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Recently it was shown that many breast cancer cell lines express leptin. In addition, leptin is increased in the plasma and adipose tissue of women with breast cancer. To investigate the role of leptin in mammary tumorigenesis, we asked whether estrogen regulated leptin expression, when during tumorigenesis leptin was first expressed, and developed tools to examine the functional consequences of leptin expression in mammary tissue. We examined leptin mRNA levels in numerous breast cancer cell lines and human tumors, confirming that leptin is widely expressed in this epithelium. There was no correlation between level of leptin expression and the mRNA levels for ER alpha or beta. Furthermore, in normal human organoids and in MCF7 cells, estrogen did not regulate the level of leptin expression. Neither DMBA-induced rat mammary tumors nor normal epithelium from DMBA treated or control rats showed any expression of leptin by RT-PCR, whereas leptin in rat mammary adipose tissue was abundant. Finally, we cloned the full-length human leptin cDNA into pcDNA3.1D/V5-His-TOPO (Invitrogen) and are making stably transfected clones for additional studies on the role of leptin in mammary tumorigenesis.

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Leptin Regulation of Mammary Tumorigenesis

INTRODUCTION:

For years we have known that increased truncal obesity is associated with post-menopausal breast cancer. Furthermore, breast size prior to pregnancy is positively correlated with postmenopausal breast cancer. Whether this is due to increased parenchyma or adipose tissue is not yet clear. In rodent carcinogen-induced models of breast cancer, loss of body fat is protective regardless of the mechanism by which adipose tissue is decreased. Due to this link between adipose quantity/metabolism and breast cancer, a number of investigators have looked for direct mechanistic links. For instance, it has been shown that specific fatty acid components (alpha-linolenic acid) in the adipose tissue appear to be protective against cancer induction. Recently, another link has formed between adipose metabolism and breast cancer. Energy metabolism within the adipose tissue is regulated, in part, by the hormone, leptin. Although leptin is usually thought of as a centrally acting mediator of satiety, leptin is also an angiogenic hormone, an activity that may relate to a physiologic function in adipose tissue (1). Tessitore et al have now shown that plasma and adipose levels of leptin are considerably higher in breast cancer patients relative to controls or patients with colorectal cancer (2). Furthermore, in these studies leptin levels correlated with elevated estrogen and progesterone receptors. Consistent with these studies, O'brien et al documented leptin mRNA levels in numerous breast cancer cell lines, including MCF-7 cells (3).

We became interested in looking at the relationship of leptin, angiogenesis, and breast cancer formation after the following study. We showed several years ago that pre-invasive breast diseases are angiogenic and that the degree of angiogenesis correlates with the risk of subsequently developing invasive breast cancer (4). To determine whether progression is dependent upon angiogenesis, we have been treating carcinogen-exposed rodents with the potent angiogenic inhibitor TNP-470. These rodents have a significantly diminished increase in body weight relative to vehicle controls. Surprisingly, along with the decrease in incidence of tumors there is nearly a complete loss of mammary adipose tissue. This correlates with a large body of chemopreventive literature that incidentally links decreased adipose tissue with decreased tumor formation.

There are several mechanisms by which leptin may be acting in breast tumorigenesis. First, leptin production may act centrally to increase satiety and therefore may be related to tumor-induced cachexia. Alternatively, leptin may act locally by stimulating angiogenesis in the adjacent adipose tissue. Angiogenesis in this setting would have two potential functions. First, angiogenesis would provide local nutritional support for the growing tumors. Second, angiogenesis is linked to triglyceride metabolism by the adipocytes, which may provide local nutritional support or stimulatory signals for the tumors. These local effects are consistent with the observation that depletion of adipose tissue, regardless of the mechanism, is associated with decreased tumor formation.

It is unclear now whether the data relating leptin and breast cancer are circumstantial or whether there is a direct link between mammary tumor formation and adipose tissue metabolism. Since mammary epithelium produces leptin and leptin expression correlates with both tumor formation

and ER/PR expression, we hypothesize that leptin expression increases during tumorigenesis and is regulated by estrogen. Furthermore, we propose that leptin expression stimulates tumor growth in a tissue site-specific fashion. To test these hypotheses we proposed the following experiments:

BODY:

Goal 1. We shall determine whether leptin is regulated by estrogen in MCF-7 cells and normal human mammary tissue.

