SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
CELL PROLIFERATION
CELL GROWTH
CANCER MODELS
BIOLOGICAL MODELS

Received
23 May 2013

Accepted
25 November 2013

Published
10 December 2013

Correspondence and requests for materials should be addressed to K.H.M. (moleyk@wudosis.wustl.edu)

Maternal high-fat diet induces hyperproliferation and alters Pten/Akt signaling in prostates of offspring

Emily C. Benesh[1], Peter A. Humphrey[2], Qiang Wang[1,3] & Kelle H. Moley[1]

[1]Department of Obstetrics and Gynecology Washington University in Saint Louis School of Medicine 425 South Euclid Ave. BJC-IH 10th Floor, Campus Box 8064 Saint Louis, MO 63110, [2]Department of Pathology and Immunology Washington University in Saint Louis School of Medicine Peters Building, 3rd Floor Saint Louis, MO 63110, [3]State key laboratory of reproductive medicine Nanjing Medical University Nanjing, China 210029.

Developing recommendations for prostate cancer prevention requires identification of modifiable risk factors. Maternal exposure to high-fat diet (HFD) initiates a broad array of second-generation adult disorders in murine models and humans. Here, we investigate whether maternal HFD in mice affects incidence of prostate hyperplasia in offspring. Using three independent assays, we demonstrate that maternal HFD is sufficient to initiate prostate hyperproliferation in adult male offspring. HFD-exposed prostate tissues do not increase in size, but instead concomitantly up-regulate apoptosis. Maternal HFD-induced phenotypes are focally present in young adult subjects and greatly exacerbated in aged subjects. HFD-exposed prostate tissues additionally exhibit increased levels of activated Akt and deactivated Pten. Taken together, we conclude that maternal HFD diet is a candidate modifiable risk factor for prostate cancer initiation in later life.

Prostate cancer (PCa) is the second most commonly diagnosed cancer in western males, and 1 in 36 American men will die of this disease[1]. In 2010, the estimated total economic burden of prostate cancer in the United States was $11.5 billion[2]. To lessen this burden on society, modifiable risk factors must be identified, but the only known risk factors for PCa – age, ethnicity, and familial history – are not modifiable[1]. The role of preventable factors such as diet and environment in the development of PCa is not clear[3,4]. For example, obesity appears to be associated with incidence of PCa, but only with advanced cases[5,6].

The Fetal Origins of Adult Disease hypothesis posits that perinatal environmental factors generationally transmit to their offspring predisposition to certain diseases that may present during adulthood[7]. Our group and others have shown that several disease conditions in offspring are influenced by maternal obesity during the gestational period, which we define as the peri-conception, *in utero*, and lactation periods. For example, maternal overnutrition and gestational diabetes mellitus contribute to a wide range of second-generation cardiometabolic disorders in both humans and rodent models[8–10]. Furthermore, disorders such as congenital malformations[11], autism[12], asthma and atopic conditions[13,14], and behavior syndromes such as attention deficit hyperactive disorder[15] are also subject to fetal programming. In murine models, maternal perinatal high-fat diet (HFD) impairs the quality of female gametes and leads to meiotic aneuploidy, embryonic loss, growth retardation, and brain defects in offspring[16]. Thus, a wide variety of embryonic and adult disorders are programmed by maternal gestational diet, which is modifiable.

One identifying marker of human and murine prostate cancers is disrupted activity of the tumor suppressor *Phosphatase and tensin homolog* (*Pten*)[17–19]. Pten, a protein- and lipid-phosphatase, is part of the Protein Kinase B (Akt) pathway, which ubiquitously regulates cell growth and survival[18]. In the canonical Akt pathway, ligand-bound receptor tyrosine kinases stimulate Phosphatidylinositol 3-kinase (PI3K) to convert phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3)[20]. Akt binds to PIP3 at the plasma membrane and undergoes post-translational modifications, such as activating Serine 473 phosphorylation[20,21]. Activated Akt then phosphorylates downstream targets to regulate cell behaviors including proliferation and programmed cell death; hyperactivation of this pathway is thus a frequent cause of tumor growth[21,22]. Pten converts PIP3 to PIP2, thereby antagonizing PI3K and inhibiting Akt signaling[17,18]. Endogenous phosphorylation of certain Pten residues, such as Serine 380, can inhibit Pten activity and permit Akt-driven proliferation[23,24].

Thus, hyperphosphorylation of Pten at Serine 380 is permissive of proliferation and is a potential mechanism of prostate hyperplasia.

Here, we examine the possibility that maternal HFD transmits a predisposition for prostate proliferation in murine adult offspring. Although murine models are thought to require genetic or chemical insults to initiate PCa[25], our data demonstrate that maternal exposure to HFD during gestation is sufficient to program hyperproliferation and increased apoptosis in dorsolateral prostate (DLP) tissue of adult offspring. This phenomenon is focally present in some acini in young adult and middle-aged mice and is dramatically intensified in older subjects. The data demonstrate that normal changes in homeostasis patterns that occur with aging are exacerbated by perinatal maternal HFD. Additionally, we demonstrate that Pten and Akt are both hyperphosphorylated in prostates from offspring of HFD-fed mothers. These data suggest that the prostate hyperproliferation occurs because of activation of the Akt pathway. Furthermore, as Pten is a cardinal prostate cancer tumor suppressor[17], these findings suggest that gestational high-fat diet is a risk factor for PCa initiation in later life.

Results

Maternal high-fat diet stimulates prostate hyperplasia in male offspring. Four-week-old C57Bl/6J female mice were fed control (chow, 13% kcal from fat) or high-fat (HFD defined by Surwit et al[26].; 59.4% kcal from fat) diets throughout life. After one month of dietary exposure, dams were mated to chow-fed C57Bl/6J studs. All resulting pups remained with their mothers until weaning at postnatal day 21 (P21) and then were kept on chow diets until sacrifice (diagrammed in Fig. 1). Chemically and genetically driven prostate cancers exhibit latency periods in murine models[19,27]. Thus, mice were aged to young adulthood (16 weeks), middle age (26 weeks), and old age (63 weeks) before sacrifice, DLP lobe isolation, and blinded expert histological analysis.

