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A marked propensity for prostate cancer to arise in the peripheral prostate with aging. The Insulin-like Growth Factor-II (*IGF2*) gene is an auto-paracrine growth stimulator that is an important positive modulator of cancer development. *IGF2* typically demonstrates monoallelic, or imprinted, expression in adult tissues and indeed this pattern is maintained in the periurethral zone, a region where cancer development is rare. In addition, *IGF2* loss of imprinting (LOI), as well as increased *IGF2* expression, are common attributes of prostate cancer. It is our hypothesis to be tested that an age-dependent loss of *IGF2* imprinting, resulting from age-dependent changes in DNA methylation, occurs specifically in the peripheral zone of the prostate and contributes to the increased risk for cancer development. To examine temporally when this loss of *IGF2* imprinting occurs and the mechanisms underlying it we propose 3 Specific Aims: (1) To determine if *IGF2* LOI in the peripheral prostate derives from stromal and/or epithelial cells; (2) To determine whether *IGF2* LOI occurs as an age-dependent process in human prostate tissues that are uninvolved with cancer; and (3) To examine DNA methylation as a mechanism for any observed changes in the imprint status in prostate tissues. This proposal is significant and unique in testing whether regional epigenetic changes occur in histologically normal prostate tissues that are destined to become neoplastic. We expect to determine whether specific age-related, peripheral zone changes in methylation and imprinting occur in the general population and whether these changes are linked to prostate cancer development.

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INTRODUCTION

Three important features of prostate cancer will be addressed in the present proposal that may provide a quantum leap in our understanding of the risk factors and development of prostate cancer. These features include: (1) A marked propensity for prostate cancer to arise in the peripheral prostate; (2) The multifocality of prostate cancer which implicates a generalized or field change in cancer susceptibility; and (3) The important role of the Insulin-like Growth Factor (IGF) axis in both aging-related and genetic-related cancers. The Insulin-like Growth Factor-II (*IGF2*) gene is a auto-paracrine growth stimulator that is an important positive modulator of

cancer development. We will provide preliminary evidence that a loss of imprinting, or biallelic expression, of the *IGF2* gene is an age-related specific epigenetic alteration that occurs in the peripheral prostate (**Figure 1**). *IGF2* typically demonstrates monoallelic, or imprinted, expression in adult tissues and indeed this pattern is maintained in the periurethral zone, a region where cancer development is rare. In addition, *IGF2* loss of imprinting (LOI), as well as increased *IGF2* expression, are common attributes of prostate cancer. Since DNA methylation is the major determinant of gene imprinting, we would anticipate that a loss of *IGF2* imprinting in the prostate will be associated with specific changes in the *IGF2/H19* promoter regions.

It is our hypothesis to be tested that an age-dependent loss of IGF2 imprinting, resulting from age-dependent changes in DNA methylation, occurs specifically in the peripheral zone of the prostate and contributes to the increased risk for cancer development. To examine temporally when this loss of *IGF2* imprinting occurs and the mechanisms underlying it we propose 3 Specific Aims: (1) To determine if *IGF2* LOI in the peripheral prostate derives from stromal and/or epithelial cells; (2) To determine whether *IGF2* LOI occurs as an age-dependent process in human prostate tissues that are uninvolved with cancer; and (3) To examine DNA methylation as a mechanism for any observed changes in the imprint status in prostate tissues. We propose to confirm our preliminary observations through a comprehensive analysis of *IGF2* LOI and DNA methylation analysis in aging prostate tissues associated with and without cancer. These studies will utilize a unique tissue bank containing normal prostate tissues of various ages. Several innovative techniques including laser capture microdissection and quantitative allele-specific imprinting assays will also be employed.

BODY

Task 1: To determine whether a LOI in *IGF2* arises from prostate epithelial cells, stromal cells or both in normal human prostate tissues.

1. *Acquisition and histologic analysis of prostate specimens, DNA production (Months 1-3):* We have histologically examined tissues to exclude concomitant cancer, made DNA and screened over 70 samples for the *Apal* polymorphism. Twenty-five have been found to contain the *Apal* polymorphism. DNA has been extracted from these tissues.
2. *Laser capture microdissection of stromal and epithelial cells from normal peripheral zone prostate tissues and RNA isolation (Months 1-12):* We have utilized laser capture to separate

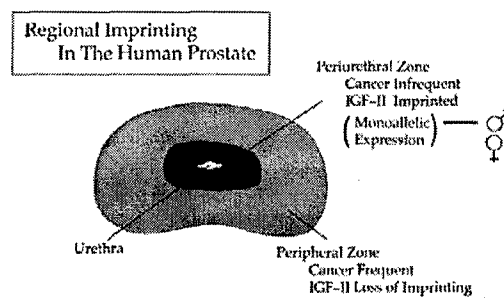


Figure 1: The *IGF2* gene demonstrates monoallelic (paternal only) expression in the periurethral zone where benign prostatic hyperplasia originates. This pattern is found in most adult tissues. In the peripheral zone, where prostate cancer arises, expression is from both inherited alleles(4).

the stroma from the epithelium in 5 tissues from the peripheral prostate of samples from men in their 60's.

3. *Imprinting analyses using RT-PCR/restriction enzyme digestion and development of quantitative allele-specific PCR assay (Months 2-14):*

We have found that in all 3 samples of normal peripheral prostate analyzed to date, both the epithelium and stroma demonstrate biallelic expression (**Figure 2**). This is remarkable since adult tissues maintain a tight monoallelic regulation of this gene. The epithelial-specific change suggests a regional epigenetic cell-specific alteration may predispose these cells to the formation of cancer. This would support our hypothesis. Furthermore, this validates the epithelium as the cell of origin for prostate cancer. The stromal expression may function through an inductive (paracrine) effect on the epithelium. Stromal-specific alterations are important in the



Figure 2. Relaxation of IGF2 imprinting in both the epithelium and stroma from normal peripheral prostate tissues. Microdissected stroma and epithelium was subjected to the Apal imprinting assay and gel analyzed. Biallelic expression is seen in both the epithelium and stroma from all three samples tested.

induction of

epithelial cancers and could possibly represent an important clonal change.

We have also developed a new assay based on SNUPE (single nucleotide primer extension) for the *IGF2* gene that is sensitive and applicable to multiple polymorphisms in the *IGF2* gene. Briefly, RNA was extracted from frozen prostate tissue and used to generate cDNA by using Omniscript reverse transcriptase from Qiagen. The region that covers the SNP site on *IGF2* exon 9 was amplified by PCR. ExoSAP-IT[®] (USB) was used to remove excess dNTPs and primers in the PCR reaction. The FluPE reaction contains 10 nM of FAM labeled primer (5'-CCAATGTTTTTCATGGTCTGAGCC), 0.75 U of HotStarTaq Polymerase (Qiagen), 200 μ M of each specific dNTP/ddNTP, up to 500 ng of ExoSAP treated PCR product and 5 mM of magnesium in a 20 μ l volume. FLuPE is performed at 95°C 15 min, 25 cycles of 95°C 30 sec and 54°C 40 sec. The reaction mix together with GeneScan[™]-120 LIZ[®] size standard (ABI) was loaded on ABI PRISM[®] 3100 Genetic Analyzer, which is a fluorescence-based DNA analysis system using the technology of capillary electrophoresis. GeneScan fragment analysis software was used to identify and size each peak relative to the internal size standard. Peak area and peak height information reflect relative intensity of each peak, which can be used for quantitation purpose. The combination of the primer and specific dNTP/ddNTP will give either 3bp or 5bp extension for imprinted allele or both if imprinting is no longer maintained. The ratio of the peak area is the indication of the degree of imprinting loss.

Task 2 : To examine the frequency of LOI in *IGF2* in aging human prostate tissues containing no associated prostate cancer.

1. *Acquisition of non-tumor associated peripheral zone prostate tissues from different ages and DNA production (Months 10-12):*

Sample Number Age	Ratio of Allele Expression		
	Adjusted Mean	SE	Precision
Equal Allele Expression	1.00	0.0072	0.91%
HPEC0011 81	0.52	0.0081	1.24%
HPEC0045 78	0.39	0.0052	1.69%
HPEC0056 85	0.17	0.0047	2.15%
HPEC0244 80	0.58	0.0147	3.21%
HPEC0345 57	0.23	0.0024	0.83%
HPEC0351 77	0.17	0.0043	2.03%

Because of RNA degradation, we had to take alternate measures to increase the number of samples. We screened a population of samples for additional polymorphisms in exon 9 of the *IGF2* gene. We have found two additional polymorphisms including a C/T (266, frequency 10%) and C/G (1926, frequency 30%). These sites do not contain cutting sites for restriction enzymes, thus we have had to develop a new assay for assessing imprinting. Using fluorescent single-nucleotide primer extension (FLuPE) we are able to specifically and sensitively detect single nucleotide base changes to detect imprinting changes in tissues (above). This assay is reproducible with a standard error of the mean (SEM) of 1-2% (Table 1). In addition, it is sensitive and able to detect 2-3% changes in allele concentration on mixing experiments (Figure 3).

It has taken 12 months (due to IRB issues and labor constraints at Pittsburgh), but we have obtained 24 samples from the University of Pittsburgh tissue bank from normal peripheral prostate tissue (12 older than 50, 12 younger than 40). This database has been

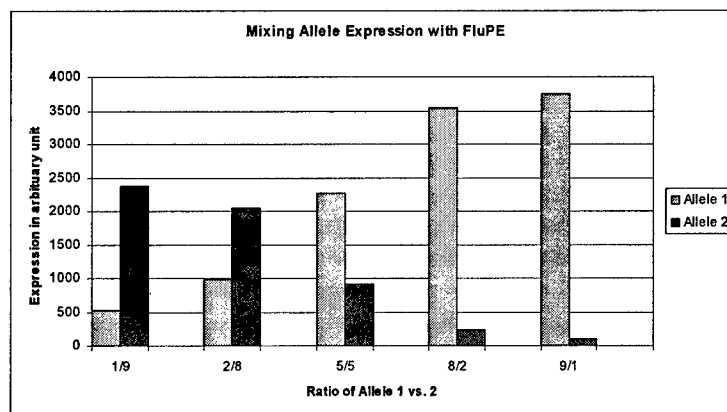


Figure 3. Mixing experiment using different concentrations of the A or G polymorphism with the FLuPE assay. The assay was performed as described above. The assay is quantitative at different allele concentrations. Some bias is seen at a 5/5 concentration for the 'allele 1'.

previously reported on as controls for cancer-specific changes in the peripheral prostate(1).

2. *Microdissection and isolation of RNA (Months 12-24):* We have validated our technique for microdissection and obtaining RNA. DNA contamination remains a problem, however we have experimented with multiple approaches and found that a double DNase digestion technique and purification on a Quiagen™ column negates any detectable DNA that remains. This yields RNA that is free of DNA contamination even at high PCR cycle number.
3. *Imprinting analyses using RT-PCR/restriction enzyme digestion and development of quantitative allele-specific PCR assay and statistical analysis (Months 12-26).* We have completed a final analysis from our tissue bank from men without prostate cancer. This consists of 6 samples, to date, all men over age 50. Degradation of RNA was found to have occurred in other samples from younger men due to a freezer problem. We find that a relaxation of imprinting occurs in all of these samples, and that it ranges from 25 to 90% (Table 1). In older patients, the loss of imprinting was almost complete in some samples. Histopathology did not show any cancer or inflammation differences between the analyzed specimens. Ongoing analyses of samples from Pittsburgh will provide data addressing this aim and a definitive conclusion regarding the loss of imprinting in aging. We expect to have this data completed in the next 2 months.

Task 3: To examine whether methylation alterations underlie differences in *IGF2* imprinting in the human prostate.

1. *Microdissection and isolation of DNA from imprinted and loss of imprinting prostate tissues (Months 24-32). Performed as described above.*
2. *Treatment of DNA with sodium bisulfite, PCR of CpG-enriched regions and sequencing (Months 24-34)*

We have identified areas within the *IGF2/H19* locus that alter their methylation status with human prostate epithelial cell (HPEC) aging in an *in vitro* model. Senescence is an *in vitro* model of aging that recapitulates many of the gene changes seen in *in vivo* aging. Utilizing collagen-coated plates and a low serum media to exclude fibroblasts human prostate epithelial cells are cultured through 15-20 population doublings before growth ceases and a characteristic senescent morphology and senescence-associated β -galactosidase staining develops. In all epithelial cultures, a complete relaxation of the *IGF2* imprint, with increased expression, was reproducibly found with passage to senescence (Figure 4).

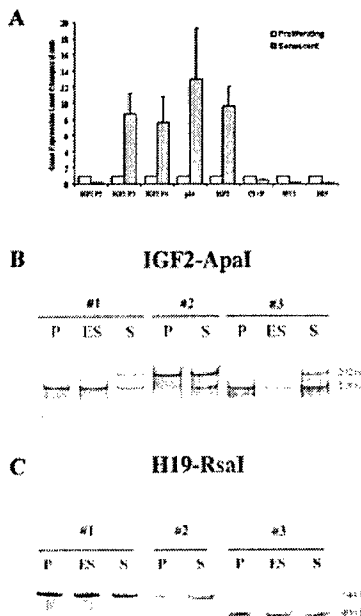


Figure 4. Gene expression and imprinting changes in proliferating and senescent epithelial cultures. *A*, gene expression level changes are shown in senescent cultures. Gene expression levels were compared in proliferating and senescent HPEC and human urothelial cells using QPCR. p16 is a cyclin-dependent kinase inhibitor and senescence marker. *IGF2* P2, P3, and P4 represent multiple promoters for *IGF2* expression. P1 expression was not detected in these cells. *WT-1* negatively regulates *IGF2* expression and is expressed minimally at senescence. *B*, loss of *IGF2* imprinting is shown in epithelial cells with passage to senescence *in vitro*. The determination of allelic imprinting was performed by reverse transcriptase-PCR of an *ApaI* polymorphism on exon 9. Restriction digest of PCR products was then performed. Uncut (292 bp) and restricted (218 bp) bands represent the two alleles. Serially passaged human prostate epithelial or urothelial cells (three individual cultures) were harvested at the passage 1 proliferating (*P*) stage and at the early senescence (*ES*) or terminal senescence (*S*) stage. Early senescent cells are arrested and contain few proliferating cells (<10%); however, they do not demonstrate the complete senescent morphology (8). With passage to senescence, the imprinted allele was re-expressed in all three cultures *in vitro*. #1, #2, and #3 represent multiple independent cultures. *C*, *H19* imprinting is maintained with senescence. cDNAs were amplified and restricted with *RsaI* to identify expression from individual alleles (544 and 406 bp, respectively).

