results were stratified by the primary study outcomes of pathologic complete response (pCR) coded as binary yes/no variables. For our analysis, MTNR1A levels in patients who achieved pCR (absence of invasive tumor in both breast and axillary lymph nodes after neo-adjuvant therapy), or experienced no relapse event (local progression, distant progression or death) were compared to those who did not achieve pCR or experienced any event.

Statistical Analysis

Data are represented as the mean ± standard error of the mean. Statistical differences between mean values in all groups at 2400hrs were assessed by the Student's t-test. Differences in the tumor growth rates among groups were determined by regression analyses and tests for parallelism (Student's t-test). All in vitro studies with breast cancer cell lines represent at least three-independent repeats. Differences were considered to be statistically significant at $p<0.05$.

RESULTS

dLAN blocks the circadian nighttime MLT nighttime signal

Peak plasma levels of MLT in rats housed in a photoperiod of LD12:12 dark during the mid-dark phase (2400 hr) were about 70-fold higher than during the mid-light phase (1200hr) (Fig. 1A). After supplementation with MLT in drinking water, plasma MLT levels at 2400hr were 142-fold higher than at 1200hr in rats exposed to a photoperiod of LD12:12 dLAN (Fig. 1B).

dLAN promotes growth and intrinsic resistance to PTX in MCF-7 tissue-isolated breast tumor xenografts.

MCF-7 breast tumor xenografts from rats exposed to dLAN showed a significantly shorter ($p<0.001$) latency-to-tumor-onset and significantly increased ($p<0.01$) growth rate (2.3 – 3 fold) compared to tumors from rats in dLAN but receiving supplemental MLT (dLAN + MLT) in the nighttime drinking water or LD12:12 (endogenous nocturnal MLT) groups (Fig. 2). Tumors from rats in dLAN showed intrinsic resistance to PTX, growing at the same rate as tumors in the dLAN group, receiving vehicle. However, xenografts from rats in the dLAN + MLT or LD12:12 groups regressed at a rate of −0.18 g/day in response to PTX.

dLAN induces and MLT (dark night) inhibits cAMP production, metabolism, and proliferative activity of breast tumor xenografts

Elevated cAMP, tumor incorporation of [^{3}H]-thymidine into DNA, tumor LA uptake and 13-HODE formation, tumor glucose and O_2 uptake, and lactate and CO_2 production levels were present in tumors at the mid-dLAN phase (2400 hr) from both dLAN + vehicle and dLAN + PTX-treated groups. Each of these groups were significantly suppressed by the presence of MLT in both dLAN + MLT or LD12:12 groups (Table 1).

dLAN stimulates and MLT inhibits the expression of key cell signaling molecules in breast tumor xenografts in female rats treated with PTX

Tumors from rats in dLAN and dLAN + PTX groups harvested at mid-dLAN phase (2400 hrs, midnight), showed strong expression of total (t) or phospho (p) HER2/HER3, AKT, AKT, ERK1/2, SRC, CREB, FASCIN, SURVIVIN, and RAS expression, while tumors from dLAN + MLT or LD12:12 (endogenous MLT) groups displayed greatly reduced expression of these signaling molecules (Fig. 3). The levels of theses signaling nodes were further reduced when PTX was administered at the onset of the circadian nighttime MLT signal.

Strong expression of pSTAT3^{Y705} was seen in tumors from dLAN and dLAN + PTX groups, but was greatly diminished in tumors from dLAN + MLT and LD 12:12 groups and even more so in the MLT + PTX group (Fig. 4). As well, SURVIVIN and FASCIN (transcriptional targets of STAT3) expression was elevated in tumors from the dLAN group, but repressed in tumors from dLAN + MLT and LD12:12 groups and to a greater extent after PTX treatment. Immunohistochemical (IHC) analysis was employed to define the expression and cellular localization of STAT3. Tumors from dLAN and dLAN + PTX groups showed strong cytoplasmic and nuclear STAT3 staining, while dLAN + MLT tumors showed diminished cytoplasmic and nuclear STAT3 staining. The dLAN + MLT + PTX tumors showed the greatest inhibition cytoplasmic and nuclear STAT3 staining (Fig. 4B).

Constitutively active SRC-D induces while MLT suppresses expression of STAT3, FASCIN, and SURVIVIN in MCF-7 breast cancer cells in vitro

To determine if SRC (induced by dLAN) and MLT (inhibited by dLAN)[38,39] differentially modulate STAT3 expression, phosphorylation, and activity in vitro, MCF-7 cells transiently transfected with a constitutively active c-SRC construct (SRC-D) and treated with diluent or MLT (10^{-8} M or 10^{-9} M) and the expression of t- and p- STAT3, SURVIVIN, and FASCIN were examined by Western blot analysis. MCF-$7_{SRC\text{-}D}$ cells exhibited a 3.1-fold increase in t-STAT3 expression, a 3.5-fold increase in pSTAT3^{Y705}, a 1.9-fold increase in FACIN, and a 14.7-fold increase in SURVIVIN expression (Fig. 5). Exposure of MCF-7 SRC-D cells to MLT in vitro for 48 h did not inhibit SRC-induced t-STAT3 expression, but did suppress pSTAT3^{Y705} by greater than 3-fold, FASCIN by 1.3-fold, and SURVIVIN expression by greater than 11-fold.

Effects of dLAN on IL-6, $_{Ac}$STAT3^{K685}, DNMT1, ARHI, and SIRT1 expression in MCF-7 Breast Tumor Xenografts

Real-time PCR and western blot analyses were employed to examine if dLAN or MLT differentially modulate IL-6 mRNA expression, or expression of $_{AC}$STAT3^{K685}, DNMT1,

ARH1, and SIRT1 which is reported to deacetylate $_{AC}$STAT3^{K685} [41]. Figure 6A and B show that tumors from dLAN and dLAN + PTX groups showed strong expression of IL-6 mRNA, $_{AC}$STAT3^{K685}, and DNMT1 proteins, but decreased SIRT1 and ARH1 expression. Tumors from the dLAN + MLT group displayed increased SIRT1 and ARH1 protein levels but diminished IL-6 mRNA and $_{AC}$STAT3^{K685}, DNMT1 protein levels, which were further reduced in response to PTX.

SIRT1 mediates MLT's suppression of IL-6-induced acetylation of STAT3 at K685 in MCF-7 breast cancer cells

SIRT1 was reported to deacetylate STAT3 at K685[41,42]. To determine if MLT, via its induction of SIRT1, suppresses IL-6 mediated acetylation (AC) of STAT3 at K685, MCF-7 cells were treated in vitro with 50 ng/ml of IL-6 followed by MLT (10^{-8} M), the SIRT1-inhibitor 6-chloro-2, 3, 4, 9-tetrahydro-1H-carbazole-1-carboxamide (EX-527, 0.5 μM), or MLT + EX-527 for 48hr. Figure 6C shows that IL-6 administration significantly increased $_{AC}$STAT3^{K685} by 1.5-fold, while MLT administration significantly suppressed IL-6 induced $_{AC}$STAT3^{K685} by 1.1-fold The SIRT1 inhibitor EX-527 repressed MLT's inhibition of IL-6 induction of $_{AC}$STAT3^{K68} by almost 0.5-fold.

IL-6 inhibition of ARHI mRNA expression is reversed by administration of MLT via SIRT1

To explore the transcriptional regulation of ARHI in MCF-7 cells by IL-6, MLT, and SIRT1, ARHI mRNA expression was measured by qPCR analysis in MCF-7 cells. Figure 7 shows that IL-6 administration significantly ($p<0.01$) suppressed ARH1 mRNA levels, but that MLT (10^{-9} M) administration blocked IL-6's inhibition of ARHI mRNA expression increasing ARHI mRNA levels by 74%. SIRT1's role in at least partially mediating MLT's promotion of ARHI expression was demonstrated by the ability of the SIRT1-inhibitor EX-527 to significantly suppress MLT-induced ARHI mRNA expression by 81%, to levels of which were similar but a little less than seen in response to IL-6 treatment.

Methylation of the ARHI promoter is induced by IL-6 but suppressed by MLT and SIRT1

Three potential CpG islands have been identified within the promoter and exons of the human ARH1 gene, with CpG island I located 1kb upstream of the transcription initiation site; CpG island II near the transcriptional initiation region; and CpG island III in the protein coding region of exon 2 (Fig. 8A)[43]. To explore the mechanism(s) by which MLT and SIRT1 reverse IL-6-mediated suppression of ARH1 mRNA expression in MCF-7 cells, we assessed the methylation status of CpG islands I and II employing the Combined Bisulfate Restriction Analysis (COBRA) approach. In MCF-7 cells, CpG island I was strongly methylated following 48 h of treatment with IL-6 (1.1-fold increase over control), but treatment with MLT significantly ($p<0.01$) suppressed IL-6-induced methylation from 1.1 to 0.3-fold (Fig. 8B and C). Administration of the SIRT1-inhibitor EX-527 reversed MLT-mediated inhibition IL-6-induced methylation of CpG island I from 0.3 to 0.7-fold. No change in methylation was observed in ARHI CpG island 2 in response to these treatments.

