Aging and Cancer-Related Loss of Insulin-like Growth Factor 2 Imprinting in the Mouse and Human Prostate

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Abstract

Loss of imprinting (LOI) is an epigenetic alteration involving loss of parental origin-specific expression at normally imprinted genes. A LOI for *Igf2*, a paracrine growth factor, is important in cancer progression. Epigenetic modifications may be altered by environmental factors. However, it is not known whether changes in imprinting occur with aging in prostate and other tissues susceptible to cancer development. We found a LOI for *Igf2* occurs specifically in the mouse prostate associated with increased *Igf2* expression during aging. In older animals, expression of the chromatin insulator protein CTCF and its binding to the *Igf2*-H19 imprint control region was reduced. Forced down-regulation of CTCF leads to *Igf2* LOI. We further show that *Igf2* LOI occurs with aging in histologically normal human prostate tissues and that this epigenetic alteration was more extensive in men with associated cancer. This finding may contribute to a postulated field of cancer susceptibility that occurs with aging. Moreover, *Igf2* LOI may serve as a marker for the presence of prostate cancer.

Introduction

Our understanding of how aging predisposes to the frequent development of prostate and other major cancers is limited. Histologic prostate cancer is found in over 60% of men in their 70s at autopsy (1). DNA damage accumulates in aging human prostate tissues that results from multiple exogenous and endogenous stressors (2). Conversely, it has been proposed that the modification of epigenetic factors, defined as inheritable changes in information passed during cellular replication that do not involve altered DNA sequence, underlie the gene expression changes that occur with aging (3). Differences in DNA methylation have been shown when identical twins are compared in older age (4). In the colon, and recently in the prostate, DNA hypermethylation with increasing age has been found within various genes (5, 6). Epigenetic modifications, including histone/chromatin modifications and genomic imprinting, seem uniquely sensitive to alterations in the cellular and organismal environment (7, 8). Degradation of these epigenetic patterns may provide insight into the susceptibility of cancer that develops with aging.

One manifestation of epigenetic modification is genomic imprinting, or the allele-specific expression of a gene based on its parental origin. The insulin-like growth factor 2 (*Igf2*) gene and its closely linked 3′ neighbor, *H19*, display imprinting and are expressed solely from either the paternal or maternal allele, respectively (9). The *Igf2*-H19 imprinted cluster shares enhancers, as well as an intergenic imprint control region (ICR) that coordinates gene expression (10). A major element in the control of *Igf2* imprinting at the ICR is the presence and binding of CTCF, a well-characterized chromatin insulator that is required for repression of the maternal *Igf2* allele (11). CpG methylation at this ICR blocks the binding of CTCF to DNA and allows the downstream enhancer to stimulate *Igf2* promoter activity across the inert boundary site. Deletion or hypermethylation of this ICR prevents CTCF binding and results in a reactivation of the maternal allele and the biallelic expression of *Igf2* (12, 13). Strict imprinting is generally maintained in normal adult tissues. However, the loss of imprinting (LOI) at *Igf2*, a potent autocrine and paracrine growth stimulator, seems to provide an important early switch in the progression of neoplasia (14, 15). This has lead to the proposition that an epigenetic loss of allelic silencing plays a role in cancer progression. In the Apc+/-Min mouse, animals displaying LOI developed twice as many intestinal tumors as did control littermates (16). Given the plasticity of epigenetic controls, we examined whether imprinting of the *Igf2* gene undergoes alteration during aging in the prostate, an organ susceptible to cancer development.

Materials and Methods

**Mice and diet.** B6 (*cast H19-p57*) mice, a congenic strain heterozygous for distal chromosome 7 sequences from C57BL/6 and *Mus castaneus*, were obtained from Dr. Shirley Tilghman (Princeton University, Princeton, NJ). Male mice homozygous for *Mus castaneus* alleles (*H19-p57*) were bred with female C57BL/6 and male mice from each litter entered randomly into aging groups. The animals were individually housed in the Veteran's Administration and University of Wisconsin Shared Aging Rodent Facility and fed on an 84 kcal/wk diet (TD91349 [Teklad]), which is ~ 10% less than the average ad libitum intake, to prevent obesity and maintain health. Ten animals per time point were euthanized at intervals (every 8 mo) beginning at ages 3 mo. Tissues were micродissected, including the coagulating glands, dorsolateral prostate (DLP), and ventral prostate (VP), and placed in RNase-free PBS and snap frozen in liquid nitrogen. Nonprostate tissues were treated identically.

