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Autologous fat grafting as a novel anti-estrogen vehicle for the treatment of breast cancer

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Abstract

Purpose—Adipose fat transfer is increasingly used for contour corrections of the tumor bed after lumpectomy and breast reconstructions after mastectomy. The lipophilic nature of the fat tissue may render adipocytes an ideal vehicle to deliver a high boost of an anti-estrogen to the tumor bed to serve as an adjunct systemic hormonal therapy. We, therefore, tested whether adipocytes could safely be loaded with an anti-estrogen and allow for release at therapeutic concentrations to treat breast cancer.

Methods—Adipose tissue was collected from patients undergoing autologous fat grafting. Influence of adipose tissue on tumorigenesis was determined both in vitro and in vivo using breast cancer cell lines. Ex vivo, adipose tissue was assessed for it's ability to depot fulvestrant and inhibit the growth of breast cancer cell lines.

Results—Adipose tissue harvested from patients did not promote breast cancer cell growth in vitro or in an in vivo mouse model. Adipose tissue was successfully loaded with fulvestrant and released at levels sufficient to inhibit estrogen receptor signaling and growth of breast cancer cells.

Conclusions—This work supports the hypothesis that adipose tissue used for autologous fat grafting can serve as a novel method for local drug delivery. As this technique is used to reconstruct a variety of post surgical defects following cancer resection, this approach for local drug delivery may be an effective alternative in therapeutic settings beyond breast cancer.

Keywords

Autologous fat grafting; breast cancer; fulvestrant; local recurrence

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CONFLICTS OF INTEREST

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INTRODUCTION

Despite many recent advances in the treatment and prevention of breast cancer, approximately 232,000 women are expected to present with the disease in the United States in 2015, resulting in nearly 40,000 breast cancer deaths (1). More than two-thirds of these women will develop tumors that express estrogen receptor and will be candidates for hormonal therapy. The two most commonly used strategies to inhibit estrogen receptor signaling are either competitive inhibition of the estrogen receptor (anti-estrogens), or inhibition of endogenous estrogen production (aromatase inhibitors) (2).

The majority of patients are now treated with lumpectomy, local radiation and radiation boost to the tumor bed to reduce the risk of local recurrence (3). With the addition of systemic and radiation therapy, cancer recurrence is effectively reduced, but still occurs in 10–30% of patients with invasive breast cancer (4–9).

Despite its documented benefits in reducing recurrence, many women cannot tolerate systemic exposure to hormonal therapy. Undesirable side effects lead to drug discontinuation rates as high as 50% (10, 11). A local approach, therefore, may represent an attractive alternative, particularly in women with high risk for local recurrence. As such, substantial work has focused on local drug delivery to the tumor site or at the desired organ/tissue. Polymer based drug delivery systems have been under investigation with the purpose of efficient local delivery and reduced systemic toxicity, but thus far have had limited clinical success (12). Autologous fat grafting is an emerging technique for reducing contour irregularities associated with breast conservation, and for reconstructive surgery following tumor resection. Adipose tissue is harvested from the patient by liposuction techniques, and then grafted to the breast (13–15). We hypothesized that an anti-estrogen could be delivered directly to the resection bed by infusing autologous adipose tissue with a highly lipophilic anti-estrogen prior to its use in reconstructing the defect. It was surmised that local drug concentration could be higher with local delivery of anti-estrogens than with systemic therapy alone, which is limited by toxicity. Thus, this delivery method may enhance the efficacy of current systemic therapy and provide an alternative for those who cannot tolerate systemic therapy. In this proof of principle study, we demonstrate that human adipose tissue harvested by liposuction can be effectively loaded with the anti-estrogen fulvestrant, and that the concentration and release of fulvestrant over time is sufficient to inhibit breast cancer cell estrogen receptor activity and proliferation.

METHODS AND MATERIALS

Chemicals and antibodies

Fulvestrant and β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Cyclin D1, estrogen receptor α and progesterone receptor antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All other chemicals were of the highest purity available.

Adipose tissue collection

All adipose tissue was collected from women at UCSF who were already undergoing autologous fat grafting for breast reconstruction. Fat was harvested from the abdomen and upper thighs via standard liposuction techniques using a 2.1 mm cannula. The adipose tissue was collected and processed using the Revolve system (LifeCell, Bridgewater, New Jersey). The Revolve system collects adipose aspirate, washes it with Lactate Ringer's solution at 37°C by gentle agitation with rotating paddles, and then removes excess liquid by vacuum aspiration. Adipose tissue was immediately used in subsequent analyses.

