Prostate cancer is a leading cause of cancer death in men over the age of 50 years, and there is a characteristic marked decrease in Zn content in the malignant prostate cells. The cause and consequences of this loss have thus far been unknown. We found that in middle-aged rats a Zn-deficient diet reduces prostatic Zn levels ($P = 0.025$), increases cellular proliferation, and induces an inflammatory phenotype with COX-2 overexpression. This hyperplastic-inflammatory prostate has a human prostate cancer-like microRNA profile, with up-regulation of the Zn-homeostasis-regulating miR-183-96-182 cluster (fold change = 1.41–2.38; $P = 0.029–0.0003$) and down-regulation of the Zn importer ZIP1 (target of miR-182), leading to a reduction of prostatic Zn. This inverse relationship between miR-182 and ZIP1 also occurs in human prostate cancer tissue, which is known for Zn loss. The discovery that the Zn-depleted middle-aged rat prostate has a metabolic phenotype resembling that of human prostate cancer, with a 10-fold down-regulation of citric acid ($P = 0.0003$), links citrate reduction directly to prostatic Zn loss, providing the underlying mechanism linking dietary Zn deficiency with miR-182-96-182 overexpression, ZIP1 down-regulation, prostatic Zn loss, and the resultant citrate down-regulation, changes mimicking features of human prostate cancer. Thus, dietary Zn deficiency during rat middle age produces changes that mimic those of human prostate carcinoma and may increase the risk for prostate cancer, supporting the need for assessment of Zn supplementation in its prevention.

Significance

Prostate cancer in man is associated with Zn loss, citrate metabolite reduction, overexpression of the miR-183-96-182 cluster, and regulation of Zn homeostasis through Zn transporter suppression. Our mechanistic study shows that a low-Zn diet upregulates this miR cluster in Zn-deficient middle-aged rat prostate, with ZIP1 mRNA/protein downregulation and a citrate-oxidizing metabolic phenotype, linking citrate reduction directly to prostatic Zn loss. The findings of this study show that the transcriptional and metabolic signal pathways induced by Zn deficiency in rats and almost certainly in men are critical for the development of human and rat prostate cancer and provide a strong rationale for including Zn supplementation in clinical trials to reduce the prostate cancer burden in the human population.
This study was conducted in rat lateral prostate (hereafter "100.025") (see Fig. 3 = 0.001) (Fig. 1 < 0.001) (Fig. 1 < 0.01 (Fig.

In this study, we recreated prostatic Zn loss in a middle-aged rat model and investigated whether with prostatic Zn loss, Zn deficiency also causes molecular alterations that may promote PCa initiation. We examined the consequences of prostatic Zn loss by analyzing Zn transporters, miRNA, and metabolomics profiles.

Results

A Zn-Deficient Middle-Aged Rat Prostate Model with Prostatic Zn Loss. This study was conducted in rat lateral prostate (hereafter called “prostate”) that is embryologically homologous to the peripheral zone of human prostate where most PCa arises (33).

Two rat strains were used: the traditional Sprague–Dawley strain and the Wistar-Unilever strain that is well characterized for prostate carcinogenesis research (34).

We sought to establish a Zn-depleted prostate model in middle-aged rats for examining molecular alterations associated with prostatic Zn loss that might lead to PCa initiation. As shown in the experimental design (Fig. 1A), 1-mo-old male Sprague–Dawley rats were fed a Zn-deficient (3–4 ppm Zn) or Zn-sufficient (~60 ppm Zn) diet for 1.5, 4, and 10 mo, forming six groups (n = 10–20 rats per group): Zn-deficient and Zn-sufficient 2.5-mo-old young-adult rats, Zn-deficient and Zn-sufficient 5-mo-old adult rats, and Zn-deficient and Zn-sufficient 11-mo-old middle-aged rats. Zn-deficient rats were fed ad libitum; Zn-sufficient rats were pair-fed with Zn-deficient rats to match their decreased food consumption (35, 36). This Zn-deficient diet mimics marked Zn deficiency in human nutrition (35). In addition, 1-mo-old male Wistar-Unilever rats were similarly fed for 9 mo to form Zn-deficient and Zn-sufficient 10-mo-old middle-aged groups (n = 20 rats per group).

Consistent with previous data (24), testis Zn levels (Zn marker) were ~40% lower in all Zn-deficient age groups (young-adult, adult, and middle-aged) than in the corresponding Zn-sufficient age groups (P < 0.001) (Fig. 1B). Prostatic Zn levels were similar in young-adult rats fed a Zn-deficient or Zn-sufficient diet for 1.5 mo (Fig. 1C). As expected (24), prostatic Zn levels were significantly reduced in Zn-deficient adult as compared with Zn-sufficient adult rats after a 4-mo dietary regimen (P < 0.001) (Fig. 1C) and in Zn-deficient middle-aged rats relative to their Zn-sufficient counterparts after an ~10-mo regimen in both rat strains [Sprague–Dawley rats, P < 0.01 (Fig. 1C); Wistar-Unilever rats, P = 0.025] (see Fig. 3D)]. These results established a middle-aged rat model with prostatic Zn loss induced by a Zn-depleted diet in two rat strains.

