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Local adipocytes enable estrogen-dependent breast cancer growth

Role of leptin and aromatase

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The importance of the microenvironment in breast cancer growth and progression is becoming increasingly clear. Adipocytes are abundant in the mammary microenvironment, and recent studies show that adipocytes produce endocrine, inflammatory, and angiogenic factors that have tremendous potential to affect adjacent breast cancer cells. Yet, the extent to which local adipocyte function contributes to the pathogenesis of breast cancer is largely unexplored. Here we describe a unique animal model to study interactions between adipocytes and breast cancer cells in the tumor microenvironment. Our results suggest that local interactions between adipocytes and tumor cells are sufficient to promote the growth of hormone-dependent breast cancer. We also demonstrate that leptin signaling in adipocytes induces aromatase expression, expected to result in higher estrogen in the microenvironment thus enabling mammary tumorigenesis.

Adipocytes are abundant in the mammary microenvironment, and produce multiple endocrine, inflammatory, and angiogenic factors, which can potentially increase adjacent breast cancer growth.[1-3] While the importance of the local microenvironment in breast cancer growth and progression is clear[4-6] the extent to which local adipocytes contribute to the pathogenesis of breast cancer is largely unexplored. We set out to establish an animal model to uncover mechanistic insights into interactions between local adipocytes and human breast cancer cells. Murine 3T3-F442A pre-adipocytes injected subcutaneously (sc) into immuno-deficient mice differentiate into mature adipocytes, form well-vascularized fat pads[7,8] and express leptin and other adipose markers at levels similar to adipose tissue.[9] We determined whether the in vivo F442A fat pad model could be used to study the effects of local adipocytes on hormone-dependent breast cancer using the estrogen receptor-positive, hormone-dependent human breast cancer cell line MCF-7. It is well known that endogenous estrogen levels in immune-deficient mice are insufficient to support the growth of MCF-7 tumors and that mice need to be implanted with estrogen pellets to enable tumor growth.[10] We tested whether F442A fat pads can support MCF-7 tumor growth in the absence of exogenous estrogen supplementation. Eight-week-old female SCID mice were injected subcutaneously (sc) with a mixture of F442A cells (2.5×10^7 per mouse) and MCF-7 cells (5×10^6 per mouse). Control mice were injected only with 5×10^6 MCF-7 cells. While no tumors grew in mice injected with MCF-7 cells alone (**Fig. 1A**), after an initial latent phase, progressive tumor growth was observed in all mice co injected with MCF-7 and F442A cells (**Fig. 1A and B**). The observed latent period is consistent with the 3 weeks required for F442A pre-adipocytes to differentiate into mature adipocytes in vivo.[8] Seven weeks after injection, mice were killed and tumors were harvested to assess histology. Tumors consisted of large clusters of breast cancer cells in contact with mature, differentiated adipocytes (**Fig. 1C**). To determine if enhanced tumor growth required proximity of adipocytes to tumor cells, we compared co-injection of F442A and MCF-7 cells with injections of F442A cells into the right flank and MCF-7 cells into the left flank. Whereas co injection resulted in progressive tumor growth, contra lateral injection resulted in well-differentiated fat pads, but no tumor growth (**Fig. 1D**). Similarly, F442A adipocytes also supported the growth of hormone-dependent human T47D breast cancer cells (data not shown), suggesting that the adipocyte-mediated effect was not specific to MCF-7 cells alone. Together these results indicate that close proximity between adipocytes and breast cancer cells in the tumor microenvironment is required for tumor growth.