Elevated expression of MT1 receptor sensitizes breast cancer cells to and MLT induction of ARHI expression to ablate PTX-resistance in vitro.

Since TSA has been reported to induce the expression of the MLT MT1 receptor and we have shown above that MLT induces ARHI expression to inhibit PTX-resistance in MCF-7 tumor xenografts from rats exposed to dLAN[47]. As shown in Fig. 9 A treatment of MCF-7 cells with TCA induces a 2.8-fold increase in MT1 protein expression compared to control cells but did not increase ARH1 proteins levels. When control cells were treated with MLT in vitro ARH1 expression was not significantly increased, but the combination of TSA + MLT induced a 3.6-fold increase in the levels of ARHI protein. Figure 9B shows that both TSA and MLT alone moderately but significantly ($p<0.05$) suppress the growth of PTX-resistant MCF-7 cells, while the combination of TSA and MLT which increased ARHI levels completely reversed PTX-resistance and induced cell killing ($p<0.005$).

Reduced expression of the MT_1 receptor predicts decreased pathological-free survival following neo-adjuvant chemotherapy in women with breast cancer

Since disruption of circadian MLT by exposure to dLAN has been associated with an increased risk of breast cancer in women working night shift work[44] and increased chemoresistance[38,39], we reasoned that low MT_1 receptor expression might be a marker for chemo-resistant breast cancer. Thus, we data-mined MT_1 mRNA expression in human breast tumors from women enrolled in the I-SPY 1 TRIAL receiving neo-adjuvant chemotherapy[45]. In the I-SPY 1 trial, patients with locally advanced breast cancer underwent tumor biopsy prior to receiving neo-adjuvant chemotherapy. Following chemotherapy, residual primary tumors were surgically resected and gene expression profiles measured on paired biopsies. Genome-wide expression array data and information on patient outcomes was available for 129 patients. As shown in Fig. 10, MT_1 receptor expression was approximately 5-fold higher in patients who experienced pathological complete response (PCR) (absence of invasive tumor in both breast and axillary lymph nodes in response to neo-adjuvant therapy), than in patients who did not experience a complete response (normalized expression = 0.171 and 0.030, respectively).

DISCUSSION

Intrinsic and acquired chemotherapy resistance, related to adaptive activation of proliferative and survival signaling pathways and other mechanisms is a serious problem for breast cancer patients[48,49]. In this study, we demonstrate that dLAN-induced disruption of the nocturnal circadian MLT signal mediates intrinsic PTX-resistance in breast cancer cells and that activation of the SRC/IL-6/STAT3/DNMT1 pro-survival pathway is critical in this process. As reported by Cos et al.[50] diminution of MLT nighttime levels in response to dLAN is not associated with changes in the rhythm of MLT secretion. Tumor xenografts from female rats exposed to dLAN with their circadian MLT signal almost completely suppressed grew significantly (2.5 to 3-fold) faster than those with a robust circadian MLT signal (LD12:12 and dLAN + MLT groups). Furthermore, xenografts from rats exposed to dLAN showed complete intrinsic resistance to PTX, while those from rats in LD12:12 (endogenous nighttime MLT signal) or receiving exogenous MLT during dLAN showed increased sensitivity to PTX and regressed at a rate of 0.18 ± 0.6 g/day, and parallels our previous

studies employing TAM or Dox[37,38]. The fact that responses to PTX, TAM, and DOX are inhibited by dLAN but promoted by MLT, despite different individual mechanisms of action, suggests a commonality through which MLT suppresses endocrine and chemotherapeutic resistance.

Exposure to dLAN drives the elevated expression of a number of oncogenes (Fig. 3) including HER2, HER3, c-SRC, and STAT3, which are all associated with poor survival in breast cancer patients[51–53]. Shown in Fig. 5, in vitro administration of MLT inhibits SRC-D-mediated induction of pSTAT3 to suppresses STAT3's downstream targets SURVIVIN and FASCIN, members of the Inhibitor of Apoptosis Protein (IAP) family and markers of poor prognosis and chemo-resistance [54–56]. Furthermore, SRC-D MCF-7 cells demonstrated resistance to PTX (data not shown) as previously reported by Hawthorne et al.[57].

Reports show that STAT3 can be activated by both phosphorylation at Y705 and acetylation at K685[23,27,40]. Elevated $_{AC}$STAT3 at K685, seen in many breast tumor biopsies, is reported to contribute to tumor progression by inducing DNMT1-mediated DNA methylation of tumor suppressor genes including ARHI[29–34]. In MCF-7 breast tumor xenografts from rats housed in a dLAN photoperiod, we observed elevated expression of IL-6, $_{AC}$STAT3^{K685} and DNMT1, but diminished levels of ARHI. This data is consistent with reports that IL-6 acetylates/activates STAT3, inducing DNMT1 expression, that then methylates the ARHI promoter[58–61]. In normal breast epithelial cells ARHI is strongly expressed, but is down regulated in approximately 70% of breast tumors[61]; its loss is correlates with tumor progression and resistance to PTX[62,63]. The ability of ARHI to block PTX-resistance has been related to its ability to bind STAT3 and prevent its nuclear localization[64]. Elevated mRNA and protein levels of ARHI induced by MLT in MCF-7 breast cancer cells along with ARHI's ability to inhibit of STAT3 nuclear localization are at least part of the mechanism behind reduced nuclear STAT3 in MCF-7 tumor xenografts exposed to both circadian MLT and MLT plus PTX (Fig. 4).

Important roles of SIRT1 in various biological processes, including apoptosis, metabolism, aging, and circadian biology, have been described[65], but its oncogenic or anti-oncogenic role in breast cancer is mixed[66]. Our data showing MLT induction of SIRT1 expression and SIRT1 deacetylation of STAT3 at K685 in breast cancer xenografts defines a viable mechanism by which MLT inhibits STAT3-mediated PTX-resistance in breast cancer and also defines how dLAN-mediated disruption of circadian MLT via epigenetic mechanisms (DNA-methylation) can promote drug-resistance in breast cancer. Our data that MLT induces SIRT1 expression, but inhibits ERK signaling are consistent with reports that SIRT1 can directly deacetylate MEK to inhibit ERK activation[67]. Future studies are needed to determine if inhibition of pERK1/2 in breast cancer is mediated by SIRT1's deacetylation of MEK.

Marotta et al.[68] described a network of 15 genes, including STAT3, that are required for proliferation in human stem cell-like breast cancer cells. As well, STAT3 appears to be a common denominator in the progression of breast tumors to a chemo-resistant, metastatic phenotype[69]. This study is the first to demonstrate that exposure of cancer host to dLAN can activate STAT3 in breast tumor xenografts via both phosphorylation and acetylation events

and that the presence of the nocturnal circadian MLT signal (endogenous or exogenous) can inhibit dLAN-induced phosphorylation and acetylation of STAT3. Our study supports that elevated expression and activity of the STAT3 oncogene is central for promoting breast tumor progression in response to dLAN and that STAT3, along with HER2/3, c-SRC, DNMT1, SIRT1, and ARHI, are circadian controlled genes/proteins (Fig. 10).

This study shows that treatment of MCF-7 cells with IL-6 induces methylation of CpG island 1 in the ARHI promoter, but not CpG island II, while MLT administration represses IL-6-mediated methylation of CpG island 1. Furthermore, MLT's suppression of ARHI CpG island I methylation can be reversed by the SIRT1-inhibitor EX-527, implicating SIRT1 as a mediator of MLT's suppression of ARHI methylation. Given that disruption of the circadian MLT signal in rats exposed to dLAN leads to activation of the IL-6/STAT3/DNMT1 pathway and decreased expression of the tumor suppressor ARH1 in human breast tumor xenografts, our study defines that dLAN epigenetically regulates ARHI expression to drive PTX resistance in breast cancer, and that the nocturnal circadian MLT signal suppresses the IL-6/STAT3/DNMT1 pathway allowing expression of the tumor suppressor ARHI. The in vitro studies showing that elevated expression of MT1 receptor by TSA administration followed by MLT administration promoted ARH1 protein expression and abolished resistance to PTX in MCF-7/PAC cells, confirm these effects of MLT are mediated via it's MT1 receptor and induction of ARHI protein expression.

