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. *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 IGF3 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,
IGF2 and H19 are expressed in a coordinate fashion that suggests, in combination with their close linkage and reciprocity, 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 paternal 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 CTCF-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 segments. Cells were trypticized and passaged at 1.2 or 1.3 divisions when confluent and underwent typically 10–15 population doublings before becoming senescent. DNA and RNA were isolated from each passage. PC2 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). 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, RNase D (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 Apat polymorphism on exon 9 (29). P1 (5'-GACACCCCTCCAGT-TGCCTCTG-3') and P2 (5'-CGGGGATGCTAAAGTATGAG-3') cross introns between exons 7, 8, and 9. The RNA-specific (1.3 kb) product was amplified 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 Apat (New England Biolabs). P3 and P4 primer sequences were 5'-CTTGGACCTTTGAGTCAATTGG-3' and 5'-GGTGTTGGCAATTATCAATG-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'-TGCACTACCTGACTAAGAGAC-3' and reverse 5'-GTGATGTCGGTAGCTTCTC-3') were used to amplify across the H19 ICR 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 preincubated by incubation with 50 µl of preimmune serum for 1 h followed by overnight incubation with 100 µl of Immunopur protein A-agarose (Pierce). Samples were incubated with 30 µl of anti-CTCF antibody (Upstate Biotechnology). Immune complexes were collected by incubation with protein G-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-µl 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'); GAGGCTTTCCTCTGCCTCTCA and GCGCTTTCCTCCAACAA, Western blot analyses 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 human tissues (33–35). The Cpg island in the upstream unmethylated cytosine to uridine domains were located on IGF2 (GenBank accession no. AF125183) at exon 4 at 21170–21525 (MR1), exon 9 at 29800–29375 (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'-ACCCACACTCAACTCCTCCCACAC), MR1-R (5'-TATTAGGATTAGTAGT), MR2-F (5'-TTGGGTGGTAGAATTTAATTAG), MR2-R (5'-CTCAAATCTAATCTC), MR3-F (5'-

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2 Primers designed for p16 IGF2, and WT-1 are available on request.
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GTAGGTTTTTTGTAGTTAGAGT, and MR3-R (5' CACCCAAAA-AACAATATCATC), which are specific for the converted DNA. The PCR products were then cloned into a pCR2.1-TOPO vector (Invitrogen). Seeds 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 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes) at 37 °C for 15 min. Cells were harvested for RNA and protein 48 h postinfection. Test 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.

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 a lentivirus vector and packaging vectors into the 293T cell line, as described elsewhere (38). Early senescent HPECs had 0.1 ml of PBS (50 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 to the cells in complete Dulbecco's modified Eagle's medium and mixed by gentle rocking. Cells were let to grow for 12 h after the initial transfection. RNA and protein were harvested at 48 h. Experiments were performed in duplicate, with similar results.

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 nonconfluent 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 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-
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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 ± 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.

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 (∼0.5-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 ± 7% in unmethylated alleles) was seen in several contiguous CpG sites ∼200 bp downstream from CTCF site 6, which contained two

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 Apal 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 regulation of 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...
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 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 ± 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**—Hypo-methylation has been documented in aging cells in vitro and in vivo (44, 45). To investigate the role of accelerated methyl-
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B

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 μM 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.

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 a 30–70% 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 in IGF2 RNA expression as well as an increase in CTCF protein level when compared with lentivirus-GFP-infected cells (Fig. 6). QPCR demonstrated a reproducible increase 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-
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

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.

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.
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 pleotropic” 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 imprinting. 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 indicates 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-
ular 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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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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