To examine the role of methylation in *IGF2* imprinting regulation and identify candidate regions to test in the human samples, we performed and have reported on several experiments (JBC, 2004). We find that DNA methylation inhibition using 2-deoxy 5'azacytidine leads to an accelerated loss of *IGF2* imprinting in HPEC cells. These changes include both a global

hypomethylation as well as regional hypermethylation of selected CpG islands (Figure 5).

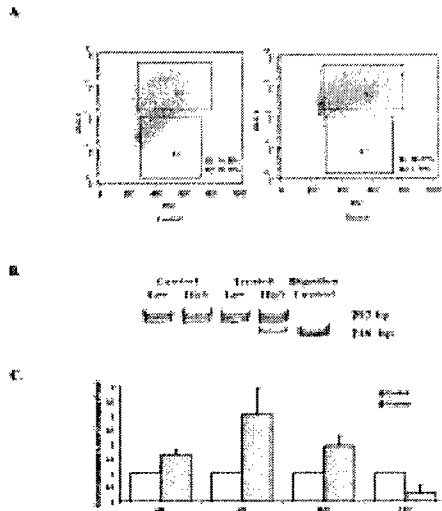


Figure 5. Loss of *IGF2* imprinting develops with premature senescence induced by D5-AzaC exposure. *A*, epithelial cells were stained with CFSE to determine cell divisions followed by treatment with 10 μ M D5-AzaC or control (Me₂SO) for 3 days. Then cultures were replated in growth medium for 5 additional days and sorted based on CFSE fluorescence intensity. Low fluorescent fractions contain cells undergoing multiple cell divisions in contrast to the high fluorescent fraction that contains cells that have undergone few cell replications. *R1* and *R2* were the gates utilized for cell sorting. *B*, biallelic *IGF2* expression develops in the high (*High*) fluorescent fraction (*R1*) treated with D5-AzaC, in contrast to monoallelic expression from all other fractions. The cells in this high fluorescent fraction contain the senescent phenotype. *C*, CTCTF is down-regulated in treated cells. Gene expression changes using QPCR are shown in sorted cell fractions. A high fluorescent cell fraction from treated cells (*R1*) is compared with untreated cells.

We have found a consistent increase in DNA methylation in a differentially methylated region in the human, intergenic between *H19* and *IGF2*, that appears to harbor a methylation imprinting mark for the *IGF2* gene. Deletion or hypermethylation of this CpG island has been demonstrated in mouse models to lead to biallelic *IGF2* expression(2). (Figure 6). An investigation of methylation at this locus is being assessed in the human prostate tissues recently obtained from Pittsburgh

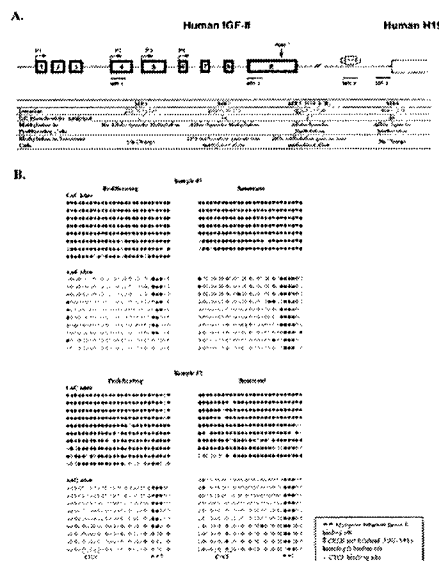


Figure 6. Methylation analysis of *IGF2-H19* in proliferating and senescent cells. *A*, a structure for the human *IGF2* gene is shown. The gene was transcribed and expressed differentially from multiple promoters (P1–P4). The polymorphism ApaI is identified on exon 9. The regions tested that contain CpG islands and putative differentially methylated regions (e.g. distinct methylation patterns on maternal and paternal alleles) are indicated as MR1–4. MR3 contains a CTCF binding site described previously. The detailed methylation analyses of all four regions are listed. *B*, allele-specific DNA methylation analysis demonstrates no alterations at the CTCF binding site within the *H19* imprinting control region (MR3) in senescent cells when compared with proliferating cells; however increased methylation is seen in adjacent regions. The methylation status of individual alleles (*each line*) was obtained by bisulfite sequencing, PCR amplification, and cloning. Each line represents an individual clone. Methylated CpGs are shown as *filled beads* and unmethylated ones as *open beads*. Two separate representative cultures are shown (*Sample #1* and *#2*), and the proliferating and senescent status is indicated. Individual alleles can be differentiated in these cultures because of the presence of a CAC/AAG polymorphism (20). One allele (CAC allele) is fully methylated, and the other (AAG allele) contains partial methylation at CpG sites 23–25 in proliferating cultures that increased consistently to complete methylation with passaging to senescence. Putative CTCF and other binding sites are indicated.

We have discovered that other epigenetic alterations may play an important role in the loss of imprinting in senescence (JBC, 2004). CTCF is an insulator protein that binds in the *IGF2/H19* intergenic region. Binding occurs on the nonexpressed allele and is hypothesized to prevent enhancer binding to the *IGF2* promoter region. We have found that CTCF expression decreases over 10-fold in senescing prostate epithelial cells. Furthermore, knockdown of CTCF

expression results in biallelic expression. This is an exciting, novel *in vivo* mechanism for IGF2 imprinting control. We are currently performing an analysis of CTCF expression utilizing immunohistochemistry in normal and cancerous prostate tissues and will correlate alterations in expression with changes in imprinting. Expression of this gene will be important to assess in aging prostate epithelial cells as well.

3. *Quantitative methylation-sensitive single-primer extension analysis of specific sites (Months 24-36)*

Given our data suggesting a more important role for CTCF binding in regulating imprinting in human prostate epithelial cells we have pursued this aspect of regulation more aggressively. We will plan to test the methylation status of the CTCF binding site and its 5' region (based on our senescence studies) in our Pittsburgh samples once the imprinting status is evaluated.

KEY RESEARCH ACCOMPLISHMENTS

- We have demonstrated that both the epithelium and the stroma express the *IGF2* gene from both alleles.
- Development of a sensitive and reproducible assay based on single-nucleotide primer extension but utilizing fluorophores and gel-based sequencing (FLuPE).
- Peripheral prostate tissues without cancer from individuals over age 50 demonstrate a significant amount of variation in loss of IGF2 imprinting. Analysis of younger tissues is ongoing.
- A loss of *IGF-2* imprinting occurs in human prostate epithelial cells undergoing senescence, an *in vitro* model of aging. *H19* imprinting is maintained consistent with the postulated enhancer competition model based on the binding of CTCF to the *IGF2-H19* intergenic region.
- Inhibition of DNA methylation results in an acceleration of loss of *IGF2* imprinting in human epithelial cells.
- Analysis of several CpG islands in aging epithelial cells *in vitro* have identified specific loci that alter methylation status with increased population doublings in the IGF2 gene.
- Analysis of the CTCF binding site in the *H19/IGF2* intergenic region demonstrates a 2-fold loss of CTCF binding at senescence. This is mediated by a multifold loss of CTCF expression by qPCR and western analysis. Downregulation of CTCF results in an increase in *IGF2* expression, and reexpression of the silenced allele. Thus, CTCF expression plays a causal role in human cells in modulating the imprinting of *IGF2*.

REPORTABLE OUTCOMES

Abstracts

1. Fu VX, Schwarze SR, Jarrard DF. Loss of IGF-II Imprinting Occurs During Senescence in Human Prostate Epithelial Cells. *J Urol* 167(4): 135, 2002
2. Schwarze SR, DePrimo SE, Grabert LM, Fu V, Brooks JD, and Jarrard DF. Novel Pathways Associated with Bypassing Cellular Senescence in Human Prostate Epithelial Cells. *J Urol* 167(4): 139, 2002
3. October 17-20, 2001, "Loss Of IGF-II Imprinting Occurs During Senescence In Human Prostate Epithelial Cells". Poster at "Epigenetics and cancer" AACR meeting. Palm Springs, CA.
4. April 11, 2002, "Loss of IGF-II Imprinting Characterizes Epithelial Cell Senescence", American Federation of Aging Research Annual Meeting, New York, NY (poster).

5. May 5, 2003, "Aging and Cancer: Are age-related alterations in genomic imprinting susceptibility factors for cancer?" Pennington Scientific Symposium on Mechanisms and Retardation of Aging, Baton Rouge, LA.

Manuscripts:

- Fu VX, Schwarze SR, Grabert L, LeBlanc S, Svaren J, and Jarrard DF. A Loss of *IGF2* Imprinting is Modulated by CTCF Downregulation at Senescence in Human Epithelial Cells. *Journal of Biological Chemistry*, 279 (50):52218-52226, 2004.
- Atwood CS, Barzilai N, Brown-Borg HM, Jarrard DF, Fu VX, Heilbronn LK, Ingram DK, Ravussin E, Schwartz RS, and Weindruch R. Pennington Scientific Symposium on Mechanisms and Retardation of Aging, *Exp Gerontology* 38(10)1217-1226, 2003.

Training:

- Postdoctoral Student: Dr. Steven Schwarz, PhD, March 2001-present. Currently interviewing for prostate cancer research positions as an Assistant Professor.

Funding:

- An RO1 (CA97131) was successfully funded based in part on data obtained from this grant. This study will look at age-related changes in imprinting in the mouse prostate and cancer susceptibility.
- An O'Brien Center for Urology program project grant entitled "Modulation of Genomic Imprinting on Oxidative Stress" has been funded based on preliminary data generated, in part, from this grant. It seeks to examine whether oxidative stress can modulate the imprinting of *IGF2* has been funded through the NIH.
- NIH/NCI 1 P20 CA103697-01 (PI: R. Weindruch) Integrating Aging and Cancer Research in NCI Designated Cancer Centers The purpose of this planning grant is to establish the "Aging and Cancer Program" within the UW-Madison Comprehensive Cancer Center (UWCCC). Our group of cancer biologists, gerontologists, oncologists, geriatricians and population scientists will address five thematic areas: Patterns of Care, Effects of Comorbidity, Psychosocial Issues, Palliative Care and Biology of Aging. Dr. Jarrard is a co-investigator on this grant in biology of aging.

CONCLUSIONS

IGF2 loss of imprinting is found in aging, senescent human prostate epithelial cells consistent with our hypothesis that it is modulated with aging. Both stroma and epithelia from peripheral prostate tissue in older men express biallelic *IGF2*. This is in contrast to strict imprinting in other adult tissues. These data suggest that this prostate-specific epigenetic alteration may be important in the remarkable frequency with which the prostate develops cancer. DNA methylation plays a role in *IGF2* imprinting control, however our data indicates CTCF expression may play a more important role in human prostate epithelial cells. Finally, additional tissue analysis to build statistical significance with the aging studies is ongoing and final conclusions will be documented in the next 6 months. Future studies will address CTCF and other mechanisms for modulating *IGF2* imprinting. In addition, we will plan to analyze other imprinted genes for alterations in peripheral prostate tissues that will support the hypothesis that global changes in epigenetic phenomenon contribute to prostate cancer development.

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A Loss of Insulin-like Growth Factor-2 Imprinting Is Modulated by CCCTC-binding Factor Down-regulation at Senescence in Human Epithelial Cells*

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The imprinted insulin-like growth factor-2 (*IGF2*) gene is an auto/paracrine growth factor expressed only from the paternal allele in adult tissues. In tissues susceptible to aging-related cancers, including the prostate, a relaxation of *IGF2* imprinting is found, suggesting a permissive role for epigenetic alterations in cancer development. To determine whether *IGF2* imprinting is altered in cellular aging and senescence, human prostate epithelial and urothelial cells were passaged serially in culture to senescence. Allelic analyses using an *IGF2* polymorphism demonstrated a complete conversion of the *IGF2* imprint status from monoallelic to biallelic, in which the development of senescence was associated with a 10-fold increase in *IGF2* expression. As a mechanism, a 2-fold decrease in the binding of the enhancer-blocking element CCCTC-binding factor (CTCF) within the intergenic *IGF2-H19* region was found to underlie this switch to biallelic *IGF2* expression in senescent cells. This decrease in CTCF binding was associated with reduced CTCF expression in senescent cells. No *de novo* increases in methylation at the *IGF2* CTCF binding site were seen. The forced down-regulation of CTCF expression using small interfering RNA in imprinted prostate cell lines resulted in an increase in *IGF2* expression and a relaxation of imprinting. Our data suggest a novel mechanism for *IGF2* imprinting regulation, that is, the reduction of CTCF expression in the control of *IGF2* imprinting. We also demonstrate that altered imprinting patterns contribute to changes in gene expression in aging cells.

Genomic imprinting is an epigenetic modification that results in the silencing of a specific allele, depending on its parental origin. Genomic imprinting plays a critical role in modulating gene expression during embryogenesis and normal

development (1). The insulin-like growth factor-2 gene (*IGF2*), an auto-paracrine growth factor located at the 11p15 chromosomal locus, is imprinted and exhibits monoallelic expression from the paternal allele in most adult tissues. However, a relaxation of the *IGF2* imprint has been found in aging-associated human cancers, including cancer of the colon and prostate (2, 3). Notably, re-expression of the silenced *IGF2* allele was also found in adjacent histologically normal tissues in these studies, suggesting that an alteration in imprinting had occurred prior to the development of these cancers. The factors that underlie this altered imprinting *in vivo* are unknown. Given the age dependence of these types of cancers, progressive cellular replication and aging may play roles in modulating genomic imprinting.