We demonstrate for the first time the disruption of the circadian MLT signal by exposure to dLAN results in the expression and constitutive activation of STAT3 through both phosphorylation and acetylation events and that $_{AC}STAT3^{K685}$ induces DNMT1 expression leading to the methylation of the ARH1 promoter and driving PTX resistance. Thus, the presence of the nocturnal circadian MLT signal appears to be critical for maintaining and/or re-establishing PTX-sensitivity in breast cancer cells. Albeit speculative, it is conceivable that elevation of STAT3 levels by exposure to dLAN may also contribute to PTX resistance by stimulating aerobic glycolysis, as STAT3 has been reported to stimulate the Warburg effect and chemo-resistance in breast cancer[38,70–72], and that MLT may mitigate the effects of dLAN on PTX resistance via inhibition of the Warburg effect through down-regulation of STAT3. It is important to point out that this work was done in luminal MCF-7 xenografts and cells, and not in basal breast cancer cells, however, once MCF-7 have become drug-resistant they are no longer luminal, but tend to show a more basal phenotype.

Given that circadian MLT blood values are not included in the clinical workup for breast cancer patients, we data-mined the multi-center I-SPY 1 Trial data set for a relationship between expression of the MT_1 receptor mRNA and pathologic complete response in patients with breast tumors ≥ 3 cm undergoing neo-adjuvant chemo-therapy. The expression of MT_1 receptor mRNA is approximately 5-fold higher in patients that obtained a pathologic complete response versus those who did not achieve a complete response. Given that loss of the MT_1 receptor would be the biological equivalent to loss of the nighttime circadian MLT signal in response to dLAN, these data highlight the clinical importance of MLT and its MT_1 receptor mediated signaling pathway in regulating the progression and chemo-resistance of breast cancer.

In conclusion, our results demonstrate that exposure to dLAN, via its suppression of the nocturnal circadian MLT signal, can function as an environmental oncogenic factor, an "environmental oncogene" if you will, promoting proliferation, progression, and PTX resistance of breast tumors through activation of the STAT3/DNMT1 signaling pathway and inhibition of SIRT1 and ARH1 (Fig. 11). Maximal efficacy and minimal toxicity with PTX therapy appears to be predicated upon its temporal administration in circadian alignment with a patient's own endogenous circadian MLT signal. As most breast cancer patients are subjected to various degrees of LAN as a result of stress, insomnia, and/or night shift work, and thus circadian MLT disruption, via activation of the STAT3 oncogene may account for intrinsic and even acquired PTX-resistance in breast cancer patients. These findings provide novel pre-clinical and clinical knowledge about MLT's role in inhibiting IL-6, STAT3, and DNMT1 in human breast cancer and highlight the critical need to investigate the clinical importance of MLT in combination with PTX for breast cancer treatment.

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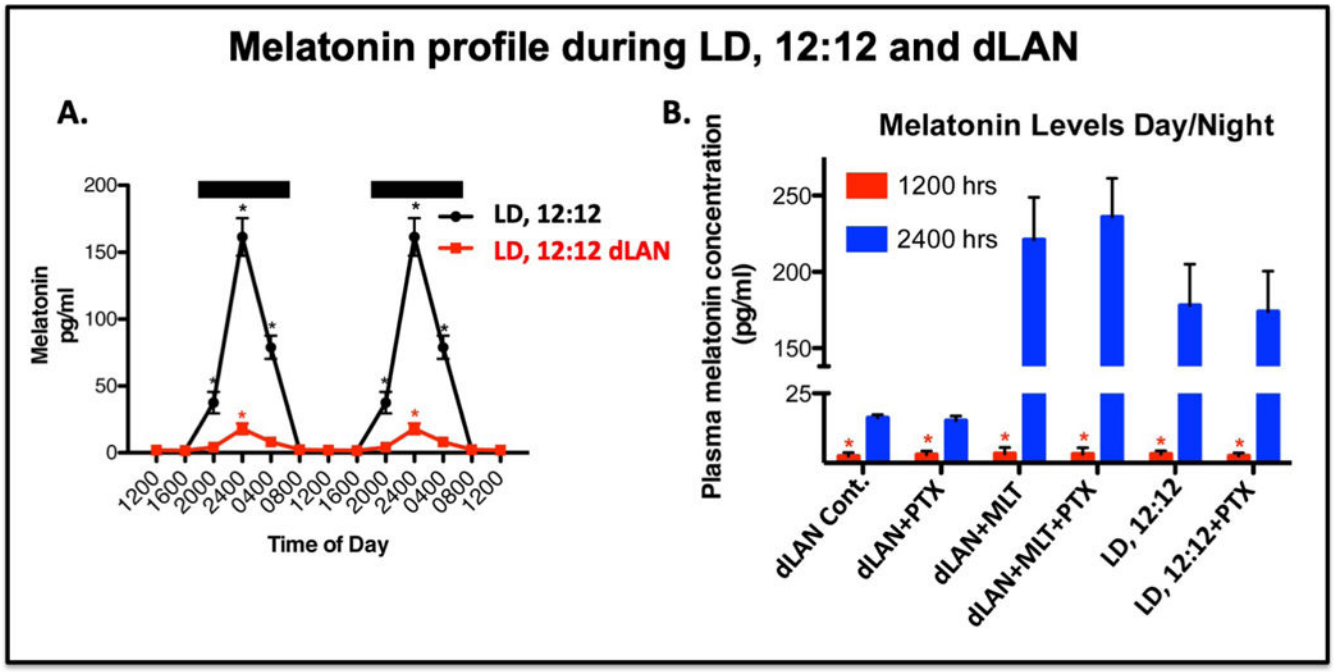

Fig. 1.

Effect of dLAN vs. LD 12:12 lighting schedules or administration of exogenous MLT during the dLAN lighting schedule on serum MLT profile in female nude rats. Female nude rats with (ERα+) tissue-isolated breast tumor xenografts were housed under control (LD, 12:12) or experimental, dLAN (with light at 0.2 lux) lighting schedules, or dLAN and supplemented with nighttime MLT, and treated with diluent or paclitaxel (PTX). (**A**) Diurnal plasma MLT levels (pg/ml; mean ± 1 SD) of female nude rats maintained in a controlled LD, 12:12 or experimental dLAN lighting cycle (n=6/group) were measured as described in "Materials and Methods". Data are double-plotted to better visualize rhythmicity (n = 6/group). Asterisks (*) denote significant differences ($p < 0.05$) in serum MLT levels in rats under the different lighting schedules. (**B**) Plasma MLT levels from dLAN at 1200 hr (red bars) and 2400 hr (blue bars) from animals maintained in dLAN and treated with vehicle (dLAN Cntl) or PTX (dLAN + PTX), or from animals on dLAN but supplemented with MLT (in nighttime drinking water) and treated with vehicle (dLAN+MLT) or PTX (dLAN +MLT+PTX), as described in "Materials and Methods". Significant differences ($p < 0.05$) in serum MLT levels in rats (n=3/group) under the different lighting schedules are denoted by an asterisk (*).

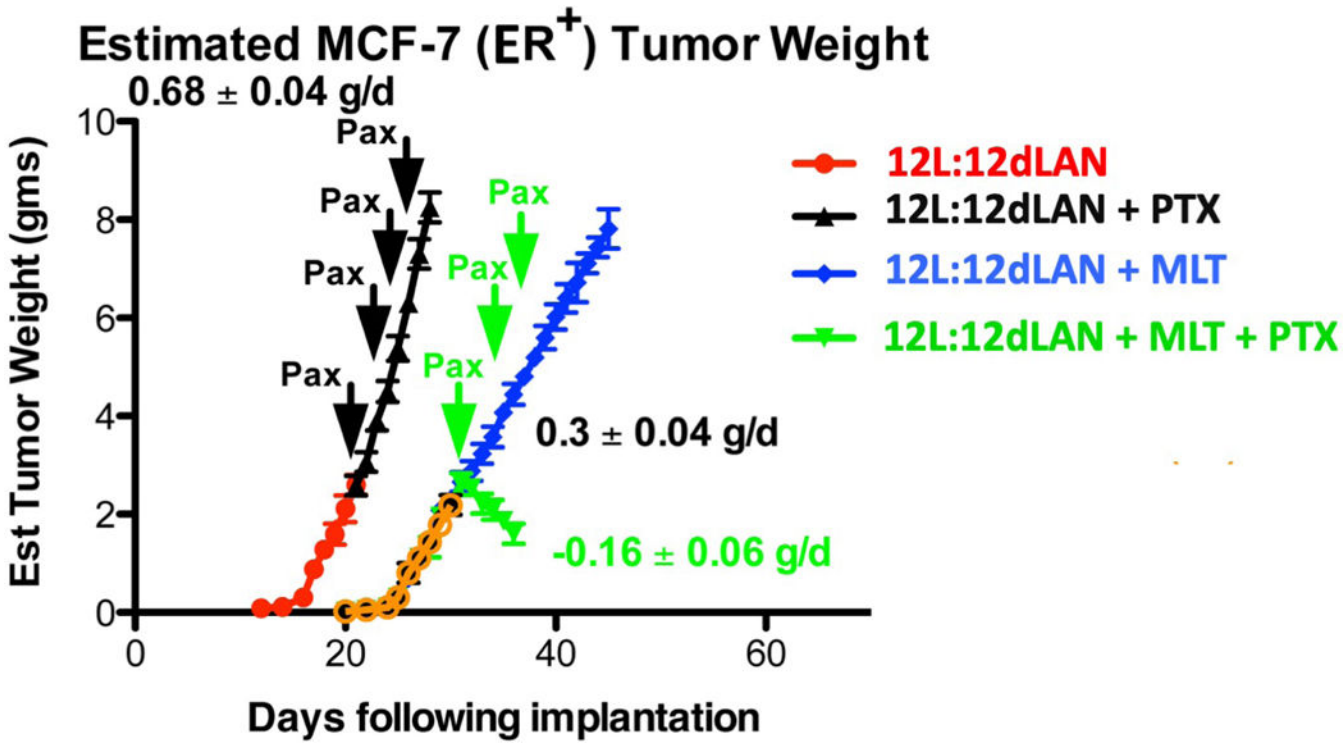

Fig. 2.