Very few of the acini in DLP samples from 16-week-old chow-exposed mice showed evidence of hyperproliferation (mean 1.92% ± 0.51%, range 0%–7.9%). Although not statistically significant, the number of hyperproliferative prostate acini was nearly two-fold higher in DLP samples from 16-week old HFD-exposed mice than in those from chow-exposed mice (Figure 2a–b; mean 3.44% ± 0.93%, range 0%–13.6%). Some regions of prostates from 16-week old HFD-exposed mice exhibited increased intraluminal secretory cells with mainly tufted architectures, without nuclear atypia (inset). By 26 weeks of age, the difference in numbers of hyperproliferative acini between chow- and HFD-exposed mice was significant (Chow: mean 3.95% ± 0.66%, range 0%–9.6%; HFD: mean 7.01% ± 1.11%, range 0%–17.24%). At this time, cribriform architecture was also detected in HFD-exposed mice (Fig. 2d, inset). The difference in hyperproliferation was even more pronounced in 63-week-old subjects (Chow: mean 2.19 ± 0.53%, range 0–8.47%; HFD: mean 4.28 ± 0.50%, range 0–7.69%). Furthermore, in some foci the gland luminal spaces were nearly filled with hyperplastic secretory cells (Fig. 2e–f, inset). These data suggest that gestational exposure to HFD stimulates hyperproliferation in the prostates of offspring and that hyperproliferation may be exacerbated in aged subjects.

To confirm these findings, tissue sections from chow- and HFD-exposed pups were assayed for expression of Ki67, a marker of proliferation. Prostates from chow-exposed 63-week-old mice exhibited significantly ($P < 0.0001$) more proliferation than those from the 16-week-old cohort, suggesting that proliferation normally increases as C57Bl6/J mice age (Fig. 3g). Additionally, as histological analysis of tissue morphology suggested, prostate samples from HFD-exposed pups demonstrated increased expression of Ki67 (Fig. 3). The difference in percent of Ki67-positive cells per image between chow- and HFD-exposed mice was significant at all time points (Fig. 3g). Most dramatically, 62.03% ± 1.51% of cells were Ki67 positive in prostates from 63-week-old HFD-exposed offspring (Fig. 3 e–g). At this time point, only 17.65% ± 0.90% of cells in prostates from chow-exposed offspring were Ki67 positive. The ranges of Ki67-positive events at this age were extensive (chow: 0.72%–51.79%; HFD: 16.71%–93.17%) and represented a 23-fold difference between HFD and chow at the lowest values. These observations suggest that normal Ki67 expression increases with age and is intensified by gestational HFD exposure.

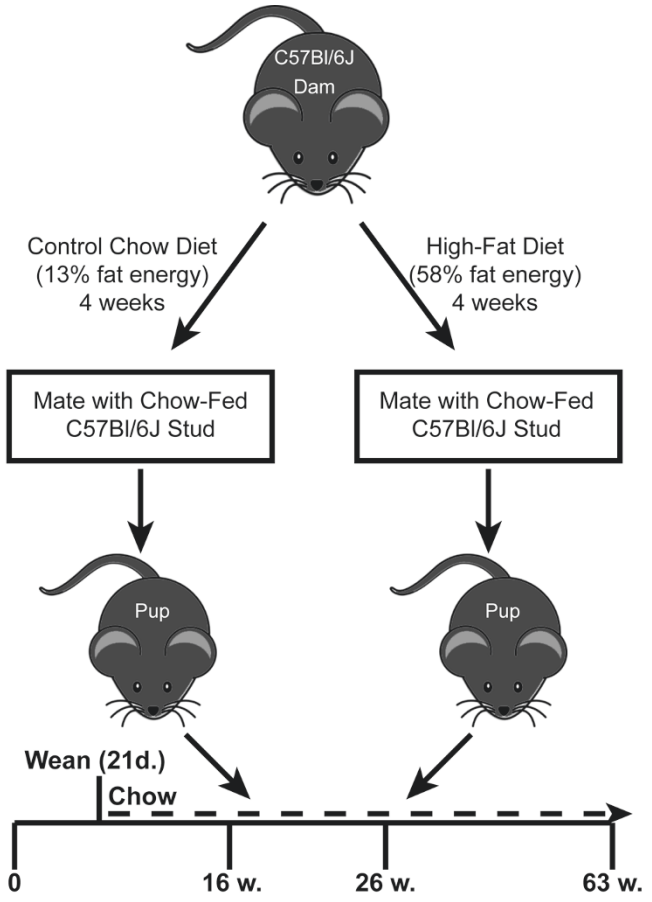

Figure 1 | A murine maternal high-fat diet model. Four-week-old C57Bl/6J female mice were fed either chow or high-fat diet for at least four weeks to expose developing oocytes to the respective diets. Starting at eight weeks of age, dams were mated to chow-fed C57Bl/6J males. Males were removed and switched back to chow diets after four days. All resulting pups remained with the mother until weaning and then were maintained on chow diets until sacrifice at 16, 26, or 63 weeks. Pups were exposed to high-fat diet during peri-conception, gestation, and lactation.

To further corroborate the findings from histological analyses and Ki67 indices, some of the mice from each timepoint were subjected to intraperitoneal BromodeoxyUridine (BrdU) injections 24 hours before sacrifice. As expected, BrdU labeling was largely absent in chow-exposed animals. Cases of BrdU positive cells in controls, which were almost exclusively observed in samples from 63-week-old mice (Fig. 4e), were generally couplets undergoing isolated proliferation events. By contrast, cases of isolated proliferation events were evident in 16-week-old HFD-exposed offspring (Fig. 4b) and were common in 26-week-old HFD-exposed animals (Fig. 4d). Strikingly, 63-week-old HFD-exposed offspring exhibited focal, intense uptake of BrdU. Whereas only proliferating couplets were detected in some acini, the majority of the cells were BrdU-positive in other acini (Fig. 4f). Taken together, these three assays indicate that maternal HFD exposure initiates prostate proliferation events in adulthood that are exacerbated in older animals.