The process that limits the proliferative potential of normal human cells is termed senescence. Senescent cells demonstrate a number of distinct characteristics, including an enlarged, flattened cytoplasm and nucleus, terminal growth arrest, and specific gene expression changes (4). Senescence can be induced by programmed or epigenetic changes resulting from repeated cell divisions and from cellular insults, including oxidative stress and DNA damage. Senescence may not be limited to an *in vitro* phenomenon because cells expressing senescence-associated β -galactosidase activity, a marker for senescent cells, accumulate with aging in human skin (5) and in a subset of prostate epithelial cells from men with benign prostatic hyperplasia (6). The examination of changes in global gene expression in senescent human cells in culture reveals additional specific genes that are altered in aging tissues, including *PAI-1*, *t-PA*, cathepsin B, activin A, tissue transglutaminase, several helicases, and members of the *IGF1* axis (4, 7, 8). Several mechanisms associated with aging *in vivo* can be found as cells undergo senescence *in vitro*, including telomere shortening (9) and changes in DNA methylation, a postreplicative addition of methyl groups within CpG dinucleotides (10). Thus, selected aspects of *in vitro* senescence are applicable to aging cells *in vivo*.

IGF2 is located within a cluster of imprinted genes on chromosome 7 in the mouse and on 11p15 in the human. The regulation of *IGF2* and its closely linked and reciprocally imprinted 3' neighbor, *H19*, has been studied intensely (11) both because of its role in human disease and as a model for understanding imprinting control mechanisms. During development,

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¹ The abbreviations used are: IGF, insulin-like growth factor; ICR, imprinting control region; CTCF, CCCTC-binding factor; HPEC, human prostate epithelial cell; QPCR, quantitative reverse transcriptase-PCR; MR, methylated region; CFSE, carboxyfluorescein diacetate succinimidyl ester; siRNA, small interfering RNA; GFP, green fluorescent protein; WT, Wilms' tumor; LOI, loss of imprinting; P, promoter; D5-AzaC, 2'-deoxy-5-azacytidine.

IGF2 and *H19* are expressed in a coordinate fashion that suggests, in combination with their close linkage and reciprocal imprinting, common transcriptional elements. One model (the insulator model) that has been developed *in vitro* (12, 13) and in mouse models (14, 15) has focused on the differential methylation of an imprinting control region (ICR) located between *IGF2* and *H19*. ICRs provide gametic marks to establish the parent-of-origin-dependent expression domains and are acquired typically in the parental germ line and persist into adulthood (16). When the *H19* ICR is methylated on the paternal allele, *IGF2* is expressed. However, expression from the maternal allele is blocked when this ICR is unmethylated. The boundaries of the mouse *H19* ICR are not precise, yet deletion (or hypermethylation) of sequences between -3.8 and 2.0 kb on the maternal allele results in the biallelic expression of the linked and reciprocally imprinted *IGF2* gene (17, 18). However, biallelic *H19* ICR methylation does not disrupt imprinting in some cases of human Wilms' tumor (19), suggesting that other mechanisms may play a role in human tissues.

Recently, it has been found (12, 20) that the repression of the maternal allele involves binding of a zinc finger CCCTC-binding factor, known as CTCF, which binds only unmethylated DNA in this ICR. This binding blocks the access of downstream enhancer proteins to the *IGF2* promoter region, which transcribes from multiple differentially expressed promoters P1-P4 (21). Conversely, the hypermethylated paternal allele does not bind CTCF, and *IGF2* is expressed from its promoters. There are clear structural differences when human and mouse sequences are compared. The mouse contains four CTCF binding sites in the *H19* ICR in contrast to seven in the human; however, only the sixth CTCF site demonstrates differential methylation (13, 22). In addition, the human *H19* ICR is not able to function when introduced as a transgene in the mouse (23). This suggests that differences in the regulation of *IGF2* imprinting may exist between species. It is also not clear what the sequential relationship between CTCF binding and methylation is in the human. In mouse embryos, mutation of the ICR leads to decreased CTCF binding and *de novo* methylation, suggesting a role for CTCF and its protein complex in maintaining a methylation-free domain (15).

Using a human model of cellular aging in which prostate epithelial and urothelial cultures were passaged sequentially, we demonstrate that a complete loss of *IGF2* imprinting develops with the onset of senescence. Senescence is associated with a decrease in CTCF binding to the *H19* ICR, an event that results in increased maternal allelic *IGF2* expression. The loss of CTCF binding is not associated with changes in methylation at the CTCF target site but is mediated by a decrease in CTCF protein expression. The present study details a novel model for examining imprinting mechanisms in pure populations of human cells during a physiologic, programmed process. The down-regulation of CTCF is a novel mechanism for imprinting regulation that may help to explain the lack of consistent correlation between methylation of the *H19* ICR and imprinting found in some human tumor tissues (20, 24). In addition, these data provide evidence that a relaxation in imprinting has a permissive effect on gene expression during cellular aging. Given that long term chronic exposure to *IGF2* appears to be important in the generation of tumors (25), the loss of imprinting and increased *IGF2* expression may be important for the development of aging-related cancers, especially in the prostate.

MATERIALS AND METHODS

Tissue Culture—Human prostate epithelial culture (HPEC) or human urothelial cells were established on collagen-coated dishes in Ham's F-12 supplemented medium containing 1% fetal bovine serum (26, 27). HPEC samples were obtained from human cystoprostatectomy specimens (ages 45–60) that did not contain prostate cancer. Urothelial

cells were generated from human ureteral specimens. Cells were trypsinized and passaged at 1:2 or 1:3 dilutions when confluent and underwent typically 10–15 population doublings before becoming senescent. DNA and RNA were isolated from each passage. PC3 and PPC-1 prostate cancer cell lines were obtained from the ATCC and maintained in supplemented Dulbecco's modified Eagle's medium.

Quantitative Reverse Transcriptase PCR—To compare gene expression levels between proliferating and senescent cells, quantitative PCR was performed using an iCycler (Bio-Rad) and SYBR Green PCR master mix (Applied Biosystems). 18 S RNA expression was used as an internal control for normalizing samples. Primers were designed for *p16*, *IGF2*, and *WT-1* using Primer Express (PerkinElmer Life Sciences).² The *IGF2* gene was spliced and transcribed by four promoters (P1, -2, -3, and -4) (28), and primers were designed to detect the expression from each specific promoter.

Imprinting Assays—To minimize DNA contamination, RQ1 DNase (Promega) treatment of total RNA and intron-crossing primers were utilized. cDNA was synthesized using murine leukemia virus transcriptase (Applied Biosystems) with random hexamers. Two pairs of primers were used to amplify an RNA-specific fragment containing the *IGF2* ApaI polymorphism on exon 9 (29). P1 (5'-GACACCTCCAGTTCGTCTGT-3') and P2 (5'-CGGGGATGCATAAAGTATGAG-3') cross introns between exons 7, 8, and 9. The RNA-specific (1.3 kb) product was separated from the DNA-specific (3.3 kb) fragment using a 1% agarose gel. The second pair of primers (P3 and P4) was used to perform nested PCR (35 cycles), and 292-bp fragments were generated for restriction enzyme digestion with ApaI (New England Biolabs). P3 and P4 primer sequences were 5'-CTTGACTTTGAGTCAAATTGG-3' and 5'-GGTCGTGCCAATTACATTTCA-3', respectively. Cell line DNA with both alleles sensitive to enzyme digestion was utilized as a control for complete restriction digestion. Mixing controls using upper and lower alleles confirmed this approach to be quantitative.

For *H19* amplification, cDNA was generated from informative samples, and primers (forward 5'-TGCACTACCTGACTCAGGAATC-3' and reverse 5'-GTGATGTCGGTCGGAGCTTC-3') were used to amplify across the *H19* RsaI polymorphism (30). The intact product length was 544 bp, and the digested fragments were 406 and 138 bp if the polymorphism was present.

Analysis of CTCF Binding and Expression—Chromatin immunoprecipitation was performed as described previously (31), with minor modifications. Cross-linking was carried out by incubating cells (1×10^7) with a final concentration of 0.4% formaldehyde for 10 min at room temperature. Cells were collected by centrifugation at 1200 rpm for 6 min, and nuclei were isolated. Chromatin was then precleared by incubation with 50 μ l of preimmune serum for 1 h followed by overnight incubation with 100 μ l of Immunopure protein A-agarose (Pierce). Samples were incubated with 30 μ l of anti-CTCF antibody (Upstate Biotechnology). Immune complexes were collected by incubation with 30 μ l of protein A-agarose for 2 h at 4 °C. Agarose beads were washed, and immune complexes were eluted twice. Cross-links were reversed, and DNA was purified by extraction with phenol/chloroform followed by precipitation with ethanol. PCR was performed using real time quantitative PCR (QPCR) (Prism 7000 sequence detection system, ABI). The product was measured by SYBR green fluorescence in 25- μ l reactions, and the amount of the product was determined relative to a standard curve generated from a titration of input chromatin. Primers for the *IGF2-H19* intergenic region were designed (5' and 3'): GAGGCTTCTCCTTCGGTCTCA and GCCACTTCCGATTCACAA. Western blot analysis was performed as described previously (32) using a polyclonal antibody for CTCF (Upstate Biotechnology, Lake Placid, NY) or anti- β -actin (Sigma).

Methylation Analyses—We examined four CpG islands in *IGF2*, of which three have been found to be methylated differentially in mice and/or human tissues (33–37). The CpG islands that were tested were located on *IGF2* (GenBank™ accession no. AF125183) at exon 4 at 21170–21525 (MR1), exon 9 at 29080–29375 (MR2), between *IGF2* and *H19*, 2 kb upstream of the *H19* start site (*H19* ICR/MR3), and at -906 to -275 in the *H19* promoter (MR4). Genomic DNA isolated from both proliferating and senescent HPEC/human urothelial cell cultures were treated with sodium bisulfite (CpGenome DNA modification kit, InterGen) to convert unmethylated cytosines to uridines while retaining methylated cytosines as unchanged nucleotides. The regions were amplified by primers MR1-F (5'-ACCCACTACAACCTCCCAAC), MR1-R (5'-TATTAGGAGTTTAGGTAG), MR2-F (5'-TTGGGTGGGTAGAGTATTAGG), MR2-R (5'-CTCAAATCACTAATCAATCAC), MR3-F (5'-

² Primers designed for *p16*, *IGF2*, and *WT-1* are available on request.

GTAGGGTTTGGTAGGTATAGAGT), and MR3-R (5'-CACTAAAAA-AACAATTATCAATTC), which are specific for the converted DNA. The PCR products were then cloned into a pCR2.1-TOPO vector (Invitrogen). For each cloning, 10–20 positive colonies were selected randomly, amplified, and analyzed on a capillary-based fluorescent sequencer (Applied Biosystems) at the University of Wisconsin Biotechnology Center DNA Sequence Laboratory.

2'-Deoxy-5-azacytidine Treatment of HPEC Cultures—Passage 1 60% confluent HPEC cultures were stained with 5 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes) at 37 °C for 15 min in Hanks' balanced salt solution. The plates were washed three times with F-12+ medium. The demethylating agent D5-AzaC (final concentration 10 μ M) (Sigma) was prepared from a stock solution (in Me₂SO) and was added to the HPEC culture, which was incubated for 3 days. Control cultures were treated with a Me₂SO vehicle. Cultures were then placed back into the Ham's F-12+ medium for 5 days before propidium iodide staining and fluorescence-activated cell sorter analysis. Examination and sorting of CFSE-loaded cells were performed with a Vantage SE fluorescence-activated cell sorter (BD Biosciences). The CFSE was excited with an argon laser (Coherent, Santa Clara, CA) tuned to 488 nm, and the emission of the fluorochrome was collected through a 530/30 band pass filter. Data acquisition analysis and sorting were performed using DiVa electronics and accompanying software (BD Biosciences). Propidium iodide staining was utilized to exclude dead cells. The high and low fluorescent fractions were defined based on CFSE staining. The same gate was applied to both treated and control cells. These sorted populations were collected, and imprinting analysis and quantitative-PCR were performed on RNA extracted from each fraction. DNA additionally was generated, and methylation analyses were performed as detailed above. The experiment was performed on three separate cultures with similar results.

siRNA Transfection—HPECs, PPC-1, and PC3 cell lines were seeded to 50% confluence on 6-well plates 24 h prior to transfection. We used LipofectAMINE 2000 (Invitrogen) for HPECs and TransIT-TKO (Mirus Corp., Madison, WI) for cancer cell line transfection reagents. 50–100 pmol of CTCF SMARTpool (Dharmacon, Inc.) siRNAs were combined in a medium with the transfection reagents following the manufacturer's protocol. The mixture was then added by drops to the cells in complete Dulbecco's modified Eagle's 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.

CTCF Lentivirus Infection—The open reading frame of human CTCF was cloned into the FUGW vector, which places the inserted DNA under the control of the ubiquitin promoter. A ubiquitin promoter was used in this experiment to express levels/cell of CTCF or green fluorescent protein (GFP) that were consistently lower than levels expressed by other more promiscuous promoters (e.g. cytomegalovirus). As a negative control and to determine infection efficiency, a similar vector expressing GFP was generated. Infectious virus was produced by transiently transfecting lentivirus vector and packaging vectors into the 293T cell line, as described elsewhere (38). Early senescent HPECs had 0.1 ml of supernatant/well (6-well plate) in the presence of 10 μ g/ml polybrene for 6 h. Cells were harvested for RNA and protein 48 h postinfection. Test infections using lentivirus-GFP showed an infection efficiency of 30–40% for senescent epithelial cell lines. Experimental results were reproduced in two independent cultures.