Cell Culture

MCF7 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Fisher Scientific, Atlanta, GA) with 10% fetal bovine serum (Sigma-Aldrich), 2 mM glutamine, and 50 unit/mL penicillin and 50 μg/mL streptomycin (Fisher Scientific), referred to as complete medium. Cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Tumor xenografts and histopathology

Athymic, nude, female mice (n=5, Taconic Farms, Inc.) were implanted with a 60-day estradiol pellet and MCF7 cells subcutaneously and bilaterally. MCF7 cells (1×10^7) alone were implanted on the left flank, and on the right flank MCF7 cells (1×10^7) mixed with an equal volume of Revolve-processed adipose tissue (100 μL) were implanted. Tumor volumes were calculated using caliper ((length × width^2)/0.5). At the conclusion of the experiment, tumors were harvested, formalin fixed, and embedded in paraffin. Hematoxylin and eosin staining was performed by the UCSF Helen Diller Family Comprehensive Cancer Center tissue core (supported by the NIH/NCI P30 CA82103 grant). Images were captured using a Zeiss PALM microbeam system in conjunction with Axiovision software (Oberkochen, Germany) and processed with Adobe Photoshop software.

Cell activity, proliferation, and viability assays

To assess relative activity of adipose tissue or MCF7 cells, a tetrazolium compound ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]; MTS) assay was performed using Celltiter 96 AQueous one solution (Promega, Madison, WI). For adipose tissue, the indicated amount of tissue was incubated with 1 mL assay reagent (1:5, Celltiter reagent to phosphate buffered saline (v/v)) at 37°C for 30 to 60 minutes. As a negative control, an equal amount of adipose tissue was lysed by repeated freezing and thawing (4 cycles). For MCF7 cells, a 96-well tissue culture plate was seeded with 2×10^3 cells. Cells were then treated with the indicated medium for 96 hours. The medium was then removed and cells were incubated with 100 μL assay reagent (1:5, Celltiter reagent to phosphate buffered saline (v/v)) at 37°C for 1 to 2 hours. Absorbance at 492 nm was measured as readout for cellular activity. To determine MCF7 cell proliferation or viability co-cultured with adipose tissue, 1×10^5 cells per well were seeded to 12-well plates. Adipose tissue collected with a 2.1 mm cannula and processed using the Revolve method was mixed 1:1 with complete medium and co-cultured with MCF7 cells for 96 hours. MCF7 cells were then harvested, counted, and evaluated for viability using a trypan

blue exclusion assay as previously described (16). All treatments were conducted in triplicate.

Western Blot Analysis

Western blot analysis of MCF7 cell proteins was conducted as previously described (16, 17).

Statistical analysis

To evaluate the correlation between adipose mass and cellular activity, a Pearson's correlation coefficient was calculated and presented as the coefficient of determination (R^2). To test the difference between two variables, a student t test was performed, with two-sided P-values ≤ 0.05 considered significantly different. Statistical evaluations were performed using SigmaPlot software (Systat Inc.).

RESULTS

Autologous fat grafting is often used following breast conservation surgery to reconstruct resultant contour deformities. In this proof of principle study, we sought to test the feasibility of loading autologous adipocytes harvested from patients with the anti-estrogen fulvestrant as a drug delivery vehicle. To assess this concept, we first developed a method for reliably evaluating the viability of adipose tissue. In cell culture models, assays employing tetrazolium salts (*e.g.* MTS) are used for measuring relative viability. To determine whether this assay could be adapted to adipose tissue, the correlation between MTS activity and grams of adipose tissue was examined. We used adipose tissue that was harvested using a 2.1 mm cannula with a Revolve system. The tissue was washed 3 times with warm saline, centrifuging (200 g, 5 minutes) and aspirating liquid between washes. Tissue was aliqouted from roughly 0.025 g to 0.2 g and assayed by MTS. A linear correlation between MTS activity and grams of adipose was demonstrated with an $R^2 = 0.847$ (Figure 1). Furthermore, repeated freeze-thaw cycles of adipose tissue significantly reduced the measured activity (data not shown), demonstrating that conversion of the tetrazolium salt to its measurable product was the result of metabolically active cells and not an artifact of adipose tissue. Thus, evaluating viability with up to ~0.2 g adipose tissue using an MTS assay and normalizing samples by mass was deemed suitable for subsequent sample evaluation.

