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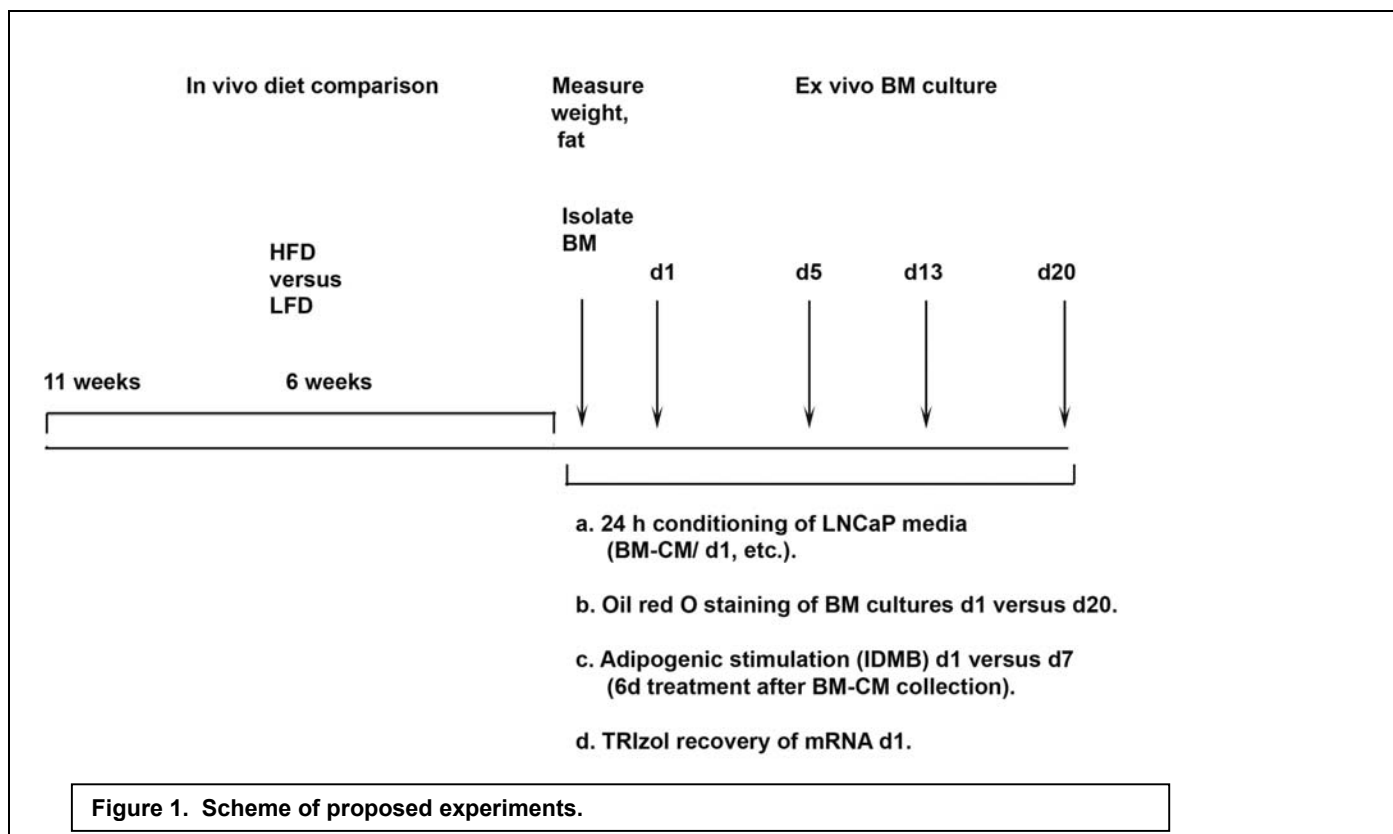
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14. ABSTRACT Obesity enhances prostate cancer (PC) metastasis to bone. Factors secreted from adipocytes enhance PC growth. We hypothesized that feeding a high fat diet to C57BL/6 mice would enhance bone marrow (BM) fibroblastic progenitor differentiation to adipocytes. Mice were fed isocaloric low (LFD) and high (HFD) fat diets for up to 11 weeks post-weaning. BM cells were isolated and cultured in serum-free RPMI 1640 media (PC media), generating BM conditioned media (BM-CM). The HFD increased the adipocyte population. BM-CM-LFD inhibited LNCaP cell growth. The HFD partially reversed this suppression. Cytochrome P4501B1 (CYP1B1), which is expressed in BM and in prostate tumor stroma and epithelia, is suspected to influence PC. CYP1B1/- BM-CM increased the proportion of DU145 cells in S-phase relative to C57BL/6 media. We conclude that BM cells secrete PC inhibitory paracrine factors, but also generate a stimulatory component in response to the HFD. CYP1B1 metabolism positively affects PC growth by metabolizing growth inhibitory factors.					
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Introduction

The goal of this study is to test the effect of fat cell differentiation in bone marrow (BM) on the proliferation of human prostate cancer (PC) cell lines. We compare mice fed low and high fat diets and then examine the effects produced by BM cells that have been maintained in culture for 0-20 days (**Figure 1**).



Body

Aim1. Characterize how *in vivo* adipogenesis in bone marrow stromal cells (BMS) affects the growth of LNCaP prostate cell lines.

1a. Primary BMS from mouse bone marrow selectively support the growth of LNCaP cell lines

BMS have been isolated from 14 week old C57BL/6 male mice through the flushing of the femur, as previously described by this laboratory (1). We originally proposed to use three co-culture methods; a) a two-compartment Boyden chamber that allows factors to transmit between prostate cancer (PC) and BMS cells; b) direct co-culture in which there is contact between PC and BMS cells; c) culture of prostate cells with enriched media. We also proposed to culture the prostate cells on plastic, laminin, or collagen 1 gels. We have found that culture of BMS cells embedded in collagen 1 gel affects the stimulation of LNCaP cells. However, the primary focus of the work has been to test, in Aim 1b, whether media from BM cells provides an enhanced stimulation of LNCaP cell proliferation. Recent work (2) has indicated that leptin and other

cytokines secreted from stromal cells, indeed, stimulate the growth of a variety of tumor cell lines. We, therefore, decided to initially limit our methods of isolating media from bone marrow in order to test the effect of dietary fat.