RESULTS

Senescence and IGF2 Expression in Human Epithelial and Urothelial Cell Cultures—HPECs were cultured on collagen-coated plates and a low serum medium to exclude fibroblasts (26). Growth of the epithelial cells is brisk through 10–15 population doublings, typically 4–6 passages, at which point the subconfluent cells undergo growth arrest and adopt a senescent phenotype (32). The epithelial senescent phenotype is characterized by morphological changes consisting of an enlarged, flattened cytoplasm and nucleus and by positive senescence-associated β -galactosidase staining. Increased expression of p16 RNA (13-fold) and protein, a cyclin-dependent kinase inhibitor, confirm the senescent phenotype (26). Roughly 70% of the cells develop this phenotype when harvested at terminal senescence. Human urothelial cells demonstrate similar growth patterns.

We had noted previously (8) on cDNA array an increase in

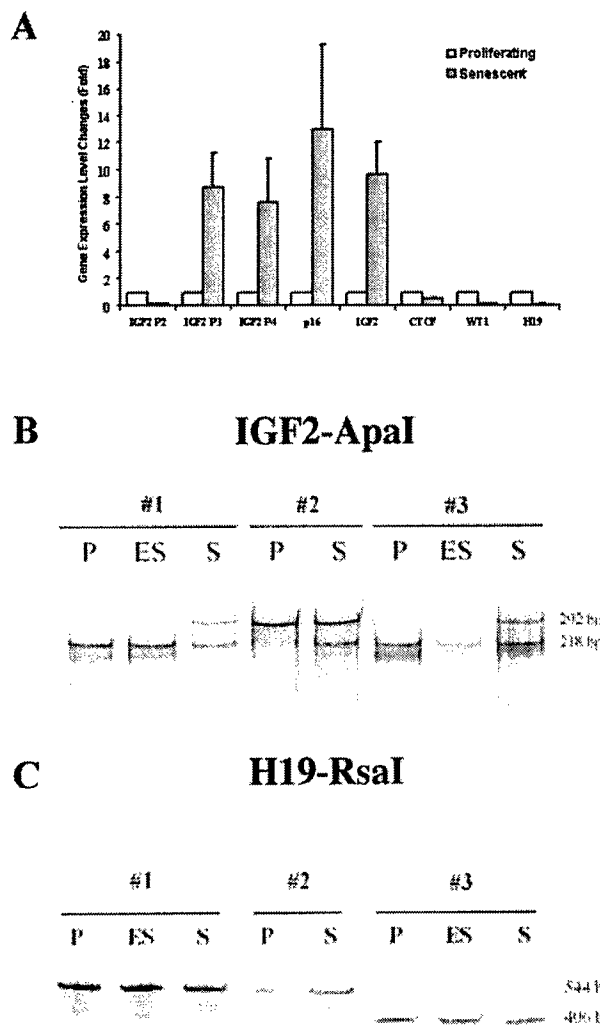
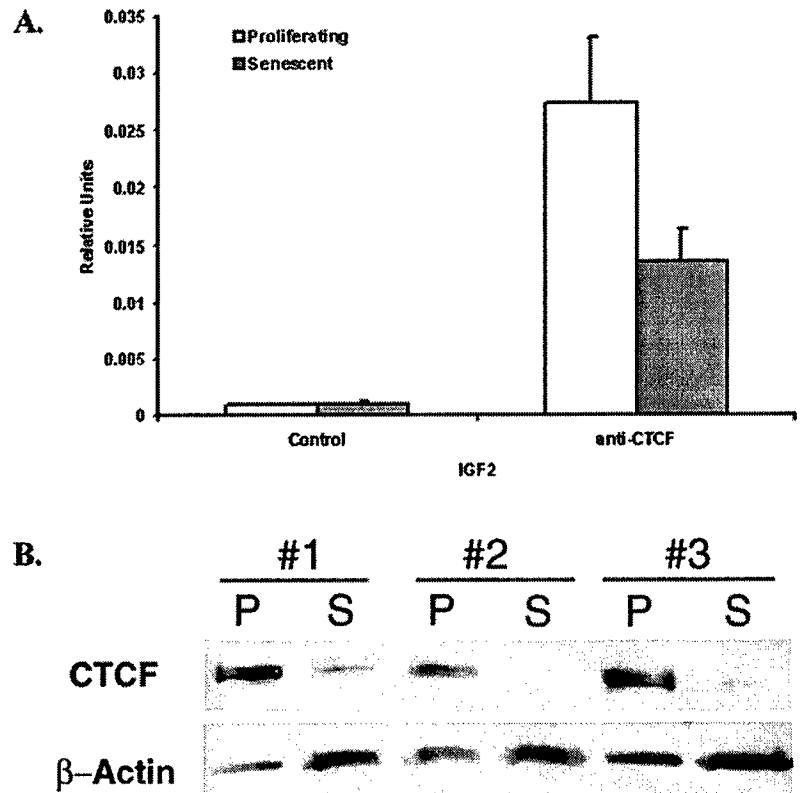


FIG. 1. Gene expression and imprinting changes in proliferating and senescent epithelial cultures. A, gene expression level changes are shown in senescent cultures. Gene expression levels were compared in proliferating and senescent HPEC and human urothelial cells using QPCR. p16 is a cyclin-dependent kinase inhibitor and senescence marker. IGF2 P2, P3, and P4 represent multiple promoters for IGF2 expression. P1 expression was not detected in these cells. WT-1 negatively regulates IGF2 expression and is expressed minimally at senescence. B, loss of IGF2 imprinting is shown in epithelial cells with passage to senescence *in vitro*. The determination of allelic imprinting was performed by reverse transcriptase-PCR of an ApaI polymorphism on exon 9. Restriction digest of PCR products was then performed. Uncut (292 bp) and restricted (218 bp) bands represent the two alleles. Serially passaged human prostate epithelial or urothelial cells (three individual cultures) were harvested at the passage 1 proliferating (P) stage and at the early senescence (ES) or terminal senescence (S) stage. Early senescent cells are arrested and contain few proliferating cells (<10%); however, they do not demonstrate the complete senescent morphology (8). With passage to senescence, the imprinted allele was re-expressed in all three cultures *in vitro*. #1, #2, and #3 represent multiple independent cultures. C, H19 imprinting is maintained with senescence. cDNAs were amplified and restricted with RsaI to identify expression from individual alleles (544 and 406 bp, respectively).

the expression of IGF2 as human prostate epithelial cells were passaged from proliferation to senescence. To confirm these findings, we harvested RNA from multiple sequential passages of human prostate epithelial or urothelial cells and performed QPCR using IGF2-specific primers (Fig. 1A). An average increase of 10-fold (± 3.8 -fold) in IGF2 was seen with the devel-

FIG. 2. Analysis of CTCF binding and expression in IGF2-H19 ICR. A, CTCF binding in the IGF2-H19 intergenic region (MR3) decreases with the passage of epithelial cells to senescence. Cross-linked chromatin was isolated from proliferating and senescent human prostate epithelial cells. The relative levels of the H19 ICR region found in the immunoprecipitate were determined by QPCR using primers flanking the CTCF binding site. Values shown are the mean $\pm$ S.D. for each PCR product, normalized to the level of the inputs. In control samples (*left*), immunoprecipitation was performed in the absence of anti-CTCF antibody. B, Western blot of CTCF expression is shown in proliferating and senescent cells. A multifold decrease in CTCF expression is seen in senescent cells (S) when compared with proliferating cultures (P). #1, #2, and #3 represent multiple independent cultures. β -Actin was applied as a loading control.



opment of senescence. *IGF2* expression is driven by multiple promoters P1–P4, and as cells were passaged to senescence, the relative expression from the *IGF2* P3 and P4 promoters increased (9- and 8-fold, respectively). The P2 promoter demonstrated decreased expression (7-fold) but contributed only relatively small amounts (15%) to the overall *IGF2* expression in senescent cells. P1 was not expressed. The P3 and P4 promoters contain several Wilms' tumor gene 1 (*WT-1*) binding sites that negatively regulate *IGF2* expression (39, 40). Consistent with a putative role for *WT-1*, we found an 8-fold down-regulation of *WT-1* RNA at senescence. Thus, the overall *IGF2* expression increased at senescence, and this was mediated by an increase in expression from the P3 and P4 promoters.

Alterations in IGF2 Imprinting with Senescence—The *IGF2* gene is imprinted, demonstrating expression solely from the paternal allele in most adult tissues (1). RNA obtained from three separate epithelial cultures was subjected to an imprinting analysis based on an *ApaI* polymorphism found in exon 9 of the *IGF2* gene (29). In prostate epithelial cultures, we found minimal expression (<5%) of the maternal *IGF2* allele in proliferating cultures (Fig. 1B, bands labeled P). As epithelial cells were passaged, a relaxation of *IGF2* imprinting occurred. Partial re-expression of the silenced allele was evident in early senescence (Fig. 1, label ES), a period at which cell growth is minimal, and the senescent morphology is found less frequently (32). A complete loss of imprinting developed subsequently in terminally arrested, fully senescent cultures.

Data obtained from human tumors and during experimental manipulation of the mouse genome indicate that the regulation of *IGF2* and its adjacent 3' imprinted gene, *H19*, are linked (41, 42). In three epithelial cultures that were informative for *H19* (one was also informative for *IGF2* imprinting), we detected no change in the monoallelic status of *H19* with the passage to senescence (Fig. 1C). *H19* expression was noted to decrease 12-fold in these cultures, as assessed by QPCR. Thus, *IGF2* and *H19* expression levels demonstrate an inverse relationship with the development of senescence in human cells, consistent

with a common regulatory mechanism proposed in mouse models (41).

CTCF Binding Decreases at Senescence—The loss of binding of the insulator protein CTCF in the intergenic *IGF2-H19* region has been demonstrated to be important in regulating biallelic *IGF2* expression in mice (12, 43). We assessed the binding of CTCF in the *H19* ICR region using chromatin immunoprecipitation in populations of senescent and proliferating cells. The relative levels of CTCF binding at this site were 2-fold lower in senescent cultures compared with proliferating cultures (Fig. 2A). Overall, CTCF expression levels were analyzed in proliferating and senescent cells using QPCR, which demonstrated a 2-fold (± 0.57 -fold) decrease in expression. Western analysis confirmed a multifold loss of CTCF protein expression in senescent cells (Fig. 2B). Therefore, diminished expression of CTCF and decreased binding to the ICR region were observed in senescent cultures demonstrating biallelic *IGF2* expression.

Methylation Analysis of the H19 ICR and Other Regions in Proliferating and Senescent Human Cells—A detailed methylation analysis was performed on multiple CpG islands in the *IGF2-H19* region as cells were passaged to senescence (Fig. 3A). The *H19* ICR (Fig. 3, A MR3) harbors an imprinting mark (13, 22) and binds CTCF, and the loss of differential methylation correlates with LOI in Wilms' tumors (19) and colon cancers (20). DNA was harvested from proliferating and senescent cells and then treated with sodium bisulfite in a reaction that converts unmethylated cytosines to uridine (then thymidine), but methylated cytosines remain unaltered. After PCR, individual alleles were cloned and sequenced. In Fig. 3B, one allele, as expected, was methylated completely at more than 25 CpG sites. As three independent epithelial and urothelial cell cultures were passaged to senescence, no methylation changes were noted across the CTCF binding site in the unmethylated allele. However, an increase in methylation (gain of $28 \pm 7\%$ in unmethylated alleles) was seen in several contiguous CpG sites ~200 bp downstream from CTCF site 6, which contained two