![Fig. 1. Establishment of a Zn-deficient middle-aged Sprague–Dawley rat model with prostatic Zn loss.](image-url)

- **A**: Study design: 1-mo-old male rats received a Zn-deficient or Zn-sufficient diet for 1.5, 4, or 10 mo to form six Zn-modulated age groups (n = 10–20 rats per group): Zn-deficient young-adult, adult, and middle-aged and Zn-sufficient young-adult, adult, and middle-aged.
- **B and C**: Tests Zn content and prostate Zn content (C) (measured in micrograms per gram dry weight) of young-adult, adult, and middle-aged rats on a Zn-deficient (3 ppm Zn) or Zn-sufficient 60 ppm Zn) diet for 1.5, 4, and 10 mo, forming six groups (n = 10–20 rats per group). The PCNA-labeling index (D) and the PCNA-labeling index in middle-aged prostate is expressed as the percent of intensely stained PCNA-positive nuclei (S-phase) per ~500 prostate epithelial nuclei evaluated in a microscope field at 200× magnification (n = 9 rats per group).
- **E**: qPCR analysis of four selected inflammation genes, S100a8, S100a9, Cxcl5, and Ptgs2, in middle-aged rat prostates (n = 6–10 rats per group, measurements were performed in triplicate with Pmb6 as a normalizer). Data are expressed as mean ± SD. All statistics are two-sided; *P < 0.05, **P < 0.01, ***P < 0.001.
Zn-Deficient Middle-Aged Rat Prostate Has a Precancerous Phenotype. Histological examination (H&E-stained sections, n = 10 rats per dietary group) showed that Zn-sufficient middle-aged rat prostate was typically growth quiescent (37), displaying a thin epithelium (Fig. 2A). By contrast, Zn-deficient middle-aged rat prostates displayed frequent epithelial hyperplasia (Fig. 2A). To evaluate prostate epithelial cellular proliferation, the proliferation marker proliferating cell nuclear antigen (PCNA) was assessed in formalin-fixed paraffin-embedded (FFPE) rat prostate tissues. PCNA-immunohistochemistry (IHC) showed that the percent of intensely stained PCNA-positive nuclei (S-phase) in Zn-deficient middle-aged prostate (9.5 ± 2.2%) was markedly increased relative to Zn-sufficient middle-aged prostate (3.9 ± 0.9%) (P < 0.001; n = 9 rats per group) (Figs. 1D and 2A), indicating that dietary Zn deficiency induces epithelial proliferation in middle-aged rat prostates.

Chronic inflammation is implicated in the pathogenesis of PCa (38), and NF-κB is a pivotal transcription factor in inflammation (39). The inflammation markers S100A8/A9 (40), CXCL5 (41), and PTGS2 (COX-2) (42) are overexpressed in human PCa. An ELISA showed that NF-κB p65 expression was significantly up-regulated in Zn-deficient middle-aged prostate compared with Zn-sufficient middle-aged prostate (P < 0.01; n = 9 rats per group) (Fig. 1E). qPCR analysis showed that these same four inflammation markers were significantly up-regulated in Zn-deficient middle-aged rats vs. their Zn-sufficient counterparts (P < 0.05) (Fig. 1F). IHC analysis in FFPE prostate tissues demonstrated that NF-κB p65 and NF-κB–regulated COX-2 were strongly expressed in proliferative Zn-deficient as compared with quiescent Zn-sufficient middle-aged rat prostate (n = 10 Wistar-Unilever rats per dietary group) (Fig. 2A). In addition, IHC analysis of prostatic PCNA and inflammation markers in Zn-deficient 2.5-mo-old young-adult and 11-mo-old middle-aged Sprague–Dawley rats showed that cell proliferation and inflammation progress with aging (n = 10 rats per age group) (Fig. 2B). Together, the data establish that Zn-deficient middle-aged rat prostates have a hyperplastic and inflammatory phenotype and that the effect of Zn depletion progresses with age.

