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Extracellular Vesicle-Mediated Reversal of Paclitaxel Resistance in Prostate Cancer

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Abstract

Prostate cancer (PCa) is the most common solid tumor in males and the second leading cause of cancer-related deaths in males in the United States. The current first line therapy for metastatic PCa is androgen deprivation therapy and is initially effective against the disease. However, castrate resistant prostate cancer (CRPC) develops in many men within 18–36 months, rendering this treatment ineffective. Chemotherapy, with a class of drugs known as taxanes is the standard-of-care cytotoxic option in metastatic castrate resistant PCa (mCRPC). However, the overall survival advantage for chemotherapy in mCRPC is only 2.2 months and the cancer cells often become resistant to these drugs as well. Once patients fail chemotherapy the progression to death is inevitable. Extracellular vesicles (EVs) are involved in cell signaling and play a role in cancer progression. Previous work has demonstrated that EVs are involved in the development of drug resistance in cancer cells. We report the reversal of taxane resistance and tumorigenic phenotype in PCa cells after EVs treatment. This study suggests that EVs represent a potentially novel therapeutic treatment option for CRPC.

Keywords

extracellular vesicles; prostate cancer; drug resistance; paclitaxel

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I. INTRODUCTION

A. Prostate Cancer

Prostate cancer (PCa) is the most common malignancy in men and the second leading cause of death in males living in developed countries.[1] The prostate is an exocrine gland in the male reproductive system that contributes about 50–70% of the semen volume.[2] The prostate is composed of luminal and basal epithelial cells surrounded by stroma.[3] Both of these cell types may become cancerous. The etiology of PCa is still not entirely known, but suggested risk factors are age, ethnicity, and family history of Pca.[4–6]

The identification of the prostate specific antigen (PSA) has greatly increased early detection of Pca.[2] However, PSA screening does not differentiate between benign or malignant PCa due to the test's poor specificity. Localized PCa is typically managed with surgery or radiation therapy options. Active surveillance has recently gained great momentum for low risk disease. Nevertheless, many of patients treated for localized PCa will eventually recur and progress. Notably, the five- and 10-year survival rates for patients diagnosed with localized PCa are 100% and 98%, respectively.[7]

There are limited treatment options for advanced PCa. In the early stages of the disease, the cells require androgens to grow. The androgen receptor (AR) of prostate cells responds to androgens produced by the body. AR signaling is required for proper development of the prostate such as cellular differentiation and gland formation. However, as PCa develops, the once beneficial AR signaling becomes a deadly driver of disease. The most effective method of treating advanced PCa is androgen deprivation therapy. However, after only 18–36 months of treatment, most patients become androgen insensitive, also known as castrate resistant prostate cancer (CRPC). In most cases of CRPC, there has been an amplification of the AR gene. Thus, the cancerous cells are able to respond to sub-physiologic levels of androgens due to the abundance of receptors.[3] As a result, androgen deprivation therapy is no longer a viable treatment option for these patients, and alternative and usually more toxic treatments are required.

CRPC is difficult to treat and can be fatal for prostate cancer patients. The next common courses of treatment for CRPC are secondary endocrine manipulations, immunotherapies, or chemotherapy with taxanes. Cytotoxic taxanes are the standard of care for advanced PCa. However, all patients eventually develop resistance to taxanes, resulting in cancer progression. Thus, a mechanism to inhibit development of resistance to taxanes would have a significant impact on therapy of men with PCa. The molecular mechanisms of taxane resistance are still being elucidated. Clusterin, B-cell leukemia/lymphoma 2 (Bcl2), AR modulation, and multidrug resistance (MDR) proteins are just a few of the known molecular pathways involved in CRPC drug resistance.[1] Taxane-resistant prostate PCa cell culture cell lines are representative of primary and metastatic tumors as well as androgen sensitivity and resistance in PCa. These PCa cell lines are important to further our understanding of how to reverse drug resistance in CRPC.