Adipocytes secrete many growth factors, hormones, and other bioactive molecules that can directly support tumorigenesis.[1-3] Since proliferation, growth, and progression of hormone-responsive breast cancer are driven by estrogen, we reasoned that F442A adipocytes may enable growth of hormone-dependent breast cancer by providing local estrogen. In postmenopausal women estrogen is derived from the activity of aromatase, an enzyme of the cytochrome p450 superfamily. Aromatase converts androgens into estrogens and preferentially mediates conversion

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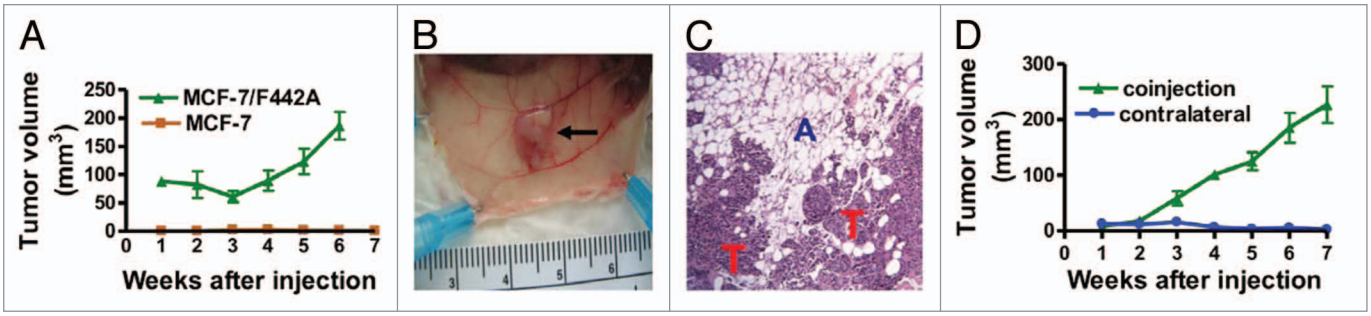

Figure 1. F442A adipocytes increases hormone-dependent MCF-7 tumor growth in vivo. (**A**) Eight-week-old female SCID mice were injected sc with F442A pre-adipocytes (2.5×10^7 cells/mouse) and MCF-7 human breast cancer cells (5×10^6 cells/mouse), or with 5×10^6 MCF-7 cells alone. (**B**) Gross morphology of MCF/F442A tumor 7 weeks after injection. (**C**) H&E stained section of MCF/F442A tumor (100×) shows fairly uniform small tumor cells (T) with violet nuclei and pink cytoplasm and mature adipocytes (A). (**D**) Comparison of co injection of F442A and MCF-7 in SCID with injections of F442A into the right flank and MCF-7 into the left flank of the same animal. Tumor growth (volume) was measured with calipers. $n = 6$, mean ± SD.