Overexpression of the Zn-Homeostasis–Regulating mir-183-96-182 Cluster in Zn-Deficient Middle-Aged Rat Prostate. We have shown by nanoString mouse miRNA assay that dietary Zn deficiency for 23 wk in adult rats causes altered miRNA expression in prostate (24). To compare miRNA expression profiles in rats growing from young adult to middle age on a Zn-deficient or Zn-sufficient diet, we employed a nanoString miRNA assay detecting >400 rat miRNAs (n = 6 Sprague–Dawley rats per group). The nanoString platform measures miRNA expression levels without reverse transcription or PCR amplification, thus eliminating enzymatic bias (43). Using a cutoff of P < 0.05 and a fold-change ≥1.4, 65 dysregulated miRNAs were identified in Zn-deficient middle-aged prostate as compared with Zn-deficient young-adult prostate. Among them, 14 up-regulated and nine down-regulated miRNAs were similarly dysregulated in human PCa (SI Appendix, Table S1). The entire miR-183-96-182 cluster that is overexpressed in human PCAs (9, 27) and that regulates Zn homeostasis of PCa cells via suppression of Zn transporters [specifically hZIP1 (9)] was up-regulated in Zn-deficient middle-aged rat prostates as compared with young-adult rat prostates (miR-183, up-regulated 1.41-fold, P = 0.006; miR-96, up-regulated 2.38-fold, P = 0.0003; miR-182, up-regulated 1.73-fold, P = 0.029) (Fig. 3A and SI Appendix, Table S1). By contrast, among the 38 up-regulated miRNAs identified in Zn-deficient middle-aged rat prostates compared with young-adult rat prostates (SI Appendix, Table S2), 11 were identical to those overexpressed in Zn-deficient middle-aged rat prostates and human PCa, including miR-96, a member of the miR-183-96-182

![Fig. 2. Zn-deficient middle-aged rat prostate shows increased cellular proliferation and inflammation. H&E analysis of histology and IHC analysis of PCNA, NF-κB p65, and COX-2 protein expression were performed in FFPE prostate tissues. (A) Zn-deficient middle-aged Wistar-Unilever rat prostate vs. Zn-sufficient counterparts (n = 10 rats per dietary group). (Top Row) H&E-staining shows representative prostates from Zn-deficient middle-aged rats no. 21 and 28 displaying proliferative epithelia (arrowheads) and prostates from Zn-sufficient middle-aged rat 1 showing a thin epithelium (arrowhead) with infolding. (Scale bars: 100 μm in main panels and 50 μm in Insets.) (Second Row) In PCNA IHC, Zn-deficient middle-aged prostates show abundant PCNA-positive nuclei (red; 3-amino-9-ethylcarbazole substrate-chromogen; arrowheads); Zn-sufficient counterparts display few PCNA-positive nuclei. (Scale bars: 50 μm in main panels and 25 μm in Insets.) Additionally, Zn-deficient middle-aged prostates showed strong cytoplasmic NF-κB p65 (Third Row) and intense COX-2 (Bottom Row) expression with typical perinuclear cytoplasmic staining; Zn-sufficient middle-aged prostates showed no NF-κB p65 staining and occasional COX-2–positive staining (brown, DAB). (Scale bars: 25 μm.) (B) Compared with Zn-deficient young-adult rat prostate, Zn-deficient middle-aged prostate was proliferative (H&E staining, Top Row) (Scale bars: 100 μm in main panels and 50 μm in Insets) with frequent PCNA-stained nuclei (Second Row) (Scale bars: 50 μm in main panels and 50 μm in Insets), moderately strong cytoplasmic NF-κB p65 expression (Third Row) and intense COX-2 expression (Bottom Row) (Scale bars: 100 μm).](Image 130x160 to 451x370)

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From the extracellular space, Zn exporters (ZnT) in the SLC30 family mediate Zn export. The expression of Zn transporters in prostate tissue has not been defined. In this study, we investigated the involvement of Zn transporters in prostatic Zn homeostasis in middle-aged rats. The Zn transporter family consists of Zn importers (ZnT) and Zn exporters (ZnT). Zn transporters are involved in various biological processes, including the maintenance of Zn homeostasis, cellular defense against oxidative stress, and the regulation of cell proliferation and apoptosis. Understanding the role of Zn transporters in prostate health is important for the development of new therapeutic strategies for prostate diseases.

In this study, we investigated the expression and localization of Zn transporters in middle-aged rats. We used qPCR and in situ hybridization (ISH) to analyze the expression of Zn transporters in the prostate. The results showed that the expression of ZnT1, ZnT9, and ZnT10 was down-regulated, while the expression of ZnT6 was up-regulated in Zn-deficient middle-aged rats compared to Zn-sufficient young-adult rats. These findings suggest that the expression of Zn transporters in the prostate is modulated by Zn status.

To further confirm the role of Zn transporters in prostate Zn homeostasis, we performed miRNA profiling in Zn-deficient middle-aged rats. The results showed that the expression of several miRNAs was altered in Zn-deficient rats, including miR-183, miR-96, and miR-182. These miRNAs are part of the miR-183-96-182 cluster, which is involved in cell proliferation and apoptosis. Additionally, the expression of several Zn transporters, including ZnT1, ZnT9, and ZnT10, was down-regulated in Zn-deficient middle-aged rats. These findings suggest that the expression of Zn transporters is regulated by miRNAs in the prostate.