B. Extracellular Vesicles

Extracellular vesicles (EVs) are secreted by all cells and contain bioactive molecules, RNA, DNA, and proteins. EVs were initially thought to be at most cellular debris. However, additional research has shown they play an important role in intercellular signaling. Cells excrete EVs into the extracellular environment and they are taken up by other cells. EVs is a broad term that encompasses a wide variety of vesicles released by the cell. Exosomses, microvesicles (MVs), and apoptotic bodies are specific types of EVs secreted by cells. The difference is based on size and origin within the cell.[2] Only exosomes and MVs are of importance for the cell communication discussed within this paper. Exosomes are 40–100 nm in diameter and originate from inward budding of endosomal membranes, which causes the accumulation of intraluminal vesicles. These intraluminal vesicles are either degraded by the lysosome or released as exosomes. Microvesicles, on the other hand, are 100–1000 nm in diameter and originate from the blebbing of cytoplasm.[8]

Identification and proper isolation of EVs are major challenges in the field. It is known that cells release exosomes, MVs, and apoptotic bodies. However, the nomenclature is imprecise due to the lack of uniformity in the field. Furthermore, there is a wide array of terms used to describe EVs such as oncosomes, exosomes-like vesicles, nanoparticles, and microparticles.[9] For the remainder of this paper, MVs and exosomes will be referred to as EVs.

The role of EVs in signaling has been demonstrated both *in vivo* and *in vitro* in different cell populations. The immune system, for example, uses exosomes to stimulate or inhibit white blood cells during antigen presentation and immune tolerance.[10,11] Meanwhile, the nervous system's microglia and oligodendroglial cells use EVs to communicate and support axons, respectively.[12,13] Most research on EVs has been conducted in cancer because they have a significant impact on cancer progression.

Cancer cells secrete more EVs than normal cells.[14] However, the reason for the increase in EV production is currently unknown. EVs are involved in tumor angiogenesis, immune suppression, drug resistance, and metastasis—important processes for cancer progression and development.[15] Additionally, the content of EVs may explain their role in cancer. EVs contain caspase 3, an apoptotic enzyme, which at a certain intracellular concentration can lead to apoptosis. Therefore, by depositing caspase 3 in EVs, cancer cells can escape apoptosis.[16] EVs have also been shown to contain Fas ligand, which can induce apoptosis in T cells. As a result, EVs released from cancer cells containing Fas ligand may inhibit T-cell mediated destruction.[17] Cancer cells are able to remove drugs in a similar fashion. Tumor cells treated with doxorubicin produced EVs containing the drug, thus preventing any cytotoxic effect on the cell.[18] Another important step in cancer development is angiogenesis, the creation of blood vessels. EVs are rich in pro-angiogenic factors such as epithelial growth factor receptor (EGFR), which stimulates pathways to create new blood vessels.[19,20] These examples provide insight into EV function in cancer.

Cancer EVs may be a new diagnostic tool and offer new therapeutic treatment options. As stated previously, in prostate cancer the PSA test is a great prevention tool, but it lacks specificity. There is a breadth of research describing the amount of bioactive molecules and

proteins in EVs. This information may lead to the use of EVs as a new biomarker in disease progression. Since EVs are a significant step in cancer progression, blocking them could be a new treatment option. Their signaling effect may also be used to help mitigate progression of cancer.

C. Prostate Cancer and Extracellular Vesicles

PCa cells excrete EVs into the extracellular environment, similar to other cancerous cells. Most of the research on PCa and EVs are from *in vitro* studies, thus the evidence is only a start to understanding the interaction of PCa and EVs. DU145 is a human prostate carcinoma cell line that shows the effect of EVs on the tumor microenvironment. EVs isolated from DU145 cells are able to transform the phenotype of a nonmalignant human prostate epithelial cell line. The nonmalignant cells, after coculture with the EVs, grew in soft agar, which is a classic sign of malignancy.[2] Noncancerous cells require adhesion signals to grow and divide. The absence of this signal causes the cells to die. However, cancer cells do not require adhesion signals. Metastatic cancers must gain the ability of anchorage independent growth to survive as it spreads to different organs in the body. Soft agar is the simplest way to test the malignant potential of cancer cells *in vitro*. Another EVs experiment in PCa demonstrated the ability of nonmalignant EVs to stop the growth of malignant cells in soft agar.[2] The role of EVs in cancer cell-to-cell communication exposes entirely new avenues of cancer treatment.