Autologous adipose tissue does not promote breast cancer cell viability and tumorigenesis

Concern has been raised about the tumorigenic potential of autologous fat grafting following tumor resection (18). It has been hypothesized that mesenchymal stem cells and associated stroma of adipose tissue could promote local recurrence by releasing adipokines that induce angiogenesis and proliferation. To evaluate this potential, we first examined the effect of co-culturing adipose tissue with breast cancer cells in vitro. When MCF7 and T47D cells were co-cultured with equal volumes of growth medium and adipose tissue for 96 hours, no significant difference was observed in breast cancer cell proliferation or viability compared to cells cultured without adipose tissue (Figure 2A and data not shown). We next extended this evaluation in vivo using a mouse xenograft model. MCF7 cells with or without equal volumes of adipose tissue were implanted subcutaneously. As expected, the initial total

volume in MCF7 tumors containing adipocyte were larger demonstrating the viability of adipocytes during tumor growth period. The tumor volumes (MCF7 cells with or without adipocytes) were not statistically distinguishable beyond the first two weeks of growth (Figure 2B) suggesting the presence of adipose tissue did not promote MCF7 cell tumorigenesis. After eight weeks of growth, however, there were significant qualitative differences in composition and structure between the tumors that are exposed to adipocyte injection and those that were not (Figure 2C and D). Implanted MCF7 cells alone produced dense, highly cellular, dedifferentiated tumors growing devoid of any mammary structure and architecture. On the other hand, MCF7 cells co-implanted with adipose tissue resulted in tumors that grew in more duct like structures, surrounded by adipocytes and the formation of new blood vessels, suggesting a more differentiated tumor. Groups of adipocytes could be seen in vein-like penetrations in the outer third of the tumors (Figure 2D, arrows). In adipocyte rich regions, “pockets of tumor” cells were observed (Figure 2D, asterisks), which were surrounded by stroma and often a substantial amount of immune cell infiltration. Furthermore, tumor pockets surrounded by adipocytes often exhibited necrosis. These findings do not suggest a tumorigeneic effect of adipocytes, but possibly increased tumor differentiation and immune cell infiltration.

Adipose tissue can effectively deliver fulvestrant to inhibit MCF7 cells

Next, we tested whether the lipophilic nature of adipocytes could be used to delivery an anti-cancer agent and provide a tumor bed treatment boost. Fulvestrant is the most potent anti-estrogen, however, its systemic bioavailability is poor, and thus not effective for systemic administration in premenopausal women. Unlike tamoxifen and raloxifene, fulvestrant is a pure anti-estrogen and does not exhibit pro-estrogenic effects. Furthermore, the relative lipophilicity of fulvestrant is high (fulvestrant logD 8.47; tamoxifen logD 6.122; raloxifene logD 5.406), and thus should partition in adipose rich tissue at higher levels. Unlike tamoxifen, fulvestrant does not require metabolism to an active metabolite and thus may be delivered locally.

To transfer fulvestrant to tumor cells, adipocytes have to take up sufficient concentrations of fulvestrant and then allow drug release and transfer to tumor cells. Following liposuction, adipose tissue (1 g) was immediately transferred to tissue culture medium containing increasing concentrations of fulvestrant (0, 1, 5 and 25 μM) and incubated at 37°C for 18 hours, rocking continuously. The tissue was then thoroughly washed with saline and adipocyte viability was evaluated (Figure 3A) to determine the toxicity of fulvestrant to adipocytes. Incubation with super-therapeutic doses of fulvestrant did not reduce viability of the adipose tissue and appeared to increase the metabolic activity and viability in treated adipocytes ($P < 0.05$). To determine whether adipose tissue can function as a vehicle for delivery of fulvestrant, fresh tissue (~1 g) was incubated with increasing concentrations of fulvestrant in complete medium for 18 hours at 37°C. Adipose tissue was then washed thoroughly with saline and transferred to a volume of complete medium equal to that used to load fulvestrant and incubated at 37°C for 96 hours to allow for fulvestrant release. Medium containing released fulvestrant was separated from adipose tissue and used to culture MCF7 cells for 72 hours, after which, MCF7 cell proliferation and estrogen receptor activity were assessed. Direct treatment of MCF7 cells with 100 nM fulvestrant was sufficient to inhibit