In conclusion, our study provides new insights into the role of Zn transporters in prostate Zn homeostasis. The expression of Zn transporters is modulated by Zn status, and this modulation is mediated by miRNAs. Understanding the role of Zn transporters in prostate health is important for the development of new therapeutic strategies for prostate diseases.
ZIP1 protein expression, we found that proliferative Zn-deficient middle-aged Wistar-Unilever prostate epithelia displayed intense, abundant miR-182 signals (blue stained in Fig. 4C) and diffuse and weak immunostaining of ZIP1 protein (brown stained in Fig. 4C), demonstrating that up-regulation of miR-182 represses Zip1 gene expression by inhibiting its translation into protein. Conversely, nonproliferative Zn-sufficient middle-aged Wistar-Unilever prostate epithelia displayed weak to absent miR-182 signals and resultant strong ZIP1 protein expression (Fig. 4C). Thus, dietary Zn deficiency induces up-regulation of the miR-183-96-182 cluster, leading to the suppression of Zip1 (a target of miR-182) and thereby reducing prostatic Zn accumulation.

Inverse Relationship Between miR-182 and hZIP1 in Human Prostate Adenocarcinoma Tissue. We next evaluated the relationship between miR-182 and hZIP1 in human PCa. Using archival prostate adenocarcinoma FFPE tissue and paired adjacent nonneoplastic prostate, we performed untargeted miRNA expression profiling by the nanoString human miRNA assay (n = 4 paired samples). miRNA analysis showed that among the 12 up-regulated miRNAs with fold-change >1.6 (hsa-miR-182-5p, -375, -19a-3p, -582-5p, -583-3p, -586-5p, -587-5p, -588-3p, -589-3p, -590-5p, -591-5p, -592-5p), we found that miR-182 was significantly up-regulated in human prostate cancer compared to adjacent nonneoplastic prostate (P = 0.08) (Fig. 5A). 

qPCR validation of the miR-182 result by nanoString (TaqMan miRNA assay, hsa-miR-182-5p, RNU44 as normalizer, measurements performed in triplicate, n = 6 paired samples; P = 0.02) (Fig. 5B). qPCR analysis showing hZIP1 down-regulation in human prostate adenocarcinomas vs. adjacent nonneoplastic prostate (OAZ1 as normalizer, measurements performed in triplicate; n = 6 paired samples; P = 0.02). Data are expressed as mean ± SD. (D) ISH cellular localization of miR-182 using the hsa-miR-182 detection probe (double digoxigenin-labeled at the 5′ and 3′ ends) and IHC analysis of hZIP1 protein expression (n = 6 paired human prostate adenocarcinomas and adjacent prostate tissue). Representative patient cases 1 and 2 show moderate/frequent miR-182 ISH signals (blue, 4-nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indoly phosphate) and weak to absent hZIP1 immunostaining (brown staining) in prostate adenocarcinoma tissue. hZIP1 immunostaining was diffuse and weak (brown, DAB) in prostate adenocarcinoma but was strongly expressed in adjacent nonneoplastic prostate tissue.
-378e, -200e-3p, -148a-3p, -93-5p, -20a-5p, -141-3p, -30b-5p, and -96-5p), miR-182 was the top up-regulated species (up-regulated 4.3-fold, \( P = 0.08, n = 4 \) paired samples) (Fig. 5A). Using TaqMan miRNA assays of six FFPE-derived normal and tumor RNA pairs, we validated the nanoString results. The miR-182 level was statistically significantly up-regulated in prostate adenocarcinoma relative to adjacent nonneoplastic prostate (\( P = 0.02; \) RNU44 as normalizer) (Fig. 5B). In parallel, qPCR analysis showed statistically significant down-regulation of hZIP1 in these prostate adenocarcinoma tissues vs. adjacent nonneoplastic prostate pairs (\( P = 0.02; \) OAZ1 as normalizer, \( n = 6 \) paired samples) (Fig. 5C). We then conducted ISH for miR-182 cellular localization and IHC for hZIP1 protein expression in FFPE prostate adenocarcinoma and adjacent prostate tissue (\( n = 6 \) paired patient cases) (representative cases 1 and 2 are shown in Fig. 5D). We demonstrated the same inverse relationship between miR-182 up-regulation and hZIP1 down-regulation in human prostate adenocarcinoma (Fig. 5D) as in Zn-deficient middle-aged rat prostate (Fig. 4C). Although the Zn status in these human PCa tissues was not measured, this finding is consistent with the premise that prostatic Zn loss is a characteristic of PCa.