EVs are involved in the development of drug resistance in PCa. DU145 cells are sensitive to camptothecin (CPT), a chemotherapeutic, and undergo CPT-induced apoptosis. EVs isolated form RC1, a PCa cell line resistant to CPT, caused the DU145 cells to become resistant to apoptosis induced by the drug. Meanwhile, the EVs from the sensitive DU145 cells caused the RC1 cells to become sensitive to CPT-induced apoptosis.[21] Another study used docetaxel-resistant prostate cancer cell EVs to confer resistance to sensitive prostate cancer cells. The results of these studies demonstrate the transfer of drug resistance mediated by EVs. It addition, EVs altered the motility, invasion, proliferation, and anchorage independent growth of the cells.[22] This evidence highlights the role of vesicles in drug resistance in PCa cells.

D. Paclitaxel

Paclitaxel (Taxol) was first discovered in the 1970s by a National Cancer Institute screen of natural compounds.[23] This compound occurs naturally and was isolated from bark of the Pacific yew tree (*Taxus brevifolia*). However, due to the scarcity of the natural Pacific yew tree and hypersensitivity reactions, paclitaxel production was halted for a decade. In the interim, there was a search for other taxane compounds with similar effects. A synthetic form of paclitaxel, created a decade later, solved the availability problem. In the 1980s, a new taxane was discovered called Docetaxel (Taxotere).[24] Docetaxel is a semisynthetic product isolated from the European yew tree (*Taxus Baccata*).[25,26] The taxanes are similar chemically and have the same effect on cells, demonstrating efficacy against many types of solid tumors.[24]

Paclitaxel is classified as an antitumor drug and microtubule-disrupting agent. The drug binds to tubulin and stabilizes microtubules during cell division. Therefore, the cell cycle is stopped at the metaphase-anaphase boundary because the metaphase plate does not form properly due to microtubule stabilization.[27,28] In a noncancerous cell, the mitotic checkpoints function properly so the cell will stop dividing. A cancerous cell, however, does not respond correctly to the checkpoints and continues to divide. The stabilized microtubules are incompatible with cell division, thus the cell undergoes apoptosis. Through this mechanism paclitaxel is able to selectively kill cancer cells without harming noncancerous cells. Low doses of taxanes also destroy endothelial cells in the tumor environment, thereby preventing tumor cell vasculogenesis.[29,30]

Here, we report the extracellular vesicle mediated reversal of Paclitaxel resistance in PCa. EVs isolated from nonmalignant prostate and human mesenchymal stem cells (hMSC) were able to reverse resistance of DU145 paclitaxel-resistant cells. This result has significant implications for future therapeutic uses of EVs in PCa treatment.

II. EXTRACELLULAR VESICLE-MEDIATED REVERSAL OF DRUG RESISTANCE IN PROSTATE CANCER

Chemotherapy plays an increasingly important role in the management of CRPC. Chemoresistance is an inherent problem, as half of all patients that receive chemotherapy will inevitably gain resistance to the treatment.[1] It has been shown that resistance to paclitaxel (Px) in taxane-resistant DU145 cells is due, in part, to the overexpression of transporter proteins such as P-glycoprotein (MDR-1/P-gp).[31] We hypothesized that in addition to P-gp, the resistance to paclitaxel may be due to the release of EVs.

To investigate the effect of EV-mediated reversal of chemoresistance in PCa, we used DU145 cells. The parent cell line DU145 undergoes apoptosis when exposed to paclitaxel. A DU145 paclitaxel-resistant (DU145 PxR) cell line was created by prolonged exposure to Px.[31] The DU145 control and DU145-PxR cell lines were cocultured with the purified RWPE EVs from the RWPE cell line, a human noncancerous prostate epithelial cell line. The cocultures were grown for seven days after which some of the cells were exposed to paclitaxel for 24 h. The effect of paclitaxel on cell viability was measured by the MTT assay, which uses a bioreductive compound only metabolized by living cells. The absorbance values of the well are directly proportional to the number of living cells in culture. As expected, DU145 PxR control cells did not die by Px induced apoptosis since these cells were grown to be resistant to the drug (Fig. 1). The DU145 PxR and EVs showed increased sensitivity to Px in a dose-dependent manner (Fig. 2). The results indicate a phenotypic reversal of drug resistance due to the effects of the EVs. As expected, parental DU145 were sensitive to the growth inhibitory effects of Px (data not shown).

To better understand the effect of EVs from other cell types, the DU145 PxR cells were cocultured with EVs from hMSC using the same procedure and experimental approach. After coculture with hMSC EVs, the DU145 PxR cells were exposed to different doses of Px and cell death was measured by the MTT assay. The resistant cells showed a significant amount (>50%) of Px induced apoptosis in a dose-dependent manner (Fig. 3). The results

demonstrate the ability of a human nonprostate cell line's EVs to reverse the drug resistance phenotype and indicate the potential therapeutic properties of hMSC EV.