As such, we have collected media from BM cells isolated from the femurs of mice maintained for 11 weeks on a diet wherein 10% (low fat diet, LFD) of the calories are provided by animal fat. The mice were placed on the diets at weaning (3 weeks of age) and sacrificed 11 weeks thereafter (14 weeks of age). Typically, each mouse provided 2×10^7 cells, which were cultured in a 10 percent serum medium for 1-20 days during which time differentiation progresses in adherent stromal cells and loosely attached hematopoietic cells. After 0, 5, and 20 days of culture, the cells were incubated for 24 hours with serum-free RPMI 1640 prostate cell growth media, which contains only buffer components and amino acids in the absence of growth factors. This conditioned media (BM-CM) was removed and used in co-incubations with human prostate cell lines. We also generated media from BM cells after culture in a collagen 1 gel. This provides a way to more closely mimic the bone environment and to eventually complete co-cultures with PC cells. We compared the effects of conditioned media to the effects of media that was not exposed to cells.

The amount of BM cells from the mice provided only modest amounts of conditioned media, thus limiting our capacity to test effects on prostate cells. We have, additionally, compare primary BM media with media conditioned by, respectively, a BM stromal line (BMS2) and a mouse embryo fibroblast line (C3H10T1/2). Each of these lines undergoes adipogenic and osteogenic differentiation. Osteogenic differentiation is substantially favored over adipogenesis for the BMS2 line, while the reverse is true for C3H10T1/2 cells. We tested the effect of activating adipogenesis (48 hours with differentiation cocktail).

We tested the effect of the media on the cell proliferation of LNCaP cells by means of the colorimetric MTT assay, which we adapted to a 12 well format. This test uses reduction of MTT to quantify mitochondrial activity. As such, the *increase* in the MTT measurement in the final 2 hours of a 24 hour conditioned media exposure is proportional to the increase in cell number. We, therefore, report the changes in MTT after 24 hour BM media exposure relative to the zero time value.

This test has allowed us to carry out triplicate assays on LNCaP cultures. In each case, we omitted androgens from the media for these otherwise androgen responsive cells, thus providing low basal growth rates. As a means to standardize our methodology, we tested the response of the cells to increasing concentrations of the synthetic androgen, R1881 (**Figure 2**). We also examined the effects on DU145 cells, an androgen-independent prostate cell line.

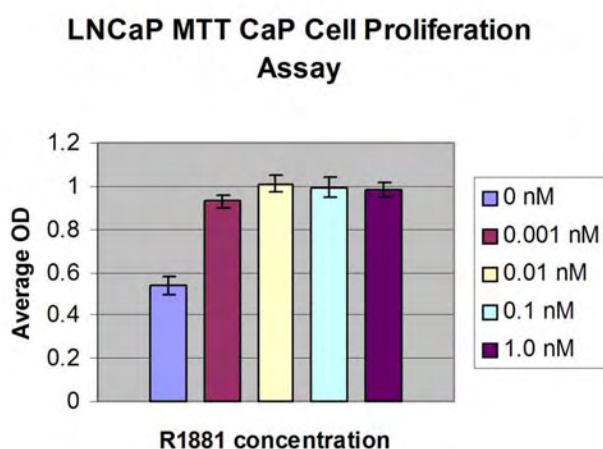
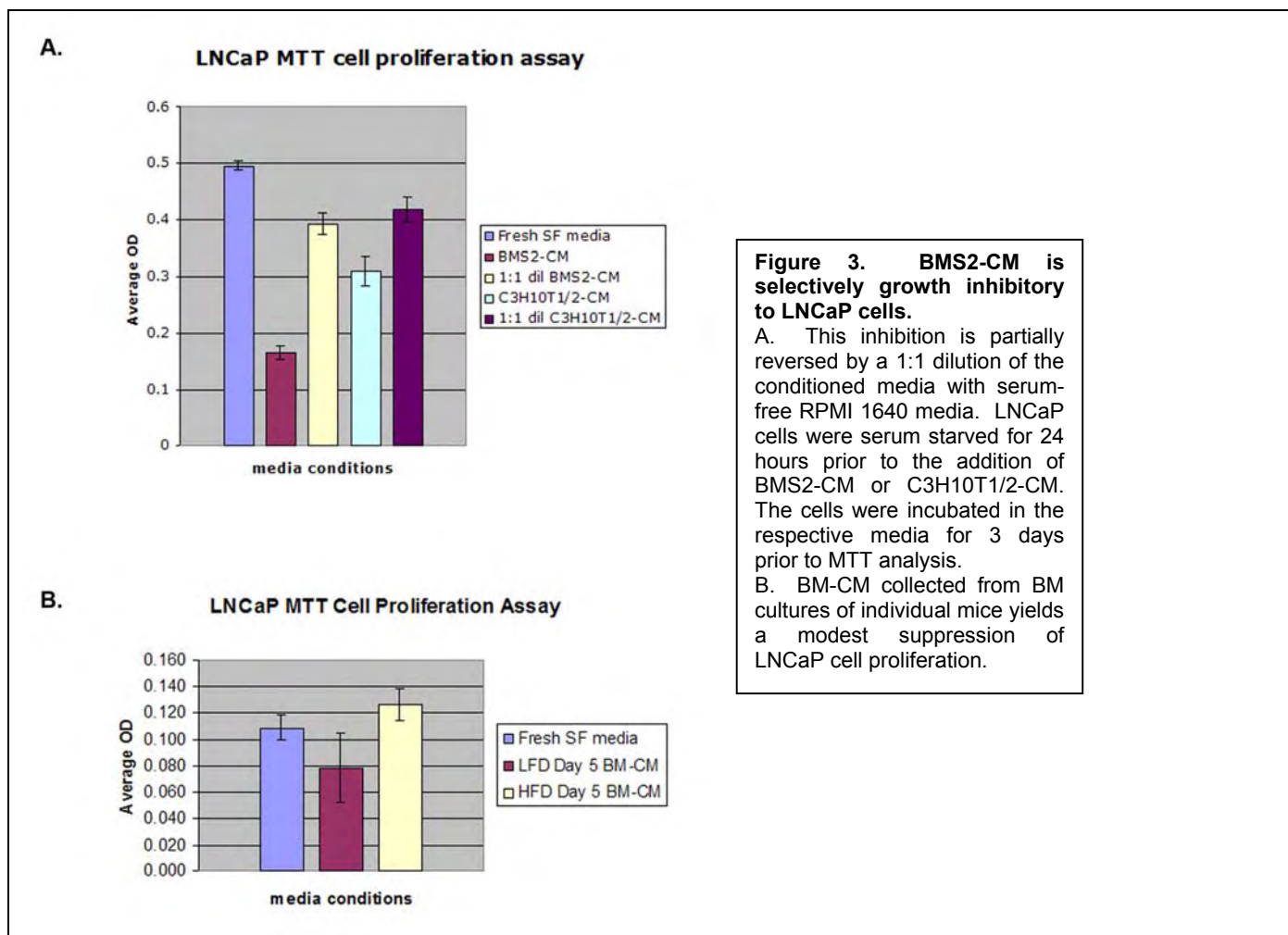