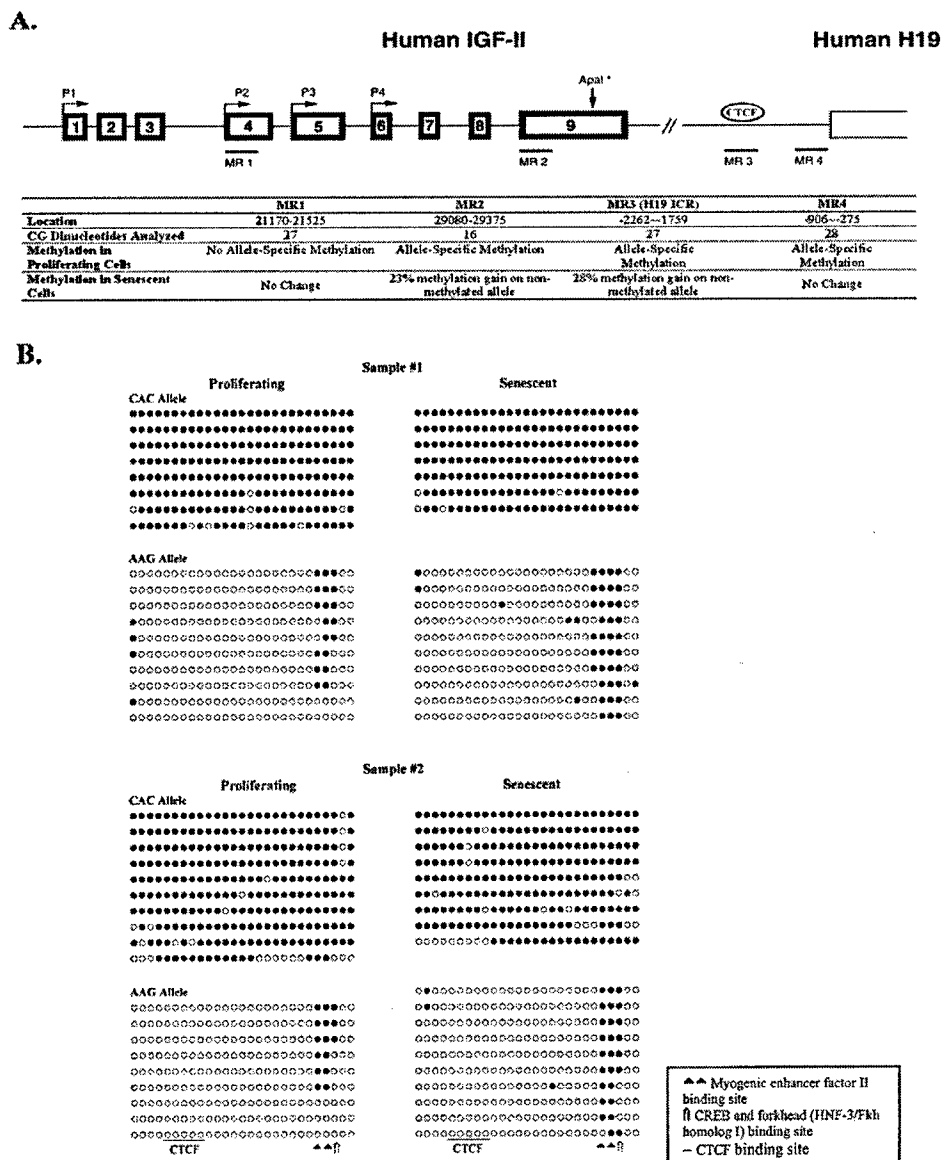


FIG. 3. Methylation analysis of IGF2-H19 in proliferating and senescent cells. *A*, a structure for the human IGF2 gene is shown. The gene was transcribed and expressed differentially from multiple promoters (P1–P4). The polymorphism Apal is identified on exon 9. The regions tested that contain CpG islands and putative differentially methylated regions (e.g. distinct methylation patterns on maternal and paternal alleles) are indicated as MR1–4. MR3 contains a CTCF binding site described previously. The detailed methylation analyses of all four regions are listed. *B*, allele-specific DNA methylation analysis demonstrates no alterations at the CTCF binding site within the H19 imprinting control region (MR3) in senescent cells when compared with proliferating cells; however increased methylation is seen in adjacent regions. The methylation status of individual alleles (each line) was obtained by bisulfite sequencing, PCR amplification, and cloning. Each line represents an individual clone. Methylated CpGs are shown as filled beads and unmethylated ones as open beads. Two separate representative cultures are shown (Sample #1 and #2), and the proliferating and senescent status is indicated. Individual alleles can be differentiated in these cultures because of the presence of a CAC/AAG polymorphism (20). One allele (CAC allele) is fully methylated, and the other (AAG allele) contains partial methylation at CpG sites 23–25 in proliferating cultures that increased consistently to complete methylation with passaging to senescence. Putative CTCF and other binding sites are indicated.

cAMP-response element-binding protein binding sites and a forkhead (hepatocyte nuclear factor-3/forkhead homolog 1) site. These sites contained partial methylation in proliferating cells. Complete methylation at these sites was also found in epithelial cultures enriched for senescent cells, as seen by sorting based on increased forward scatter (data not shown). Thus, increased methylation did not occur within the CTCF binding site or widely across the H19 ICR on the unmethylated allele; however, a focal increase was seen in a downstream region.

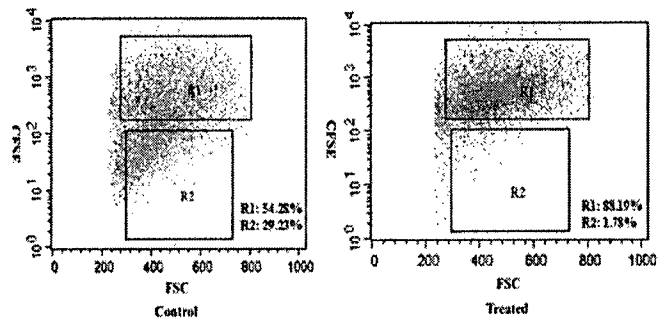
We also analyzed several other CpG islands that have demonstrated (34–37) allele-specific methylation patterns in the mouse and are putative ICRs (Fig. 3A). At MR2, located within IGF2 exon 9, we found that 50% of the alleles contained fewer

methylated sites (<30% of total) than the remaining alleles (>70%), indicating that allele-specific methylation exists in this region (data not shown). Analysis of the methylation status of individual alleles demonstrated a reproducible $23 \pm 5\%$ increase in methylation at senescence, occurring primarily across an HpaII site on the less methylated allele. Analysis of methylation demonstrated no significant changes in either MR4, a region containing allele-specific methylation patterns, or MR1, a region showing no allele-specific methylation.

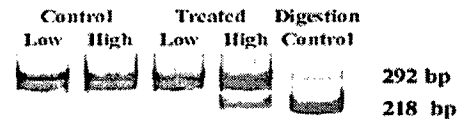
Treatment of Epithelial Cells with Methylation Inhibitors Results in Senescence and Biallelic IGF2 Expression.—Hypomethylation has been documented in aging cells *in vitro* and *in vivo* (44, 45). To investigate the role of accelerated methyl-

A.

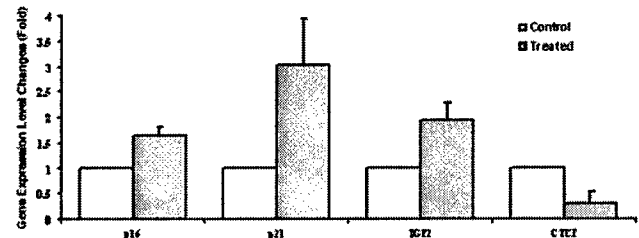
FIG. 4. Loss of IGF2 imprinting develops with premature senescence induced by D5-AzaC exposure. A, epithelial cells were stained with CFSE to determine cell divisions followed by treatment with 10 μ M D5-AzaC or control (Me₂SO) for 3 days. Then cultures were reapplied in growth medium for 5 additional days and sorted based on CFSE fluorescence intensity. Low fluorescent fractions contain cells undergoing multiple cell divisions in contrast to the high fluorescent fraction that contains cells that have undergone few cell replications. R1 and R2 were the gates utilized for cell sorting. B, biallelic IGF2 expression develops in the high (High) fluorescent fraction (R1) treated with D5-AzaC, in contrast to monoallelic expression from all other fractions. The cells in this high fluorescent fraction contain the senescent phenotype. C, CTCF is down-regulated in treated cells. Gene expression changes using QPCR are shown in sorted cell fractions. A high fluorescent cell fraction from treated cells (R1) is compared with untreated cells.



B.



C.



ation loss, we exposed epithelial cultures to D5-AzaC, a compound that binds and inhibits multiple DNA methyltransferases. Informative epithelial cultures were stained initially with CFSE (46), an inert lipophilic fluorescent compound that incorporates into the plasma membrane and is divided evenly between daughter cells after each cell division. The resultant intensity is proportional to the number of cell divisions a culture undergoes. For 3 days, epithelial cells were treated with 10 μ M D5-AzaC or vehicle alone and then released for 5 days followed by sorting for high CFSE intensity (*i.e.* cells that have divided rarely) or low intensity (*i.e.* cells that have divided frequently) (Fig. 4A). The percentage of cells in the low intensity population was minimal in the treated group (1.8%) compared with control group (29%), demonstrating the ability of the drug to induce cell growth arrest.

In treated cultures, a complete relaxation of imprinting occurred in the high intensity (few divisions) group (Fig. 4B). This growth-arrested subset up-regulated markers of senescence, including p16, p21, and IGF2 as assessed by QPCR (Fig. 4C). We found that a 3-fold (± 0.6 -fold) decrease in CTCF expression occurred reproducibly in treated cultures in the high intensity group (Fig. 4C). When controlling for cell proliferation, these studies demonstrated that inhibiting methylation leads to an accelerated loss of IGF2 imprinting and senescence. A decrease in CTCF expression was demonstrated, implicating the loss of CTCF binding in the IGF2 LOI. No methylation alterations were identified at the CTCF binding site (data not shown) in senescent *versus* proliferating cells.

Down-regulation of CTCF Leads to Increased IGF2 Expression and Loss of Imprinting—To examine the role of CTCF in IGF2 imprinting, CTCF expression was reduced in proliferating HPECs, as well as the PC3 and PPC-1 prostate cancer cell lines, by transfecting pooled CTCF siRNAs. After 48 h, no

morphological changes were noted in the transfected cells. Western blot demonstrated a decrease of greater than 50% in CTCF protein expression in transfected cancer cell lines (Fig. 5A). This down-regulation was less marked in proliferating HPECs (20–40%). Quantitative PCR demonstrated ~40–70% silencing of CTCF RNA expression in siRNA-transfected cultures when compared with controls (data not shown).

RNA analysis demonstrated a consistent increase in IGF2 RNA expression in cultures transfected with siRNA (Fig. 5B). Imprinting was unable to be assessed in the HPECs because of a lack of the ApaI polymorphism; however, for both PPC-1 and PC3, an increased expression of the silenced and imprinted IGF2 allele was demonstrated after siRNA transfection (Fig. 5C). These data showed that down-regulating CTCF expression results in an increased IGF2 expression and a relaxation of the imprinted IGF2 allele.

Induction of CTCF Expression in Senescent HPECs Results in IGF2 Expression Increases—Lentiviruses were utilized to express CTCF in infrequently dividing senescent epithelial cells. We verified the infection efficiency by infecting parallel cultures with an equivalent titer (see "Materials and Methods") of virus expressing GFP. At the titers used, the lentiviruses transduced ~40% of HPECs in senescent cultures. Longer infection periods led to cell death (data not shown). Cultures infected with lentivirus-CTCF had a mean 3-fold increase of in CTCF RNA expression as well as an increase in CTCF protein level when compared with lentivirus-GFP-infected cells (Fig. 6). QPCR demonstrated a reproducible decrease in IGF2 RNA expression in lentivirus-CTCF infected cultures.

DISCUSSION

Alterations in the imprinting status of IGF2 are important in the regulation of gene expression that occurs during develop-

A.

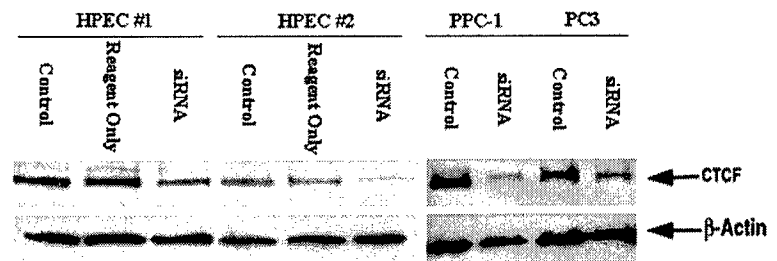
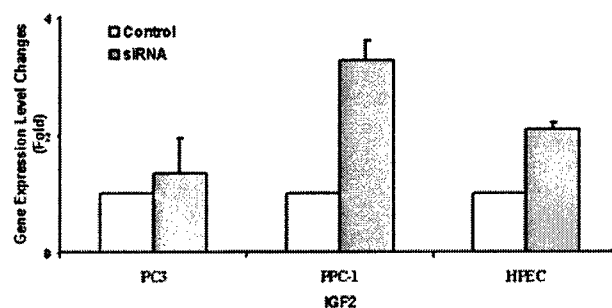


FIG. 5. Down-regulation of CTCF using siRNA transfection results in increased IGF2 expression. Pooled anti-CTCF siRNAs (50 nM) were transfected into HPEC, PPC-1, and PC3 human prostate cancer cell lines. RNA and protein were harvested 48 h after transfection. **A**, Western blot demonstrates that CTCF protein levels decrease post-transfection. β -Actin was utilized as a loading control. **B**, *IGF2* RNA expression increases with CTCF gene knockdown in primary prostate epithelial cultures and prostate cancer cell lines. **C**, loss of imprinting occurs with CTCF siRNA transfection in cancer cell lines. The determination of allelic imprinting was performed by reverse transcriptase-PCR of an ApaI polymorphism on exon 9. Uncut (upper, 292 bp) and restricted (lower, 218 bp) bands represent the presence of two alleles. PC3 contains some lower allele expression prior to transfection, and siRNA treatment results in the equal expression of both alleles. PPC-1 demonstrates detectable expression from the lower allele after CTCF down-regulation.

B.



C.