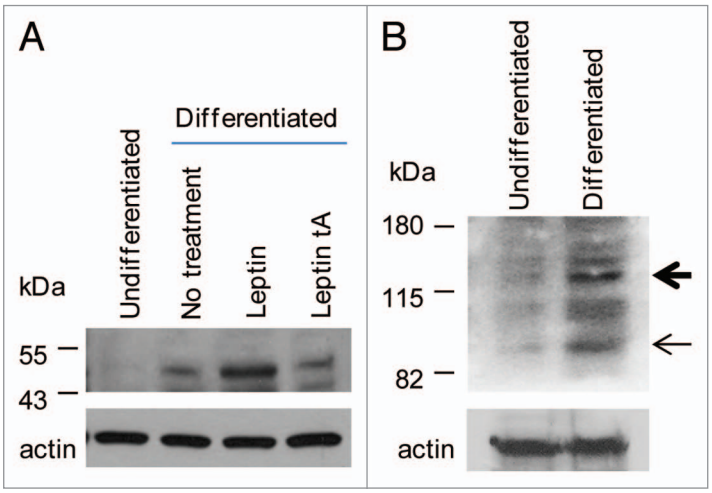

Figure 2. Aromatase and leptin receptor expression in F442A cells in vitro. (**A**) Western blot of basal and leptin-induced aromatase protein expression in F442A undifferentiated pre-adipocytes and differentiated adipocytes. F442A pre-adipocytes or confluent, differentiated adipocytes in 24-well plates were incubated with mouse leptin (PeproTech, 100 nM), leptin-triple mutant antagonist (leptin TA; Prospec, 250 nM) or buffer as control for 6 h under typical cell culture conditions. Aromatase protein expression was determined by western blot analysis using a standard protocol with anti-CPY19 (C-16; Santa Cruz Biotechnology) as primary antibody to detect aromatase. Western blot for actin is shown as loading control. (**B**) Leptin receptor expression in F442A pre-adipocytes vs. F442A adipocytes indicating an increase in the long form of LepR (LepR-1) in adipocytes compared with pre-adipocytes. Antibody to detect mouse LepR by western blotting was NB120-5593 (Novus Biologicals).

of adrenal androstenedione into estrone.[11,12] While aromatase is highly expressed in ovarian follicles, in postmenopausal women the primary site of aromatase activity and estrogen production is the adipose tissue. Thus after menopause, peripheral aromatase activity and plasma estrogen levels correlate with body-mass index,[13] and adipose-derived estrogen is thought to be a major contributor to obesity-related postmenopausal breast cancer risk and progression. Lowering of plasma estrogen levels with aromatase inhibitors has therapeutic benefit as adjuvant treatment in women with estrogen receptor-positive breast cancer[14] and as first line treatment in postmenopausal women with advanced cancer.[15] However, the extent to which plasma estrogen levels are sufficient to support hormone-dependent breast cancer is unclear. In postmenopausal women, local estrogen levels in breast cancer tissue are 10-fold higher than in plasma, suggesting increased local estrogen production.[16] Indeed, breast cancer tissues have considerable aromatase activity[17,18] and express aromatase in different cell types including tumor, stromal, endothelial cells, and adipocytes.[19] Interestingly, tissue-specific promoters and different transcription factors control aromatase expression in different cell types including tumor cells and adipocytes.[20] In mouse models, overexpression of aromatase in mouse mammary epithelia leads to hyperplasia in the absence of circulating estrogen[21] and modulates tumor development in HER2/neu transgenic mice.[22] Overexpression of aromatase in the human breast cancer cell line MCF-7 enables breast cancer growth in nude mice, which can be blocked by aromatase inhibitor.[23] While these observations argue for a role of local aromatase activity in breast cancer, the contribution and regulation of adipocyte-produced aromatase in the tumor microenvironment and the extent to which adipocyte aromatase contributes to cancer pathogenesis is not known. We determined aromatase expression in vitro in undifferentiated F442A pre-adipocytes and in F442A adipocytes differentiated using a cocktail of 1 μM dexamathasone and 1 μg/ml insulin. Western blot analysis showed that undifferentiated F442A pre-adipocytes express very low levels of aromatase whereas high levels of aromatase was observed in fully differentiated F442A adipocytes (**Fig. 2A**).

Although adipose tissue is known to express aromatase, little is known about the mediators that regulate its expression and activity in this tissue. The hormone leptin is primarily produced by adipocytes and regulates adipose tissue mass and energy balance. Since obesity increases the number and size of adipocytes in the mammary adipose tissue, we hypothesized that autocrine actions of leptin might induce aromatase expression in adipocytes. Leptin, an approximately 16 kD, secreted polypeptide, is a member of the type I helical cytokine family and related to growth hormone, prolactin and interleukins.[24,25] In addition to its roles in the regulation of appetite and metabolism, leptin also