Anchorage independent growth, also referred to as soft agar colony formation, is the most stringent *in vitro* assay to measure tumorigenic properties of cells. We measured soft agar colony formation in DU145 PxR cells after coculture and treatment with Px (Fig. 4). Our results indicate that hMSC EV treatment was able to significantly inhibit soft agar colony formation.

III. CONCLUSIONS

The experimental conditions require further investigation to fully elucidate the cause of the reversal of drug resistance. The next step in this experiment will be to analyze the proteins involved in the Px induced apoptosis of the cells. We hypothesize that apoptotic proteins are involved such as poly ADP ribose polymerase (PARP) and caspase 3. PARP repairs single-stranded breaks in the DNA. An initial step of apoptosis is to cleave PARP, thus cleaved PARP is a classic indicator of apoptosis. Caspase 3 belongs to a family of proteins, called caspases, which cause apoptosis. Caspase 3 along with caspase 7 can lead to all the features of apoptosis on their own.

Further protein analysis is necessary on the P-gp/MDR transporter proteins of the experimental cells. As stated previously, it is known that the Px resistance of DU145 cells is a result of MDR overexpression. Therefore, it is logical to assume that the reversal of the drug resistance is due to a change in MDR expression. A decrease in the ability to pump the drug out of the cell would lead to higher cytotoxicity and result in more cell death. In addition, we will analyze the transporter channels of the cells using flow cytometry. The difference in drug efflux pumps is a known difference between the control and drug-resistant cell lines. An analysis of the cell after exposure to EVs will highlight any physical changes in the transporter proteins of the cells. Hoescht FACS is another technique we will use to analyze the membrane permeability of the cells.

The effect of the EVs on anchorage independent growth is important for understanding the phenotype change. DU145 cells are a metastatic human prostate cancer cell line and will exhibit invasive growth in soft agar. Nonmalignant cells require adhesion signals in order to proliferate. However, malignant cells do not and are able to grow in a suspended medium. The results of the MTT assay showed the reversal of drug resistance by EVs, but it is unknown if the EVs have changed other phenotypic characteristics of the cells. The soft agar experiment has demonstrated the ability of EVs to change multiple phenotypes of a cancer cell during exposure.

The most important questions for the future of EVs are proper isolation and identification of vesicle contents to know what type of vesicle is being transferred. Exosomes and MVs are similar, but molecularly different. Further standardization is needed to be able to successfully classify the exact EV populations that are being transferred. This clarification will benefit the precision of the research as well as help to standardize the practice of EV isolation across the field.

Proteomics is the key to learning about the contents of the vesicles. As stated previously, there has been some work done on the molecules and proteins contained within the EVs. However, more research is needed to address the specific factors in each case. Insight into a possible uniform mechanism for placing molecules into EVs, if EV contents are specific to a particular cancer, and if the contents of the vesicles correlate with the stage of the disease are only a few of the many questions that must be addressed as we consider EVs as a therapeutic tool. The analysis of cancer cell vesicles has been done in the PC3 prostate cancer cell line, but no such analysis has yet been done on DU145 cells.

Specifically for this experiment, the main question is what in the EVs is inhibiting resistance. Proteomics analysis of EVs will allow for the characterization of the reversal of drug resistance. An initial hypothesis would be the change of MDR expression in the Px resistant cells. However, this differential expression could occur at either the protein or RNA level. Thus, we can only further the knowledge of EVs role in intercellular signaling by knowing the identity of transferred material. These questions must be fully addressed before we can begin to use EVs as a possible therapeutic treatment for PCa.

The results of this study show the potential therapeutic uses of EVs against taxane resistance in PCa. The reversal of a drug resistance phenotype in PCa cells is an important step to understanding transfer of information between cells. Horizontal gene transfer is defined as any mechanism where genetic material is transferred from nonparent donors to recipient cells. Methods include cell-to-cell interactions with cell fusions or mobile genetic elements such as plasmids and bacteriophages.[32] Successful gene transfer requires many elements to work together simultaneously that depend on the signaling, recognition, molecular triggers, and the surrounding environment. Thus, EVs present a novel and surprising vehicle to cause phenotype change in cancer cells.