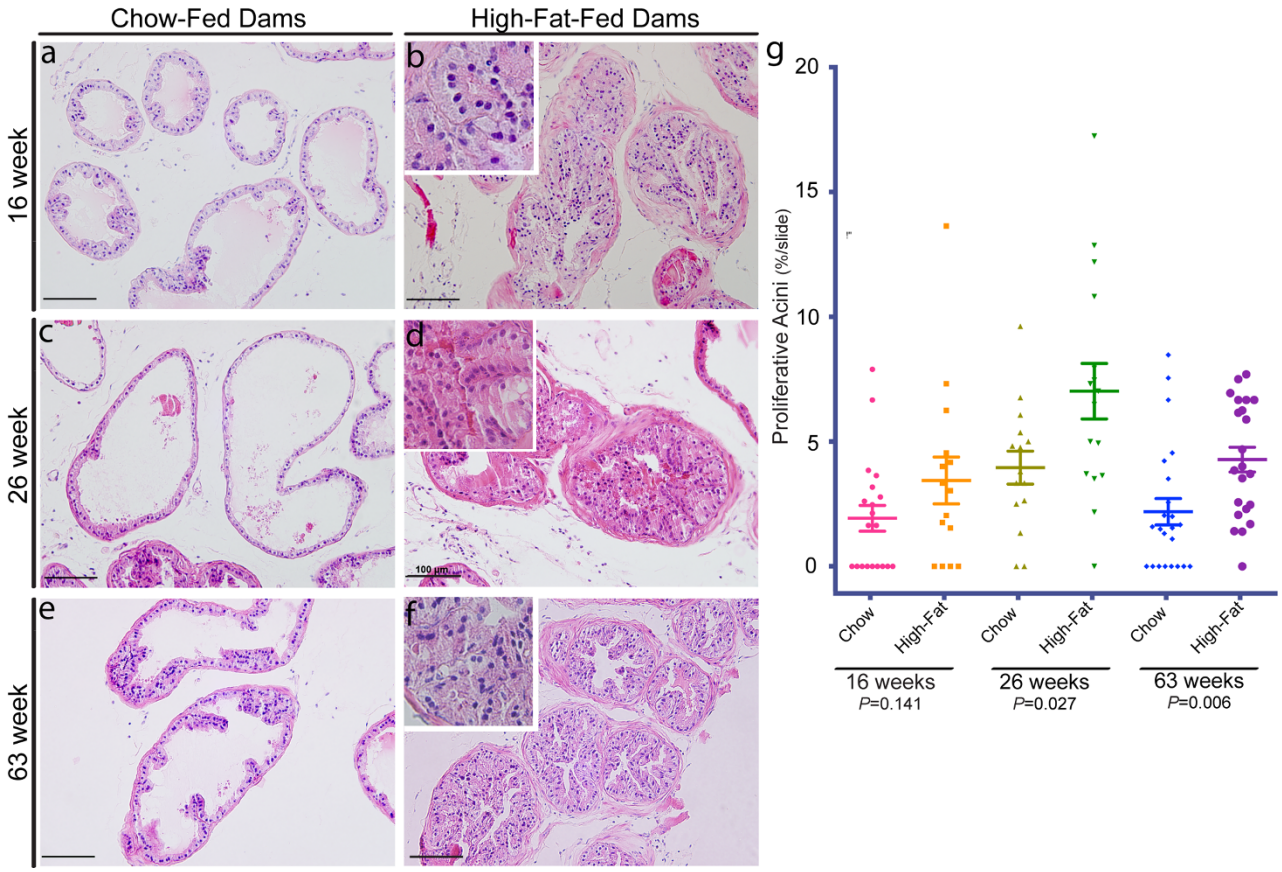

Figure 2 | Gestational high-fat diet stimulates hyperproliferation in the prostate. (a–f) H & E staining of prostate acini from chow- (a, c, and e) and HFD-exposed (b, d, and f) offspring at 16 (a and b), 26 (c and d), and 63 (e and f) weeks of age. Insets are high-magnification images to illustrate the most marked morphological defects in prostates from HFD-exposed animals, including tufted (b), and cribriform growth patterns (d and f), but a lack of nuclear atypia. (g) Quantification of the numbers of hyperproliferative acini per slide in prostates from chow- and HFD-exposed offspring at 16, 26, and 63 weeks of age. N ≥ 2 samples per mouse from ≥7 mice, scale bars = 100 μm.

Maternal HFD exposure stimulates hyperapoptotic compensatory mechanisms. Given the hyperproliferation observed in prostates from HFD-exposed offspring, we investigated whether prostates were enlarged in these animals. Body weights were not significantly different between those exposed to chow or HFD during gestation (Table 1). Similarly, weights of the *en bloc* excised urogenital sinus (UGS) regions and the DLP lobes showed no correlation with gestational diet exposure at any time point (Table 1).

Wild-type C57Bl/6J mice do not spontaneously develop prostate tumors; they require genetic and/or chemical insults to initiate and recapitulate human prostate cancer[19,25,27,28]. Thus, C57Bl/6J HFD-exposed murine models might maintain homeostatic mechanisms that protect against environmental insults such as maternal high-fat diet. To assess this possibility, we used terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) to calculate apoptosis indices in prostates from 16-, 26- and 63-week-old mice born to chow- or HFD-exposed mothers. At 16 weeks, apoptosis was slightly more frequent in prostate acini from HFD-exposed mice than in those from chow-exposed mice (Fig. 5a–b, g; chow: mean 10.65% ± 2.12%, range 0%–32.54%; HFD: mean 17.42% ± 2.63%, range 0%–48%). At 26 weeks, no significant difference in apoptosis was observed in prostates from HFD- and chow-exposed offspring (Fig. 5c–d, g). Strikingly, HFD-exposed offspring in the oldest age group demonstrated a mean apoptosis index of 27.53% ± 2.38% (Fig. 5f, g; range 0%–73.4%). In contrast, the mean apoptotic index of 63-week-old chow-exposed animals was significantly lower (Fig. 5e, g; 7.86% ± 1.06%, range 0%–24%). Thus, trends in apoptotic indices of adult HFD-exposed animals were exacerbated in older animals, mirroring the hyperproliferation phenotypes described above. Taken together, these data demonstrate that in young and middle-aged animals, gestational HFD initiates focal hyperproliferation. In older animals, increased levels of apoptosis may compensate for the highly elevated hyperproliferation such that no net tissue growth occurs.

Maternal high-fat diet induces activating Akt and inhibitory Pten post-translational modifications in prostate tissue. We sought to identify the mechanism by which hyperplasia is stimulated in prostates from HFD-exposed animals. As the tumor suppressor Pten inhibits proliferation via the Akt pathway[21] in non-cancerous prostate tissue, we investigated Akt phosphorylation at Serine 473[21] in offspring by two methods, western blot detection and fluorescent intensity analysis. At 16 weeks, western blot detection demonstrated that, whereas there was no difference in levels of total Akt, prostates from HFD-exposed mice had 4.35-fold more Phospho-(Serine 473) Akt than prostates from chow-exposed mice (Fig. 6a–c). Similarly, fluorescent intensity analysis demonstrated that prostates from 63-week-old HFD-exposed animals had equivalent levels of total Akt but higher levels of Phospho-(Serine 473)-Akt than those from chow-fed animals, (Fig. 6d–i). Together, these data demonstrate that maternal gestational high-fat diet activates Akt signaling in prostate tissue.