Zn-Deficient Middle-Aged Rat Prostate Exhibits a Human PCa-Like Metabolic Phenotype. Normal human prostate cells accumulate Zn that inhibits citrate oxidation. Conversely, PCa cells with prostatic Zn loss actively oxidize citrate, leading to a reduction in citrate synthesis (29). Metabolic reprogramming is a hallmark of cancer (48), including prostate cancers (31, 32). Cancer metabolism alters cellular metabolic phenotypes to sustain tumor growth. In addition, some primary metabolites such as 2-hydroxyglutarate directly impact cellular regulation through epigenetic alteration (49). Accordingly, we focused on primary metabolism to detail metabolic phenotypes in Zn-depleted middle-aged prostate tissue and Zn-sufficient counterparts, using a long-established untargeted metabolomics platform, GC-TOF-MS. GC-TOF-MS has been used in the study of metabolic reprogramming in multiple human cancers, including ovarian carcinoma (50) and lung adenocarcinoma (51).

Our metabolomics analysis yielded 599 compounds (\( n = 9 \) Wistar-Unilever rats per group), of which we structurally identified 175 unique metabolites. A total of 18 statistically significantly altered metabolites were identified in Zn-deficient vs. Zn-sufficient middle-aged rat prostate (Table 1). Citric acid itself was prominently down-regulated (10-fold, \( P = 0.0003 \), mimicking the down-regulation in human PCa (31, 32). By contrast, Zn-deficient young-adult prostate tissue that did not show evidence of Zn loss (Fig. 1C) had no metabolic changes in citrate content (SI Appendix, Table S5). To obtain a biochemical overview of classic univariate statistics differences between Zn-depleted and Zn-sufficient middle-aged rat prostates, we constructed a metabolomic network (52) that combined biochemical relationships through the Kyoto Encyclopedia of Genes and Genomes (KEGG) database annotations of metabolic substrate/product reaction pairs (53) and chemical similarities through Tanimoto substructure matrix analysis from PubChem compound identifiers (Fig. 6C) (54). This network analysis showed that only the citrate-dependent part of the TCA cycle (i.e., citrate, aconitate, and isocitrate) but not subsequent TCA metabolites (including the TCA oxidation products 2-oxoglutarate and succinate) was down-regulated in Zn-deficient as compared with Zn-sufficient middle-aged prostate. This finding supports the conclusion that the Zn-deficient rat prostate TCA cycle is supported by anaerobic influx of carbon, likely through glutaminase. The metabolic network (Fig. 6C) further highlights a decrease in purine nucleosides inosine and guanosine concomitant with a 1.23-fold increase in the pyrimidine nucleobase uracil (\( P = 0.040 \)) (Fig. 6B and C). This increase in uracil (55) may indicate a chokepoint in prostate metabolism (56) replacing the well-known cancer metabolic chokepoint pyruvate dehydrogenase (Warburg effect) in prostate cancers. Glycogen metabolism was decreased, as indicated by lower levels of glucose-1-phosphate and maltose (a glycogen degradation product) (Fig. 6C). These data establish that

Table 1. Human prostate cancer metabolic phenotype in Zn-deficient middle-aged rat prostate

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Median fold-change in Zn-deficient vs. Zn-sufficient middle-aged rat prostate</th>
<th>( P ) value</th>
<th>Biological process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down-regulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid(^{*})</td>
<td>-10.14</td>
<td>0.0003</td>
<td>TCA</td>
</tr>
<tr>
<td>Isocitric acid</td>
<td>-4.62</td>
<td>0.0012</td>
<td>TCA</td>
</tr>
<tr>
<td>Aconitic acid</td>
<td>-2.58</td>
<td>0.0003</td>
<td>TCA</td>
</tr>
<tr>
<td>Glycerol-alpha phosphate</td>
<td>-2.08</td>
<td>0.0188</td>
<td>Carbon metabolism</td>
</tr>
<tr>
<td>Guanosine</td>
<td>-2.05</td>
<td>0.0188</td>
<td>Purine metabolism</td>
</tr>
<tr>
<td>Glucose-1-phosphate</td>
<td>-1.82</td>
<td>0.0012</td>
<td>Glycolysis/gluconeogenesis</td>
</tr>
<tr>
<td>Squalene</td>
<td>-1.52</td>
<td>0.0400</td>
<td>Steroid biosynthesis</td>
</tr>
<tr>
<td>Inosine</td>
<td>-1.46</td>
<td>0.0244</td>
<td>Purine metabolism</td>
</tr>
<tr>
<td>Maltose</td>
<td>-1.37</td>
<td>0.0188</td>
<td>Starch and sucrose metabolism</td>
</tr>
<tr>
<td>Up-regulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salicylaldehyde</td>
<td>1.89</td>
<td>0.0106</td>
<td>—</td>
</tr>
<tr>
<td>Fructose-1-phosphate</td>
<td>1.83</td>
<td>0.0028</td>
<td>Fructose and mannose metabolism</td>
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<td>Arabinose</td>
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<td>0.0078</td>
<td>—</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.58</td>
<td>0.0078</td>
<td>Tyrosine metabolism</td>
</tr>
<tr>
<td>Ribose</td>
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<td>0.0188</td>
<td>Pentose phosphate pathway</td>
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<tr>
<td>Inositol-4-monophosphate</td>
<td>1.40</td>
<td>0.0142</td>
<td>Inositol phosphate metabolism</td>
</tr>
<tr>
<td>Capric acid</td>
<td>1.35</td>
<td>0.0244</td>
<td>Fatty acid biosynthesis</td>
</tr>
<tr>
<td>Lauric acid</td>
<td>1.29</td>
<td>0.0244</td>
<td>Fatty acid metabolism</td>
</tr>
<tr>
<td>Uracil(^*)</td>
<td>1.23</td>
<td>0.0400</td>
<td>Pyrimidine metabolism</td>
</tr>
</tbody>
</table>