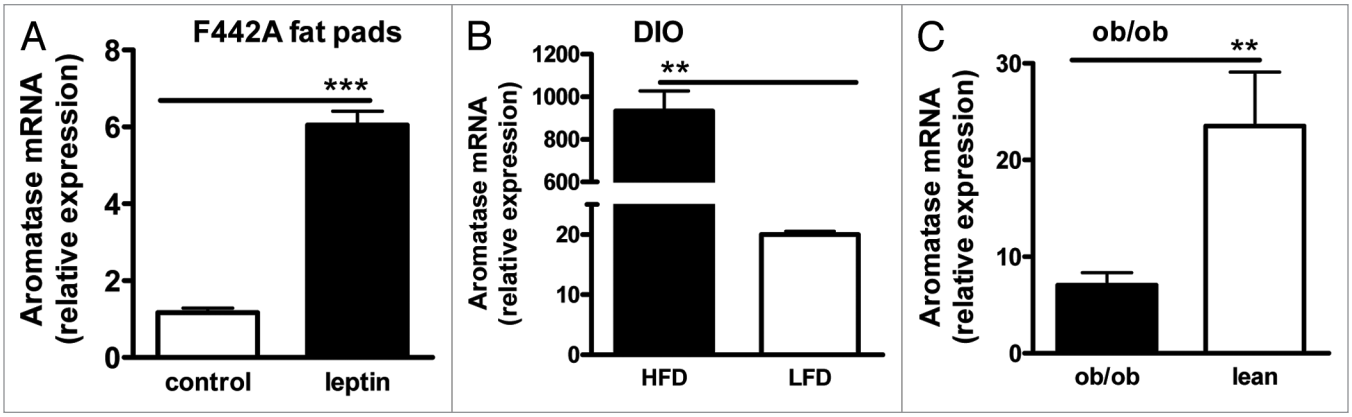

Figure 3. Aromatase gene expression in vivo. (**A**) Aromatase mRNA in F442A fat pads in vivo. Mice were injected sc in the proximity of the F442A fat pad with 10 μg leptin or with vehicle and sacrificed 3 h later. Gene expression was measured using real-time quantitative PCR. $n = 4$, mean ± SD; ***$P < 0.001$. Changes in aromatase mRNA levels in para-uterine fat pads from female C57BL/6J mice fed either a HFD or LFD for 16 weeks (**B**), and in 8- to 10-week-old female lean and genetically obese ob/ob mice (**C**). (**B and C**) $n = 6$, mean ± SD **$P < 0.01$.

has important roles in developmental and physiological processes in promotion of linear growth, onset of puberty, neural development, bone development, lung development and immune and thyroid function.[25] Leptin acts through the cell surface leptin receptor (LepR), which exists in several splice variants, only one of which, referred to as LepR-1[24] or LepRb[25] has a 300 amino acid cytoplasmic domain that mediates intracellular signaling.[25] LepR is expressed in the brain and also in lung, kidney and adipose tissue.[26] Expression of leptin and LepR has been described in breast cancer tissues but not in normal breast epithelium or benign tumors,[27-29] and a number of studies have described contributions of leptin to cancer growth. Thus, in breast cancer cell lines, leptin functions as a mitogen[28,30,31] and increases expression of genes associated with angiogenesis, cell proliferation, and breast cancer growth including aromatase.[31-33] Blocking leptin signaling with a peptide antagonist inhibits breast cancer-associated angiogenesis[34] and growth of estrogen receptor-positive and -negative breast tumors. Genetically obese leptin-deficient ob/ob mice as well as leptin receptor-deficient db/db mice fail to develop oncogene-induced mammary tumors.[35,36] While these studies clearly suggest the relevance of leptin signaling in mammary tumorigenesis, they cannot distinguish between local and systemic effects, and they also do not address the role of leptin signaling in adipocytes in the mammary microenvironment.

In F442A adipocytes aromatase expression is increased by incubating cells with recombinant mouse leptin (PeproTech) but not with leptin-triple mutant antagonist (leptin TA, Prospec) as shown in **Figure 2A**. Autocrine leptin signaling in F442A cells is supported by expression of the signaling long form of LepR (LepR-1) in differentiated F442A adipocytes but not in undifferentiated F442A pre-adipocytes (**Fig. 2B**). We also determined the effect of leptin on aromatase expression in F442A fat pads. To generate fat pads, F442A pre-adipocytes were expanded in vitro and injected sc into the flank of 6–8-week-old SCID mice (3×10^7 cells per mouse). After 21 d when fully differentiated and vascularized fat pads are established,[8] groups of mice were injected sc in the proximity of the F442A fat pad with 10 μg leptin or with vehicle. Total RNA was extracted from F442A fat pads 3 h later and analyzed by real-time PCR for aromatase expression as described.[37] As shown in **Figure 3A**, leptin injection resulted in a 6-fold increase of aromatase in F442A fat pads.