Given that Pten normally antagonizes PI3K and inhibits Akt signaling, we next investigated whether Pten was deactivated in these animals. At 16 weeks, there was no difference in levels of total Pten, but Phospho-(Serine 380)-Pten was 3.79-fold higher in prostates

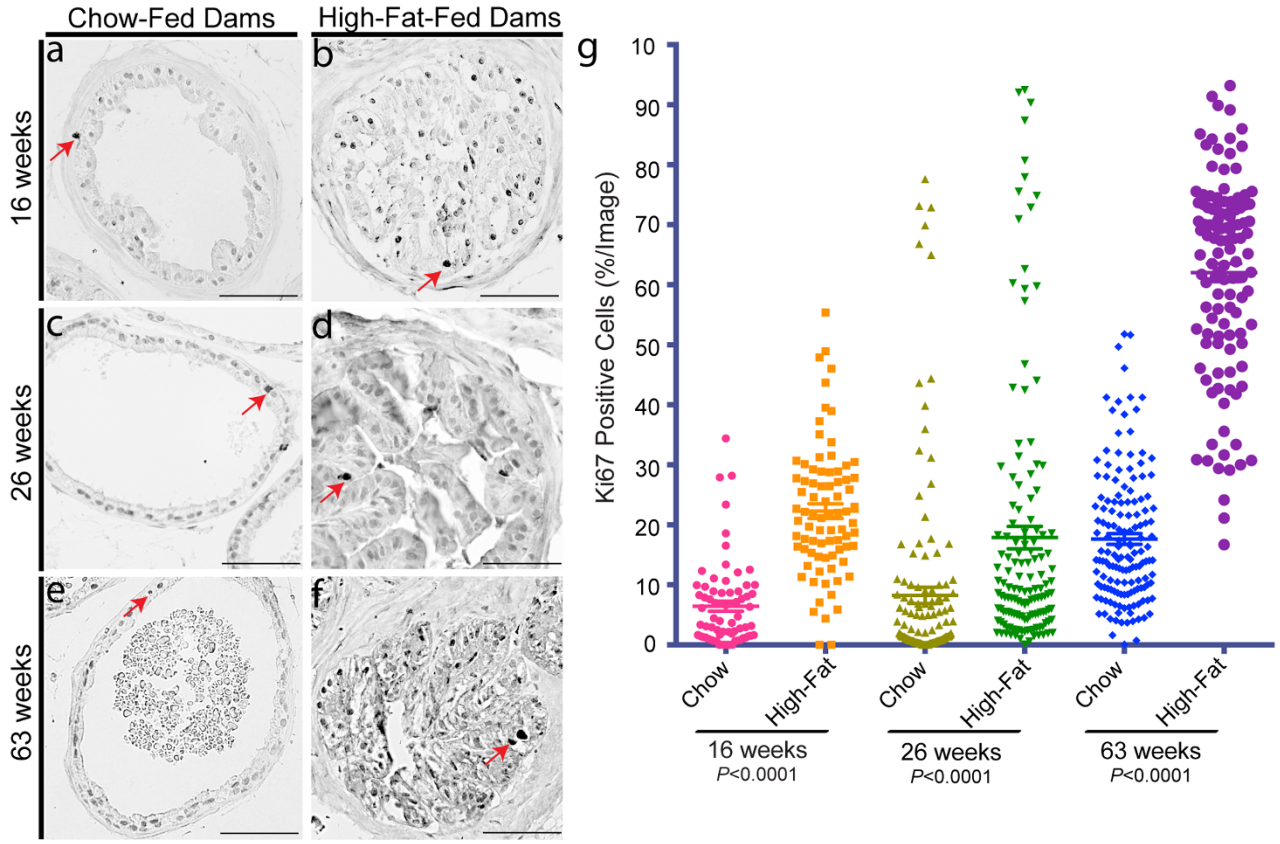

Figure 3 | Maternal high-fat diet exposure stimulates expression of Ki67. (a–f) Ki67 staining (black) of prostate acini from chow- (a, c, and e) and HFD-exposed (b, d, and f) offspring at 16 (a and b), 26 (c and d), and 63 (e and f) weeks of age. Arrows indicate Ki67-positive nuclei. (g) Quantification of the percentage of Ki67-positive cells per image. N ≥ 71 images from ≥7 animals per condition, scale bars = 100 μm.

from HFD-exposed offspring than in those from chow-exposed offspring (Fig. 6j–l). Similarly, prostates from 63-week-old HFD-exposed animals had equivalent levels of total Pten but higher levels of Phospho-(Serine 380)-Pten than chow-exposed animals (Fig. 6m–r). These collective data indicate that Pten deactivation and Akt activation accompany the hyperplasia observed in prostates from HFD-exposed offspring.

Discussion

Here, we identify a novel risk factor for prostate hyperproliferation: maternal high-fat diet from periconception to weaning. Our observations indicate that hyperproliferation occurs throughout the life-course and is exacerbated in aged subjects. Gestational HFD exposure did not result in altered prostate weights, due in part to compensatory apoptosis. Pten and Akt hyperphosphorylation in these animals provides one explanatory mechanism for the observed hyperplastic phenotypes. Collectively, these data introduce gestational HFD exposure as a novel risk factor for prostate cancer in later life.

This work provides a relatively simple murine model to initiate prostate hyperproliferation. To our knowledge, this is the first study to consider the effects of gestational HFD on fetal programming of prostate cancer predisposition. Importantly, the phenotype stimulated by gestational high-fat diet resembles early phases of prostate dysplasia: focal hyperplasia that in some cases, after a long latency period, results in abnormal and widespread proliferation[25]. Although we did not observe tumor formation, we found that gestational HFD deactivates the tumor suppressor Pten, permitting atypical Akt activity. Pten is commonly lost in aggressive prostate cancers[29,30]; thus, our Pten/Akt signaling data imply that gestational HFD exposure predisposes murine offspring to potentially aggressive prostate cancers, rather than benign foci. If this is the case, we expect that concomitant dietary exposure and genetic or chemical 'second hits' would result in aggressive prostate intraepithelial neoplasia (PIN) and carcinoma *in situ* phenotypes. Such investigations are a critical avenue of future research.