Differential effects of PTX on the growth and regression of (ERα+) MCF-7 tissue-isolated breast tumor xenografts in female nude rats housed in a lighting schedule of dLAN, or dLAN supplemented with MLT during dLAN. Estimated tumor weight [g/day] of MCF-7 human breast tumor xenografts from nude rats exposed to a dLAN lighting schedule and treated with diluent (red circles) or PTX (black triangles) [6 mg/kg/day] or a dLAN lighting schedule and supplemented with MLT during dLAN (gold circles, blue diamonds) or dLAN + MLT + PTX (green inverted triangles). Tumor weights were estimated daily as described in “Materials and Methods.”

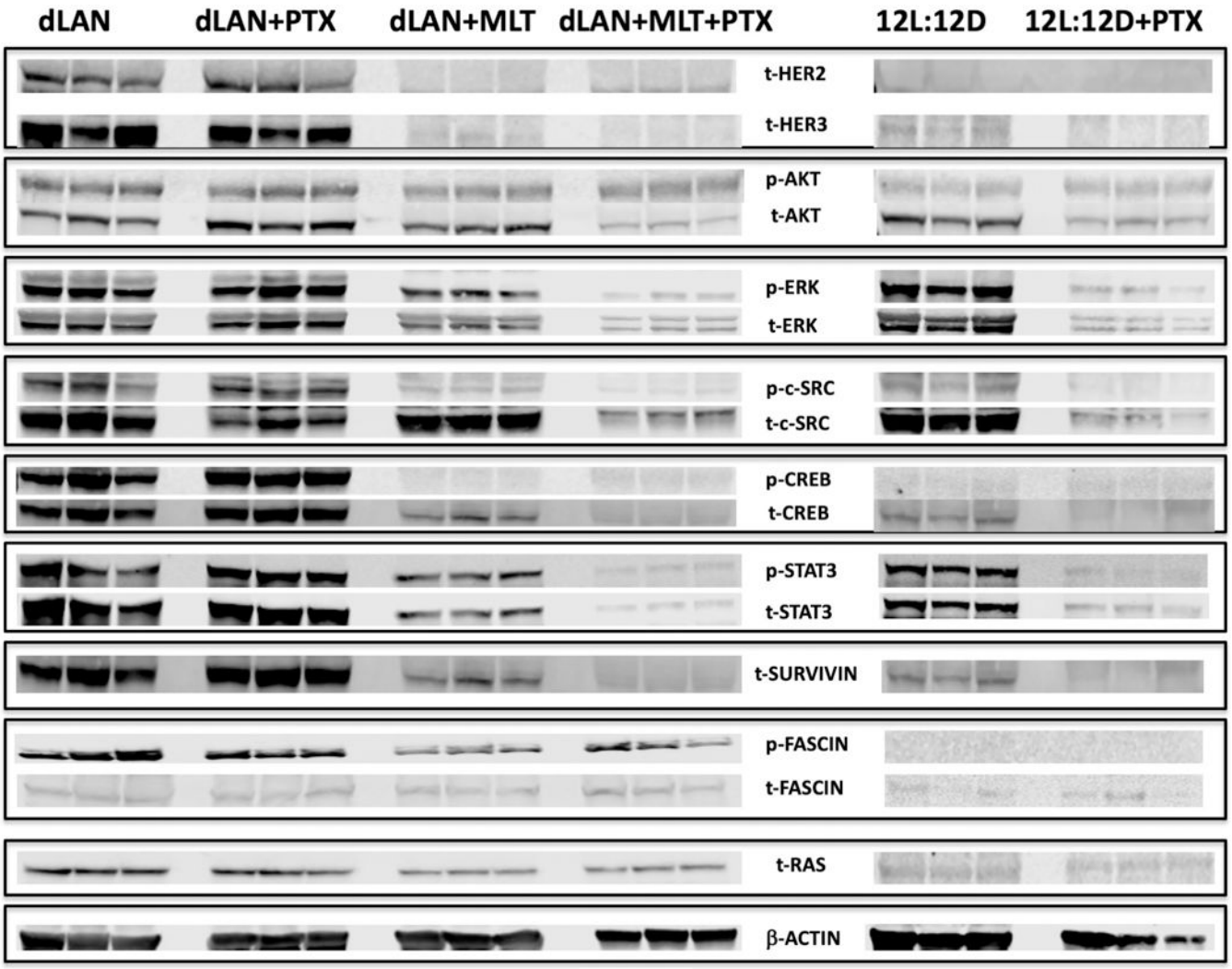

Fig. 3.

dLAN induction and LD, 12:12 or exogenous MLT suppression of cancer-associated signaling kinases and transcription factors in tissue-isolated (ER+) MCF-7 human breast tumor xenografts in female nude rats treated with or without PTX. Western blot analysis of total tissue lysates from tissue-isolated MCF-7 breast tumor xenografts harvested rats in dLAN lighting schedule and treated with vehicle (dLAN), PTX (dLAN+PTX), MLT during dLAN (dLAN+ MLT), MLT plus PTX during dLAN (dLAN+MLT+PTX) or a LD, 12:12 lighting schedule and treated with vehicle (LD, 12:12) or PTX (LD, 12:12+PTX). All tumors were harvested at 2400 hr (mid-dLAN phase) from 3 animals in each group. Total cell lysates (120 μg of protein per sample) from each tumor were analyzed by Western blot for expression of total and/or phosphorylated forms of HER2, HER3, ERK1/2, P AKT, ERK, SRC, CYCLIN D1, DJ-1, CREB, PKCα, and RAS. β-actin was used as a control for equal loading.

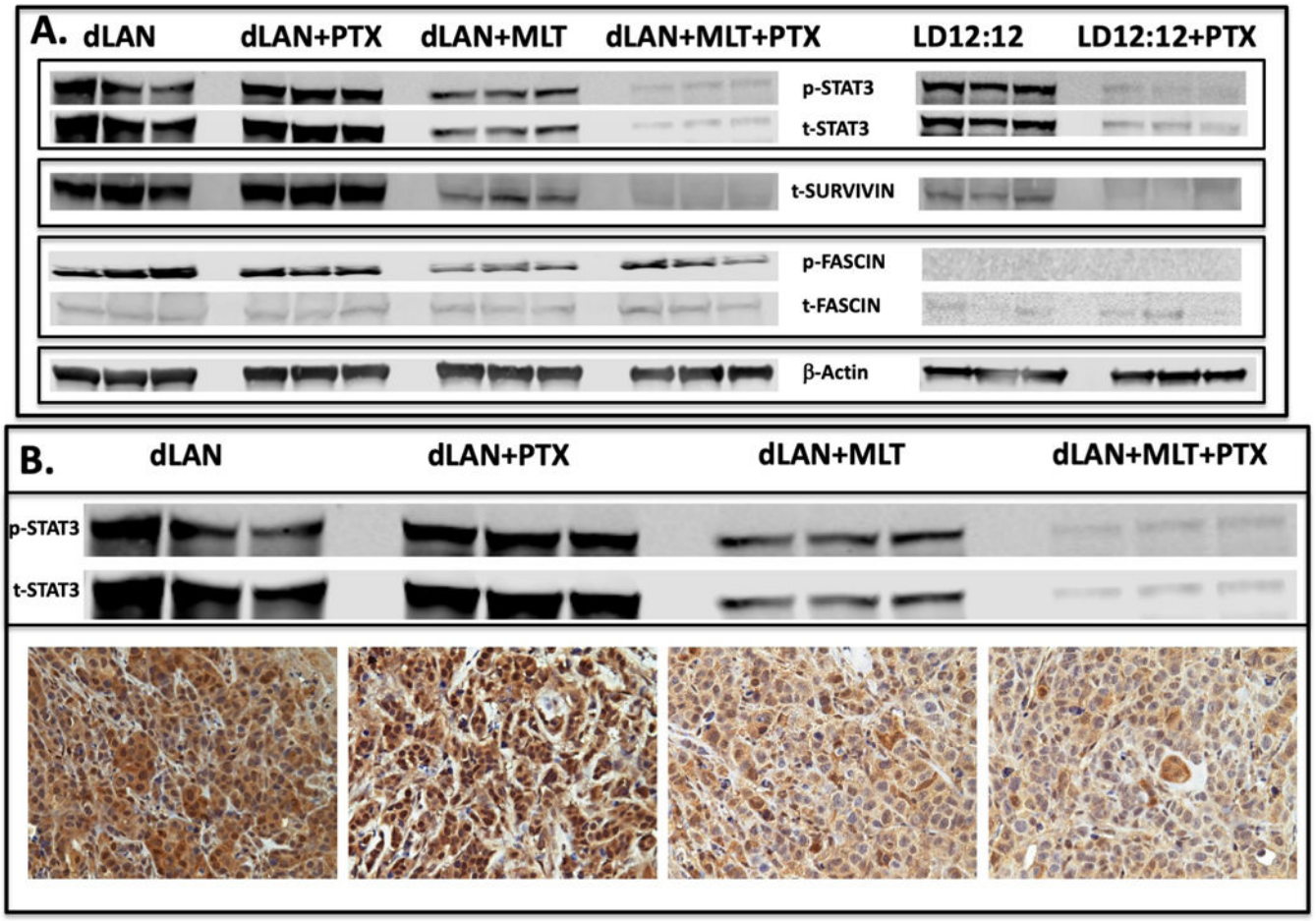

Fig. 4.