Figure 2. Androgen-dependent LNCaP cells respond to increasing concentrations of R1881. LNCaP cells were treated with increasing concentrations of the synthetic androgen, R1881, for 24 hours prior to MTT analysis.



We have also developed a BrdU-incorporation method, based on fluorescence activated cell sorting (FACS) to assay changes in the cell cycle and apoptosis. In this assay, LNCaP cells are incubated with the conditioned media for 24 hours prior to fixation and staining. BrdU is added to the media for the final 2 hours. In Aim 1b, these assays have been used to compare the effects of conditioned media from mice maintained on low and high fat diets.

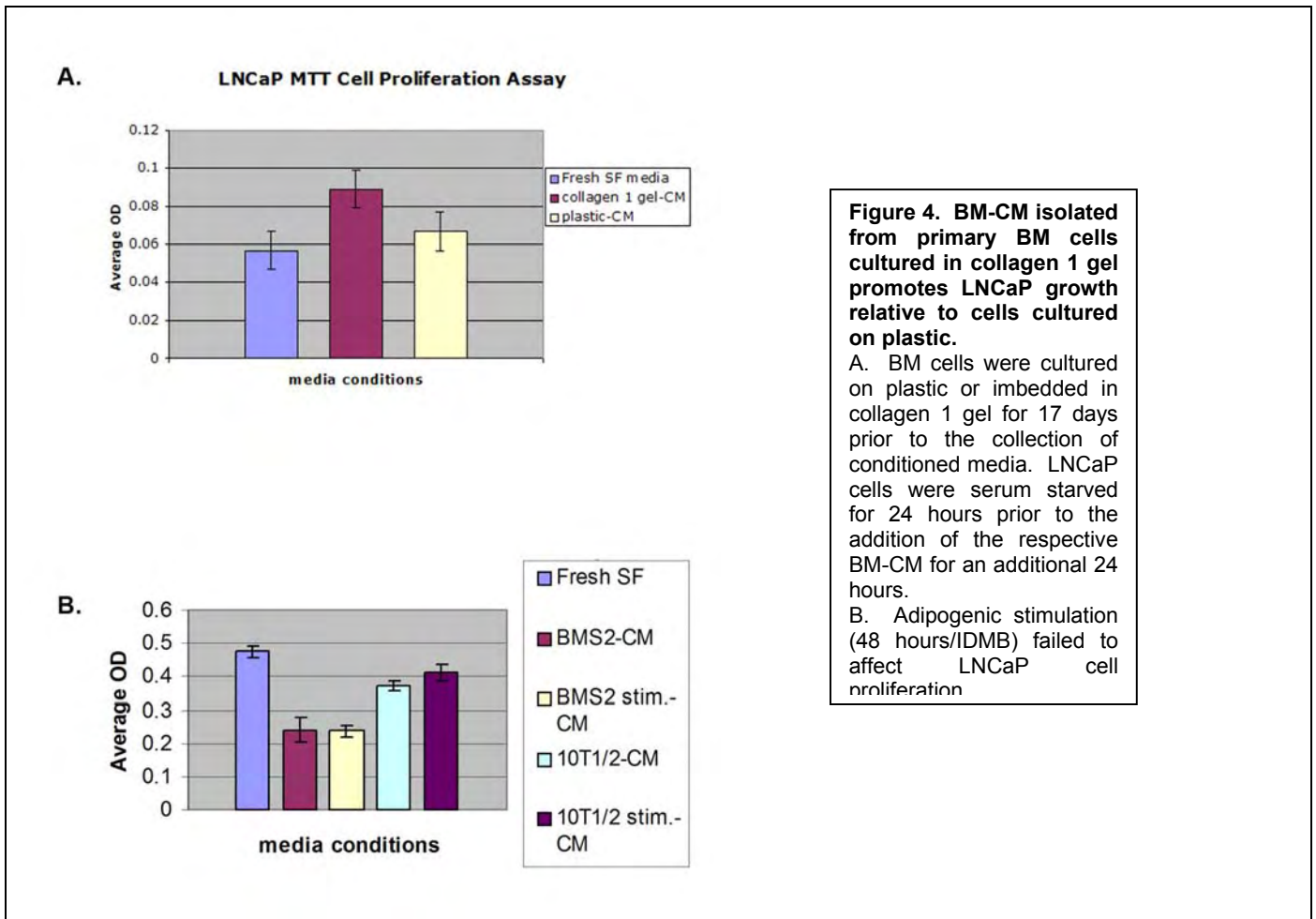
Outcomes

Figure 2 shows that the LNCaP cells were stimulated by low concentrations of androgen, but that a reversal occurred at high concentrations. This reproduces previously published responses (3).

