Increased intragenic IGF2 methylation is associated with repression of insulator activity and elevated expression in serous ovarian carcinoma

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INTRODUCTION

Insulin-like growth factor-II (IGF2) encodes a potent autocrine and paracrine mitogen that is important to early growth and development (Hoyo et al., 2012; St-Pierre et al., 2012), but is also often deregulated in cancers, including colon (Cui et al., 2003) and ovarian cancers (Sayer et al., 2005; Lu et al., 2006; Murphy et al., 2006; Dammann et al., 2010; Qian et al., 2011) where it has been shown to modulate taxol resistance (Huang et al., 2010). The H19 gene is located adjacent to IGF2 and produces a long non-coding RNA molecule whose function is not completely understood. Both genes are regulated by genomic imprinting, resulting in the production of IGF2 transcripts from the paternally derived chromosome and H19 RNAs from the maternally derived chromosome. Epigenetic marks dictate this pattern of expression, and are established during gametogenesis in a process that involves the differential marking of the parental chromosomes with DNA methylation. These marks are then “read” such that expression occurs from only one of the two parental alleles, and this pattern of expression and epigenetic marking is stably transmitted throughout somatic cell division.

Prior studies have determined that a region upstream of the H19 promoter is methylated on the paternally derived chromosome, while the maternally derived chromosome is hypomethylated. Within this differentially methylated domain, referred to as the imprint control region (ICR), are multiple binding sites for the CTCF binding factor (CTCF) protein. CTCF is a highly conserved zinc finger protein that binds to genomic DNA at numerous sites throughout the genome (Sanyal et al., 2012; Wang et al., 2012) and has played a key role in the evolutionary diversity of metazoans (Heger et al., 2012). One of the functions of CTCF is the remodeling of chromatin structure to form insulator elements (Filippova, 2008), and CTCF has been shown to co-localize with cohesion in the control of chromatin architecture and gene regulation (Lee and Iyer, 2012). The importance of CTCF binding to genomic
imprinting was demonstrated by several groups who showed that CTCF binds the unmethylated maternal chromosome, upstream of the promoter region of the H19 gene within the ICR. CTCF binding prevents enhancers downstream from H19 from activating IGF2 on the maternal chromosome, while the methylation present on the paternally derived chromosome prevents CTCF binding. IGF2 transcription is thus positively influenced by the unencumbered cis-acting enhancer elements (Wolffe, 2000).

Prior studies, including our own, have identified a consensus binding motif, 5’-CCGCNNGNNGC-3’ for the CTCF protein (Wylie et al., 2000; Rosa et al., 2005). We identified a sequence within IGF2 intron 3 that matches this consensus motif, and our preliminary analysis showed a strong relationship between methylation of this putative binding site and expression of IGF2 in ovarian cancer tissues. Herein we examined the methylation status of this site, including parental origin, and show that this novel consensus sequence binds to CTCF and mechanistically functions as an insulator element in ovarian cancer cells.

MATERIALS AND METHODS

SPECIMENS
De-identified primary ovarian cancer tissues for this study were obtained from the Duke Gynecologic Oncology Tissue Bank (DCOTB) under a protocol approved by the Duke University Institutional Review Board. The DCOTB collects and banks specimens for research purposes following acquisition of informed consent from patients undergoing surgery for epithelial ovarian cancer under a separate protocol approved by the Duke University Institutional Review Board. Surgical specimens were processed immediately after removal from the patient and stored at −80°C. Human spermatozoa were from the Duke Division of Reproductive Endocrinology and Fertility and used under a protocol approved by the Duke University Institutional Review Board. Conceptual tissues were provided and stored at −80°C for 3 min, 35 cycles of 94°C for 30 s, 62°C for 45 s, with a final 5 min extension at 72°C. The amplicons were resolved by agarose gel electrophoresis, excised, and purified using Sigma GenElute Spin columns (Sigma Aldrich). Bisulfite sequencing was performed using the Radiolabeled Terminator Cycle Sequencing Kit (USB Corporation) according to the manufacturer’s directions. Sequencing products were resolved by denaturing polyacrylamide gel electrophoresis and the gels were dried under vacuum followed.