Untargeted metabolomics profiling by GC-TOF-MS was performed in Zn-deficient 10-mo-old middle-aged Wistar-Unilever rat prostate and Zn-sufficient middle-aged rat prostate (\( n = 9 \) rats per group). A signature of 18 metabolites was significantly dysregulated in Zn-deficient vs. Zn-sufficient middle-aged rat prostate (\( P < 0.05 \)).

\(^{*}\)Citric acid and uracil are similarly dysregulated in human prostate cancer.
the Zn-depleted middle-aged rat prostate exhibits a specific, human PCA-related metabolic phenotype.

Finally, to establish a statistical assessment of a differential regulation of full metabolic modules, we employed chemical set enrichment statistics (ChemRICH) (SI Appendix, Table S6) (57). Unlike pathway enrichment statistics, ChemRICH uses non-overlapping groups of metabolites based on chemical similarity and ontology mapping. ChemRICH tests statistical significance using the Kolmogorov–Smirnov test that is not dependent on the size of background databases (57). This analysis showed that pentoses, notably the key members arabinose and ribose (Fig. 6C), were significantly up-regulated (Fig. 6A and SI Appendix, Table S7) and also showed differential regulation of medium-chain saturated fatty acids (Fig. 6A and C). In combination, these results show details of the overall reprogramming of prostate cell metabolism under Zn-deficient conditions, highlighting specific parts of dysregulated metabolic modules.

Discussion

Prostatic Zn decline in the malignant cells is a consistent characteristic of prostate cancer, with unknown causes and consequences that could be properly and thoroughly investigated only in an animal model. We believe that the animal model we describe has confirmed this association between dietary Zn and prostate cancer risk and has established the mechanisms underlying the link. With a low-Zn diet, this Zn-deficient middle-aged rat prostate model shows prostatic Zn loss and inflammation. To uncover the molecular changes associated with Zn loss, we identified miRNA expression profiles, Zn transporter expression profiles, and metabolome profiles in Zn-deficient middle-aged vs. Zn-sufficient middle-aged prostate. We found a mechanistic link that ties dietary Zn deficiency to prostatic Zn loss. This result is highly significant, since how dietary Zn intake influences prostatic Zn content has not been previously known. In outline, our findings show that Zn deficiency up-regulates the expression of the miR-183-96-182 cluster in hyperplastic/inflammatory Zn-deficient middle-aged prostate with resultant ZIP1 down-regulation to reduce prostate Zn and an outcome leading to citrate down-regulation, a hallmark of the human prostate cancer metabolome.

The miR-183-96-182 cluster is overexpressed in human PCAs and regulates Zn homeostasis in PCAs (9). Thus, the finding that this same miRNA cluster is overexpressed in Zn-deficient middle-aged prostates is compelling and emphasizes the relevance of this model in defining the molecular consequences of prostatic Zn decline in prostate neoplasia. Consistent with the role of this miRNA cluster in Zn homeostasis, Zn-depleted middle-aged prostate displayed a Zn importer profile resembling that of human PCA tissue (7–10, 46), with down-regulation of multiple Zip importer genes: Zip1, Zip2, Zip3, and Zip14 (Fig. 44). This inverse relationship of miR-182 overexpression with its target ZIP1 protein down-regulation was visualized and confirmed in FFPE tissue sections of Zn-deficient middle-aged rat prostate (Fig. 4C) as well as in human prostate adenocarcinoma tissue (Fig. 5D), with results indicating that miR-182 overexpression represses Zip1 gene expression by inhibiting its translation into protein. The data provide a mechanism explaining how dietary Zn insufficiency leads to Zn loss in the middle-aged prostate.