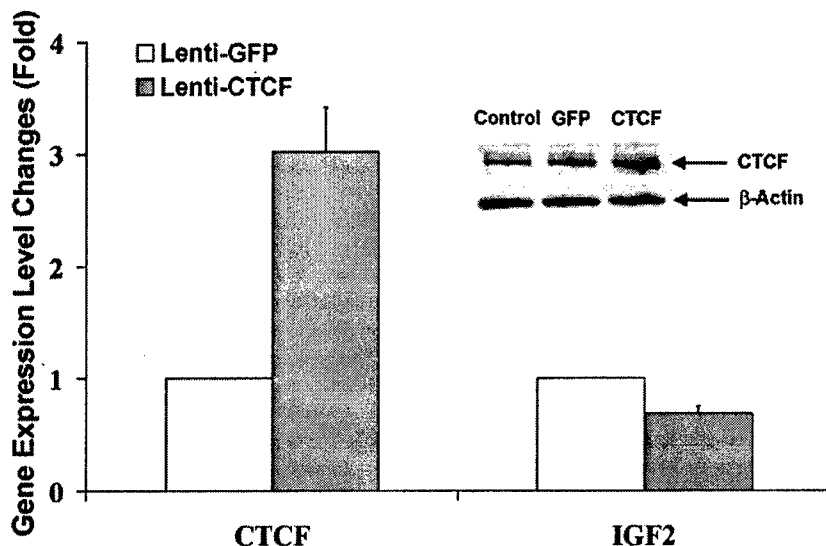
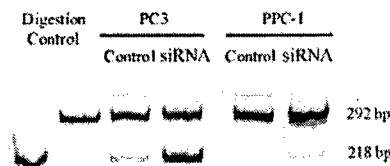


FIG. 6. Induction of CTCF expression in senescent cells leads to decreased IGF2 expression. The open reading frame of human CTCF or GFP was cloned into the FUGW vector, which places the inserted DNA under the control of the ubiquitin promoter. Viral supernatant was placed on the cells and then harvested for RNA and protein 48 h postinfection. Test infections using lentivirus-GFP (*Lenti-GFP*) showed an infection efficiency of 40% for these senescent epithelial cells. CTCF RNA expression was assessed by QPCR and demonstrated a 3-fold increase in lentivirus-CTCF (*Lenti-CTCF*)-infected cultures compared with lentivirus-GFP cells. Protein levels of CTCF were increased reproducibly (inset). A down-regulation of *IGF2* was observed consistently in lentivirus-CTCF-infected senescent primary cultures analyzed by QPCR.

ment, in cancer, and potentially in other biologic processes (33). In the present study, we demonstrate that *IGF2* also undergoes a loss of imprinting with the development of replicative senescence in human prostate epithelial and urothelial cells. Senescence is an *in vitro* model of aging that mimics mechanistically several aspects of *in vivo* aging (4, 7, 8). Given the finding that

a loss of imprinting in colon and prostate tissues occurs in older patients (3, 2), alterations in imprinting may be an important mechanism of gene regulation in aging epithelial cells. A second finding is that the loss of imprinting of *IGF2* at senescence is associated with a decrease in CTCF binding, an enhancer insulator, within the *H19* ICR. This is consistent with mouse

models and indicates a primary role for CTCF in the *IGF2* imprinting control pathway in genetically intact human cells. Third, we demonstrate that this decrease in CTCF binding is mediated by a decrease in CTCF expression, rather than DNA hypermethylation. The forced loss of CTCF expression leads to an increase in *IGF2* expression and loss of imprinting. Conversely, we find that the increased expression of CTCF in senescent cells decreases *IGF2* expression. CTCF down-regulation is a novel mechanism that may help explain the lack of consistent correlation between methylation of the *H19* ICR and imprinting found in human tumor tissues (20, 24).

The finding that changes in imprinting occur with senescence has not been reported previously. Senescence is a terminal phenotype that is important as a tumor suppressor in limiting the growth of cells but may also function in an "antagonistically pleiotropic" manner to overexpress proteins, such as *IGF2* or proteases, that may be detrimental to aging tissues (4). The chronic endogenous/exogenous exposure of cells to higher levels of *IGF2* generates multiple tumor types in mice (25); thus, strict mechanisms are needed to regulate the paracrine and autocrine mitogenic activity of *IGF2* (48). In our *in vitro* model of cellular aging, biallelic expression was linked to an increase in *IGF2* expression. At senescence, the reactivated maternal allele demonstrated expression levels equivalent to those of the paternal allele (Fig. 1), yet total concentrations of *IGF2* increased 10-fold. Clearly, other transcriptional factors or the loss of repressors contributed to this amplified response. One candidate down-regulated at senescence in HPECs that may amplify P3 and P4 promoter expression consists of several well described WT-1 binding sites that negatively regulate *IGF2* expression (39, 40). These data indicate that imprinting plays a primary role in regulating *IGF2* expression in human cells and that the loss of imprinting results in a permissive environment with the subsequent multifold increase in *IGF2* expression.

This human system presents a unique opportunity to examine imprinting and its regulation in genetically intact cells undergoing a programmed and sequential cellular process. This complements and has advantages over single point analyses of heterogeneous fetal tissues and tumors, which have provided often conflicting and contradictory data (20, 24). Prostate epithelial cells represent a relatively homogenous group of cells that, once established in culture, have characteristics of a basal, stem cell phenotype (26, 49). With passage to senescence, we demonstrate that *IGF2* LOI is associated with the maintenance of *H19* imprinting consistent with a common regulatory mechanism for these two genes. An inverse relationship in expression is also noted, with *H19* RNA decreasing significantly at senescence. This is consistent with a transcriptional model involving access to a common set of enhancers shared between *IGF2* and *H19*. One proposed mechanism for this reciprocal imprinting is binding of the enhancer-blocker protein CTCF to the *H19* ICR, located between *IGF2* and *H19* (50). On the unmethylated chromosome, CTCF acts as a transcriptional insulator and blocks activation of the *IGF2* promoters by distal enhancer elements. As cells progress to senescence and re-expression of the silenced maternal allele occurs, we find that a 2-fold decrease in the binding of CTCF to this region supports CTCF in human imprinting control.

We demonstrate in immortalized human prostate cancer cells and HPECs that the down-regulation of CTCF leads to an increase in *IGF2*. Furthermore, in cancer cells, a relaxation of imprinting was found. The HPECs utilized in this experiment were not informative for the *Apal* polymorphism. This suggests that in cancer cells and possibly in normal human epithelial cells CTCF plays a critical role in *IGF2* expression and imprint-

ing. Alterations in the expression of CTCF would help explain a number of diverse findings in human tissues, notably the presence of *IGF2* LOI in colon tumors containing hypomethylation of both alleles (24). However, recent data in human osteosarcomas suggest that other mechanisms exist that may bypass the CTCF boundary (51). The proposed down-regulation of CTCF represents a novel mechanism for altering the imprinting of *IGF2*, and the current model is the first to identify this as a mechanism in human cells. In addition, the loss of CTCF expression, which plays a vital role in survival and proliferation (43), may represent an important pathway in the maintenance, and possibly inception, of senescence.

Our results indicate that *IGF2* LOI in genetically intact human cells occurs in the absence of alterations in methylation at the *H19* ICR. Hypermethylation of this region in the mouse leads to biallelic *IGF2* expression, and methylation has been considered to be the primary event in the regulation of *IGF2* imprinting (1). Our analysis focused on methylation changes surrounding the sixth CTCF binding site, which contains allele-specific differential methylation in the human, and a minor gain of methylation at this site has correlated with *IGF2* LOI in colon, bladder, and Wilms' tumors (19, 20, 22). Alterations in CTCF binding would explain the LOI at senescence in the absence of hypermethylation of the unmethylated allele. We did find a reproducible increase in methylation at several partially methylated CpG sites downstream from the CTCF binding site that spans two cAMP-response element-binding protein sites and a forkhead (hepatocyte nuclear factor-3/forkhead homolog 1) site. This change may reflect senescence-associated *de novo* hypermethylation, propagating potentially from the edges of the CpG island (52), an age-related phenomenon seen at selected CpG islands (e.g. estrogen receptor) (53). Notably, these sites were methylated partially in fully imprinted proliferating cells, suggesting they do not have a major regulatory role in the imprint of *IGF2*.

We do not discount completely a role for methylation alterations in the control of imprinting in human cells undergoing senescence. Indeed, our data demonstrate that exposure of HPECs to the DNA methyltransferase inhibitor D5-AzaC indicate an important role for methylation loss. However, we did not document alterations in methylation at the *H19* ICR region. Global losses of methylcytosines are associated with aging both *in vitro* and *in vivo* in humans and have been postulated to represent a mitotic clock signaling senescence (10, 44, 45). In the aging human prostate, the overall methylcytosine content of normal prostate tissues from younger men (mean age 33 years) is significantly higher than that in benign prostatic hyperplasia and cancer tissues from older men (mean age 76 years) (54). We did find that, in HPECs treated with D5-AzaC, CTCF expression was reproducibly down-regulated in cells containing *IGF2* LOI. The effect of inhibiting methyltransferases appears to be indirect, by modulating the transcription of CTCF or other genes that may modify imprinting.

The present study demonstrates for the first time that development of the senescent phenotype, an *in vitro* model of aging, is characterized by the up-regulation and biallelic expression of *IGF2* in normal epithelial cells. This study examines the regulation of *IGF2* imprinting in a genetically intact, homogenous cell population during a programmed process. The loss of CTCF expression as a mechanism in cells for regulating *IGF2* imprinting is novel. Our data suggest a model in which a loss of CTCF binding mediates *IGF2* LOI; however, the majority of *IGF2* expression increases occurs because of altered transcriptional binding. In human and rat prostate tissues, *IGF2* levels increase with aging (47, 55). Based on these data, we speculate that alterations in imprinting may occur during cel-

lular aging *in vivo* and result in changes in gene expression. If so, these findings may have profound implications for the molecular basis of aging, as well as the propensity of the prostate and other organs for developing age-associated diseases.

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Meeting report

Pennington Scientific Symposium on Mechanisms and Retardation of Aging[☆]

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The Pennington Scientific Symposium on Mechanisms and Retardation of Aging was held in Baton Rouge, Louisiana, USA from May 4–6, 2003. The aim of this symposium was to gather leaders in the field of aging to discuss the latest theories and mechanisms of aging as well as the life prolonging effects of calorie restriction (CR). The overall program and list of speakers is presented in Appendix A. To encourage lively discussion, speakers were invited to bring one guest who had either similar or opposing views to their own. During the first session, several views of aging with respect to the endocrine system, brain function, cardiovascular function, immune system and susceptibility to cancer were presented. The second session focused on mechanisms of life extension by CR induced alterations in neuro-endocrine pathways, gene expression, mitochondrial energy metabolism and oxidative stress. The program ended with a lengthy round-table discussion to facilitate communication on how these studies could translate into

CR research in humans. During the round-table discussions, speakers and guests identified a few noteworthy highlights of the presentations to be further emphasized in the present report. More specifically, this report will focus on three major hypotheses of aging presented during Session 1; hormonal regulation, DNA methylation and nutrient sensing. From Session 2, we report on potential biomarkers of aging and the latest results from the ongoing CR trials in non-human primates.

Session 1: Theories of Aging in Tissues and Organs

Aging may be defined as the gradual and progressive deterioration in function of an organism. However, it is unclear exactly why organisms age. Steven Austad began the symposium with an insightful lecture about the many existing theories of aging. He focused on the major causal theories including: (1) for the good of the species, (2) somatic inevitability and (3) evolutionary aging. He also presented several mechanistic theories of aging; including those implicating oxidative stress, telomeres and endocrine dysfunction, and hypothesized that both causal and

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mechanistic approaches must be combined to most clearly address these questions in the future.

A. Hormone Profile and Longevity

The influence of the endocrine system on aging processes and life span has garnered major attention by biogerontologists over the past few years. This is because many hormones display a gradual, progressive decline with aging, sometimes beginning years before the post-reproductive stage of life. Examples include testosterone (T), dehydroepiandrosterone sulfate (DHEAS), growth hormone (GH), and insulin-like growth factor-1 (IGF-1). It is currently hypothesized that declines in the serum concentrations of these hormones may lead to decline in physiologic function. A new theory, the 'Mitogenic–Differentiation Theory of Aging', challenging this view was also presented at this symposium (Bowen and Atwood, submitted). This theory proposes that the hormones regulating reproduction act in an antagonistic pleiotrophic manner to control aging via cell cycle signaling, promoting growth and development early in life in order to achieve reproduction. However, later in life, in a futile attempt to maintain reproduction, these same systems become dysregulated and drive senescence.

The decline in the levels of hormones in elderly patients frequently do not meet the defined criteria for hormone deficiency suggesting that the term supplementation (versus replacement) is more often correct with regard to potential clinical intervention. Testosterone replacement in younger hypogonadal males corrects the reduced muscle and bone mass, loss of strength, increased fat mass, decreased libido and the altered mood and cognitive abilities (Wang et al., 2000). Although this symptom complex is similar to that commonly observed in aging males, few T supplementation/replacement studies have been conducted in older men, where T levels are more likely to be in the low–normal range. The androgen, DHEAS declines ~80% by age 80 and is a well-known biomarker of aging (Roth et al., 2001a). The low levels reached in almost all older individuals have been correlated with cognitive impairment, atherosclerosis and insulin resistance. Clinical trials using supraphysiologic replacement doses of DHEA have reported harmful side effects similar to those observed with anabolic steroid excess. However, trials in older adults using lower replacement doses revealed potentially important benefits such as increases in bone mineral density, fat-free mass and improved insulin action (Villareal et al., 2000). Because the completed studies have all been short term and not well controlled, new studies are currently being conducted to study the effects of DHEAS replacement in men and women in longer, double-blind, randomized, placebo-controlled trials (R.S. Schwartz, conference communication). These important trials will address the effects of DHEAS on body composition, insulin action and physical function in older individuals.

The decline in the GH axis that is observed with aging and body composition and metabolic changes that may be associated with this process are termed the somatopause. An increase in central adiposity, a decline in lean body mass, strength, bone mass, slow wave sleep and renal blood flow may all be associated with somatopause, since many of these resemble those changes found in adult GH deficiency. Replacement studies in growth hormone deficient adults show substantial reversal of these symptoms following treatment with GH. While GH supplementation in older individuals has produced improvements in body composition (lean and fat mass, as well as fat distribution), relatively few changes in function or strength have been demonstrated (Brill et al., 2002). In addition, the side effects of GH treatment are many and include carpal tunnel syndrome, insulin resistance and concern regarding exacerbation of tumor growth. A recent clinical trial in older women using growth hormone releasing hormone revealed fewer side effects, a 25–30% increases in IGF-1 levels, improvement in lean and fat mass as well as fat distribution (R.S. Schwartz, conference communication). However, these beneficial effects disappeared following completion of the hormone treatment. The overall concern is that little is known about the long-term physiological outcomes of hormone administration to older adults.