Table 1 | Oligonucleotide primers.

| Primer | Application | Sequence (5’–3’) |
|--------|-------------|-----------------|
| Int3-IGF2-BS-F | Bisulfite sequencing | GTTTTTTTGTGTGGGATTAGAGAG |
| Int3-IGF2-BS-R | Bisulfite sequencing | AACCACACAAAAACAAAAAAACATC |
| Int3-IGF2-R-seq | Bisulfite sequencing | CAAACCCACCTACCTCCCTTG |
| Int3-CTCF3-PS-F | Pyrosequencing | TGGGAAAGTTTTGTGGTGTGTTAGT |
| Int3-CTCF3-PS-R | Pyrosequencing | Biotin-ACCTCTACAACCCAAAC CCAAACCTT |
| Int3-CTCF3-PS-seq | Pyrosequencing | GGAAGTTTTTTGGTTTGTAAGT |
| Int3-CBS-chip-F | Chromatin IP | ACTCACTCCCTCCTCAC |
| Int3-CBS-chip-R | Chromatin IP | AGCAAACACCCCCGTCG |
| MYC-chip-F | Chromatin IP | CCTGAAAGAATAAACAGAGAG |
| MYC-chip-R | Chromatin IP | GTGGCGTGGAAACTTCG |
| Nhel-CTCFF2 | Luciferase reporter | GCAAATTTACTCTGCTTCAC |
| R2-CTCF-Xhol | Luciferase reporter | ATATAGTCGACCATCCCA |
| GAPDH-Smal-F | Luciferase reporter | AAAGCTCGAGAGAGAGGAG |
| GAPDH-BglII-R | Luciferase reporter | TAATTGCAAGATTAAGAGCA |

DNA EXTRACTION

Tissue culture cells were washed with 1× PBS and collected after they reached 70–80% confluence. Frozen tumor tissues were homogenized using a FastPrep SP120 Homogenizer (Bio101 Thermo Savant; Logan, UT) and collected into microcentrifuge tubes. DNA was extracted using Gentra Puregene Reagents with protocols provided by the manufacturer specific to the sample type (Qiagen, Valencia, CA, USA). Quality and quantity of extracted DNA was evaluated using a Nanodrop 1000 spectrophotometer (Thermo Scientific; Wilmington, DE, USA). Aliquots were prepared and stored at −80°C. The A260/A280 absorbance ratios were >1.8 for all DNA used.

METHYLATION ANALYSIS

Two methods were used for CpG methylation analysis of the IGF2 intragenic region. Quantitative radiolabeled bisulfite sequencing was used for frozen ovarian cancer tissues. Because of the manufacturer’s permanent discontinuation of the radiolabeled isotope used for quantitative bisulfite sequencing, bisulfite pyrosequencing was used for all other analyses.

Bisulfite sequencing

Genomic DNA (1 μg) was modified with sodium bisulfite as previously described (Murphy et al., 2003) and eluted in 25 μl of nuclease-free water. Forty nanograms of the bisulfite modified DNA (assuming complete recovery) was used in a 25 μl PCR reaction that included 3 mM MgCl2, 1 mM each dNTP, and 0.4 μM each primer with 1.25 μ Platinum Taq (Invitrogen; Carlsbad, CA, USA). A 290 bp PCR product specific to intron 3 of IGF2 was generated with primers listed in Table 1. PCR conditions were 94°C for 3 min, 35 cycles of 94°C for 30 s, 62°C for 45 s, with a final 5 min extension at 72°C. Sequencing products were resolved by denaturing polyacrylamide gel electrophoresis and the gels were dried under vacuum followed.
by exposure to a storage phosphor screen. Methylation was quantified using the Molecular Dynamics Storm Phosphorimaging System and ImageQuant software (GE Healthcare; Pittsburgh, PA, USA). The percent cytosine methylation was calculated as follows: \[ \frac{C^M}{C^M + C^U} \times 100 \], where \( C^M \) is the measurement for cytosine at a given CpG dinucleotide position and \( C^U \) is the measurement for thymine at the same position of the cytosine base (which reflects unmethylated cytosine after bisulfite conversion and PCR amplification).