In **Figure 3A**, MTT analyses show that LNCaP cells exposed to media from BMS2 cells (BMS2-CM) exhibit appreciable growth suppression compared to media from C3H10T1/2 cells. Dilution of the BMS2 media (1 part BMS2-CM: 1 part serum-free RPMI1640 media) decreased the suppression.

Cultures from individual mice on LFD produced variable, modest suppression of LNCaP growth proliferation, similar to that seen with C3H10T1/2 cells (**Figure 3B**). An increase in the culture time prior to the 24 hour media conditioning (5 days to 20 days) had no effect.

We have analyzed DU145 cells cultured with standard media and BM-CM using BrdU labeling and fluorescence activated cell sorting (FACS). The FACS analysis of the cell cycle distribution with control and LFD BM-CM had no effect on proliferation. We are currently doing the equivalent BrdU/FACS experiment with LnCAP cells.



We suspected that the effects of the BM cells would be very susceptible to the surrounding extracellular matrix. **Figure 4A** shows that conditioned media isolated from BM cells substantially enhances LNCaP proliferation when the BM cells are cultured in collagen 1 gel compared to plastic. This stimulation was comparable to androgen stimulation. By contrast, adipogenic stimulation of BMS2 or C3H10T1/2 cells to pre-adipocytes (48 hours/IDMB) had no effect on the activity (**Figure 4B**).

Experiments as yet not completed.

We have not tested the effects of BM-CM on alternative LnCAP sublines.

We have not used alternative methods of co-culture, including transwells and direct co-culture.

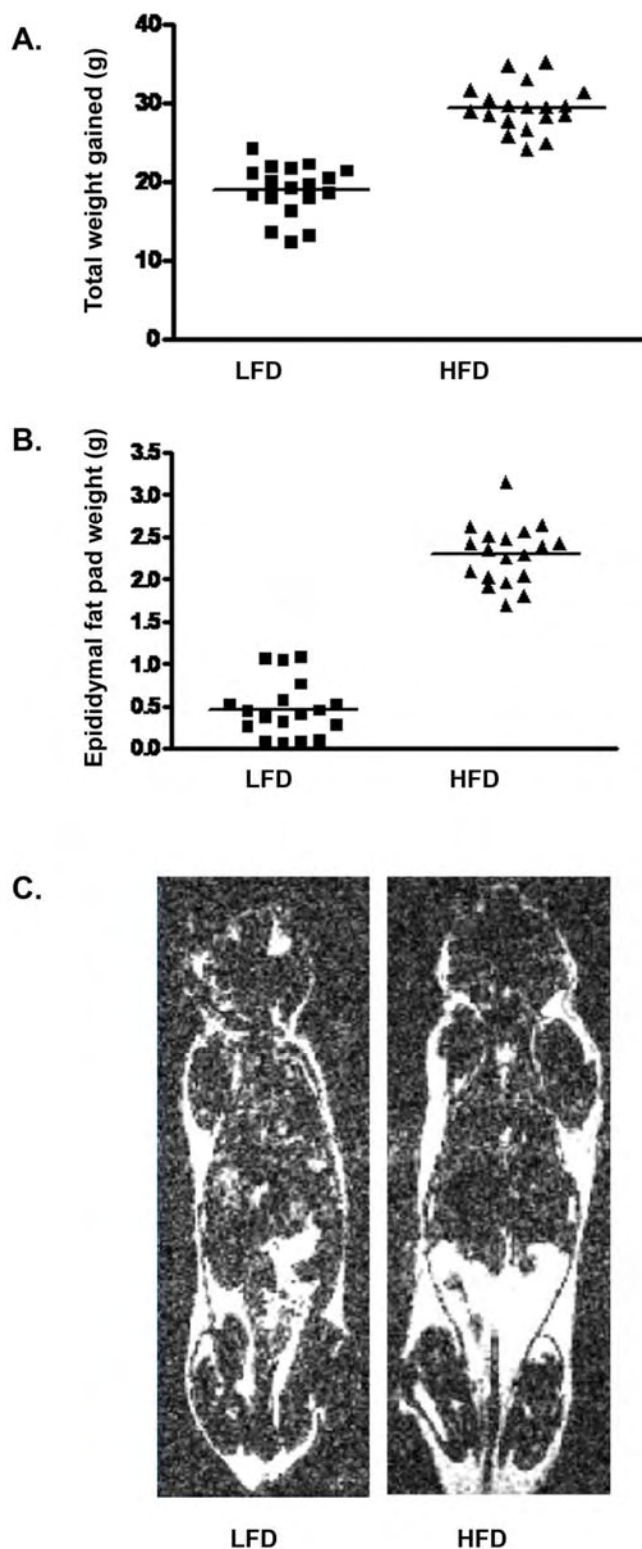


Figure 5. Weight accumulation on the HFD corresponds to increased overall fat pad development..

A. Total weight gain in mice fed the LFD or HFD for 11 weeks post-weaning. Each symbol represents an individual animal, with the average weight gain indicated by the solid line.

B. Epididymal fat pad weight in each of the animals presented in panel A.

C. MRI image analysis of mice fed the LFD or HFD for 11 weeks post-weaning. The adipose tissue is visualized as the white areas in the image.

1b. Increased *in vivo* adipogenesis of BMS isolated after a high fat diet affects proliferation of LNCaP cells in a co-culture model.

We first established the obesity mouse response (body weight and fat pad increases) to elevated dietary fat. We compared male mice fed from weaning (pnd21) on the LFD with mice fed a 60% high fat diet (HFD). Measurement of the food intake showed that the mice had similar caloric intake (less carbohydrate intake). Mice were sacrificed to obtain BM cells, which were used for the generation of conditioned media and for mRNA isolation. The BM cells were cultured for the first 24 hours in serum-free RPMI1640 media. We have used this media, as described in Aim 1a, to test the impact of dietary fat and obesity on BM-CM PC proliferation.