Evaluation of STAT3, FASCIN and SURVIVIN expression, and histologic staining in breast tumor xenografts in response to dLAN, MLT, and PTX. Modulation of the STAT3 transcription factor and its downstream targets FASCIN and SURVIVIN in tissue-isolated (ERα+) MCF-7 human breast tumor xenografts from female nude rats housed in a dLAN photoperiod and treated with vehicle (dLAN), PTX (dLAN + PTX), MLT during dLAN (dLAN + MLT), MLT plus PTX during dLAN (dLAN + MLT + PTX) or a LD, 12:12 lighting schedule and treated with vehicle (LD, 12:12) or PTX (LD 12:12 + PTX). (**A**) Western blot of total (t) and phospho (p) STAT3, FASCIN, and t-SURVIVN from total tissue lysates of breast tumor xenografts harvested from rats in each group above. All tumors were harvested at 2400 hr (mid-dLAN phase) from 3 animals in each group. Total cell lysates (120 μg of protein per sample) from each tumor were analyzed by Western blot. β-actin was used as a control for equal loading. (**B**) Characterization of cytoplasmic and nuclear staining of STAT3 in MCF-7 tissue-isolated breast tumor xenografts from dLAN, dLAN + PTX, dLAN + MLT, and dLAN + MLT + PTX treatment groups. Representative IHC of tumor xenografts using anti-STAT3 (Cell Signaling) (1000 x magnification).

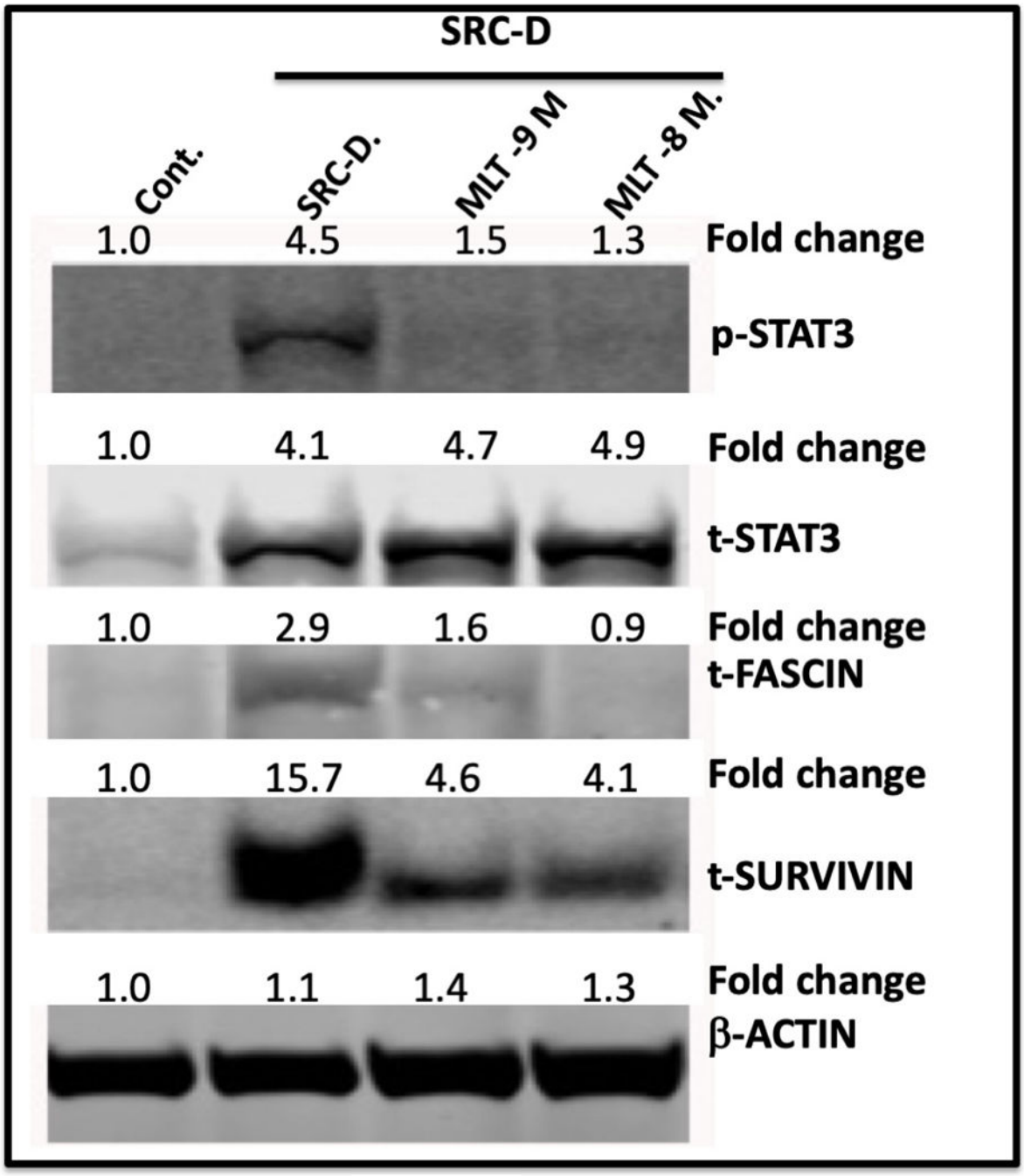

Fig. 5.

Induction of STAT3, FASCIN and SURVIVIN expression by c-SRC and suppression by MLT in MCF-7 breast cancer cells. MCF-7 breast cancer cells stably transfected with the constitutively active SRC-D construct and treated with MLT (10^{-9} M or 10^{-8} M) for 24 h, after which the expression of t-STAT3, pSTAT3, t-FASCIN, and t-SURVIVIN levels were measured by Western blot. β-actin was used as a control for equal loading.