**Bisulfite pyrosequencing**

Genomic DNA (800 ng) was modified with sodium bisulfite using the Zymo EZ DNA methylation kit (Zymo Research Corporation, Irvine, CA, USA). For pyrosequencing of the intron 3 region of \( IGF2 \), we amplified bisulfite modified DNA using 0.12 \( \mu \)M of each primer (all primers are listed in Table 1) in a 25 \( \mu \)l reaction containing 40 ng of bisulfite modified template DNA, 1.5 \( mM \) \( MgCl_2 \) and using the PyroMark PCR Kit (Qiagen; Valencia, CA, USA). Cycling conditions were 95°C for 15 min, then 55 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s, followed by 10 min at 72°C. Pyrosequencing was performed using PyroMark Gold Q96 Reagents according to the manufacturer’s recommendations (Qiagen). The sequence analyzed includes two CpG dinucleotides; one located within the consensus CTCF binding motif and a second CpG outside the consensus motif (5’-gaccCGcagggtggc-3’; consensus underlined).

To validate the pyrosequencing assay, we used plasmids containing bisulfite modified methylated and unmethylated copies of the region analyzed, as previously described (Wong et al., 2006; Murphy et al., 2012). The plasmid DNAs were quantified, defined mixtures prepared, and run in quadruplicate. Pyrosequencing assay design and validation for the \( IGF2 \) DMR was as described previously (Murphy et al., 2012).

**RNA ANALYSES**

Cells were cultured until 70–80% confluent and washed with cold PBS. Total RNA was isolated from these cells and from frozen human conceptual tissues with RNA Stat-60 reagent (Amsbio, Lake Forest, CA, USA). One microgram was reverse transcribed using Superscript II according to the manufacturer’s protocol (Invitrogen). The resulting cDNA was amplified using a TaqMan assay for \( IGF2 \) expression (Assay ID: Hs04188276_m1) with a Rotor-Gene Q Real-Time PCR thermocycler (Qiagen). Beta-2-Microglobulin (B2M) (Assay ID: Hs00187842_m1) was used as a control for input. Expression values were determined using the delta delta \( C_t \) method. \( IGF2 \) mRNA expression values from cell lines were obtained from our previously published Affymetrix U133A microarray data (Gene Expression Omnibus Accession GSE25428) and expression values were validated using quantitative real-time RT-PCR.

**PROTEIN ANALYSIS**

Western blotting was performed to determine the endogenous levels of CTCF protein in the ovarian cancer cell lines. Briefly, 30 \( \mu \)g of total cell lysate was obtained using cell lysis buffer from cells that were cultured to 70–80% confluence (Cell Signaling). Antibodies used were anti-CTCF (Millipore #07-729, diluted 1:2000) and anti-α-Tubulin (Millipore #05-829, diluted 1:200). The anti-mouse HRP conjugated secondary antibody (Bio-Rad; Hercules, CA, USA) was used at a dilution of 1:3000 and the proteins were detected with the ECL kit (GE Healthcare) following the manufacturer’s instructions.

**CHROMATIN IMMUNOPRECIPITATION**

Chromatin Immunoprecipitation (ChIP) was performed with the ChIP-It kit (Active Motif; Carlsbad, CA, USA) using 25 \( \mu \)g of sheared chromatin per IP and 2 \( \mu \)g of each antibody. The rabbit anti-human CTCF polyclonal antibody used was from Millipore (#07-729), the anti-Brother Of The Regulator of Imprinted Sites (BORIS) antibody was from Abcam (Ab18337; Cambridge, MA, USA) as well as the rabbit IgG control antibody (ab37415). Real-time PCR was performed using SYBR green reagents (Qiagen) in an Applied Biosystems 7900HT Real-Time PCR System. Primers are listed in Table 1. The 5’-insulator site in the \( c \)-MYC gene constitutively binds CTCF and was used as a positive control for CTCF binding as previously described (Pugacheva et al., 2005).

**INSULATOR ASSAYS**

The DNA fragment containing the intron 3 CTCF consensus sequence was PCR amplified using the primers listed in Table 1. The product was cloned into the pGL3-control Luciferase Reporter Vector (pGL3-P-control; Promega, Madison, WI, USA) using the Nhe1 and Xho1 restriction sites. This plasmid contains the luciferase gene under control of the SV40 promoter with SV40 enhancer sequences located downstream. The multi-cloning site is upstream of the SV40 promoter region. A sequence fragment from within the \( GAPDH \) locus was also used for comparative purposes (see Table 1). This fragment was cloned into the pGL3-P-control using SmaI and BgII restriction sites. The recombinant plasmids were confirmed by restriction digestion and by nucleotide sequencing. Constructs were named pGL3 (vector only), pGL3-CTCF, and pGL3-GAPDH. HEY, CAOV2, and OVCAR2 cells were grown to 70–80% confluency and transfected with the various pGL3 plasmid constructs using FuGENE®HD reagent according to the manufacturer’s protocol (Promega, Madison, WI, USA). Luciferase activity was measured 48 h and 72 h post-transfection in sextuplicate according to the protocol from the manufacturer (Promega). Luciferase activity was normalized for cell number.