Work Accomplished: The first challenge was to identify RT-PCR primers that worked in human tissues. We tried primer sets from several publications, using human adipose tissue as a positive control. Many did not work at all under any conditions of Mg^{++} or temperature. Eventually we settled on the primers published by Sobhani et al (5), which worked well in our hands, yielding a 224 basepair product. The sequences are as follows:

Forward: CCT GAC CTT ATC CAA GAT GG

Reverse: GAG TAG CCT GAA GCT TCC AG

PCR cycles were: Hot start followed by 95°C for 60 sec, 62°C for 30 sec, and 72°C for 2 min with a final extension of 72°C for 10min after 35 cycles. Reaction conditions included 1mM $MgCl_2$.

Using these primers we screened a number of breast cancer cell lines to confirm published data and to determine which cell line(s) expressed little or no leptin, for our future transfection studies. Semi-quantitative leptin RT-PCR was performed using actin for normalization. Each reaction was initiated using the same amount of cDNA (usually 10 or 20 ng) as measured by PicoGreen. As shown in **Figure 1**, leptin was abundantly expressed in all cell lines tested, with very low expression in SKBr3 and Hs 578T. In all cases we also performed RT-PCR for ERα and β. **Table 1** shows summary data from several experiments; data are expressed as the ratio of SybrGreen-stained band intensity for each unknown relative to actin. Leptin expression occurred independent of the expression of either estrogen receptor.

Figure 1

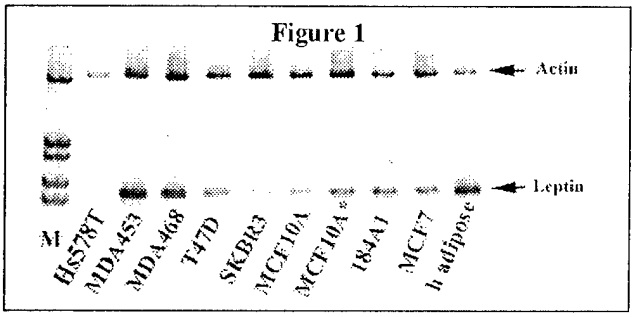


Table 1

	Hs578T	MDA-MB-453	MDA-MB-468	T47D	SKBR3	MCF7	MCF10A	MCF10A*	184A1	Adipose
Leptin	0.09	0.98	0.75	0.39	0.06	0.18	0.35	0.79	0.51	4.96
ERα	0	0	0.03	0.92	0.23	1.90	0.10	0.01	0.02	-
ERβ	0	0.06	0	0.02	0.40	-	0	0.05	0.04	-

Next, we isolated normal epithelial organoids from 11 mastectomy and reduction mammoplasties and prepared epithelium from 3 tumors. These were tested for leptin and ERα and β, as above. In the two cases in which we had matched normal and tumor tissue, leptin was 5 and 25 fold higher in the tumor tissue, consistent with leptin expression being driven by the process of tumorigenesis. **Table 2** shows an example of these data; again data are the ratio of the unknown to actin in SybrGreen-stained gels. "M" means the specimen came from a mastectomy; "R" means the tissue was from a reduction mammoplasty.

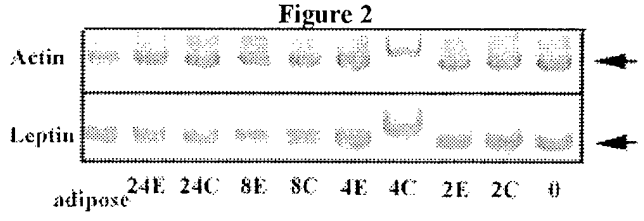
Table 2

	M1	M2	Tumor2	M3	M4	Tumor4	Tumor5	R15	R18	R25	Adipose tissue
Leptin	1.12	0.94	24.79	1.17	0.68	4.93	1.93	1.43	3.34	1.48	44.80
ERα	0.80	0.84	0.06	0.66	0.62	0.13	0.42	0.47	0.14	0.40	-
ERβ	1.09	1.43	0.39	1.02	1.02	0.10	0.53	0.74	0.10	0.81	-