The implications for the reversal of drug resistance in CRPC are important for treating advanced disease. Previous work on taxane resistant cells showed that EVs from resistant cells could change the phenotype of sensitive cells to drug resistant. The DU145 docetaxel-resistant EVs transferred drug resistance to the sensitive DU145 cells. As a result, MDR-1 expression was acquired in the newly resistant cells.[22] That experiment demonstrated the reverse of the experiment presented within this paper. The reciprocal nature of EV signaling in drug resistance is a surprising result. On completion of the future analysis of this experiment, more conclusions may be drawn between the two experiments involving taxane resistant prostate cancer cell lines. In conjunction, these results begin to reveal the role of EVs in taxane resistance PCa.

Since the progression of PCa *in vivo* leads to all cancer cells becoming chemoresistant, there is a discrepancy with these *in vitro* results. The experiment presented in this paper raises the possibility of using sensitive EVs to reverse drug resistance. Thus, it is reasonable to think that sensitive cells within the tumor could possibly reverse drug resistance *in vivo*. The reciprocal nature of EV signaling implies that there is a competition occurring *in vivo* during PCa. Further investigation is required to fully understand the nature of this battle of EVs and why it appears that the resistant EVs win out over time.

IV. FUTURE DIRECTIONS

A. Current Challenges in Prostate Cancer Treatment

CRPC is the second leading cause of cancer death in men in the developed world. Androgen deprivation therapy is effective initially; however, nearly all patients become castrate resistant, leading to a fatal outcome.[33] Advances have been made in understanding the mechanism of PCa development, but this has only translated to a modest improvement of therapeutic outcomes. Enzalutamide and Abiraterone, two androgen receptor pathway inhibitors, represent breakthroughs in CRPC treatment. However, approximately 20–40% of patients have either no response to these agents or develop resistance within two years of treatment.[33–35] Successful PCa therapy may require selective targeting of the PCa cells for delivery of agents that either kill PCa cells or reduce their tumorigenic/metastatic phenotype in a clinically meaningful manner. Therefore, novel strategies aimed to directly target cancer cells and inhibit PCa cell survival pathways must be explored for their potential to improve therapy.

B. Human Mesenchymal Stem Cell Extracellular Vesicle (hMSC EV) Therapy for Pca

hMSC EVs are versatile bioactive molecules that can inhibit tumor growth.[21,36,37] On the basis of our findings, we propose to investigate the application of hMSC EV to reverse CRPC and inhibit PCa tumorigenicity and metastasis and provide therapeutic benefit for CRPC patients. Importantly, hMSC EVs are well tolerated and, therefore, can be utilized as a therapeutic delivery platform. To date, the use of hMSC EVs for PCa therapy has not been explored. Given the rapid advances that have been made in EV field within the last few years (e.g., exRNA communication), there is great potential for novel application(s) to anticancer therapeutic development.

hMSC EVs have diverse biological functions that are implicated in cell-cell signaling and in transferring protein and gene products. We hypothesize that hMSC EV therapy may alter AR/IGF-1 (insulin-like growth factor 1) signaling resulting in the inhibition of PCa tumor growth and progression. Persistent AR transcriptional activity underlies resistance to AR-targeted therapy and progression to lethal CRPC. CRPC invariably develops due to aberrant reactivation of the androgen signaling axis. Blocking the AR axis by androgen deprivation or treatment with enzalutamide-induced autophagy in androgen-responsive CRPC cell lines.[38] IGF-1 has been implicated in prostate cancer progression and the IGF-1/IGF-1R pathway represents a significant target in Pca.[39–41] IGF-1 can potentiate androgen signaling through AR activation. However, the role of IGF-1 in the reversal of enzalutamide resistance is currently unknown. Therefore, hMSC EV treatment may result in the inhibition of AR and IGF-1 PCa survival signaling.

Following interaction with their target cells, hMSC EVs may influence cell behavior in several ways including stimulating cell surface interaction,[42] transferring receptors between cells,[43,44] delivering target proteins, and inducing epigenetic changes in target cells by transferring specific genetic information.[45,46] Following these events, hMSC EV treatment may confer phenotypic changes resulting in the inhibition of PCa tumorigenicity and metastasis.