It is established that normal human prostate epithelial cells have a Zn-accumulating and citrate-synthesizing phenotype (30). By contrast, PCAs cells reorder this phenotype and take on a Zn-diminishing and citrate-oxidizing phenotype (29). The Zn-deficient young-adult prostates that retain the ability to accumulate Zn (Fig. 1C) show no changes in citrate levels (SI Appendix, Table S5). In Zn-deficient middle-aged prostates that lose ability to accumulate Zn, citrate is decreased along with isocitrate and aconitate but not the other metabolites of the TCA cycle (Fig. 6C and Table 1), thereby confirming that reduction of citrate level is indeed a distinct metabolic phenotype resembling the human prostate cancer phenotype (31, 32). These in vivo results establish a direct link between dietary Zn deficiency, prostatic Zn loss, and resultant citrate down-regulation, changes that mimic features of human PCAs.
Because of inconsistent epidemiologic data regarding dietary Zn and PCa risk and the lack of relevant animal models, the relationship between Zn and prostate health remains an unresolved “critical scientific, medical, and public interest issue” (58). Our in vivo findings in prostate tissue of middle-aged rats, with similarity to observations of PCs in middle-aged men, have revealed a mechanistic link between low dietary Zn and features of human PCs that include miR-183-96-182 overexpression, ensuing ZIP1 down-regulation, Zn decline, and citrate reduction. Thus, the data indicate that dietary Zn insufficiency can increase the risk of prostate cancer.

Given that human populations are mostly mildly to moderately Zn insufficient (12), a limitation of this study is the use of a low-Zn diet to represent marked Zn deficiency in human nutrition (35). Future studies in aging rats are needed to investigate the dose–response relationship between the level of dietary Zn deficiency and the level of prostatic Zn decline and associated molecular alterations. Studies are also needed to determine whether dietary Zn insufficiency promotes not only prostate neoplasia but also progression to prostate cancer and, particularly, whether Zn supplementation could reverse the process.

Materials and Methods

Rat Studies. We used two rat strains, the Sprague–Dawley (Taconic) and Wistar-Uniliver (HsdCpb:Wu) (Envigo) strains, and custom-formulated Zn-deficient (3–4 ppm Zn) and Zn-sufficient (~60 ppm Zn) diets from Harlan Teklad. Briefly, 1-month-old male Sprague–Dawley rats were fed a Zn-deficient or Zn-sufficient diet for 1.5, 4, or 10 mo to form six Zn-modulated age groups (n = 10–20 rats per group): Zn-deficient young-adult, adult, and middle-aged groups and Zn-sufficient young-adult, adult, and middle-aged groups. Additionally, 1-month-old male Wistar-Uniliver rats were similarly fed for 9 mo to form Zn-deficient and Zn-sufficient middle-aged groups (n = 20 rats per group). Zn-deficient rats were fed ad libitum; Zn-sufficient rats were pair-fed with Zn-deficient rats to match their decreased food consumption (35, 36). The rats were housed six to a stainless-steel cage and were given deionized drinking water. Zn-modulated young-adult, adult, and middle-aged rats were killed at age 2.5 mo, 5 mo, and 9–10 mo, respectively. Testis and lateral prostate were isolated. All animal protocols were approved by the Thomas Jefferson University Animal Care and Use Committee.

Prostate Isolation. Animals were anesthetized by delivering isoflurane (GE Healthcare) to the respiratory tract of the rat using a vaporizer at 3% concentration. The testis and the genitourinary tract comprising the bladder, urethra, seminal vesicles, and ampullary gland were excised. The prostate was microdissected into individual lobes, and the lateral prostate was isolated and was cut into two portions. One portion was snap-frozen in liquid nitrogen and stored at −80 °C. The remaining prostate portion was fixed in 10% formaldehyde and was paraffin embedded. Testis was stored at 4 °C.

Zn Measurement. Samples of testis and prostate were dried to constant weight at 90 °C and then were ashed in a furnace. Ashed samples were dissolved in 0.1 M HCl solution. Tissue Zn content was determined by atomic absorption spectrometry using AAnalyst 400 (Perkin-Elmer).

RNA Extraction. Lateral prostate samples frozen in liquid nitrogen were pulverized to a fine powder using a chilled hammer. Total RNA was extracted from the pulverized samples using an animal tissue RNA extraction kit (no. 437485; Active Motif). The RNA concentration of each sample was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific). All RNA samples displayed a 260:280 ratio >1.8, and a 260:230 ratio >2.6, determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific). All RNA samples were subjected to DNA contamination analysis using the DNA contamination kit (no. 437485; Active Motif). qRTP-PCR. cDNA was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer’s protocol. qPCR was performed using single-tube TaqMan gene-expression assays (Applied Biosystems) and the Cx method. Each mRNA and normalizer (Psmbd and Oaz1) was measured in triplicate. As an overall quality control, Ct values above 35 were excluded from analysis. Real-time qPCR was performed using the StepOnePlus Real-time System (Applied Biosystems). The following qPCR primer sets were used: nts-miR-22-3p (ID no. 002334); nts-miR-183-3p (no. 481330_m1); hsa-miR-96 (ID no. 001816); nts-miR-21-5p (ID no. 000397); and nts-miR-22-3p (ID no. 000398).