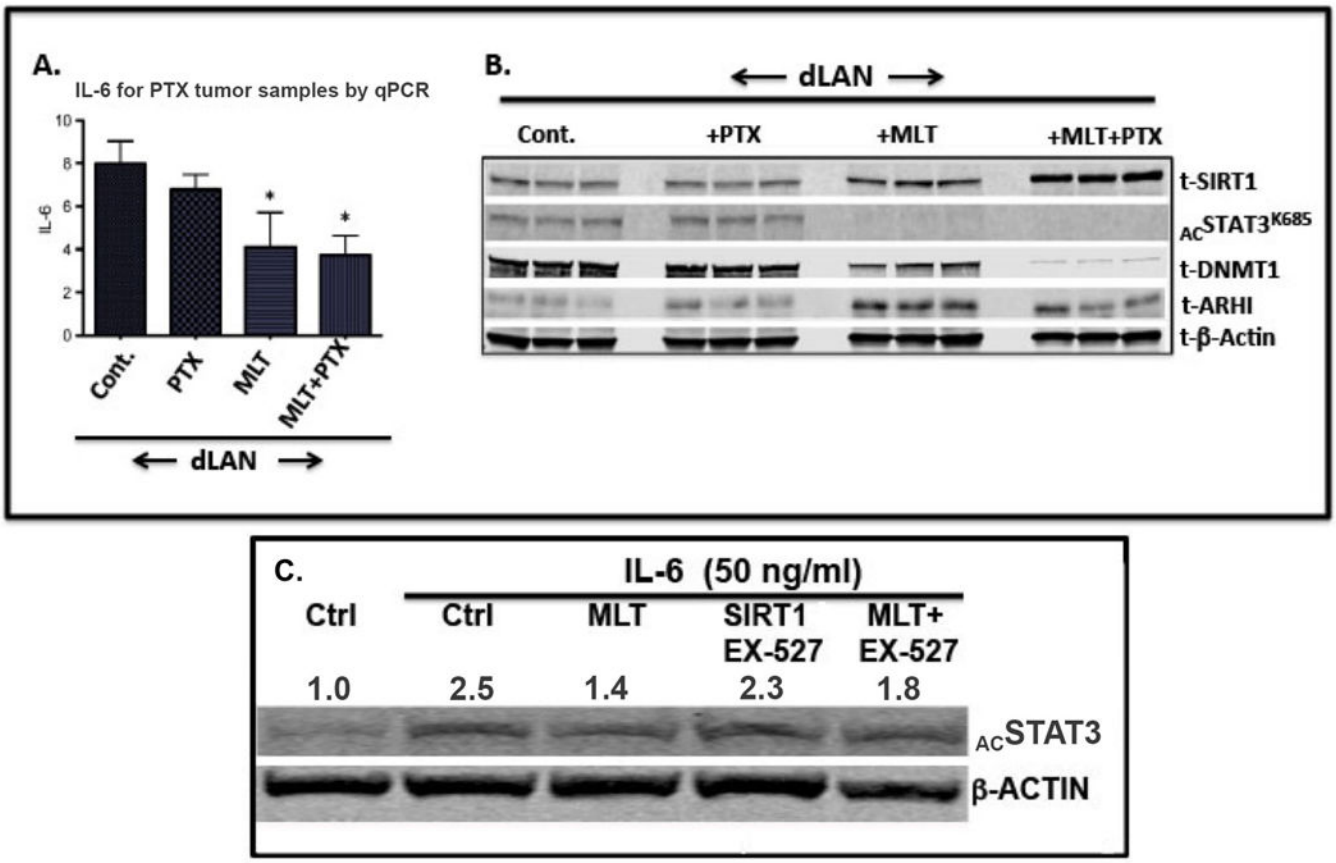

Fig. 6.

Modulation of the cytokine IL-6, acetylated STAT3, DNMT1, SIRT1, and the tumor suppressor ARHI by dLAN and MLT. (**A**) Expression of IL-6 mRNA in tissue-isolated (ER +) MCF-7 human breast tumor xenografts from female nude rats in the dLAN, dLAN+PTX, dLAN+MLT and dLAN+MLT+PTX groups as measured by quantitative real-time PCR. (**B**) Western blot analysis of total tissue lysates from tissue-isolated MCF-7 breast tumor xenografts harvested rats in the dLAN, dLAN+PTX, dLAN+MLT and dLAN+MLT+PTX groups. All tumors were harvested at 2400 hr (mid-dLAN phase) from 3 animals in each group. Total cell lysates (120 μg of protein per sample) from each tumor were analyzed by Western blot for expression of STAT3 acetylated at K685 ($_{AC}STAT3^{K685}$) and DNMT1, SIRT1, ARHI. β-actin was used as a control for equal loading. (**C**) Western blot analysis of STAT3 acetylation at K685 in MCF-7 total cellular protein harvested following treatment with IL-6 (50 μg/ml), IL-6 + MLT (10^{-9} M), or IL-6 + MLT + EX-527 (0.5 μM) for 24 h. Total protein (120 μg per sample) from each treatment group was analyzed by Western blot for expression of STAT3 acetylated at K685 ($_{AC}STAT3^{K685}$). β-actin was used as a control for equal loading.

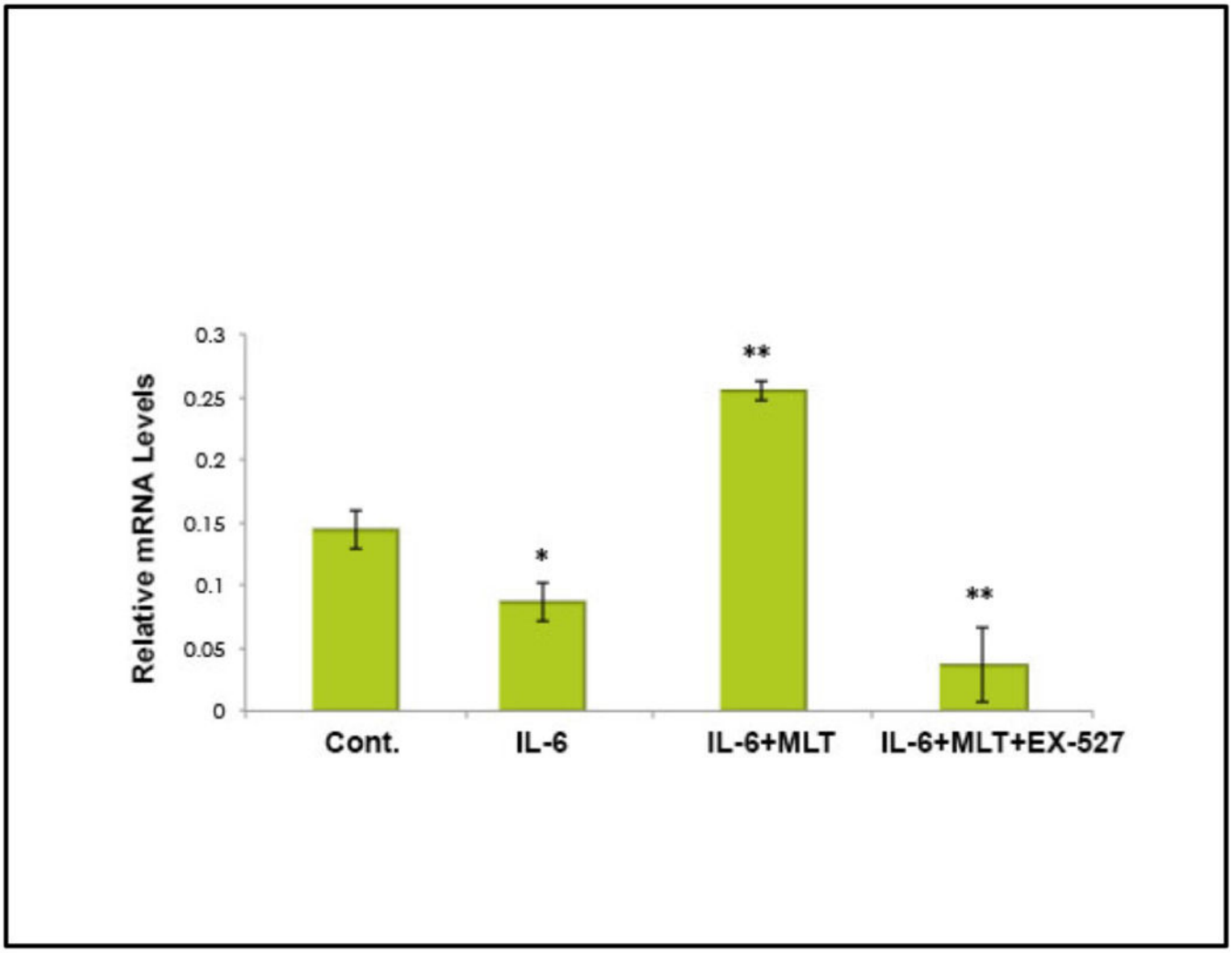

Fig. 7.

Induction of STAT3 acetylation by IL-6 and inhibition by MLT and SIRT regulate the in vitro expression of ARH1 mRNA in breast cancer cells. Following treatment of MCF-7 cells in vitro with IL-6 (50 ng/ml), IL-6 + MLT (10^{-9} M), and IL-6 + MLT + the SIRT1-inhibitor EX-527 for 48 h. Total RNA was extracted by using the TRIzol eagent and real time PCR (qPCR). All PCR analyses were performed in triplicate in a volume of 20 μl, using 96-well optical-grade PCR plates and optical sealing tape (Bio-Rad). Differences in the expression of the ARHI transcripts were normalized with respect to GAPDH expression.