V. CONCLUDING REMARKS

The potential for therapeutic use of EVs is a possible solution to the current issues in mCRPC. The results from our experiments put forth the potential use of EVs as a potential biomarker and therapeutic option in PCa. While still undefined, there is some factor inherent to these vesicles with the potential to change the phenotype of PCa cells. A full analysis of the content of EVs will further our knowledge of disease progression.

The therapeutic applications of EVs may be the most promising in CRPC. As the results from this study indicate, it is possible to use EVs to reverse drug resistance in CRPC. We must be careful to not over interpret the results of this *in vitro* study. However, these results do deserve further evaluation to understand the possible benefits of EV therapy. EVs hold the potential to help slow progression of disease and, therefore, negative outcomes in advanced PCa.

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ABBREVIATIONS

PCa	prostate cancer
PSA	prostate specific antigen
AR	androgen receptor
CRPC	castrate resistant prostate cancer
mCRPC	metastatic castrate resistant PCa
EV	extracellular vesicles
MV	microvesicles
CPT	camptothecin
hMSC	human mesenchymal stem cells
PxR	DU145 paclitaxel resistant cell line
Px	paclitaxel
MDRP/P-gp	multidrug resistance protein/P-glycoprotein
PARP	poly ADP ribose polymerase
IGF-1	insulin-like growth factor 1

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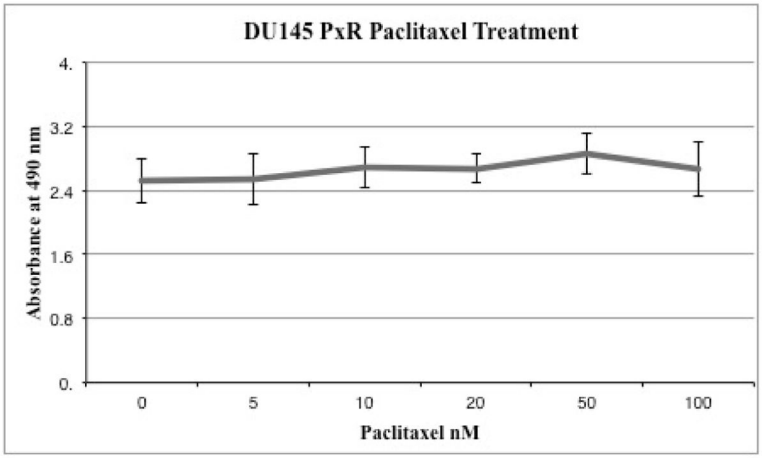

FIG. 1.

DU145 PxR cells do not respond to Px treatment. DU145 PxR cells were treated with the indicated doses of Px. Cytotoxicity was measured using the MTS assay. The results represent the average ± standard deviation of two independent experiments performed in quadruplicate.

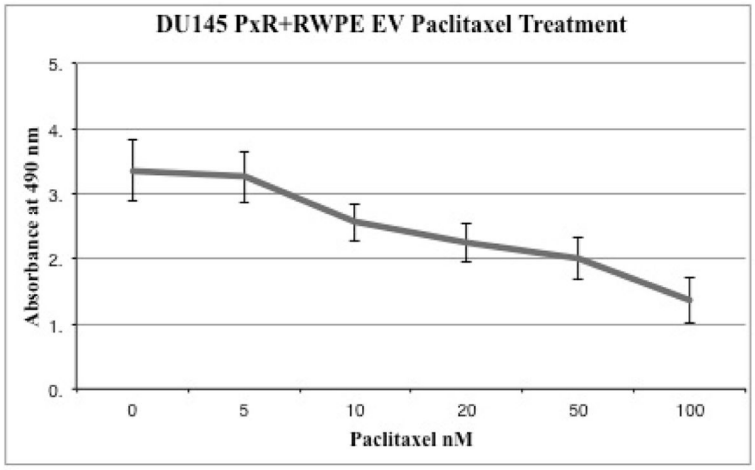

FIG. 2.

Nonmalignant prostate cell EVs restore sensitivity to Px. DU145 PxR cells were cocultured with EVs isolated from RWPE cells for five days. Cells were then treated with Px. Cytotoxicity was measured using the MTS assay. The results represent the average ± standard deviation of two independent experiments performed in quadruplicate.