**cDNA preparation and quantitative PCR.** Total RNA was extracted from frozen tissues using the RNeasy Mini kit (Qiagen) as described by the manufacturer. Total RNA was treated with DNase before reverse transcription. cDNA was made from 1 µg of total RNA, and oligo (dT), using Omniscript RT reagents (Qiagen) per the manufacturer's protocol. Reverse transcription without reverse transcriptase was carried out to detect genomic DNA contamination. Quantitative PCR (QPCR) was performed with
SYBR Green (Applied Biosystems) on a MyQ QPCR machine (Bio-Rad; ref. 17). Both cyclophilin and GAPDH were used as internal controls and neither showed altered expression with aging (data not shown). Primer sequences for Igf2, H19, p57, CTCF, GAPDH, and cyclophilin are available on request.

**Fluorescent primer extension assay.** Primer extension assays were performed as previously described (18). A single nucleotide polymorphism was identified on Igf2 exon 6 (G/A) and used to identify individual alleles. A 700-bp intron spanning PCR product was generated from cDNA using primer sequences CTCTCAGGCCGTACTTCCGGAC (forward) and GGCCCGGAATTAGTTGATTT (reverse). ExoSAP-IT (U.S. Biochemical) was used to remove excess deoxynucleotide triphosphates (dNTP) and primers GCGCCGAATTAGTTGATTT (reverse). ExoSAP-IT (U.S. Biochemical) was used to remove excess deoxynucleotide triphosphates (dNTP) and primers from the PCR reaction. A FLAM-labeled fluorescent primer extension assay (FluPE) primer was generated (5′-ACATCGGCCAAGGGGATCTCAGC). One hundred nanograms of PCR product were added to 10 nmol/L of FLAM-labeled primer, 0.75 U of HotStarTaq Polymerase (Qiagen), 200 μmol/L of each specific dNTP/dNTPs (dATP/dTTP, dCTP, and dGTP) and primers extension used to differentiate allelic expression with high sensitivity (<5 parts in 100) in the linear range.

**DNA methylation analyses.** Bisulfite treatment of DNA, cloning, and sequencing was performed as we have previously described (17). Primers used to amplify bisulfite treated DNA within the H19 ICR for cloning included CTCF 3 and CTCF 4 (100 μmol/L of each) (20). Samples were run in triplicate and the mean used for statistical comparison.

Chromatin immunoprecipitation assays.** Fresh tissues were cross-linked by mincing in 1% Formaldehyde-PBS solution for 20 min. Tissue samples from 2 mice for each age group were pooled; washed in PBS; resuspended in 150 mmol/L NaCl, 10% glycerol, and 50 mmol/L Tris 8.0 (with 1:2000 dilution of Sigma protease inhibitor cocktail); and homogenized using the Tissue Tearer (Biospec Products). Crosslinked chromatin was prepared, sonicated, and immunoprecipitated using 2 μg of anti-CTCF (Upstate); or mouse monoclonal antibodies to cyclophilin (Upstate), or mouse monoclonal antibodies to GAPDH (Oncogene Research Products) were diluted in blocking buffer at a 1:500 ratio (the linear range of detection) and incubated at 4°C overnight. Negative controls included no primary antibodies. After washing, Alexa 494- conjugated anti-rabbit (Invitrogen) and FITC-conjugated anti-mouse (Becton Dickson) secondary antibodies were diluted 1:2000 in blocking buffer containing 10 μg/mL Hoechst 33342 (Molecular Probes) as a DNA counter stain and incubated at room temperature for 1 h. Sections were then washed twice in blocking buffer, aspirated, mounted beneath coverslips, and allowed to dry overnight (21).

Stained tissues were analyzed by quantitative fluorescence microscopy as described (21, 22). Using an Olympus BX51 microscope, digital images were acquired using a RT Color digital camera and ProSPOT Advanced software (Diagnostic Instruments, Inc.). Using identical camera settings, images from five different random fields were acquired per section and the integrated density of each whole single-color image was measured using NIH ImageJ. Each IGF2 measurement was normalized to that of α-tubulin, a constitutively expressed, cell-associated control.

siRNA transfection. Human prostate epithelial cells (HPEC) and mouse prostate epithelial cells were harvested and cultured as described (23). Cells lines were seeded to 50% confluence on 6-well plates 24 h before transfection. One hundred to 200 pmol of CTCF SMARTpool (DHARMACON, Inc) siRNAs (mouse or human, respectively) were combined in medium with Lipofectamine 2000 (Invitrogen Life Technologies) transfection reagent following the manufacturer’s protocol. The mixture was then added dropwise to the cells in complete DMEM medium and mixed by gentle rocking. Cells were retreated with siRNAs 12 h after the initial transfection. RNA and protein were harvested at 48 h. Experiments were performed in duplicate with similar results.