Outcomes:

Figure 5A shows the differences in total weight gain and **Figure 5B** the increase in epididymal fat pads after 11 weeks on the diet. There was a three-fold increase in the weight of the fat pad during this period. We also used MRI imaging, **Figure 5C**, to demonstrate that these increases were uniform across all fat pads. These obesity responses are typical of those obtained in other studies (4).

We compared the effect of BM-CM from LFD and HFD on LNCaP cells using the MTT test. In various experiments, we have collected BM-CM at days 1, 5, 13, and 20 of culture. **Figure 6** shows results from days 5 and 20. Day 13 provided similar results, while day 1 remains to be analyzed. The data shows that individual animals vary in their responsiveness. However, a similar trend is observed in all animals wherein the BM-CM isolated from animals on the LFD diet substantially inhibits LNCaP cell proliferation, while the HFD BM-CM partially alleviates this suppressive effect.

Table 1 compares the cell cycle effects of the BM-CM (LFD versus HFD) on the cell cycle distributions of DU145 cells. This shows that the BM-CM from the mice fed the HFD led to a 5% increase in the arrest of the cells in S-phase relative to the animals provided the LFD, with a complementary increase in the percentage of apoptotic cells.

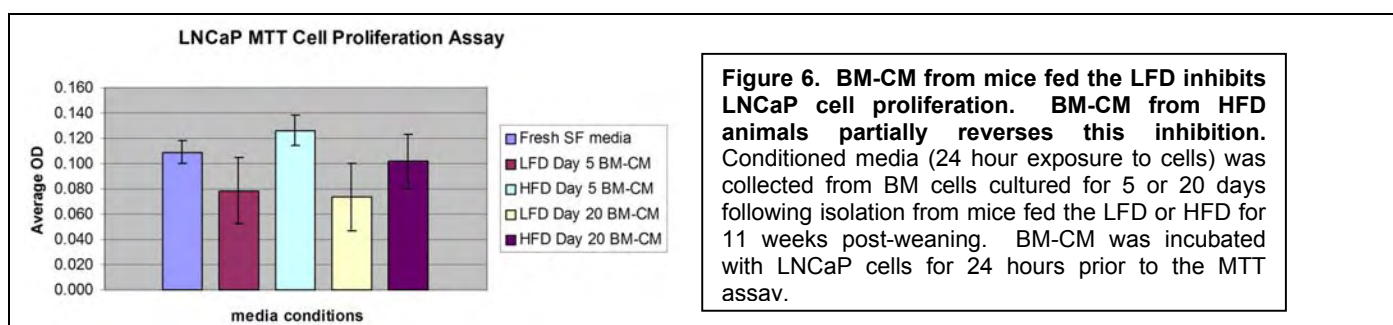


Table 1. BrdU Incorporation in DU145 Cells Exposed to C57BL/6 BM Conditioned Media.

Diet	G0/G1	S	G2/M	Apoptosis
Contol SF media	61.2	26.9	11.9	10.1
LFD	59.3	28.9	11.7	10.9
HFD	58.6	34.01	7.39	17.8

1c. Assess the increase in BMS adipogenesis produced *in vivo* by high dietary fat *in vivo*.

In order to assess the effect of diet on the formation of adipocytes, we stained the BM cultures with Oil Red O, which is taken up by lipid droplets found in adipocytes. We also tested whether the HFD diet affected precursor cells, which differentiate to adipocytes with more extensive culturing.

Outcomes:

We found that there were very few adipocytes in the eluted BM cells immediately after isolation from the animals maintained on the diets for 11 weeks (14 weeks of age). In contrast, BM cultures from mice maintained on the diet for only 6 weeks (sacrificed at 9 weeks of age) demonstrated significant numbers of adipocyte clusters after 24 hours of culture. We identified the number of adipocyte colonies forming per well using duplicate cultures of each of three mice (**Figure 7**). This points to a need to test the effects of 6 week BM-CM on PC proliferation (media has been collected). Clearly, the HFD is generating more adipocytes in these younger mice. The response is lost in the more mature animals.

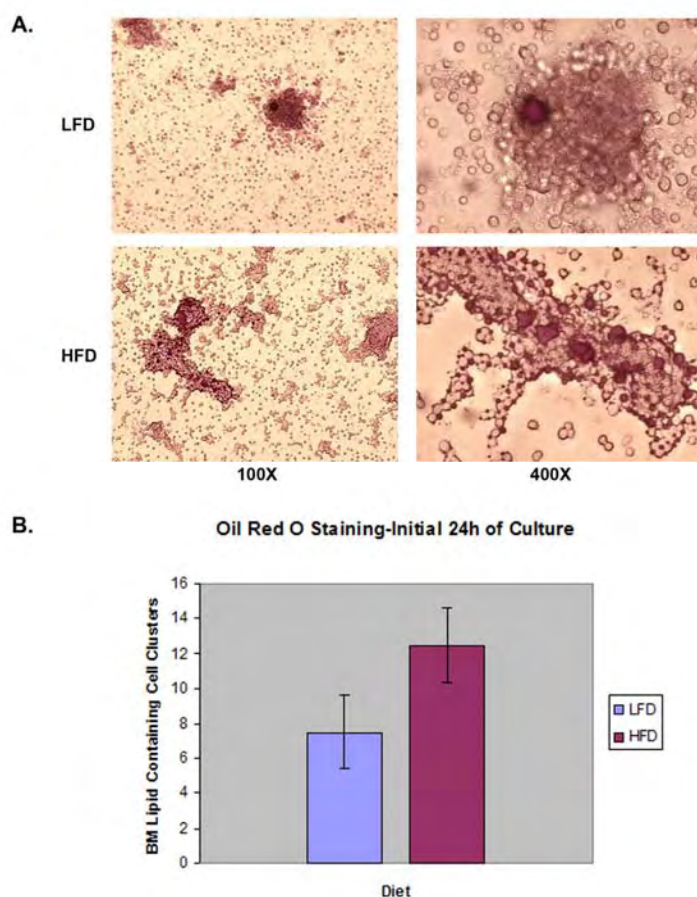


Figure 7. HFD increases the proportion of adipocytes in the BM.