Disruption or loss of *Pten* gene products (either genetically or post-transcriptionally) is very common in human and murine aggressive prostate tumors[29–31,17,18]. However, specific alterations in Pten post-translational inhibition, such as phosphorylation as observed here, provide an attractive mechanism to explain individual disparities in prostate cancer outcomes. The Pten-Akt pathway is an established mediator of insulin receptor signaling[18,32]. Evidence has also linked Pten-Akt signaling to lipid metabolism through sterol-regulatory element-binding proteins[33]. Pten additionally regulates apoptosis and senescence in prostate cells, particularly after oxidative stress[34]. All of these pathways are disrupted by gestational overnutrition[35]. It is possible that post-translational regulation of the Pten-Akt pathway, and thus cell homeostasis, depends on gestational exposure conditions. This may explain the diverse array of outcomes observed in prostate cancer incidence. Obesity rates are rising in the western world; in 2010, approximately 30% of reproductive-age American women were obese[36]. If individual gestational exposures contribute to human prostate cancer risk, then we speculate that future generations will present a more severe, and possibly unpredictable, array of prostate cancer outcomes. However, careful characterization of gestational exposures may identify specific dietary risk factors and abrogate this outcome.

Our data revealed that hyperplasia was stimulated by high-fat diet at all of the tested timepoints. In particular, we observed significantly more hyperplasia in HFD-exposed 63-week-old animals than in younger counterparts. These older animals also exhibited a statistically

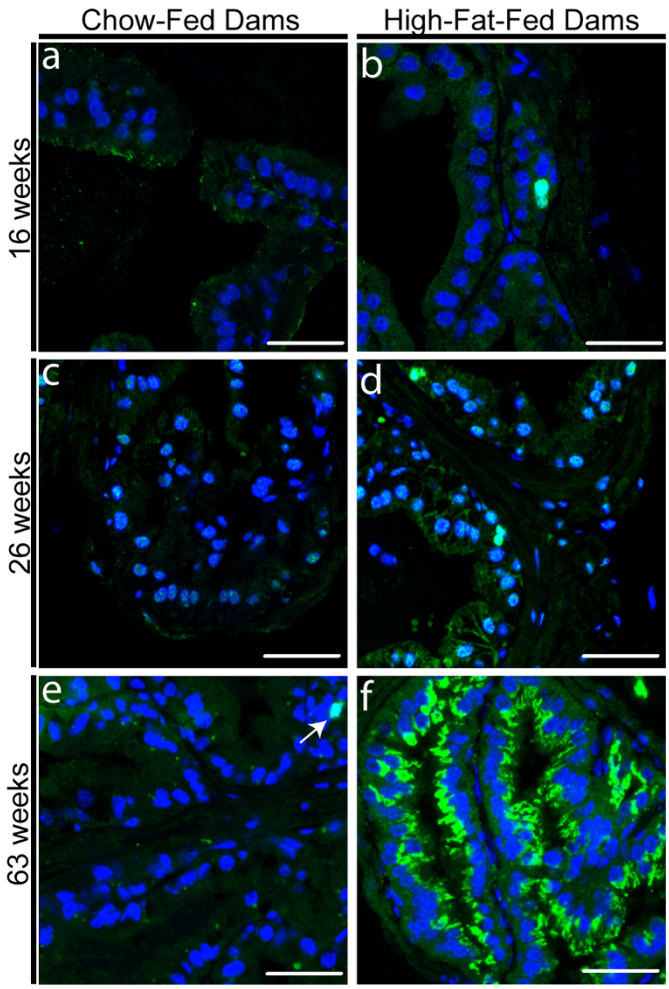

Figure 4 | Maternal high-fat diet exposure stimulates increased uptake of BrdU in prostates. (a–f) BrdU (green) incorporation into prostates from chow- (a, c, and e) and HFD-exposed (b, d, and f) offspring at 16 (a and b), 26 (c and d), and 63 (e and f) weeks of age. Nuclei are stained with TO-PRO-3 (blue). Arrow indicates BrdU-positive nuclei, scale bars = 50 μm.

significant rise in apoptosis, which presumably accounted for the lack of difference in prostate size between these animals and chow-exposed controls. HFD-induced upregulation of apoptosis could be explained by several mechanisms: First, apoptosis mechanisms could be operating normally in these animals. In this case, HFD-driven Akt signaling and hyperplasia could trigger checkpoints that cause an apoptotic response. This would be most prevalent in older subjects, which had highly elevated hyperplasia rates. Second, given that Pten regulates apoptosis[37], HFD could concurrently deregulate hyperplasia and apoptosis by deregulating Pten activity. Finally, gestational diet could deregulate both Pten/Akt-driven proliferation and another independent pathway, leading to an upshift in apoptosis. Studies in conditional murine genetic models with impaired checkpoints or apoptosis could identify the prevailing mechanism underlying this observation.

This study identified a relatively broad developmental window that was influential in programming prostate hyperproliferation. Animals were exposed to HFD during the entire perinatal period because mice do not spontaneously exhibit prostate cancer. Thus, the strong phenotype reported herein was an unexpected result. During periconception, gestation, and early post-natal growth, multiple developmental events occur that could be perturbed by HFD exposure. One possibility is that maternal HFD disrupts the oocyte or zygote. *De novo* DNA methylation is established during maternal folliculogenesis, and then modified again at fertilization[38]. Methylation of mono-allelically expressed imprinted genes is perturbed in prostate cancer cell models and aggressive prostate cancer foci[39–42]. For example, expression of the imprinted gene Insulin-like Growth Factor 2 (*IGF2*) is deregulated in prostate cancer lesions[43]. IGF2 signals through IGF receptors 1, 2, and insulin receptor. IGFR1 and insulin receptor stimulate signaling through multiple pathways including the Akt cascade[44], which we observed to be disturbed by gestational HFD-exposure. Thus, we speculate that gestational diet could deregulate IGF2 imprinting which would in turn alter signaling at its receptors, Pten/Akt signal transduction, and subsequent homeostasis in offspring. It will be important to explore the influence of gestational diet on both gene-specific and global epigenetic signatures in future research.