Obese women have a higher risk for hormone-dependent breast cancer development, recurrence and death[38,39] and obesity has also been found to hinder breast cancer treatment.[40] To address the issue of aromatase expression in obesity we compared aromatase expression in the adipose tissue of lean and obese mice in a model of diet-induced obesity (DIO). Lean six-week-old female C57BL/6J mice were placed for 16 weeks on either a high fat diet (HFD; D12492; Research Diets) in which 60% of the total calories were derived from fat or a control low fat diet (LFD; D12450B) in which 10% of the total calories were derived from fat. The HFD induced obesity whereas LFD resulted in moderate age-appropriate weight gain. After 16 weeks para-uterine adipose tissues were removed, RNA was prepared and aromatase mRNA determined by real-time PCR. Aromatase mRNA was increased more than 45 fold in adipose tissues of HFD induced obese mice compared with LFD fed lean mice (**Fig. 3B**). HFD-induced obesity is a model that closely resembles human obesity including a marked increase in circulating leptin. Therefore the increase in aromatase expression in HFD mice could potentially be due to higher leptin levels. To address this question further we tested aromatase expression in leptin-deficient genetically obese ob/ob mice. Aromatase mRNA in the para-uterine adipose tissue of adult obese C57BL/6J ob/ob mice was significantly lower than in matched lean wild-type control mice (**Fig. 3C**), suggesting that leptin drives aromatase gene expression in adipose tissue. To further test this hypothesis, we injected mice with recombinant leptin and found that leptin increased aromatase expression in the adipose tissue of leptin-deficient ob/ob mice and also in the adipose tissue of lean mice (**Fig. 4**). Together our results not only demonstrate that leptin signaling in the adipose tissues increases aromatase expression, but also suggest that leptin and aromatase in the local tumor microenvironment play a crucial role in development and growth of hormone-dependent breast cancer. These

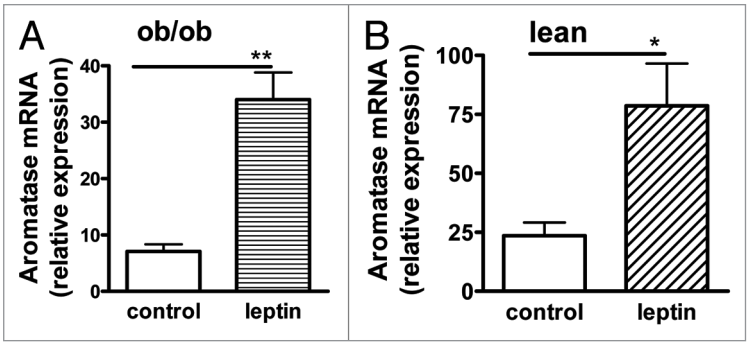

Figure 4. Induction of aromatase expression by leptin. Aromatase mRNA expression in para-uterine fat pads from ob/ob (**A**) and wild-type C57BL/6 mice (**B**) 3 h after ip injection with saline (control), or leptin (10 μg; R&D Systems). (**A and B**) $n = 6$, mean ± SD; $^{*}P < 0.05$ $^{**}P < 0.01$. Gene expression was measured using real-time quantitative PCR.

findings provide a foundation to understanding the regulation of aromatase in adipose tissues in general and in the tumor microenvironment specifically and can provide a rationale for targeting the adipocyte leptin-aromatase axis in the prevention and/or treatment of hormone-dependent breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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