**STATISTICAL ANALYSIS**

All statistical analyses were performed using Prism 5 for Macintosh (Graphpad Software, Inc.) with \( p \) values less than 0.05 considered significant.

**RESULTS**

**IDENTIFICATION OF A CTCF BINDING MOTIF IN IGF2 INTRON 3**

The genomic structure of \( IGF2 \) is presented in Figure 1. Given the importance of CTCF binding upstream of the \( H19 \) promoter to the imprinting and expression of \( IGF2 \) and \( H19 \) and the ubiquity of \( IGF2 \) overexpression in serous epithelial ovarian cancer (Murphy et al., 2006), we wished to determine if there are other CTCF binding sites within the \( IGF2/H19 \) imprinted domain that contribute to regulation of \( IGF2 \) in this disease. By searching the genomic sequence, we identified a binding motif within \( IGF2 \).
intron 3 having sequence matching that of the consensus motif for CTCF binding (5′-CCGCNNGGNGGC-3′) (Wylie et al., 2000; Rosa et al., 2005). Remarkably, the position of this motif is ∼350 bp downstream of an inverted repeat element within the same intron, in a region that was identified as being conserved among mammals that exhibit imprinted IGF2 expression (Weidman et al., 2004). The inverted repeat has subsequently been found to encode a microRNA, MIR483 (NR_030158.1) that has been implicated in angiogenesis, oncogenicity, and adipose tissue programming (Veronese et al., 2010; Qiao et al., 2011; Ferland-McCollough et al., 2012; Ma et al., 2012).

**INTRAGENIC IGF2 METHYLATION CORRELATES WITH EXPRESSION**

To determine if IGF2 expression levels are associated with intragenic methylation of IGF2 at this region, we analyzed 35 primary serous epithelial OVCA specimens using quantitative radiolabeled bisulfite sequencing. We found highly variable methylation at the region adjacent to and within the IGF2 intron 3 consensus CTCF binding motif. Methylation of two CpGs, including one within the core binding motif, was higher in 18 OVCAs with elevated IGF2 expression versus 10 with low expression (average 68.2% versus 38.5%; p < 0.0001) (Figure 2A). We also examined the relationship between expression and methylation in a panel of tissues from a 108 day human female conceptus, and found that most tissues with higher methylation also had higher IGF2 expression (gut, liver, umbilical cord, brain, pancreas and heart versus thymus, decidua, placenta, spleen, muscle, and kidney; p = 0.03 for t test between these groups; data presented in Figure 2B is by individual tissue for clarity). These results support that the methylation status of these intronic CpG dinucleotides is positively associated with IGF2 expression and may therefore be involved in regulating levels of IGF2.

**GERMLINE METHYLATION**

The CpG dinucleotides within the CTCF binding motifs of the IGF2/H19 ICR are methylated on the paternal chromosome and this methylation profile is established during gametogenesis. Because the ICR and intron 3 CpGs are located greater than 100 kb apart (Figure 1), we analyzed the IGF2 intron 3 CpG sites in 16 independent human spermatozoa specimens to determine if these CpGs are also methylated in sperm. These and subsequent analyses were performed by bisulfite pyrosequencing after validating the linear performance of the assay using defined mixtures of fully methylated and unmethylated DNAs (input versus measured methylation $R^2 = 0.99$; Figure 3). We found that the intron 3 CpG sites exhibited high levels of methylation in sperm from all 16 individuals (Figure 4A; results from replicate measures shown). However, comparison of the two intron 3 CpGs revealed significant differences in methylation levels despite being located just 13 bp apart (CpG 1: mean 93.2%, SD = 1.7%; CpG 2: 79.7%, SD = 1.4%; paired t test p < 0.0001). Human fetal liver tissue run in parallel was 58.9 and 44.0% methylated at these CpG sites, respectively.

Analysis of human conceptual tissues from up to nine individuals showed intermediate levels of methylation at these same sites.