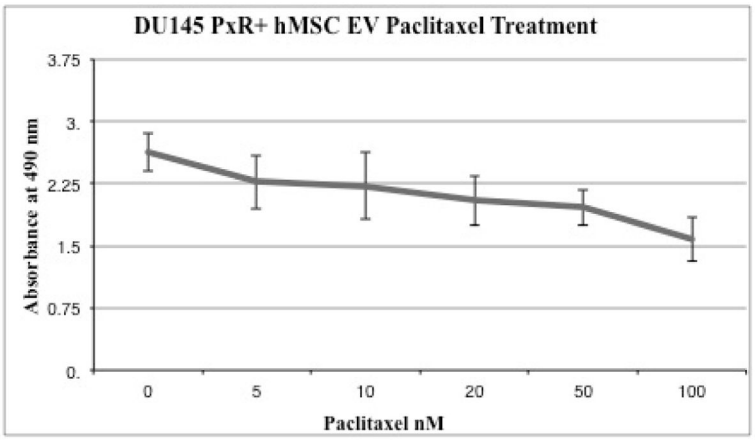

FIG. 3.

hMSC EVs restore sensitivity to Px. The same experimental approach was used as described in Fig. 2. Cells were then treated with Px. Cytotoxicity was measured using the MTS assay. The results represent the average ± standard deviation of two independent experiments performed in quadruplicate.

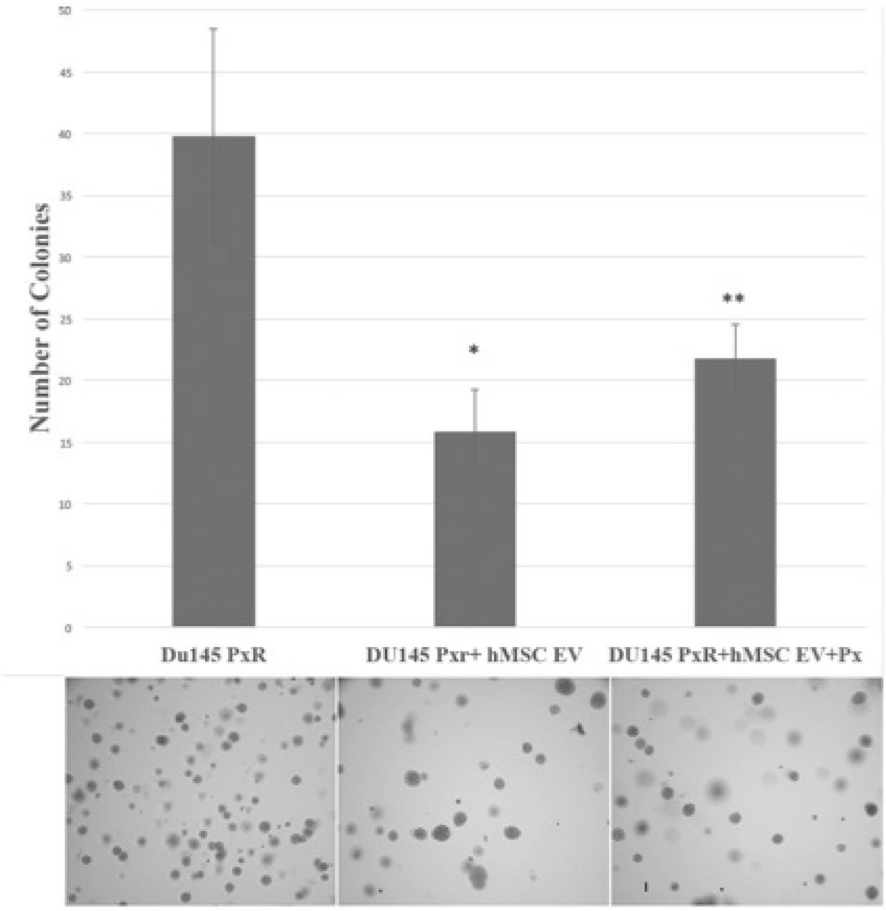

FIG. 4.

hMSC EV-mediated reduction of soft agar growth. hMSC EVs were isolated and cocultured for seven days with DU145 PxR cells. Soft agar colon forming assay was performed for two weeks. The data represents the mean ± standard deviation of two independent experiments performed in triplicate. There were five dishes/condition. A paired *t*-test was performed to analyze the decrease in soft agar colony formation of DU145 PxR + hMSC EV cells (*$p <$ 0.001), and DU145 PxR cells + hMSC Ev + Px (*$p < 0.00$) when compared to untreated DU145 PxR cells.