As a further test for estrogen regulation of leptin expression, we treated MCF-7 cells, cultured in dextran charcoal stripped serum, with estradiol (10^{-8} M), tamoxifen (10nM), or a combination of the two. Depending on the experiment we examined leptin mRNA at various intervals between 2 and 24 hours. In all cases human adipose tissue was used as a leptin positive control. In no case did we see any evidence of estrogen regulation of leptin levels.

Finally, we treated normal human mammary organoids with 10^{-8}M estradiol and examined leptin/actin mRNA levels over a 24-hour period. An example is in **Figure 2**. Labels for each sample indicate whether the organoids were control "C" or estrogen treated "E" and the hours of estrogen treatment. As with the MCF7 data, we saw no evidence of estrogen regulation of leptin message levels.

Figure 2



Conclusion: Leptin is expressed in most breast cancer tumor cell lines and in primary isolates of human tumors. Leptin expression does not correlate with expression of ERα or β. Furthermore, there is no evidence for estrogen regulation of leptin mRNA levels.

Goal 2: We shall examine leptin expression during the course of tumorigenesis in the DMBA rat mammary carcinogenesis model system used in our laboratory.

Work Accomplished: The human primers did not amplify leptin from rat adipose tissue; therefore, we again tried several primer sets, settling on sequences from Jin et al (6). The sequences and conditions are as follows:

Forward: CCT GTG GCT TTG GTC CTA TCT G

Reverse: AGG CAA GCT GGT GAG GAT CTG

PCR conditions: Hot start followed by 95°C for 30 sec, 64°C for 30 sec, and 72°C for 45 sec with

a final extension of 72°C for 5 min after 35 cycles. Reaction conditions included 1mM $MgCl_2$.

Despite excellent amplification of leptin from rat adipose tissue, from multiple DMBA-induced tumors and from pooled normal epithelial organoids from either control or DMBA treated rats, we never saw any expression of leptin in mammary tissue.

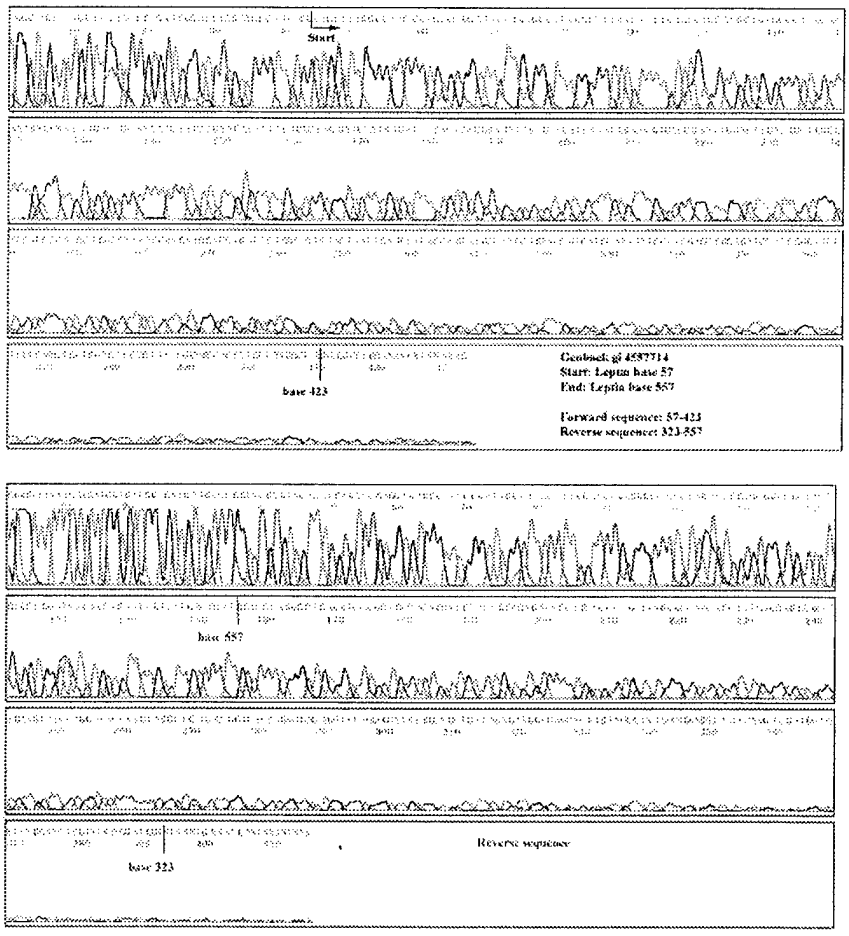
Conclusion: In this model system, leptin does not play a role in mammary tumorigenesis.