**FIGURE 1 | Schematic of the IGF2/H19 imprinted domain.** The IGF2/H19 imprinted domain is located at chromosome 11p15.5 and encompasses ∼170 kb. The insets show the genomic structure of the IGF2 gene (middle) and H19 gene (bottom), adapted from the UCSC Genome Browser, GRCh37/hg19 (Kent et al., 2002). Green ovals, CpG islands; yellow ovals, CTCF binding sites; tall vertical lines/boxes, coding sequence; short vertical lines/boxes, non-coding sequence. The promoters with direction of transcription are indicated by arrows. The IGF2 locus also produces a paternally expressed antisense transcript (IGF2AS), indicated by the purple lines/boxes. The IGF2 intron 3 CTCF binding motif is shown as underlined sequence with the CpG dinucleotides analyzed herein in capital letters. DMR, differentially methylated region; ICR, imprint control region.
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FIGURE 2 | IGF2 intragenic methylation and IGF2 transcription.
(A) Methylation of the CpG within the IGF2 intron 3 CTCF binding motif (CpG 1) and adjacent CpG (CpG 2) in invasive serous ovarian cancers with very low IGF2 expression (Affymetrix U133A MAS5 values between 9 and 71, average = 26; labeled as “low”) compared to those with higher expression (MAS5 values between 128 and 24,056, average = 5,343; labeled as “high”).
(B) IGF2 expression shown as a bar graph (+SD for replicate measures; left y axis) as detected in tissues from a 108-day gestation human female conceptus with the corresponding level of methylation, given as a percentage, at the CpG within the intron 3 CTCF binding motif (right y axis, circles ±SD of replicate measures). The data are arranged from left to right in increasing order of IGF2 expression.

FIGURE 3 | Validation of the IGF2 intron 3 region pyrosequencing assay. Methylation was measured for both CpG dinucleotides within the intron 3 region under study and averaged (±SD for triplicate measures) for defined mixtures of plasmids (x axis) containing the bisulfite modified methylated and unmethylated intron 3 region sequences.

(FIGURE 4B: range 36.3–72.6%), suggesting that the methylation in somatic tissues may be parent-of-origin-dependent, although this was not formally determined in this study. We also analyzed the IGF2 DMR in the same spermatooza specimens. The IGF2 DMR and P2–P4 promoter region have previously been reported as methylated on the maternally derived chromosome (Issa et al., 1996; Sullivan et al., 1999), although subsequent studies have found that methylation is present on the paternally derived chromosome (Murrell et al., 2008). We observed that the IGF2 DMR is highly methylated in sperm (mean, 93.6%; SD = 3.3%; N = 16; not shown), indicating that this region is also a paternal germline methylation imprint, and together indicating that the entire IGF2/H19 imprinted domain may acquire the paternal methylation marks present in somatic tissues during spermatogenesis.

CTCF BINDING WITHIN IGF2 INTRON 3
We performed mechanistic analyses of the intragenic CTCF binding site in three ovarian cancer cell lines, including HEY, OVCA2, and CAOV2. HEY cells have the lowest IGF2 transcript levels, as do many other established ovarian cancer cell lines (Matsumura et al., 2011), while the other two lines exhibit higher IGF2 expression (FIGURE 5A). By pyrosequencing, methylation of the intron 3 region was lowest (66%) in the HEY cells, while the other lines showed >85% methylation at these CpG dinucleotides (FIGURE 5B).

Chromatin immunoprecipitation was used to determine if CTCF binds to the region containing the consensus CTCF binding motif. As a positive control, the anti-CTCF antibody was used to test for binding at the MYC locus, known to have high CTCF binding activity (Pugacheva et al., 2005). ChIP results showed enrichment of CTCF binding at the MYC CTCF binding site relative to the no antibody control (FIGURE 5C).

We next examined CTCF binding to the putative CTCF consensus binding sequence within IGF2. Although CAOV2 and OVCA2 were obtained from different sources, our testing showed that they are genetically identical (Korch et al., 2012). Despite this, CAOV2 has much higher endogenous CTCF mRNA transcript levels (not shown) and protein expression as measured by Western blotting than OVCA2 (FIGURE 5D), providing the unique opportunity to evaluate the role of differential CTCF expression in these otherwise genetically identical cells. OVCA2 cells did not exhibit enriched binding of CTCF to the intron 3 CTCF binding motif relative to the control, while CAOV2 and HEY showed ~2.2 to 3.8-fold enrichment of CTCF binding (FIGURE 5E).