Another possibility is that maternal HFD affects prostate development postnatally. Rodent prostate development is incomplete until postnatal day 15[45], and early postnatal exposures have been demonstrated to induce prostate hyperplasia and PIN[46]. Thus, HFD exposure during lactation may program proliferation rates, perhaps by influencing Akt-dependent cell signaling. Future studies must parse out the specific exposure window by honing down the timing of HFD exposure in these animals.

Consumption of a high-fat diet is an established modifiable risk factor for many types of cancer, and obesity leads to adverse outcomes in patients with aggressive prostate cancer[47]. Unfortunately, specific time windows in the patients' lifespans and individual dietary risk factors for aggressive prostate cancer initiation have not been clearly delineated[5,6,48], and the influences of the perinatal and early developmental time windows on prostate cancer health are relatively unstudied[49]. The data presented here provide evidence that the perinatal time window is important for patterning prostate health. Exposures during this timeframe can now be tracked in human subjects and manipulated in animal models. It should be noted that other cancer predispositions are also subject to perinatal dietary

Table 1 | Maternal high-fat diet does not change weight of offspring prostate tissue

		CHOW ± sd	n	HFD ± sd	n	*P*
16 week	Body Weight (g)	27.93 ± 2.19	16	26.46 ± 2.18	15	0.062
	UGS[a] Weight (g)	0.53 ± 0.13	16	0.49 ± 0.05	15	0.225
	DLP[b] Weight (mg)	0.0232 ± 0.005	15	0.0252 ± 0.008	12	0.453
26 week	Body Weight	29.85 ± 1.99	6	29.52 ± 3.58	5	0.853
	UGS Weight	0.67 ± 0.11	6	0.64 ± 0.11	5	0.697
	DLP Weight	0.0269 ± 0.008	6	0.0274 ± 0.007	5	0.909
63 week	Body Weight	38.26 ± 5.45	8	37.28 ± 4.12	10	0.661
	UGS Weight	1.19 ± 0.50	8	1.13 ± 0.38	10	0.785
	DLP Weight	0.0293 ± 0.001	8	0.0272 ± 0.009	6	0.687

[a]Urogenital Sinus;
[b]Dorsolateral Prostate.

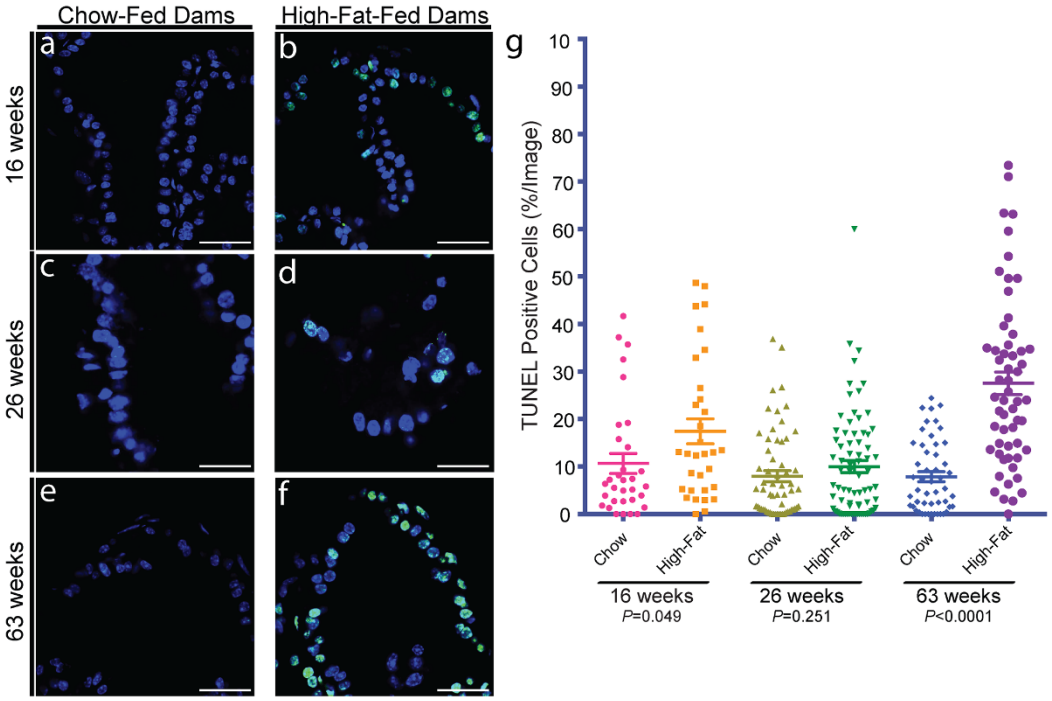

Figure 5 | Maternal high-fat diet stimulates apoptosis in 63-week old prostate tissue of male offspring. (a–f) TUNEL (green) of prostate acini from chow- (a, c, and e) and HFD-exposed (b, d, and f) offspring at 16 (a and b), 26 (c and d), and 63 (e and f) weeks of age. Nuclei are stained with TO-PRO-3 (blue). (g) Quantification of the percentage of TUNEL-positive cells per image. N ≥ 32 images from ≥7 mice, scale bars = 50 μm.

exposures. For example, gestational diet programs mammary tumor development in both first- and second-generation female rodent offspring[50,51]. Multigenerational effects of perinatal HFD also predispose offspring to reproductive and pituitary tumors; these studies have indicated that maternal diet is particularly influential during the early postnatal period[52,53]. Recent epidemiological work found that the distance between the pelvic and iliac crests, which is hormonally controlled, in the mother positively correlated with prostate cancer incidence in male offspring[54]. The current data provide an intriguing counterpart to these previous findings; a gestational high-fat environment may program hyperplasia in many hormone-responsive tissues. Taken together, these findings could lead to a recommendation that maternal diet be more tightly regulated to prevent adverse cancer outcomes in offspring as they age.