cell proliferation (~60%, Figure 3B) and down-regulate estrogen receptor expression and activity (Figure 3C). Adipose loaded with medium containing 1 μM fulvestrant resulted in sufficient release to inhibit cell growth and expression of the progesterone receptor. Further reduction in estrogen receptor expression and cyclin D1 were seen with higher loading concentrations of fulvestrant (Figure 3D).

DISCUSSION

Following surgical resection, the risk of breast cancer recurrence is mitigated by radiotherapy and adjuvant hormonal therapy. However, adverse effects related to systematic adjuvant hormonal therapy limit compliance, with fewer than half completing the recommended five-year treatment course (10). For women with disease with low metastatic potential, the primary concern is prevention of local recurrence. For these patients, local delivery of hormonal therapy would likely provide a less toxic alternative by avoiding toxicities associated with systemic treatment. Fat grafting is an emerging technique that is used in women with lumpectomy to correct contour deformities. Based on this rationale, we sought to test whether the adipose tissue used in autologous fat grafting could be used to provide local delivery of the anti-estrogen fulvestrant. To this end, we have shown that adipose tissue can be loaded with fulvestrant *ex vivo*. Further, high levels of fulvestrant do not significantly impact adipocyte viability and the fat cells remain viable for reinjection within 18 hours. The release of fulvestrant from adipose tissue is suitable to inhibit estrogen receptor function and breast cancer cell proliferation.

Substantial effort has been made to develop novel approaches for the sustained local delivery of therapeutics. For breast cancer, approaches have included implantable hydrogels, wafers, rods, and films that act as depots (12), which are often designed to biodegrade and thus eliminate the need for surgical removal, topical applications that facilitate transdermal delivery (19, 20), intraductal administration (21, 22), and iontophoretic devices that utilize electric fields to drive and direct drug diffusion (23, 24). An adipose depot has several advantages over these other approaches. It makes use of an increasingly accepted procedure, autologous fat grafting for reconstruction, avoiding the need for an additional procedure and its associated morbidity. Adipose tissue can be loaded within a reasonable time frame and excessive tissue is generally available even if adipocyte viability after drug treatment is reduced. Novel freezing technologies will allow storage and reloading for repeat procedures. Drug-loaded adipose tissue would be grafted into the resection cavity, maximizing drug contact with the surgical margins and potential residual tumor cells transforming a corrective technique into a therapeutic intervention. Finally, using autologous tissue as a drug carrier, rather than foreign bodies such as microbeads, minimizes the risk of encapsulation or undesirable immune response, which has been a significant concern for devices designed for local drug delivery.

Several concerns have arisen with the advent of autologous fat grafting for reconstruction of contour deformities following breast cancer tumor resection. A major oncologic concern is the promotion of tumor growth when introducing adipose tissue (15, 25). Although some studies support this concern (26–29), increased risk of breast cancer has not manifested in the clinic (25, 30, 31). Consistent with clinical findings, we found that co-culturing breast

cancer cells with adipose tissue for 96 hours did not effect MCF7 proliferation or viability. Furthermore, when co-implanted with adipose tissue in a mouse xenograft, MCF7 cell tumor formation was not significantly affected by the presence of adipose tissue. From these previous studies, it has been shown that experimental context plays a key role. Tumor type, tumor cell line model, adipose tissue processing, and tissue donor all have been shown to affect how adipose tissue influences tumorigenesis. These contextual variances likely all contribute to the difference in our findings compared to others. Furthermore, for this study, whole tissue was used when evaluating the effect of adipose on tumor growth, to best model its application when engrafting following drug loading. This is in significant contrast to most preclinical studies that focus on the influence of the stromal vascular fraction or the further purified adipose derived stem cells from whole tissue. The stem cell containing stromal compartment has been shown *in vitro* and *in vivo* to promote angiogenesis, metastasis, and tumor cell proliferation in a variety of tumor types (32–36). In whole tissue, however, these cells constitute a relatively small fraction and likely exert significantly less pro-growth activity (37), as witnessed in this study.