**Results** To test whether IGF2 undergoes a LOI during aging in the prostate, we determined the imprinting status of mouse prostate tissues using a sensitive primer extension assay. C57BL/6 mice containing a Mus castaneus Igf2-P57 locus were used to allow differentiation between the paternal and maternal alleles based on a polymorphism (G/A) within IGF2 exon 6. Mice have multiple prostate lobes. The DLP most closely corresponds to the human peripheral prostate, where prostate cancer regionally develops, based on anatomic and RNA signature analyses (24). We observed that DLP tissues from 3-month-old sexually mature mice mostly retain the imprinted status of IGF2 (Fig. 1A). IGF2 and its closely linked 3′ neighbor H19 display imprinting in adult tissues and are expressed solely from either the paternal or maternal alleles respectively (9). In contrast, we found older cohorts developed progressively higher average expression from the once inactive IGF2 chromosome. To establish whether the marked LOI for IGF2 observed in the DLP of aging mice is unique to that prostate lobe, and to the prostate in general, we analyzed VP, liver, and kidney in aging C57BL/6 (Cast IGF2-P57) cohorts (Fig. 1B). A relaxation of imprinting was not observed in any of these tissues indicating this age-associated LOI was lobe-specific for the mouse DLP.

Relaxation of IGF2 imprinting has been linked to increases in IGF2 gene expression (12, 25). IGF2 functions as a potent autocrine...
and paracrine growth stimulator (15) and is important in the progression to neoplasia (14). We used qPCR initially to detect and quantify mRNA levels in the 3-, 11-, 19-, and 24-month cohorts. We observed age-associated increases in \( \text{Igf2} \) mRNA levels in DLP tissues (Fig. 2A). \( \text{H19} \) cDNA expression decreased and approached significance (\( P = 0.07 \)) with aging. Both results are consistent with coordinate regulation from the \( \text{H19} \) ICR (10). In the aging cohorts, \( \text{Igf2} \) protein expression was evaluated in the DLP using quantitative immunofluorescence. DLP tissues from 24-month-old mice express significantly more \( \text{Igf2} \) (30%; \( P = 0.01 \)) when compared with 3-month-old mice (Fig. 2B). The prostate and other tissues examined did not show macroscopic morphologic changes or tumors (Supplementary Fig. S1A). However, histologic analysis of the DLP revealed both epithelial and stromal hyperplasia within the individual glands, as well as rare glandular atrophy. Inflammation, as assessed by CD45 a marker of lymphocytes (26), was minimally increased in older mouse prostate tissues on this moderate caloric restriction protocol used to engender longevity and health (Supplementary Fig. S1B). To understand the mechanism involved in the relaxation of \( \text{Igf2} \) imprinting seen in the prostate, we assessed CpG methylation at multiple CTCF binding sites within the \( \text{H19} \) ICR (Fig. 3A). Previous studies in mice showed that methylation or deletion of the ICR results in re-expression of the maternal allele and biallelic \( \text{Igf2} \) expression (12, 13). Utilizing MS-qPCR after bisulfite treatment of DNA, we found no significant change in methylation at CTCF binding sites between young (3 months) and old (24 months) DLP tissues. These results were additionally confirmed by sequencing after bisulfite treatment. Other regions, such as the \( \text{H19} \) promoter, show increased methylation in the older cohort (28% ± 4% versus...
38% ± 2%;  *P* = 0.04) consistent with the hypothesis of aging-related regional DNA hypermethylation (6).

We next examined the extent to which CTCF protein binding is altered by aging at binding sites 2, 3, and 4 within the *H19* ICR in DLP tissues. CTCF acts as an insulator by binding to the *H19* ICR and preventing enhancer binding to and expression of the *Igf2* promoter (10). Using chromatin immunoprecipitation (ChIP) assays, a significant age-associated decrease in binding was observed at both CTCF 3 (41%;  *P* = 0.01) and CTCF 4 (40%;  *P* = 0.01; Fig. 3B). No alteration in binding was seen at CTCF 2, a site that does not regulate *Igf2* promoter activity (27). In VP tissue from the same animals, which does not show an age-related LOI, no alteration in CTCF binding was found (Fig. 3C).

This decrease in CTCF binding to the ICR directed us to inquire whether CTCF expression levels change in mouse DLP tissues with aging and whether they modulate *Igf2* imprinting. Expression analyses showed an age-associated decrease of CTCF mRNA as well as protein levels in mouse DLPs (Supplementary Fig. S2A). To determine the effect of CTCF expression on *Igf2* imprinting, we knocked down CTCF levels in mouse prostate epithelial cells cultured from the DLP and HPECs using siRNA. CTCF protein expression was reduced 30% to 50% in transfected cell lines using pooled CTCF siRNAs specific for the human or mouse gene (Supplementary Fig. S2B). After 48 h, no morphologic changes were noted in the transfected cells. QPCR showed a ~30% to 70% silencing of CTCF RNA expression in siRNA-transfected mouse and human cultures when compared with controls (data not shown). Increased expression (*A/A*′ = 8–18%) of the silenced/imprinted *Igf2* allele was reproducibly shown after siRNA transfection using FluPE (Supplementary Fig. S2). Hence, a loss of CTCF expression and binding to the *H19* ICR resulted in biallelic *Igf2* expression. To understand better the implications of this prostate-specific epigenetic change, we next focused on determining whether *Igf2* imprinting alterations occur in the peripheral prostate of aging men.