Methods

Animal husbandry. All animals were handled with IUCAC approval by the Washington University Animal Studies Committee, Animal Welfare Assurance protocol # A-3381-01. Three groups of 10 C57Bl/6J four-week old male and female mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Mice were housed five per cage in standard 12-hour light/dark conditions with *ad libitum* access to food and water. For each breeding cycle, five age-matched female mice were fed either control chow diet (PicoLab Rodent Diet 20; 13.2% of calories from fat) or standard high-fat diet (TestDiet, 58R3; 59.4% of calories from fat)[26]. High-fat- and control-fed dams were mated to age-matched males (that were diet-exposed for ≤4 days). Resulting pups remained with their mother until weaning, after which they were maintained on control chow diets until sacrifice. Male offspring were sacrificed at 16, 26, and 63 weeks of age. five animals per diet were given intraperitoneal injections of 5-Bromo-2-Deoxyuridine (BrdU [30 mg/kg]; Sigma-Aldrich, St. Louis, MO) for 24 hours prior to sacrifice. At sacrifice, UGS regions were removed *en bloc* to cold PBS (pH 7.4), and DLPs were disassociated (as previously described[55,56]) and fixed in 4% paraformaldehyde overnight. Prostates were dehydrated to 70% ethanol, processed overnight, and paraffin embedded. Four levels were sectioned onto glass Posi-Slides (Lab Storage Systems, Inc.).

Hematoxylin and Eosin (H & E) staining. Sections (6 mm) were deparaffinized in three changes of xylene for 5 minutes (5′) each and rehydrated for 2′ in each of the following: 100% Ethanol (EtOH), two changes of 95% EtOH, and two changes of water. Sections were stained in CAT Hematoxylin (BioCare Medical) for 7′ and washed for 2′ in running water. Slides were differentiated with one dip in 0.5% acid-alcohol solution, washed for 1′ in running water, blued with 1 dip in 0.2% ammonia water, washed for 5′ in running water, rinsed in 95% alcohol for 2′, and counterstained for 30 seconds in Accustain Eosin Y Solution (Sigma-Aldrich, St. Louis, MO). Slides were dehydrated for 2′ in each of the following: 70% EtOH, 95% EtOH, and two changes of 100% EtOH. Slides were then cleared for 5′ in xylene and mounted in Cytoseal XYL (Richard-Allan Scientific). Two slides per animal were analyzed for percent hyperproliferative acini per slide (quantification modified from[57]) by an investigator blinded to the feeding regimen.

Immunohistochemistry (IHC) and immunofluorescence (IF). Sections (6 mm) were processed as previously described[58]. Briefly, sections were deparaffinized, rehydrated, quenched in H_2O_2/methanol solution, and boiled for 30′ in 10 mM sodium citrate solution for antigen retrieval. Sections were permeabilized in 0.2% TritonX-100 in PBS for 15′, blocked in 10% goat serum/5% BSA in PBS, and incubated with α-Ki67 antibody (Abcam, 1 : 250), α-Pten (Cell Signaling Technology 1 : 200), α-Phospho (Ser380) Pten (Cell Signaling Technology, 1 : 200), α-Akt (Cell Signaling Technology, 1 : 200), or α-Phospho (Ser473) Akt (Cell Signaling Technology, 1 : 200) overnight at 4°C (all antibodies were raised in rabbit). Slides were washed in PBS and incubated for 1 hour at room temperature in either HRP- (Santa Cruz Biotechnology, 1 : 2500) or Alexa Fluor 488/568-conjugated (Molecular Probes,1 : 1000) secondary antibodies raised in goat. For IHC: Slides were washed, developed for 5′ by using a Vector DAB Kit (Vector Laboratories) per manufacturer's protocol, counterstained for 30 seconds in CAT Hematoxylin, rehydrated, and mounted with Cytoseal-XYL. Sections were imaged on a Nikon Eclipse E800 upright microscope with an Olympus DP71 camera and manufacturer's software. The average percent of Ki67-positive cells was determined by counting cells in 15 images per slide and two slides per mouse. For IF: Sections were counterstained with TO-PRO-3 iodide 642 and mounted with Vectashield. Imaging was performed by confocal microscopy (Nikon D-Eclipse-C1 E800; Nikon EZ-C1si image Capture software Bronze Version 3.80). Prostate epithelium regions were selected in Photoshop CS6, and pixel intensities were measured in ImageJ and normalized to total cell count.

BrdU labeling. Sections (3 μm) were baked at 55°C for 10′ directly before labeling. Sections were deparaffinized with two xylene washes for 5′ each and rehydrated with sequential 2′ incubations in each of the following: two changes in each of 100% EtOH, 95% EtOH, and 70% EtOH, and then incubated in PBS for 5′. Antigen retrieval was performed as described above. Slides were cooled, treated with DNaseI (5 U/mL, Life Technologies) for 30′ at 37°C, washed, and blocked in 3% BSA/0.1% Tween-20/PBS for 30′ at room temperature. Sheep anti-BrdU (Abcam, 1 : 200) was applied in 1 : 10 dilution of blocking buffer overnight at 4°C. Slides were washed in PBS and labeled with Alexa Fluor 488 secondary antibody (Life Technologies, 1 : 100) for 30′ at room temperature, and TO-PRO-3 iodide 642 (Life Technologies, 1 : 500) was applied for 5′