Reduced signaling through the GH/IGF-1 pathway results in life span extension in multiple species including mammals, flies, worms and yeast (Brown-Borg et al., 1996; Clancy et al., 2001; Fabrizio et al., 2001; Flurkey et al., 2001; Kenyon et al., 1993; Tatar et al., 2001). Of the mammalian longevity mutants, the Ames and Snell dwarf mice are GH deficient resulting in low to non-detectable levels of plasma IGF-1 [reviewed by (Bartke and Turyn, 2001)]. Another mutant, the growth hormone receptor knockout mice (GHR KO) are also long-lived, but have high levels of endogenous GH (Coschigano et al., 2000). Bartke and coworkers (Bartke and Turyn, 2001) have focused on insulin signaling in both the Ames dwarf and the GHR KO mice. In these animals, peripheral insulin and glucose levels are reduced and glucose responses to injected insulin are enhanced, suggesting increased insulin sensitivity. However, administration of both glucose and insulin suggest that these animals may exhibit insulin resistance in skeletal muscle and have reduced insulin release by the β -cell, but have increased insulin sensitivity of the liver. Thus, suppression of somatotrophic signaling, along with secondary changes in insulin release and actions may lead to delayed aging and prolonged longevity in laboratory mice.

The mitogenic–differentiation theory of aging puts forth a novel alternative mechanism by which these hormones function and may help explain aging in all sexually reproductive life forms. This theory suggests a new definition of aging—*any change in an organism over time*. This definition includes not only the changes associated with the loss of function (i.e. senescence; the commonly accepted definition of aging), but also

the changes associated with the gain of function (growth and development). Using this new definition, the rate of aging would be synonymous with the rate of change. The rate of change (aging) is most rapid during the fetal period when organisms develop from a single cell at conception to a multi-cellular organism at birth. Therefore, fetal aging is determined by the rate of mitogenesis and differentiation. This theory suggests that mitogenesis and differentiation also are responsible for aging throughout life. Since life-extending modalities consistently affect reproduction, and reproductive hormones are known to regulate mitogenesis and differentiation, it is proposed that aging is primarily regulated by hormones controlling reproduction. By having hormones that are sensitive to environmental conditions regulate both reproduction and aging, an organism is able to modulate its fertility and its rate of aging based on environmental conditions. Therefore, under hostile conditions (i.e. CR, cold, stress), fertility is preserved and longevity extended awaiting a friendlier reproductive environment.

In mammals, reproduction is controlled by the hypothalamic–pituitary–gonadal (HPG) axis. The centrally produced hormones may represent the mitogenic factor(s) while the peripherally produced hormones may represent the differentiation factors. Longevity inducing interventions, including CR, decrease fertility by suppressing centrally produced HPG axis hormones thereby affecting signaling through the IGF-1 pathway, which may regulate longevity in some animal models. This is exemplified by genetic alterations in *C. elegans* where homologues of the HPG axis pathways, as well as the *daf-2* and *daf-9* pathways, all converge on *daf-16*, the homologue of human Forkhead, that is known to be important in the regulation of cell cycle events. Therefore, the dysregulation of cell cycle events caused by changes in reproductive hormones is a common mechanism, which could drive degenerative changes during senescence. The most obvious of these is cancer, however, Alzheimer's disease may be also due to an aberrant re-entry of neurons into the cell cycle (Raina et al., 2000; Yang et al., 2001). Interestingly, it has been shown that individuals with Alzheimer's disease have elevated serum concentrations of gonadotropins (Bowen et al., 2000) and elevated neuronal luteinizing hormone concentrations corresponding to those regions of the brain most damaged by the disease (Bowen et al., 2002). Luteinizing hormone also modulates the processing of the amyloid- β precursor protein and the generation of A β , the major component of amyloid plaques that deposit in the Alzheimer's brain (C.S. Atwood, conference communication).

One intriguing commonality among the endocrine work presented is the relationship between the reproductive hormones, growth hormones (GH, IGF-1) and aging. The evidence indicates that the mutant mice that live longest exhibit the greatest degree of gonadal suppression and interestingly, do not exhibit the age-related decline in cognitive function normally observed in wild type mice

(Bartke et al., 2002; Brown-Borg et al., 1996; Kinney et al., 2001b). Fertility is also quantitatively reduced in GHR KO mice, animals that retain cognitive function in old age when compared to normal control mice (Kinney et al., 2001a). In sharp contrast, the short-lived, large, GH transgenic mice enter puberty significantly earlier than wild type animals, and exhibit several indices of premature aging including signs of early cognitive impairment [reviewed by (Bartke et al., 2002)]. Therefore, maintenance of several aspects of brain functioning with age and the relationship to the gonadotropins and growth and development are deserving of future research efforts in the quest to delay aging in humans.

B. Age-related Alterations in Genomic Imprinting: DNA Methylation

Epigenetic alterations have been postulated to modulate the aging processes and represent susceptibility factors that may underlie the increase in cancer rates in the elderly. In the case of prostate cancer, the most commonly diagnosed solid tumor in US males, deaths are expected to increase with the increasing geriatric population. The molecular events that underlie this disease are not well understood, however, several observations provide insight into its development. It is a disease of aging with the latent form of the disease found in ~75% of men by age 80 (Haas and Sakr, 1997). Another striking observation is that the majority of prostate cancers (over 75%) arise in the peripheral region of the prostate. Even more remarkable is the observation that in radical prostatectomy specimens removed for cancer, typically four independent foci of cancer can be found (Epstein et al., 1994). This suggests that in the peripheral prostate, a field effect has occurred that has lead to cancer promotion in multiple cells.

One candidate underlying both this field defect and regional predilection of prostate cancer is the observation that a loss of Insulin-like Growth Factor-II (*IGF-II*) imprinting occurs in histologically normal peripheral prostate tissues (Jarrard et al., 1995). The *IGF-II* gene is stringently imprinted in adult tissues, and biallelic expression, associated with *IGF-II* upregulation, has been implicated in cancer development (Feinberg, 2000). Given this age-dependency of prostate cancer, and the fact that an imprint control mechanism (i.e. DNA methylation) is altered with aging, loss of *IGF-II* imprinting may be an acquired molecular event that can be reversed or modulated.

Genomic imprinting is an epigenetic modification in the gamete or zygote that results in the silencing of a specific parental allele in the offspring. Thus, in contrast to most genes, imprinted genes (roughly 40 are known) express only from one allele (Morison et al., 2001). *IGF-II* is located at 11p15 and exhibits monoallelic expression from the paternal allele in most adult tissues (Pfeifer, 2000). Exceptions include specific regions of the brain and possibly the liver. *IGF-II* is an embryonic mitogen with a clear role

as a paracrine and autocrine regulator of cell proliferation in the human prostate (Cohen et al., 1994). It is part of the IGF axis, a multi-component network of molecules that includes the ligands IGF-I, IGF-II, cell-surface receptors and binding proteins. Epidemiological studies have implicated the IGF axis as a predisposing factor in the pathogenesis of both human breast and prostate cancer. Repeatedly, higher tissue levels of *IGF-II* have been found in early prostate cancers, when compared to normal tissues, and in more pathologically advanced stages of the disease (Fichera et al., 2000; Ho and Baxter, 1997; Tennant et al., 1996). Notably, long-term, chronic endogenous or exogenous exposure appears to be especially important in the generation of tumors. Transgenic mice engineered to re-express *IGF-II* as adults develop diverse carcinomas only after a long latency period (Rogler et al., 1994).

Loss of imprinting, or a re-expression of the silenced allele, has been implicated in the pathogenesis of cancer (Rainier et al., 1993). Loss of imprinting of *IGF-II* was first demonstrated in Wilms tumors (Rainier et al., 1994), an embryonic tumor of childhood, but the same abnormality has also been noted in colon (Cui et al., 1998) and prostate cancers (Jarrard et al., 1995). A number of these studies demonstrate that a relaxation of *IGF-II* imprinting correlates with higher levels of *IGF-II* expression.

The mechanisms that regulate *IGF-II* imprinting are complex and include upstream promoters, downstream enhancers, as well as DNA methylation a post-replicative addition of methyl groups to the 5-carbon position of cytosines primarily within CpG dinucleotides. An important aspect to understanding imprinting regulation has focused on imprinting control regions (ICRs) (Reik et al., 2000). ICRs provide gametic marks that establish parent of origin dependent expression domains. The ICR between *IGF-II* and *H19* expresses paternal *IGF-II* in the methylated state, yet blocks expression in the maternal allele when unmethylated. The repression of the maternal allele involves binding of the chromatin insulator factor CTCF, which binds only to unmethylated DNA in this ICR and blocks the access of downstream enhancer proteins to the promoter. It is not clear whether the loss of CTCF binding leads to de novo methylation or the converse, however, DNA methylation certainly plays an important role. Disruption of DNA methylation, using a methyltransferase-knockout mouse (embryonic lethal), leads to a loss of the normal imprint status of *IGF-II*, *H19* and *p57* (Li et al., 1993), and the treatment of non-immortalized cells with methyltransferase inhibitors results in biallelic *IGF-II* expression (Hu et al., 1996).

It has been well documented that with aging, global hypomethylation occurs associated with regional hypermethylation (Issa, 1999). It has been proposed that DNA methylation changes seen in aging may contribute to altered imprinting of the *IGF-II* gene. This process is further modulated by environmental or somatic causes that may accelerate the relaxation of *IGF-II* allelic imprinting. In this

model, a threshold level for *IGF-II* imprinting may be reached in aging. However, with additional stimuli (e.g. dietary methyl deficiency, oxidative stress due to caloric intake or hormonal exposure), loss of imprinting of *IGF-II* will be accelerated in prostate tissues. As evidence that aging can modulate imprinting, Jarrard and colleagues have recently demonstrated that a complete loss of imprinting of *IGF-II* occurs with the passage of human prostate epithelial cells to senescence in vitro (unpublished data). This is associated with a multifold increase in *IGF-II* expression levels. In the aging rat prostate, an eight-fold increase in *IGF-II* protein expression has been noted (Slater et al., 2000) but, to date levels in the aging human prostate have not been reported. Given data demonstrating a role for increased *IGF-II* levels in the promotion of cancer, including prostate cancer, it is postulated that the relaxation in *IGF-II* imprinting may play a role as a switch to initiate the development of a field of susceptibility that is ultimately manifest by the multifocal tumor generation seen in prostate cancer specimens.

A mechanism for retarding these changes in methylation, and potentially imprinting is diet. In animals, a number of factors implicated in aging have been shown to accelerate the loss of DNA methylation. These include free radical accumulation (Weitzman et al., 1994), carcinogens (Wilson and Jones, 1983), and dietary deficiencies in folate, methionine, vitamins B₆ and B₁₂, and serine (Slattery et al., 1997). Dietary increases in vitamin B₁₂ and B₆, which increase levels of the methylation reaction substrate S-adenosylmethionine (SAM), have been linked to decreased prostate cancer risk in humans (Key et al., 1997; Tollefsbol and Andrews, 1993). It has also been proposed that caloric restriction retards aging by restricting methylation metabolism, thus slowing the rate of loss of methylation (Cooney, 1993). Therefore, should the proposed hypothesis be validated, a number of options for reversing these methylation and imprinting changes are available for testing.

C. Master Regulators of Aging

The 'metabolic syndrome of insulin resistance' represents a constellation of defects that are important risk factors for age-related diseases (Hansen, 1999; Reaven, 1988). This syndrome is linked to excess nutrients since it is most commonly associated with obesity, particularly abdominal obesity, and is a risk factor for all-cause mortality (Calle et al., 1999). It is also commonly associated with the development of type 2 diabetes mellitus, the incidence of which increases dramatically with aging (Harris et al., 1998). Furthermore, this syndrome increases the risk for thrombosis and coronary artery occlusion. Other manifestations of the metabolic syndrome, such as hypertension and dyslipidemia, contribute to the marked increase in risk of developing atherosclerosis. Of interest, this syndrome has been characterized as a pro-inflammatory

state with increased plasma levels of various adipose associated cytokines and acute phase reactants. The latter may in turn be implicated in the etiology of several age-related diseases. Finally, insulin resistance and central obesity might also be a risk factor for a variety of cancers.

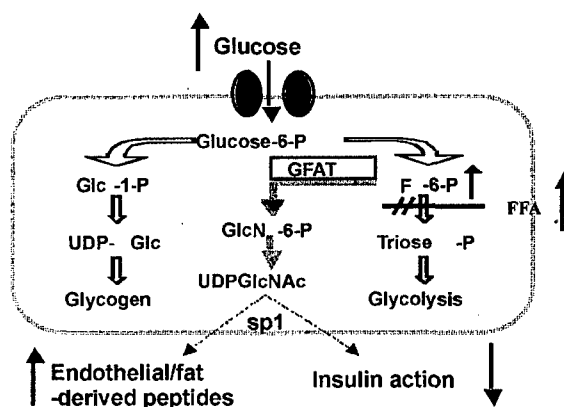
Thus, the metabolic syndrome is likely to play a central role in biological processes that link it to shorter life span in humans. It is generally postulated that insulin resistance plays a causative role in the various components of the metabolic syndrome (*linear model*). However, recent studies have suggested that chronic excess of nutrients leading to the activation of nutrient-sensing pathway/s may play a direct role in the pathogenesis of both insulin resistance and various other components of the metabolic syndrome (*parallel model*). It has been suggested that these direct effects of nutrients are likely to be partly mediated via enhanced glycosylation and altered gene regulation of key proteins.

Under normal circumstances, the deleterious effects of the excessive availability of nutrients are countered by the prompt activation of nutrient 'counterregulatory' systems. The latter include (but are not limited to) hypothalamic neuro-circuitries partly under the control of leptin. The activation of these nutrient 'counterregulatory' systems should prevent the excessive storage of energy and the onset of insulin resistance. Thus, there appears to be a functional feedback loop mediated by the hypothalamus, which normally prevents visceral adiposity, insulin resistance, and other metabolic features of aging from developing.

Importantly, decreased caloric intake has been associated with increased life span and a decreased incidence of age-related illness in many species (Barzilai and Gupta, 1999). Indeed, diseases associated with obesity in humans are mirrored by ad libitum (AL) feeding in other species. For example, AL fed models are insulin resistant and have shorter life spans than CR models. Since fat tissue has previously been viewed as an inert depot for nutrient storage, prevailing scientific opinion has supported a dominant role for decreased nutrient intake per se in the protective effects of CR. However, adipose tissue is an active *endocrine* organ whose secretory products include hormones, cytokines, and complement factors that can be measured in physiological levels in plasma. These observations have alerted investigators to the possibility that increased fat mass, might in turn lead to negative sequelae in remote organs. Furthermore, nutrients may directly activate metabolic pathways that have important systemic effects and that may initiate age-associated diseases.