Prostate tissues from men ages 17 to 81 years old with and without associated prostate cancer were analyzed. Given the predilection of human prostate cancer for the peripheral zone, we focused our analysis on this region. Using FluPE, a polymorphism (G to C) within the *Igf2* coding sequence was used to quantitate allele-specific expression. Overall, a significant trend toward LOI with aging was seen when all samples were included and statistically analyzed in a linear fashion ( *P* = 0.02; Fig. 4A). We then analyzed only those samples without associated prostate cancer. The imprint status of peripheral prostate tissue from a cohort of older men (55–81 years; mean, 64 years) without cancer (*A/A*′ = 28%) was suggestively more relaxed than from younger (18–40 years; mean, 27 years) patients (*A/A*′ = 24%;  *P* = 0.2). When the allelic expression ratios were compared for older men with associated cancer to the age-matched group without prostate cancer (60% versus 28%), a significant difference ( *P* < 0.001) was seen. Thus, we find a more extensive relaxation in imprinting develops in the prostates of men who have associated cancer.

**Discussion**

Our findings confirm that the *Igf2* imprint is not stable in adult mammals, but rather undergoes degradation with aging. Furthermore, in humans, it is associated with a “field effect” that develops in the peripheral prostate containing cancer. Several factors implicated in aging may play a role in this LOI including oxidative stress, diet, hormones, and environmental toxicants (7, 8, 28). There is remarkable variation in the rate of epigenetic degradation within and among organs with aging. Although the extent of this LOI with aging in the mouse prostate is extensive (>60%), we do not show a significant change in the kidney or liver. Minor changes in *Igf2* imprinting (<6%), not linked to expression, have been recognized in the heart tissue from aging mice (18). We also find that *Igf2* LOI with aging extensively involves the DLP of mice but not the VP. This is of interest, owing to the anatomic homology of the DLP to the peripheral prostate in the human (24, 29), the region where prostate cancer most commonly develops. One observation that has
led to the development of the hypothesis that tissue-specific imprinting degrades with aging was our finding that Igf2 LOI is limited to the peripheral zone of human prostates, and not found in the transition zone where benign prostatic hyperplasia occurs (30).

A number of factors are involved in Igf2 imprinting control, including DNA methylation, the covalent modification of histone proteins, and the binding of the insulator protein CTCF (10, 11). One target of this observed loss of epigenetic control is the chromatin-associated protein CTCF, a zinc finger binding protein. We show that a forced decrease in CTCF expression in both human and mouse prostate epithelial cells leads to a re-expression of the paternal Igf2 allele. In the mouse prostate, a decrease in CTCF expression with aging was associated with a loss of CTCF binding at the H19 ICR. This decrease in CTCF expression has also been recognized in vitro with the development of cellular senescence (17). Our finding that substantial changes Igf2 imprinting occur in the absence of significant methylation changes at the H19 ICR, an observation seen in other human genitourinary tissues (31), challenge the central dogma that DNA methylation solely controls Igf2 imprinting in the human (13). Imprinting in Wilms' tumors may also be maintained in the presence of aberrant methylation at CTCF core sites within the H19 ICR (32). It has been also found that polycomb group proteins expressed from the maternal genome, not DNA methylation, control paternal MEA silencing (33). These data emphasize the emerging importance of chromatin factors, including CTCF, in the regulation of imprinting.

Histologic prostate cancer is pervasive in aging men (1). Our finding of a significantly greater relaxation of Igf2 imprinting in histologically normal tissues from men with associated prostate cancer compared with men without the disease suggests a role for Igf2 LOI in the postulated field effect encompassing prostate tissues and influencing them to develop cancer (Fig. 4B). Further support for this field effect is evidenced by the typical finding of 5 separate foci of cancer in found in human prostates removed for malignancy (34). Other gene expression alterations may mark this global tissue defect in the peripheral prostate (35). The finding that Apc+Min mice that biallelically express Igf2 have enhanced intestinal tumor development compared with monoallelic expression suggests LOI is a risk factor for tumor development in the susceptible organ (16). We anticipate this epigenetic alteration involving LOI may find use as a marker for patients with prostate cancer. Furthermore, it is likely that men who are better able to maintain Igf2 imprinting status with aging have a decreased risk of prostate cancer development.

Disclosure of Potential Conflicts of Interest

R. Weindruch is a founder of LifeGen Technologies, LLC. The other authors disclosed no potential conflicts of interest.

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