Mice were fed the LFD or HFD for 6 weeks post-weaning. BM cells were isolated and immediately placed in culture for 24 hours for the generation of conditioned media.

A. Cells were subsequently fixed and stained with Oil Red O.

B. Quantification of the average number of foci containing greater than 10 adipocytes under each dietary condition.

Aim 2. Identify how BMS adipogenesis *in vitro* changes the growth support for LNCaP cells. Do factors from LNCaP cells affect adipogenesis or osteogenesis in ways that alter the release of factors that influence cell growth.

We have established that a standard differentiation cocktail, when applied to BM cells, indeed induces adipogenesis. We have examined this response in the 14 week old mice, where adipocytes were not seen under basal culture conditions. In our protocol, we initiated adipogenesis with IDMB (insulin, dexamethasone, isobutylmethylxanthine after the 24 hour generation of media for the LNCaP co-culture. The BM cells are exposed to IDMB for 48 hours and insulin for the subsequent 6 days. After this period, the cells were incubated in serum-free RPMI 1640 media for 24 hours to obtain BM adipocyte conditioned media (BM-CM/Ad). Our goal here is to test whether *in vivo* exposure to HFD compared to LFD enhances the response to the standard adipogenic cocktail. An increased response would suggest the increased presence of BM adipogenic progenitor cells. We have collected TRIzol extracts to test the extent of differentiation. The following real time PCR primer pairs have been generated in our laboratory and have been shown to be effective on our adipocyte mRNA extracts: PPAR γ , C/EBP α , perilipin, FABP4, Glut4, and leptin.

The response of these BM primary cultures was compared to differentiation of BMS2 cells after an equivalent treatment. Conditioned media was also obtained from these differentiated BMS2 cultures (BMS2-CM/Ad).

The BM-CM/Ad media are being tested for effects on LnCAP proliferation comparison to control and pre-differentiation CM (MTT assay, BrdU incorporation).

Outcomes:

Oil Red O staining identified increased adipocyte clusters in response to the IDMB treatment in BM cultures generated from animals maintained on either the LFD or HFD (**Figure 8**). Importantly, the adipogenic differentiation was substantially increased by the HFD. The extent of HFD differentiation was similar to that seen with BMS2 cells. The comparison of these adipogenic responses (# of foci containing greater than 10 adipocytes) for replicate treatments of BM cells from three separate mice are shown in **Table 2**.

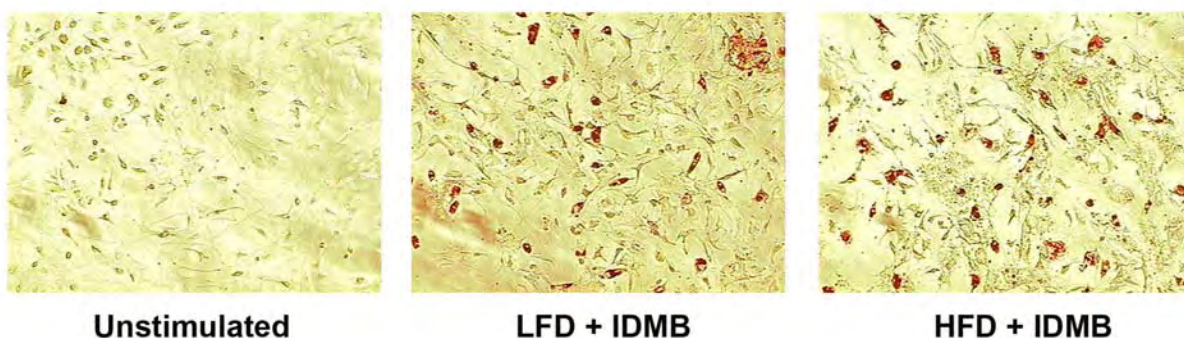


Figure 8. HFD increases the pre-adipocyte population in the BM.

BM cells were cultured from mice fed the LFD or HFD for 11 weeks post-weaning. Pre-adipocytes were stimulated to adipocyte differentiation by the addition of IDMB, as quantified in Table 2.

Table 2. IDMB-Stimulation of Cultured BM Cells Following 11 Weeks on the Low or High Fat Diets.

Mouse ID	Diet	# of Foci Containing > 10 Adipocytes
I49	LFD	1
I50	LFD	0
I51	LFD	0
I45	HFD	1
I46	HFD	3
I47	HFD	2

Aim 3. Use micro-arrays and PCR to identify factors released by BMS that stimulate DNA synthesis in LNCaP cells and determine how this release is affected by the high fat diet or *in vitro* adipogenesis. Identify factors released from LNCaP cells that influence adipogenesis.

Our goal is to use Affymetrix mouse arrays to measure the expression profiles of mRNA isolated from BM stromal cells that have been obtained from least 6 different mice after *in vivo* administration of HFD and LFD. The BM stromal cells were cultured for 24 hours and separated from less adherent hematopoietic cells prior to isolation of mRNA using the TRIzol reagent. These expression arrays will be used to identify increases in secreted proteins that may affect LNCaP or DU145 proliferation.

To broaden the assessment of the effects of B-MCM on LNCaP and DU145 cells, we have also isolated mRNA following control and BM-CM treatments. We have developed the following real time PCR primer pairs to assess the cell responses: prostate specific PSA, androgen receptor (AR), ornithine decarboxylase (ODC).

In order to test the array methodology, we have compared the expression profiles of control BMS2 and C3H10T1/2 cells. These cells produce media which have very different effects on LNCaP cells.

Outcomes :

Over 200 genes exhibited at least 5-fold differences in basal expression between BMS2 cells and C3H10T1/2 cells. These included several growth factors, cytokines, and chemokines. Some key differences are shown in **Figure 9**.