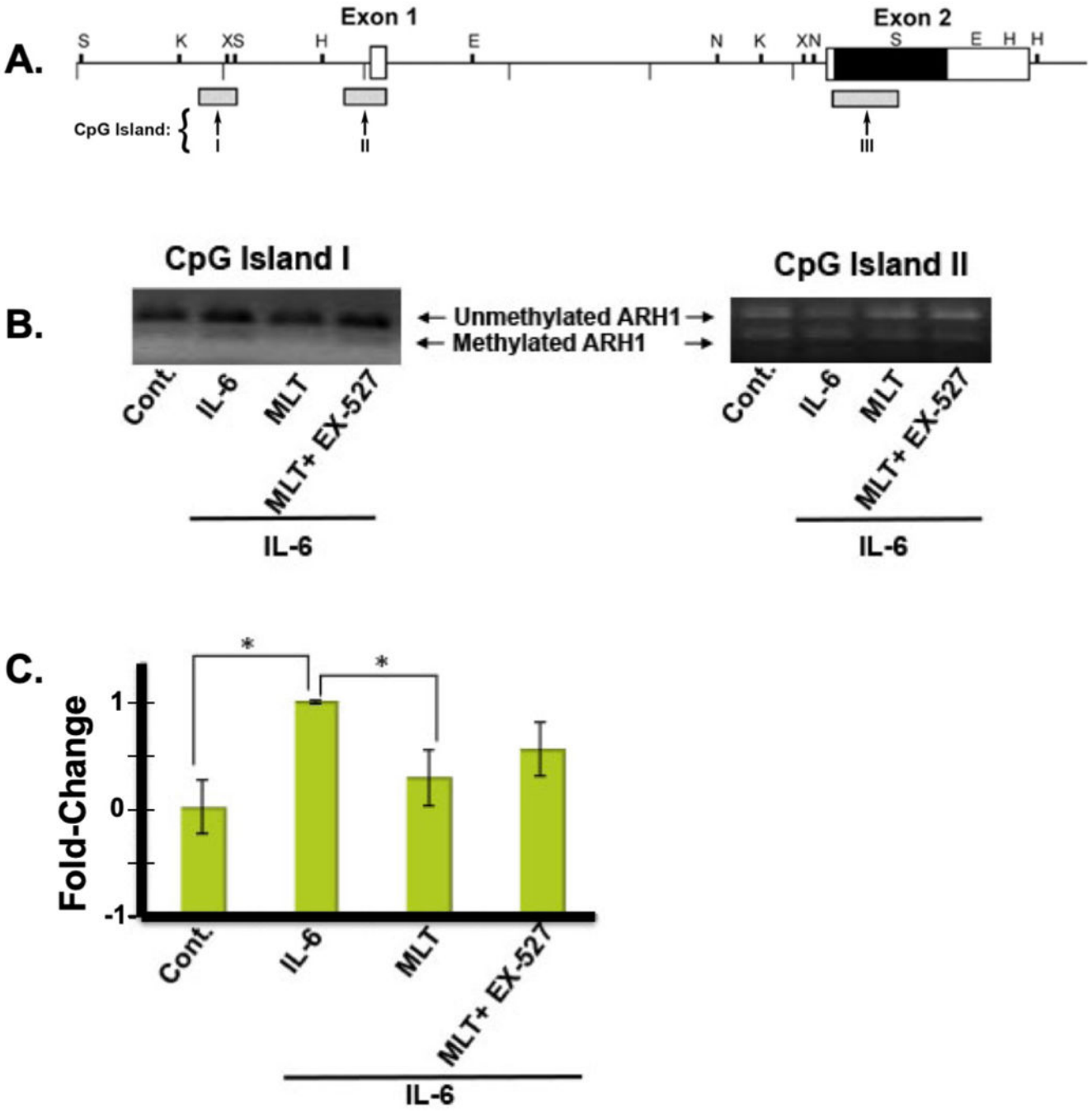

Fig. 8.

Induction of ARHI promoter methylation by IL-6 is suppressed by MLT and SIRT1 in MCF-7 breast tumors. (**A**) A schematic overview of CpG islands I, II, and III (denoted by hatched boxes) in the promoter and Exon2 region of the ARHI gene. (**B**) The methylation of CpG islands I and II in the ARHI promoter of MCF-7 cells were analyzed using Combined Bisulfite Restriction Analysis (COBRA) with previously described in Materials and Methods. in all treatment groups (Cont., IL-6, IL-6 + MLT, and IL-6+MLT+ EX-527). The DNA methylation status of the CpG island I of the ARHI promoter in tumors from Control,

IL-6, IL-6 + MLT, and IL-6+MLT+ EX-527 treatment MCF-7 breast cancer cells were analyzed. Graphic analysis (**C**) of methylation of CpG I showing that IL-6 promotes the methylation of CpG island I in the ARHI gene, that MLT administration can significantly ($p<0.01$) suppress IL-6-mediated methylation of CpG island I, and that the SIRT1 inhibitor EX-527 can reverse the effects of MLT on the methylation if CpG island 1. No significant effects were observed in the methylation of CpG island II. Methylated and non-methylated bands are noted by the arrows.

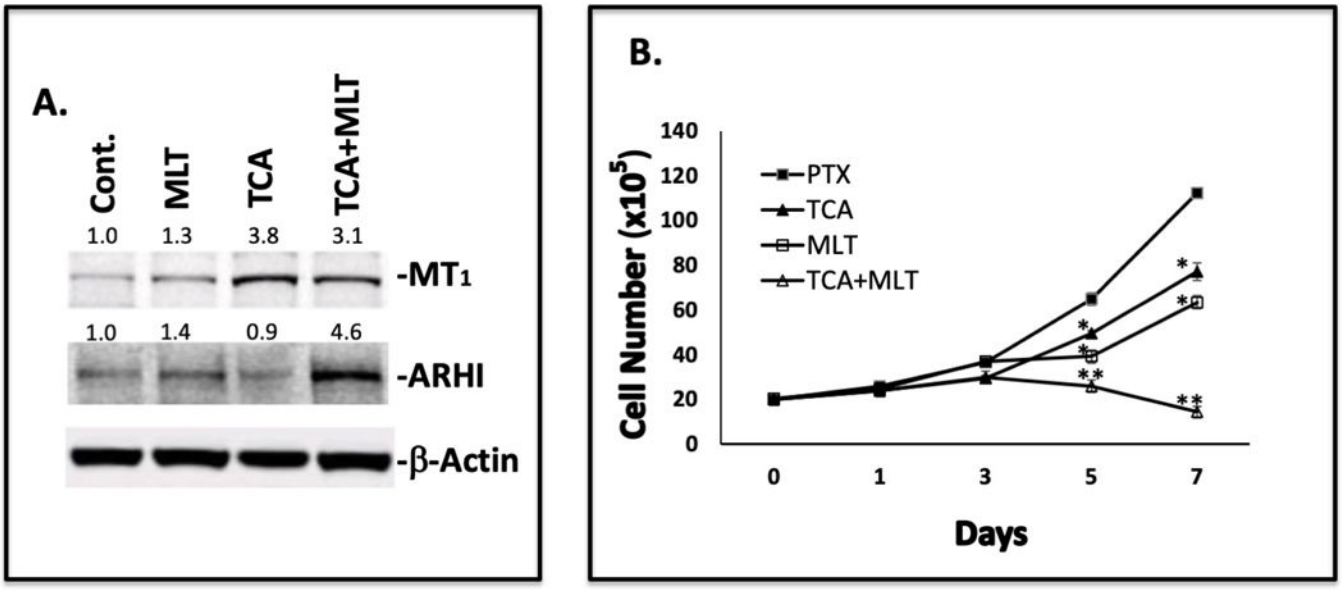

Fig. 9.

Induction of MT1 receptor and ARHI expression in MCF-7 breast cancer cells in response to trichostatin A and MLT, and the impact of elevated MT1 receptor and MLT on PTX-resistant MCF-7 breast cancer cells. (**A**) MCF-7 breast cancer cells were administered TSA (0.7 μM), MLT (10 nM), or TSA + MLT for 24 h after which the expression of MT1 and ARHI protein levels were measured by Western blot. β-actin was used as a control for equal loading. (**B**) MCF-7/PAC cells were plated at a density of 20 × 10^5 cells per ml in six-well plates and 5 hours after seeding, cells were treated with PTX (5 μM/ml), PTX, TCA (0.7 μM), MLT (10 nM), or TCA + MLT. On specific days total and viable cells counted on a haemocytometer. n = 3 independent studies, and * - $p<0.05$ and ** - $p< 0.005$