Although proof of concept was demonstrated, significant questions remain regarding the feasibility of translating this approach to the clinic. For example, can the therapeutic dose be regulated and can local delivery of an active dose be sustained to achieve a clinical benefit? Will the presence of drug affect successful engraftment of tissue? Does added time required to load adipose tissue with drug significantly impact the fat grafting procedure and patient care? To demonstrate the robustness of this approach, we are evaluating efficacy with more varied breast cancer models, both *in vitro* and *in vivo*, as well as adipose tissue from multiple donors. Furthermore, quantitative mass spectrometry studies of drug uptake and release are ongoing to characterize the capacity to depot fulvestrant in adipose tissue and to determine the release rate kinetics to assess the potential for sustained therapeutic delivery and dose modification. Finally, we are exploring methods for banking adipose tissue, which may allow tissue initially obtained to be used for multiple engraftments, thus potentially reducing patient burden (*e.g.* fewer rounds of liposuction and less anesthesia) while providing multiple therapeutic applications.

CONCLUSION

In this proof of principle study, we demonstrated that adipose tissue intended for autologous fat grafting may act as a vehicle for the local delivery of drug to the breast and moreover to deliver a therapeutic boost to the tumor bed with the possibility to directly impact residual tumor cells. The increasing use of autologous fat grafting in surgery may provide unique and novel opportunities for this method to transform a predominantly cosmetic approach into a therapeutic intervention by delivering short-term high doses of anti-cancer agents to the tumor bed to decrease tumor recurrence.

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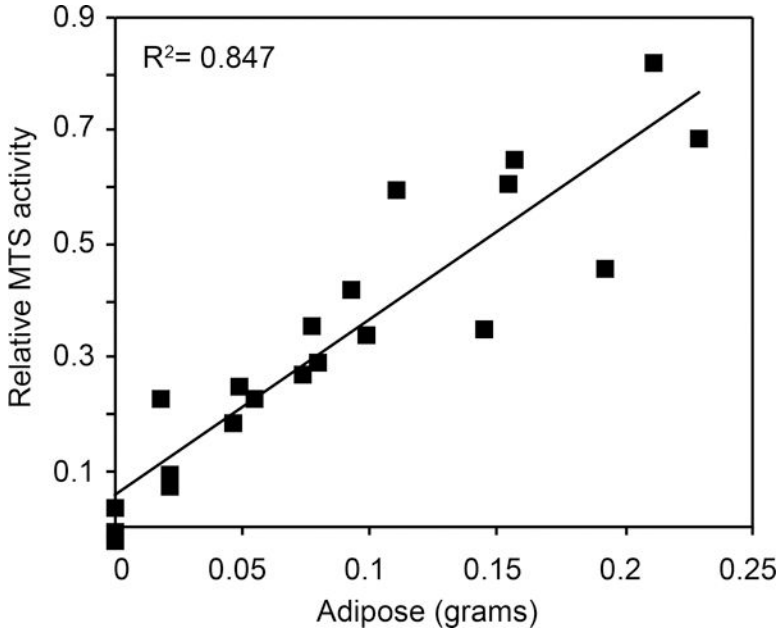

Fig. 1.

Evaluation of adipose tissue viability using an MTS assay. Increasing amounts of adipose aspirate were assayed by MTS. Absorbance at 492 nM is plotted versus grams of tissue. Linear correlation was assessed by Pearson's correlation coefficient, with the R^2 presented.