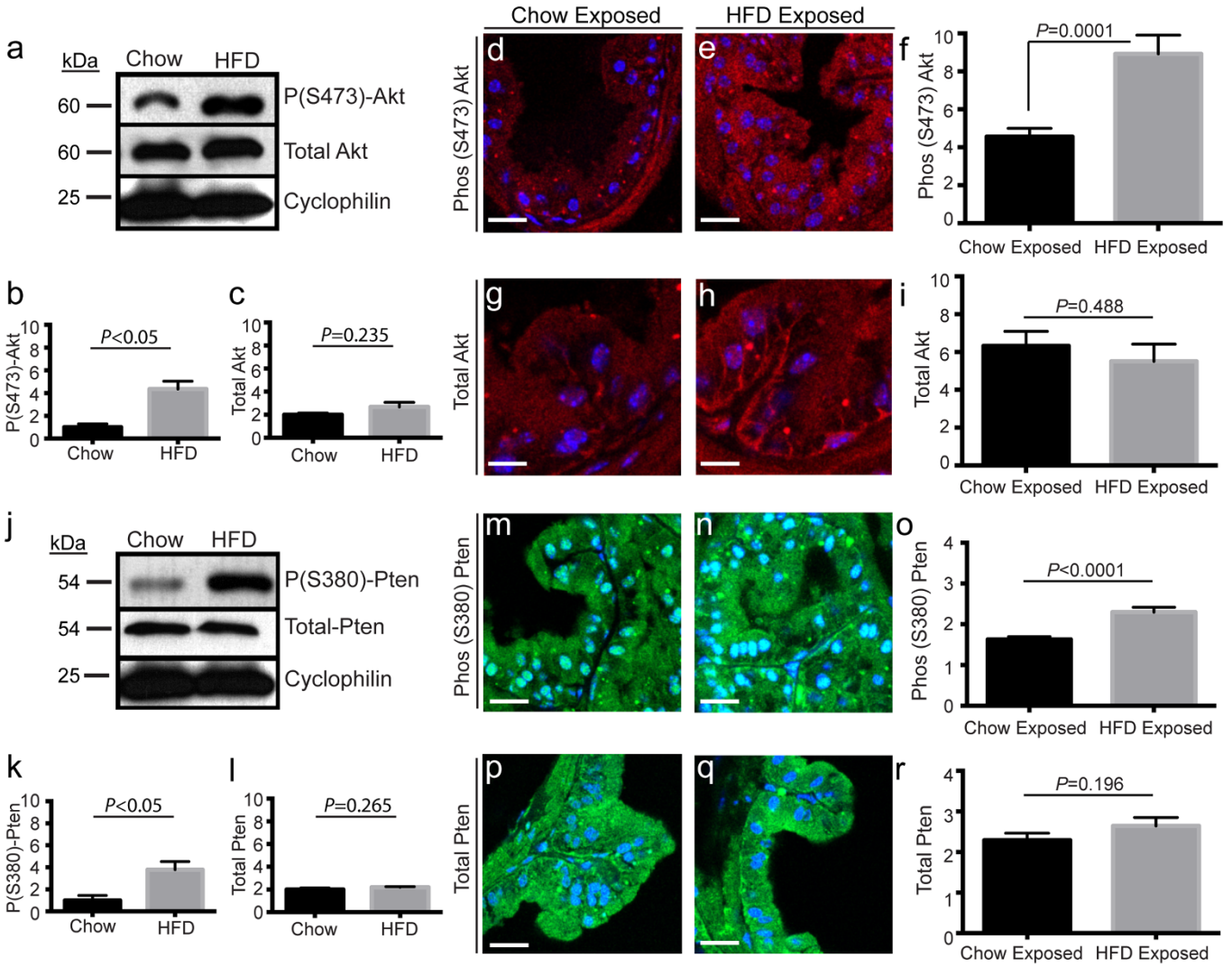

Figure 6 | Akt (Serine 473) and Pten (Serine 380) are hyperphosphorylated in prostates from offspring of HFD-exposed mothers. *From 16-week old chow and HFD-exposed animals*: Representative images are given of Phospho-(Serine 473) Akt, total Akt (a), Phospho-(Serine 380) Pten, total Pten (j) and cyclophilin (loading control; a & j) proteins present in 16-week old dorsolateral prostates from chow and HFD-exposed animals. Quantifications of normalized Phospho-(Serine 473) Akt (b), Total Akt (c), Phospho-(Serine 380) Pten (k) and Total Pten (l) protein signal are presented, n = 3 animals per condition. *From 63-week old chow and HFD-exposed animals*: Representative images of Phospho-(Serine 473) Akt (d–e) Total Akt (g–h), Phospho-(Serine 380) Pten (m–n) and Total Pten (p–q) from chow- and HFD-exposed dorsolateral prostate tissue sections. Normalized fluorescence intensities are quantified in each subsequent graph (f, i, o, r), n = 5 animals per condition, all scale bars = 10 μM.

at room temperature. Slides were mounted in Vectashield mounting medium (Vector Laboratories Inc.). Imaging was performed on a confocal microscope as above.

TUNEL assay. TUNEL assays were performed by using a Fluorescein In Situ Cell Death Detection Kit (Roche Diagnostics) according to the manufacturer's protocol. Sections were counterstained with TO-PRO-3 iodide 642 and mounted with Vectashield. Slides were visualized by confocal microscopy as above, and the average percent of TUNEL positive cells was calculated from 15 images from each of 2 slides per animal.

Western blotting. Whole 16-week murine dorsolateral prostates were excised, washed in PBS, and homogenized in RIPA buffer supplemented with Protease (Roche) and Phosphatase Inhibitors (Sigma). Samples were rotor-stat homogenized, spun at 14,000 RPM for 5′, and supernatants were aliquotted and stored at −80°C. BCA assays (Thermo Scientific) were used to calculate total protein concentrations. Standard western blotting methodologies were used, with acrylamide gels separated for 3 hours at 50 V. After transfer, blots were blocked in 5% non-fat dry milk solution for 1 hour at room temperature. Membranes were incubated in primary antibody overnight at 4°C in heat-sealed bags. Primary antibodies were the same as used above; all were diluted 1 : 1000. Membranes were washed and incubated with HRP-conjugated secondary antibodies (Santa Cruz, 1 : 10,000) for 1 hour at room temperature. Membranes were treated with ECL reagent (Bio Rad). Films were exposed for <30 seconds. Bands from each condition were quantified with ImageJ. Data were normalized to cyclophilin loading controls and statistically compared.

Statistical analyses. Data are from ≥7 chow- or high-fat-diet-exposed animals at each timepoint. Each data point (n) is individually represented in the graphs; horizontal bars represent the mean. All errors values are the SEM. *P* values were determined in two-tailed unpaired students t-tests with Prism 6 Software (GraphPad).

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Acknowledgments

K.H.M. and E.C.B. were supported by the Washington University Transdisciplinary Research in Energetics and Cancer Program Grant (NCI; U54 CA 155496). E.C.B. was funded on the Washington University Department of Pathology Training Grant (5T32 DK 7296-33). We thank Drs. Graham Colditz, Jason Weber, and Deborah Frank for suggestions on the data analysis and manuscript preparation. We also thank Dr. Len Maggi and Raleigh Kladney for instruction on the dissection technique, Dr. Kelsey Tinkum and Lynn White for instruction on mouse husbandry, and Alma Johnson and Daniel Cusumano for help with tissue preparation.

Author contributions

E.C.B. and K.H.M. designed the study and wrote the manuscript. E.C.B., Q.W. and K.H.M. developed and oversaw the feeding and dissection protocols. E.C.B. collected the data and interpreted it with K.H.M. P.A.H. performed the histological analyses.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Benesh, E.C., Humphrey, P.A., Wang, Q. & Moley, K.H. Maternal high-fat diet induces hyperproliferation and alters Pten/Akt signaling in prostates of offspring. *Sci. Rep.* **3**, 3466; DOI:10.1038/srep03466 (2013).