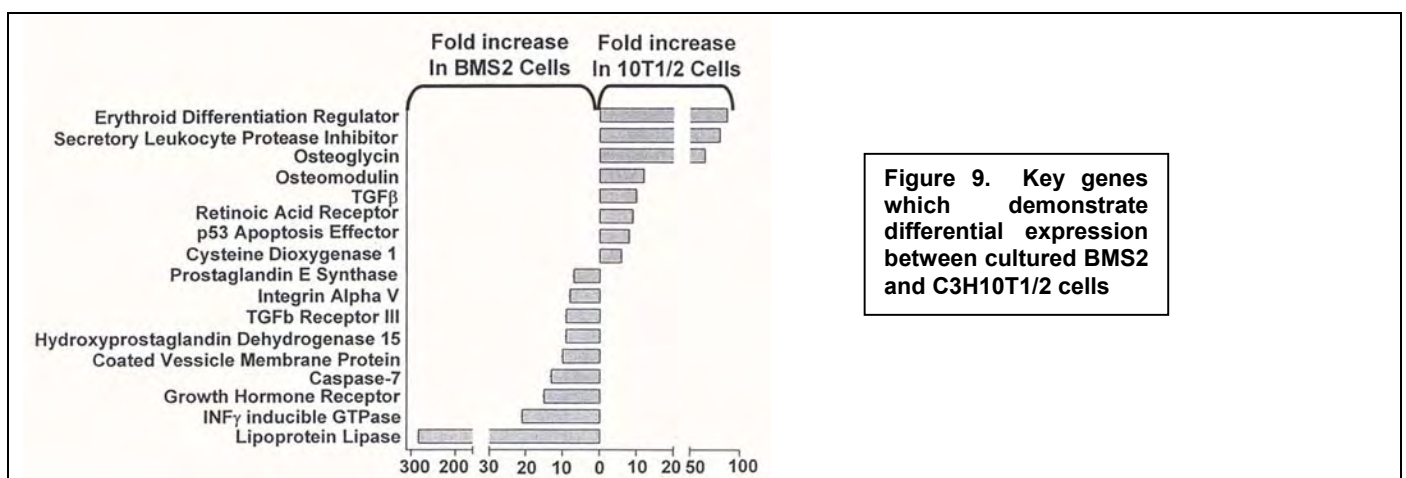
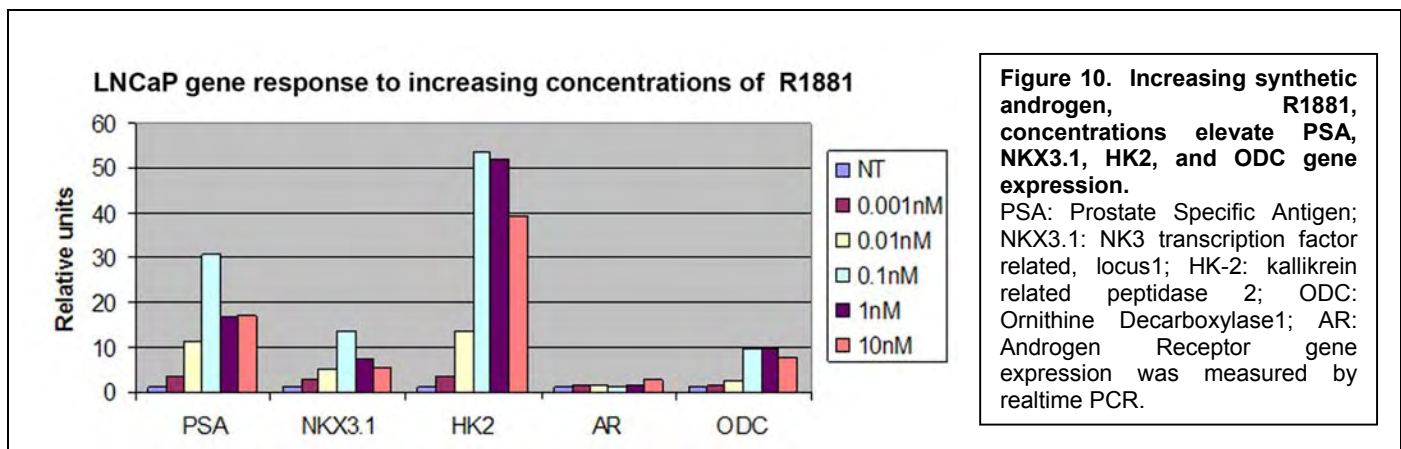


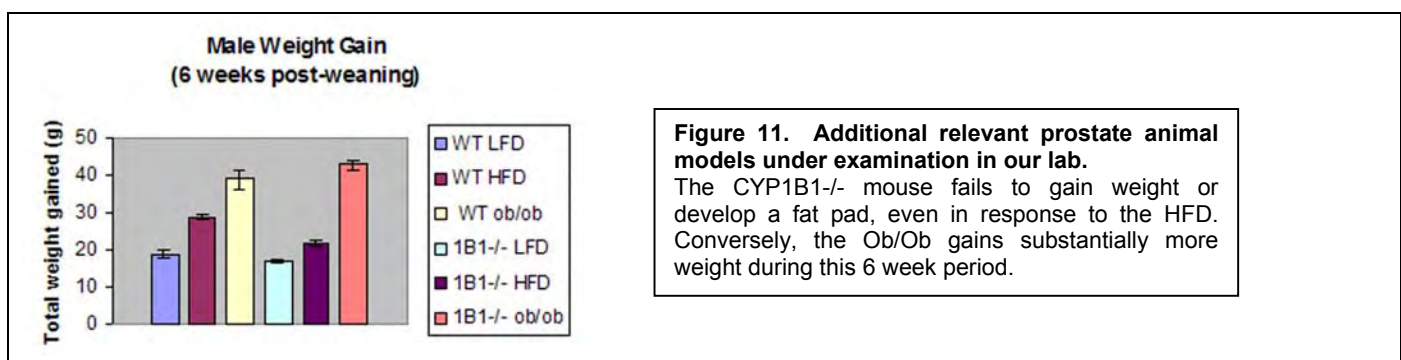
Figure 9. Key genes which demonstrate differential expression between cultured BMS2 and C3H10T1/2 cells

We have validated the PCR primers by testing androgen responses in LNCaP cells (**Figure 10**).