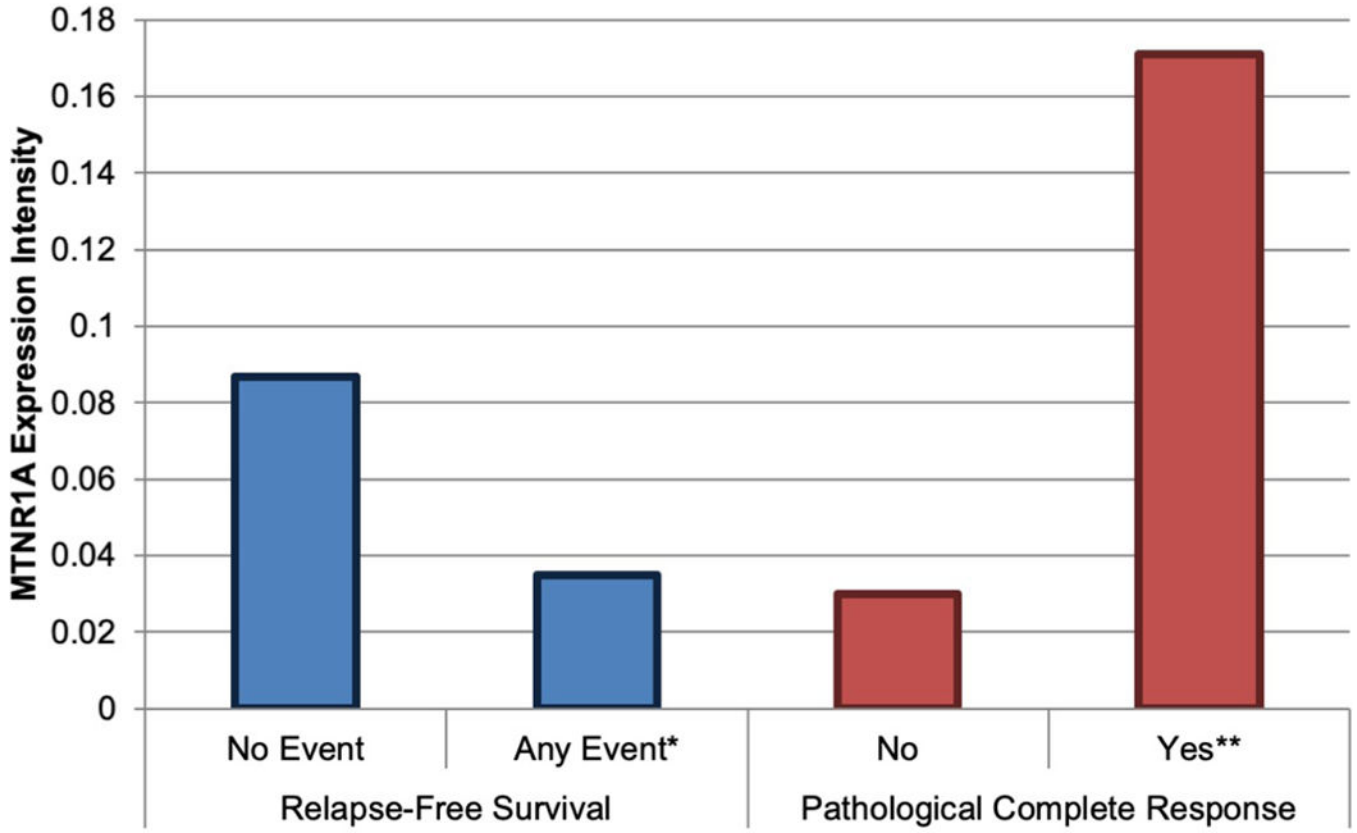

Fig. 10.

I-SPY1 trial, association of MT_1 expression levels with 5-yr pathological complete response and relapse-free survival. Genome-wide expression array data on pre-treatment tumor samples, linked with complete clinical outcome data was available for 129 patients. In patients who achieved pathological complete response (absence of invasive tumor in both breast and axillary lymph nodes in response to personalized neo-adjuvant therapy), MT_1 receptor expression was approximately 5-fold higher than in patients who did not achieve a complete response (normalized expression = 0.171 and 0.030, respectively). In addition, MT_1 receptor expression was associated with better relapse-free survival, with lower melatonin receptor levels in patients experiencing any event (local progression, distant progression or death) during the study period, and higher levels among patients experiencing no event (0.035 and 0.087, respectively).. Given that loss of MT_1 would be equivalent to the loss of the nighttime circadian MLT signal in response to dLAN, these data provide compelling support for the clinical importance of MLT and its MT_1 receptor in suppressing drug resistance and metastasis of advanced breast cancer.

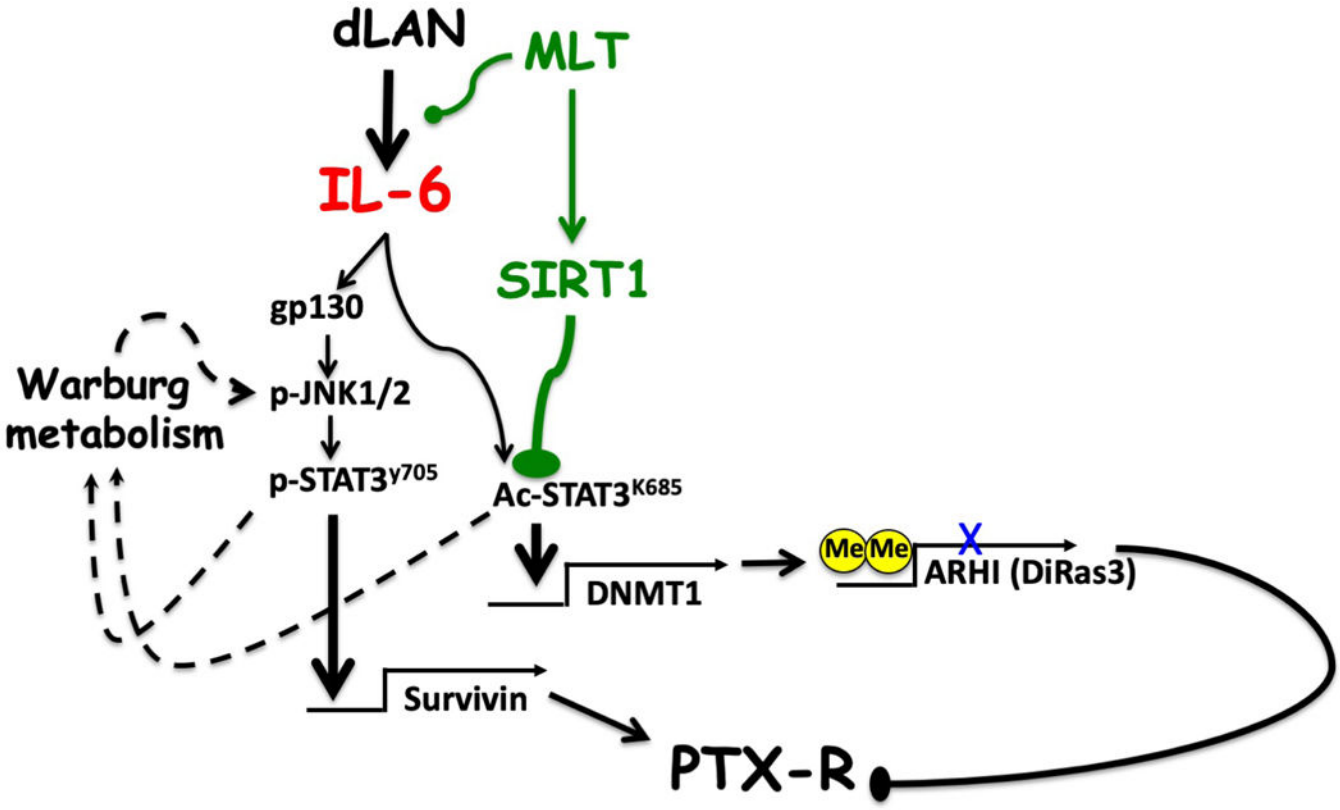

Fig. 11.

Diagram of pathways by which dLAN regulates resistance to paclitaxel via activation of the IL-6/STAT3/DNMT1 pathway to methylate the ARHI promoter and inhibit ARHI mRNA expression, and how circadian MLT can induce SIRT1 to block the IL-6/STAT3/DNMT1 pathway to allowing ARHI expression and paclitaxel sensitivity in breast cancer. Included in this diagram is the interplay between STAT3's reported induction of aerobic glycolysis in tumor metabolism (Warburg effect) and the ability of the Warburg effect to also drive expression of the activation of the STAT3 oncogene.

Table 1.

Tumor cAMP levels, ^{3}H-thymidine incorporation into tumor DNA, and tumor DNA content *in vivo* during dark phase (2400 – 0400 hrs) of MCF-7(SR^{+}) human breast tumor xenografts maintained in 12L:12dLAN-Diluent Controls, -Paclitaxel (4μg/Kg), –Melatonin (1.7μg/d), -Paclitaxel + Melatonin, 12L:12D Controls, and 12L:12D-Paclitaxel. Values are means ± SD (n = 3/group).

Group Conditions (3/group)	cAMP (nmol/g tissue)	^{3}H-Thymidine Incorporation (dpms/μg DNA)	DNA Content (mg/gm)
12L:12dLAN Controls (2400 hrs)	1.87 ± 0.24	69.0 ± 1.8	3.79 ± 0.20
12L:12dLAN+Paclitaxel (2400 hrs)	1.72 ± 0.24	70.4 ± 2.7	3.81 ± 0.19
12L:12dLAN+MLT (2400 hrs)	0.07 ±0.03[a]	7.0 ± 1.0[a]	2.17 ± 0.10[a]
12L:12dLAN+PX+MLT (2400 hrs)	0.08 0.02[a]	6.7 ± 1.0[a]	2.12 ± 0.20[a]
12L:12D Controls (2400 hrs)	0.08 0.03[a]	7.0 ± 1.0[a]	2.27 ± 0.20[a]
12L:12D + PX (2400 hrs)	0.07 0.03[a]	6.9 ± 1.4[a]	2.35 ± 0.20[a]

[a]P< 0.05 vs. dLAN Control group and dLEN+PAX+MLT group.