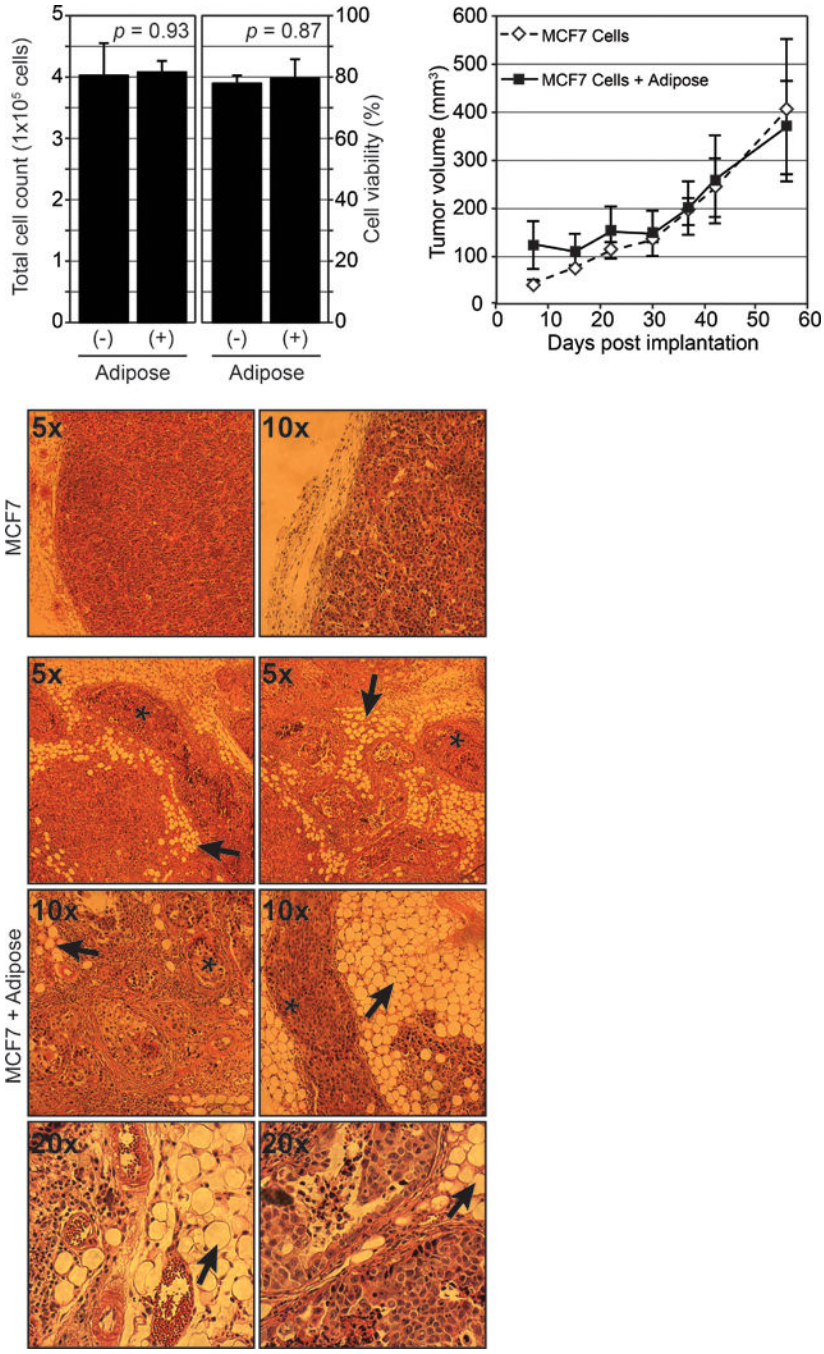

Fig. 2.

Affect of adipose tissue on MCF7 cell tumorigenesis. **a** MCF7 cells co-cultured for 96 hours with (+) or without (−) adipose tissue and evaluated for total cell count and viability. **b** Tumor formation of MCF7 cells implanted with or without adipose tissue. c Hematoxylin and eosin stained sections from MCF7 cell tumors established with or without adipose tissue. Image magnification is indicated in upper left corners. Arrows indicate adipocytes and asterisks indicate tumor pockets. For **a** and **b**, averages are presented with bars indicating the standard error of the mean.