Supplementary Experiments (mice not funded by DOD). Effects of conditioned media isolated from BM of CYP1B1^{-/-} mice and Ob/Ob (leptin-deficient) mice.

In carrying out our prescribed experiments, another research program in the laboratory (gene effects on obesity in mice fed HFD) has provided the opportunity to expand our study. C57BL/6 mice deficient in cytochrome P450 1B1 (CYP1B1) and leptin (Ob/Ob) expression are of particular interest since each impact on prostate tumor growth (5-6). The CYP1B1 ^{-/-} mice fail to increase weight or develop extensive fat pads on the HFD. By contrast, the Ob/Ob mouse becomes more obese in 6 weeks than the standard C57BL/6 mouse, which has attained most of the weight gain by the 6 week mark (**Figure 11**). We have examined adipogenesis and isolated BM-CM in exactly the same way as for the LFD and HFD treatments of wild type C57BL/6 mice.



We have stimulated BM cultures with IDMB, as described in Aim 2, in order to evaluate the effect of CYP1B1 deletion on the formation of pre-adipocytes following Oil Red O staining. Maintaining the mice on the HFD for 11 weeks post-weaning (14 weeks of age) resulted in a greater than 2-fold increase in the number of foci containing greater than 10 adipocytes relative to the C57BL/6 animals (**Table 3**). This stimulation was substantially enhanced by isolating the BM cells from younger animals, which had been exposed to the LFD for only 6 weeks (9 weeks of age). This data indicates that although CYP1B1 deletion prevents fat pad development, enhanced pre-adipocyte development is occurring in peripheral tissues, such as the BM.

Table 3. IDMB-Stimulation of Cultured C57BL/6 and CYP1B1^{-/-} BM Cells Following 6 or 11 Weeks on the Low or High Fat Diets.

<u>Genotype</u>	<u>Diet</u>	<u>Average # of Foci Containing > 10 Adipocytes</u>
11 weeks on diet		
C57BL/6	LFD	1
1B1 ^{-/-}	LFD	0
C57BL/6	HFD	6
1B1 ^{-/-}	HFD	14
6 weeks on diet		
1B1 ^{-/-}	LFD	24

We have, likewise, examined the BM the Ob/Ob mice after 13 days in culture (standard protocol in the IDMB stimulation) and found that the BM contained such large numbers of mature adipocytes, in the absence of IDMB stimulation, that counting foci was not feasible. We have, however, quantified the number of positively-staining foci in the BM cells on the day of isolation (day 0) and have found no difference between the Ob/Ob mouse with or without CYP1B1 expression (**Table 4**).

Table 4. Oil Red O Analysis of Ob/Ob and Ob/Ob CYP1B1^{-/-} BM Adipocytes on the day of BM isolation (d0) Following 6 Weeks on the LFD.

<u>Genotype</u>	<u>Average # of Foci Containing > 10 Adipocytes</u>
Ob/Ob	10
Ob/Ob 1B1 ^{-/-}	12

We have also examined the effect of BM-CM collected from day 5 cultures of wild type C57BL/6 or CYP1B1^{-/-} mice maintained for 11 weeks of the HFD on DU145 cell BrdU incorporation. **Table 5** shows that CYP1B1 deletion substantially increases the number of cells in S-phase, independent of the dietary conditions. We are currently evaluating the effect of this BM-CM on LNCaP cell proliferation in both the MTT and DU145 proliferation assays.

Table 5. BrdU Incorporation in DU145 Cells Exposed to C57BL/6 and CYP1B1^{-/-} BM Conditioned Media.

<u>Diet/Genotype</u>	<u>G0/G1</u>	<u>S</u>	<u>G2/M</u>	<u>Apoptosis</u>
Contol SF media	61.2	26.9	11.9	10.1
<u>LFD</u>				
CYP1B1 ^{-/-}	48.5	40.7	10.9	14.0
C57BL/6	59.3	28.9	11.7	10.9
<u>HFD</u>				
CYP1B1 ^{-/-}	46.9	41.9	11.2	16.7
C57BL/6	58.6	34.01	7.39	17.8

Collectively, the CYP1B1^{-/-} model demonstrates that CYP1B1 deletion increases the proportion of adipocytes in the BM and the BM-CM stimulates the progression of DU145 cells to S-phase.

Key Research Accomplishments

- Culture of BM stromal cells embedded in collagen 1 gel affects the stimulation of LnCAP cells.
- BMS2-CM is selectively growth inhibitory to LNCaP cells.
- BM-CM from mice fed the LFD inhibits LNCaP cell proliferation. BM-CM from HFD animals partially reverses this inhibition.
- HFD increases the proportion of pre-adipocytes and adipocytes in the BM.
- CYP1B1 deletion prevents fat pad development and mediates enhanced adipocyte development in the BM.
- CYP1B1 deletion substantially increases the number of cells in S-phase relative to C57BL/6, independent of the dietary conditions.

Reportable Outcomes

This funding has provided the seed for additional support for this research by the University of Wisconsin Comprehensive Cancer Center O'Brien Fund for prostate cancer research. To date, insufficient data has been generated for a publication. However, we have submitted an abstract for the September 2007 DOD prostate cancer meeting in Atlanta, GA.

Conclusion

This one year project has provided ample evidence in support of our initial hypothesis that there is a relationship between adipocyte differentiation in the BM and prostate tumor cell growth. Ongoing studies are examining the effect of *in vivo* diet and CYP1B1 status on the expression of genes that secrete proteins, which could modulate PC growth. This project can eventually impact prostate cancer research by identifying tumor growth factors that are secreted from the bone marrow that respond to dietary fat consumption and CYP1B1 metabolism, and which could potentially affect prostate cancer metastasis to the bone. The frequent, functional human CYP1B1 polymorphisms may also affect this response.

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