Based on ongoing investigations in Dr Barzilai's laboratory, it is hypothesized that the hexosamine biosynthesis pathway (HBP) is a central nutrient-sensing mechanism (see Figure). Activation of HBP induces insulin resistance and regulates the secretion of several adipose-derived peptides (McClain and Crook, 1996; Rossetti, 2000; Wang et al., 1998). While most intracellular glucose is directed toward glycogen synthesis or glycolysis,

approximately 1–3% of the incoming glucose that is converted to fructose-6-phosphate (F-6-P) enters HBP (see Figure). This occurs by the action of the rate limiting enzyme glutamine, F-6-P amidotransferase (GFAT), and results in the O-glycosylation of intracellular proteins by *N*-acetylglucosamine. As an example, this glycosylation pathway activates the transcription factor SP1, which is involved in the nutrient-mediated modulation of the expression of numerous proteins. With excess nutrient intake, HBP flux may increase several folds, making HBP an ideal 'sensing' mechanism for nutrient availability.



Besides the effect of HBP on insulin resistance (Hawkins et al., 1997; Rossetti et al., 1995), acute induction of the HBP increases the gene expression of a variety of adipose tissue-derived peptides and cytokines (Gabriely et al., 2001, 2002). During aging, the chronic increase in the levels of plasma glucose and free-fatty acids leads to increased intracellular concentrations of F-6-P. This in turn results in further increased activity of the HBP. The HBP has been recently implicated in some of the vascular complications of diabetes, which have striking similarities to those seen with aging (Du et al., 2000). Indeed, advanced glycosylation end products increase with aging, suggesting that this pathway is already up-regulated and may be more sensitive to nutrient flux. Because a chronic increase in nutrients can directly increase carbon flux through the HBP, it may have a potential role in the metabolic syndrome of aging and on the mechanisms mediating the beneficial effects of CR.

Session 2: Mechanisms of Life Extension

Evidence that CR retards aging and extends median and maximal life span was first described nearly 70 years ago (McCay et al., 1935). Since then, similar observations have been made in a variety of species including rats, mice, fish, flies, worms and yeast (Barrows and Kokkonen, 1982; Weindruch and Walford, 1988). From these studies numerous potential biomarkers of aging have been found.

Based on the biomarkers obtained from other species, the results obtained from the CR trials in non-human primates are consistent with the theory that CR also is likely to increase life span in longer-lived species (Bodkin et al., 1995; Kemnitz et al., 1993; Lane et al., 1998).

A. Biomarkers of Aging

The term 'biomarkers of aging' implies parameters, which allow one to reliably estimate biological or 'functional' age, as opposed to chronological age, for any subject. A good biomarker or set of biomarkers might also be expected to offer predictive information on the future longevity of an individual. There is no clear distinction between physiological changes that occur with age and what may be labeled 'biomarkers of aging.' It is obviously not sufficient to follow a parameter that undergoes a change with age and treat that as a biomarker.

CR slows the rate of change of numerous parameters that are normally observed with aging. These include parameters that are not clearly disease related, such as collagen in rats (Everitt, 1971) and immune responses in mice (Walford, 1974; Walford et al., 1973). Other CR- and age-sensitive measures are liver enzymes, response to hormones, eye lens proteins, protein turnover, age pigment, behavioral and psychomotor indexes, and others (Weindruch and Walford, 1988; Yu, 1990).

Do accurate 'biomarkers of aging' exist? In animals with a relatively short life span (such as rodents), average and maximum life span data can serve as indicators of whether a preventative intervention has been successful. Indeed, whether or not an intervention increases maximum life span in laboratory rodents is widely viewed as the 'gold standard' for testing the efficacy of an intervention to retard the aging process. However, there are serious shortcomings with this assay including its cost, the time required and its inability to provide direct information on the biological age of individual tissues, especially since different tissues age at different rates.

In longer-lived species, such as humans, longevity determination is not practical, and methods to determine over a relatively short space of time whether the rate of aging is being influenced are required. The National Institute on Aging has long supported development of biomarker research (Baker and Sprott, 1988; Reff and Schneider, 1982). Desirable features of good biomarkers include: (1) it be measurable without harming the subject, (2) it be highly reproducible and reflect physiologic ('functional') age, (3) it shows significant age-related changes within a relatively brief time period (in relation to the organism's life span), and (4) the clinical functions should be crucial to the health of the species (Reff and Schneider, 1982).

The recent series of publications describing increased longevity in several types of mutant mice (reviewed by

Barger et al. [submitted]) which include Ames and Snell dwarf mice, 'little' mice, IGF-1 receptor mutants and $p66^{shc-/-}$ mice suggest the importance of investigating putative biomarkers of aging in these models. This takes on additional importance as it can be argued that, for most of these models, the effects on longevity are not too impressive as compared to that exerted by CR.

A new area of inquiry is the attempt to develop drug or nutrient interventions (mimetic) that may mimic the actions of CR (Lane et al., 2002; Weindruch et al., 2001). A challenge in that regard is the aforementioned significant shortcomings associated with the current 'gold standard' of life extension for screening interventions that may retard aging. Besides the long time frame required, this life extension assay provides imprecise data on the rate of aging in individual organ systems (e.g. did the candidate mimetic retard aging in the heart, the brain?). Importantly, advances in genomics provide exciting new opportunities and have created the new field of 'nutrigenomics' which can be applied to this problem. It has been argued that the use of microarrays to generate gene expression profiles (which consist of hundreds of markers of aging at the transcriptional level) can be used to determine the biological age of a tissue and thereby allow the determination of whether interventions can retard aging on an organ-specific basis (Weindruch et al., 2002). Further, one can quantify the ability of a candidate mimetic to induce the transcriptional reprogramming that is similar to what is associated with CR. Proteomics is expected to offer similar opportunities.

B. Calorie Restriction in Non-human Primates

To address the relevance of CR to humans, non-human primate studies were initiated at the National Institute on Aging (NIA) in 1987 (Ingram et al., 1990) and at the University of Wisconsin (UW) in 1989 (Kemnitz et al., 1993). The studies were intended to provide the experimental control that would be difficult to achieve in human studies, while expanding knowledge about the biological effects of CR beyond the rodent model. The total number of monkeys involved in the NIA longitudinal study is about 120 with roughly equal numbers of control and CR monkeys. The UW study began with only mature adult male rhesus monkeys and later (1994) added additional mature adult male and female monkeys for a total of 64 monkeys roughly divided among control and CR groups. Knowledge about CR in non-human primates will therefore be exclusively derived from studies of rhesus monkeys that have a usual median life span of around 25 years and a maximum life span of 40 years.

Both studies targeted a 30% reduction in caloric intake. For the UW study this reduction was made from baseline measurements of intake for individual monkeys. Because the NIA study incorporated a wide age range including

the development stage, caloric intake for the CR monkeys was based on a reduction from age- and weight-matched control monkeys. Food allotments for the control group were derived from tables developed by the National Research Council (NRC) regarding recommended levels of caloric intake for rhesus monkeys of specific age and body weights. Details on the diet (standardized natural ingredient diet for NIA and purified diet for UW) and study design can be found elsewhere (Ingram et al., 1990; Kemnitz et al., 1993; Lane et al., 1999). CR regimens in both studies have proven to be generally safe in their implementation with virtually no untoward health effects reported.

CR in both studies has produced the expected alterations in body composition, including reduced body weight and fat mass (Blanc et al., 2003; Weindruch et al., 1995). For juvenile monkeys in the NIA study, lean mass and height were also reduced in CR monkeys, and their development, as measured as delays in puberty and bone growth, was retarded (Lane et al., 1995b). In general, physiological effects of CR have paralleled most of those reported in rodent models (Roth et al., 2000). As examples, in CR monkeys body temperature is reduced (Lane et al., 1996) and plasma glucose and insulin levels are lower (Lane et al., 1995a). Metabolic rate is also reduced during the early phases of CR, but generally equilibrates to control levels as body composition is altered after many months on CR (Lane et al., 2000, 1996). UW adult CR monkeys exhibit some reductions in metabolic rate measured primarily as resting energy expenditure (Blanc et al., 2003).

Both studies have reported that CR monkeys manifest lower risk factors for age-related diseases. For example, monkeys in the NIA study have lower heart rates, systolic blood pressure, and serum triglycerides while maintaining higher levels of specific HDLs that would indicate reduced risk of heart disease (Lane et al., 1999). CR monkeys in both the NIA and UW studies have enhanced glucose tolerance and insulin sensitivity (Cefalu et al., 1999; Lane et al., 1995a) with few reported cases of diabetes (NIA = 2; UW = 1).

CR has also been shown to alter age-related hormonal changes. For example, the usual declines in the adrenal steroid, dihydroepiandrosterone, and the pituitary hormone, melatonin, are attenuated in older CR monkeys in the NIA study (Lane et al., 1997; Roth et al., 2001b). The UW study has focused on molecular markers of oxidative stress and has identified reduced production of protein carbonyls reduced mitochondrial DNA deletions in muscle biopsies taken from CR monkeys (Zainal et al., 2000) and reduced cytokine responses to xanthine oxidase in monocytes from CR monkeys (Kim et al., 1997). In the NIA study, despite reports of reduced number of lymphocytes occurring during early stages of CR, the ability of monocytes to deal with an inflammatory challenge appears to be enhanced. Specifically, the age-related decline in production of interferon-gamma is

attenuated in older monkeys on CR (Mascarucci et al., 2001). The UW study has also utilized gene array technology to assess whether CR alters age-related changes in the pattern of gene expression (Kayo et al., 2001). Finally, new techniques continue to emerge from these studies. For example, the NIA study has developed an *in vitro* assay to assess possible mechanisms of CR. Specifically, cells grown in serum from CR monkeys exhibit enhanced survival and stress responses when subjected to various challenges (de Cabo et al., 2003).

While findings emerging from these studies appear encouraging regarding the potential beneficial effects of CR on aging processes in monkeys, it remains premature to reach a final conclusion. Conclusive evidence that CR retards the rate of aging in these studies will require three vital elements of proof as follows: (1) reduction in mortality rate; (2) reduced incidence of age-related disease and pathology; and (3) enhanced function at older ages. Regarding mortality and disease incidence, trends in the studies appear to support the beneficial effects of CR; however, these trends are presently not statistically significant. Regarding function, data have demonstrated age-related declines in visual accommodation and auditory acuity in the NIA study, but CR had not attenuated these changes (Torre et al., 2003). In the UW study, some elements of auditory function appeared to be enhanced in the CR group (Fowler et al., 2002). In simple learning tasks, no beneficial CR effects have been noted thus far in the NIA study. Further analyses are ongoing including correlating behavioral function with *in vivo* brain imaging. Age-related declines in the volume of the striatum as well as reduced binding potential of dopamine D2 receptors in this brain region have been documented in the NIA monkeys (Ingram et al., 2001). Similar studies are being planned for the UW study. New studies currently underway for the NIA study including further analyses of reproductive physiology, oral health, immune function, skeletal structure, and chromosomal aberrations, all of which have emerged from a recently approved grant program to outside collaborators.

In summary, both the NIA and UW studies have demonstrated the feasibility and potential utility of conducting long-term studies of aging in rhesus monkeys involving CR as an intervention. Both studies are approaching critical stages in their evolution as their monkeys approach geriatric ages that will offer many opportunities for further elucidation of the long-term effects of CR and possible mechanisms that regulate these effects.

Summary

The Pennington Symposium on Aging was a successful forum for focused discussions on mechanisms of aging and the impact of CR on these mechanisms. In this report, we

presented the current thinking on a few theories of aging as well as the latest findings of the CR studies in non-human primates. During the round-table discussions, it was concluded that future works need to be directed on determining robust biological markers of aging in animals and humans.

Appendix A

Pennington Symposium 'Mechanisms and Retardation of Aging' Chairs: Eric Ravussin, PhD, Robert S. Schwartz, MD Richard Weindruch, PhD

Keynote 1: Theories of Aging

Steven N. Austad, PhD

Session 1: Aging in Tissues and Organs

Age-Related Changes in Brain Biochemistry and Pathology: Causes and Consequences
Craig S. Atwood, PhD

Cardiovascular Aging in Humans: Key Features, Underlying Mechanisms, and Promising Interventions
Douglas R. Seals, PhD

The Aging Endocrine System: To Supplement or Not to Supplement
Robert S. Schwartz, MD

Aging and Cancer: Are Age-Related Alterations in Genomic Imprinting Susceptibility Factors for Cancer?
David F. Jarrard, MD

Age-Related Immune Dysregulation: Molecular Mechanisms and Reversal by Nutrient Intervention
Simin N. Meydani, D.V.M., PhD

Keynote 2: Genetics of Longevity from Yeasts to Humans

S. Michal Jazwinski, PhD

Session 2: Mechanisms of Life Extension

Molecular Mechanisms of Life Extension in *Drosophila*
Stephen L. Helfand, MD

Endocrine Pathways for Life Extension in Rodents
Andrzej Bartke, PhD

Role of Oxidative Stress in the Aging Process

Arlan G. Richardson, PhD
New Mechanistic Pathways of Caloric Restriction
Suggested by Gene Expression Profiles
Richard Weindruch, PhD

Nutritional Retardation of Aging in Rhesus Monkeys
Donald K. Ingram, PhD

Caloric Restriction and Cellular Fuel Sensing
Nir Barzilai, MD

Caloric Restriction and Mitochondrial Energy Metabolism
Jon J. Ramsey, PhD

Strategies of Retardation of Aging: Caloric Restriction vs. Increased Physical Activity
John O. Holloszy, MD

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