Goal 3: We shall determine whether leptin confers a growth advantage to MCF-7 cells in a site-specific manner.

In this specific aim we planned to acquire the leptin cDNA, express it in MCF-7 cells and look at the angiogenic capacity and growth rate of these transfected cells dependent upon site of cell injection (flank vs. mammary fat pad).

Work accomplished: Several aspects of this specific aim changed over the year. First, we determined that MCF-7 would be an inappropriate cell line for two reasons. 1. It expresses significant levels of leptin constitutively and therefore, overexpression would be less likely to have an effect than if we used a negative cell line. 2. It is highly angiogenic and therefore addition of another angiogenic factor would potentially be difficult to detect. Based upon our studies in Goal 1, we decided to use SKBr3 for our transfection studies.

Figure 3



The second aspect that changed was the lack of an available cDNA that we could acquire from the research community for our expression studies. Therefore, we cloned the full-length human leptin cDNA from MCF-7 cells and directionally inserted it using TOPO cloning into pcDNA3.1/V5-His-TOPO from Invitrogen. This vector uses the CMV promoter for high level eukaryotic expression. In addition it has V5 epitopes for protein

expression varification and a polyhistidine region for protein isolation. Colonies were screened by PCR using vector based primers and restriction digestion of the insert to ascertain fidelity. **Figure 3** shows the forward (top) and reverse (bottom) sequencing of this construct using plasmid primers, as determined by our core sequencing facility. Both forward and backward sequencing confirmed the integrity of the construct.

We are currently working on the transfection of this construct into SKBR3 to make stable transfectants. Based upon previous work, transiently transfected clones often give insufficient expression of growth factors to screen clones for in vitro angiogenesis. This is an assay that we routinely use in the lab in which cell lines in transwells are co-cultured with human umbilical vein endothelial cells to determine whether the cell line stimulates endothelial tubule formation (7). We hope to have stable clones soon, to test them for in vitro angiogenesis, and to move onto in vivo studies in the near future.

Conclusion: The complete leptin cDNA was PCR cloned into a CMV-driven expression vector for transfection studies.

KEY RESEARCH ACCOMPLISHMENTS:

- Confirmation that leptin is widely expressed in human breast cancer cell lines and human tumors.
- Discovery that leptin expression is unrelated to the expression of ER α or β.
- Discovery that leptin expressio is not regulated by estrogen.
- Discovery that leptin is not expressed in DMBA-induced rat mammary tumors.
- Cloning of the full-length human leptin cDNA into a eukaryotic expression vector.

REPORTABLE OUTCOMES:

None to date.

CONCLUSIONS: Leptin is widely found in human breast tumors and cell lines, but it is also highly expressed in isolated normal human breast organoids derived from mastectomies or reduction mammoplasties. Its expression is not regulated by estrogen nor is it related to expression of the estrogen receptors. Therefore, it seems likely that leptin may relate to normal mammary physiology. Whether it plays a role in mammary tumorigenesis is unclear. To continue exploring the role of this hormone in mammary tumorigenesis, we have cloned the human leptin cDNA into a eukaryotic expression vector.

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APPENDICES: None

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