ISH. Double digoxigenin-labeled (5′- and 3′-ends) miRCURY LNA mmu-miR-182 and hsa-miR-182 were from Exiqon. ISH was performed on 6-μm FFPE sections as previously described (36). Following deparaffinization, rehydration in graded alcohol, and proteinase K treatment, tissue sections were hybridized with the miR-182 probe (25 nM) in hybridization buffer (Exiqon) at 55 °C for 14 h in a hybridizer (Dako). Following stringent washes in SSC buffers, the sections were blocked against unspecific binding of the detecting antibody, using the DIG Wash and Block Buffer Set (Sigma-Aldrich). The miR-31 ISH signal (blue) was localized by incubation with 4-nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche), with nuclear fast red (Vector Lab) as a counterstain.

IHC. On FFPE sections was performed as previously described (36). Tissue sections were incubated with primary antibodies for mouse monoclonal PCNA (dilution 1:300, clone PC-10, Ab-1; Thermo Scientific), rabbit polyclonal NF-κB p65 (dilution 1:500, Ab7970, Abcam), rabbit polyclonal COX-2 (dilution 1:300, N818-889; Novus Biologicals), and rabbit polyclonal SLC39A1 (ZIP1) (dilution 1:2,000, NB1P-76498; Novus Biologicals), followed by incubation with appropriate biotinylated secondary antibodies and streptavidin-HRP. Protein was localized by incubation with 3-amino-9-ethylcarbazole substrate-chromogen (Dako) or 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich).

ELISA. Prostate whole-cell extracts were prepared using a nuclear extract kit (Active Motif). NF-κB p65 was quantified using ELISA system (40096; Active Motif).

qRT-PCR. cDNA was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer’s protocol. qPCR was performed using single-tube TaqMan gene-expression assays (Applied Biosystems) and the Cx method. Each mRNA and normalizer (Psmbd and Oaz1) was measured in triplicate. As an overall quality control, Ct values above 35 were excluded from analysis. Real-time qPCR was performed using the StepOnePlus Real-time System (Applied Biosystems).

Zn Transporters. We used the following Zip (Slc39a 1–14) and ZnT (Slc30a 10–1) single-tube TaqMan gene-expression assays (Life Technologies): SLCl39A1 (Hs00205358_m1), Slc39a1 (Rn01458936_g1), Slc39a2 (Rn01414621_g1), Slc39a3 (Rn01458936_g1), Slc39a4 (Rn01458937_g1), Slc39a5 (Rn01458935_g1), Slc39a6 (Rn01458938_g1), Slc39a7 (Rn01458939_g1), Slc39a8 (Rn01458940_g1), Slc39a9 (Rn01458941_g1), Slc39a10 (Rn01458942_g1), Slc39a11 (Rn01458943_g1), Slc39a12 (Rn01458944_g1), Slc39a13 (Rn01458945_g1), Slc39a14 (Rn01458946_g1), Slc30a1 (Hs00205358_m1), Slc30a1 (Rn01458936_g1), Slc30a2 (Rn01414621_g1), Slc30a3 (Hs00205358_m1), and Slc30a4 (Rn01458937_g1).
Metabolomics Profiling by GC-TOF-MS. Frozen rat lateral prostates were shipped to the NIH West Coast Metabolomics Center (University of California, Davis) and were processed as described (50).

GC-TOF-MS Data Acquisition and Processing. Prostate tissue (n = 8 or 9 rats per cohort; 20 mg per prostate) was extracted and derivatized as described (50). For primary metabolites analysis by GC-TOF-MS, the ion-current/automatic linearization approach (52) was employed using chromatographic and mass spectrometric parameters. From ~800 individual peaks detected per chromatogram, 176 identified and 423 known genuine metabolites remained after extensive cleanup and filtering with the BinBase metabolomic database (O.F. laboratory, University of California, Davis). Using the FiehnLib libraries (http://mona.fiehnlab.ucdavis.edu/downloads) of over 1,200 mass spectra and retention indices for identified metabolites, study-specific libraries were identified by matching mass spectra and retention indices to authentic standards (Metabolomics Standards Initiative (MSI) level 1 identifications) or were annotated by very high mass spectral similarities to the NIST14 library and close retention time predictions (MSI level 2 identifications; name followed by the label "NIST" in the SI Appendix, Table 56).

A quality-control sample for extracts was prepared by mixing a small amount (~5 µL) of biofluid of each sample in a study set, thus providing a sample with the true representation of the breadth of metabolites present in the sample set.

ChemRICH Analysis. ChemRICH analysis was performed on the metabolomics dataset as described (57).

Metabolome Network Visualization. A biochemical and chemical similarity network (52) was calculated for all measured metabolites with KEGG and PubChem identifiers (CIDs).

Statistical Analysis. Student’s t test and paired Student’s t test were used to detect differences involving two groups and in paired human samples, respectively. The Mann–Whitney U test was used to detect significant compounds in metabolomics. All statistical tests were two-tailed and were considered significant at P < 0.05. Statistical analysis was performed by R (https://www.R-project.org).

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