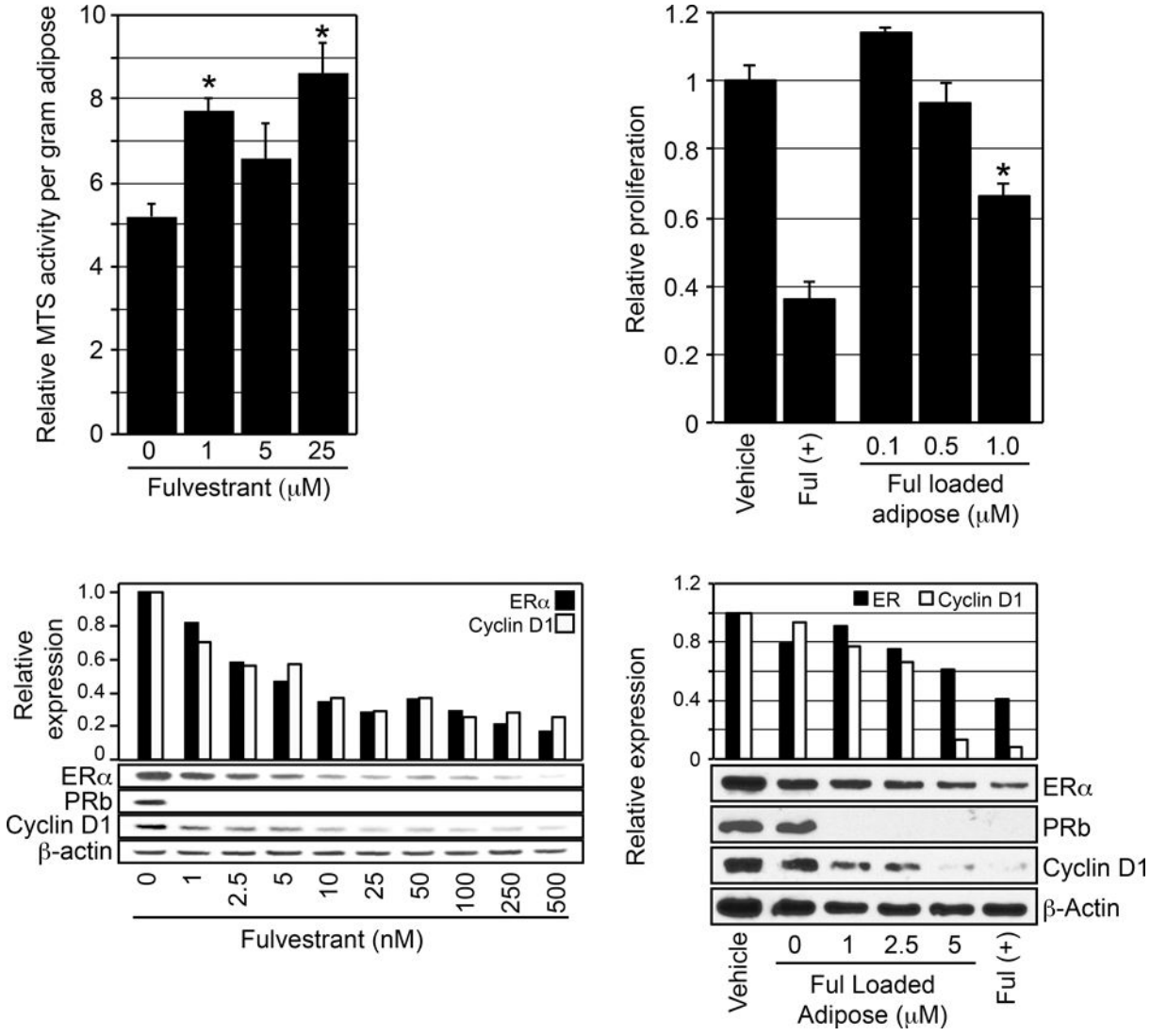

Fig. 3.

Adipose tissue can be loaded with and deliver sufficient fulvestrant to inhibit breast cancer cells. **a** Adipose tissue (~1 g) incubated in medium containing increasing concentrations of fulvestrant for 18 hours. Adipose was either assayed by MTS immediately **a** or washed with saline and incubated in fresh medium for 96 hours to collect released fulvestrant. Medium containing released fulvestant was then separated from adipose and used to culture MCF7 cells for 72 hours. MCF7 cells were then assayed by MTS **b** or western blotted for estrogen receptor and estrogen receptor response gene expression d. In **b** and **d**, MCF7 cells were directly treated with vehicle or 0.1 μM fulvestrant (Ful (+)) for 72 hours and evaluated as controls. **c** MCF7 cells were treated with increasing concentrations of fulvestrant for 72 hours and western blotted for estrogen receptor and estrogen receptor response gene expression. For **c** and **d**, estrogen receptor and cyclin D1 protein bands were quantified by densiometry and normalized to β-actin expression. Asterisk indicates significant difference compared to 0 μM fulvestrant or vehicle.