BORIS BINDING AT THE IGF2 INTRON 3 CTCF SITE
We also determined if the BORIS protein (encoded by CTCFL) binds to the CTCF motif. BORIS and CTCF are paralogs that have the same binding specificity, with BORIS normally expressed during spermatogenesis. BORIS has been shown to become deregulated in cancers, and to compete with CTCF for binding occupancy.


**FIGURE 4 | Characterization of IGF2 intron 3 CTCF binding motif methylation in spermatozoa and human fetal tissues.** (A) Measurement of % methylation (y axis) for both CpG dinucleotides (CpG Site 1, CpG within the CTCF binding motif; CpG Site 2, CpG adjacent to the CTCF binding motif) in 16 individual human spermatozoa specimens (1–16 on x axis) and in human fetal liver (FL). The results from each of two assays are shown. (B) Box and whisker plot showing average percent methylation for the two CpG dinucleotides in the IGF2 intron 3 region across a panel of human conceptual tissues (boxes, 10th to 90th percentile). The number of individuals analyzed for each tissue type is indicated.

(Klenova et al., 2002). Indeed, by ChIP, BORIS was enriched at the intron 3 CTCF site in HEY and OVCAR2 (in which CTCF does not bind) but BORIS was not enriched at the intron 3 CTCF site in CAOV2 cells (Figure 5F).

**REPRESSOR/INSULATOR ACTIVITY OF IGF2 INTRON 3**

We used a luciferase reporter assay to determine how the presence of the IGF2 intron 3 CTCF binding motif influences transcriptional activity. We tested three constructs, including the vector alone, the vector containing the CTCF binding motif, and the vector containing an intronic sequence from within the GAPDH locus with similar nucleotide composition but that does not contain a consensus CTCF binding motif. Luciferase activity was normalized to that obtained for the pGL3 vector alone.

In all three of the cell lines, the presence of the GAPDH sequence fragment was not inhibitory to transcription, with increased luciferase activity relative to the vector control (Figure 6). In the OVCAR2 and CAOV2 lines that have no or relatively low CTCF binding activity, respectively, the presence of the IGF2 intron 3 CTCF binding region reduced luciferase activity, suggesting that this sequence element may function as an insulator or repressor in these cells. In the HEY cells, which have the highest level of CTCF binding activity, lowest IGF2 transcription, and lowest methylation among the three lines tested, the CTCF region was not inhibitory to transcription.

**DISCUSSION**

We have identified a novel consensus binding sequence for the CTCF protein within an intron of IGF2. The IGF2/H19 imprinted domain contains an intensively studied cluster of CTCF binding sites that are located upstream from the H19 promoter region. Hypermethylation of the paternally derived allele and hypomethylation of the maternally derived allele at this ICR is required for the appropriate establishment of imprinted expression in mice, and CTCF binding to the hypomethylated maternally derived chromosome maintains the unmethylated state of this allele in somatic tissues (Schoenherr et al., 2003; Szabo et al., 2004). CTCF binding to the orthologous region in humans may not be as critical in regulating expression of IGF2 or H19 (Ulaner et al., 2003).

We had previously shown a positive correlation with methylation at the IGF2/H19 ICR and IGF2 expression that was not related to loss of imprinting (Murphy et al., 2006). In the present study, we also determined that there is a significant positive correlation between methylation of the intron 3 CpG sites and IGF2 transcription in serous epithelial ovarian cancers. Our assay measured methylation of two CpG dinucleotides, including one within the CTCF consensus binding sequence and another located 13 bp away, just outside the consensus sequence. The CpG within the CTCF binding motif showed consistently higher methylation than the one positioned outside the binding motif in all tissues analyzed, but the significance of this observation is presently unclear.
Regardless of the difference in methylation between the two sites within a given tissue, they both showed higher methylation in normal tissues and tumors with higher IGF2 transcript levels, suggesting that this region may contribute to regulating IGF2 activity.

A hallmark of imprinted genes is parental allele-specific methylation that is established during gametogenesis. Methylation at the IGF2/H19 ICR is present on the paternally derived chromosome and is one of only several regions in the human genome known to contain a paternally derived gametic imprint mark. Most of the methylation associated with genomically imprinted genes is established on maternally derived chromosomes. We analyzed 16 independent human spermatozoa specimens to determine if the methylation at the intron 3 region of IGF2 was established during spermatogenesis. The spermatozoa all showed very high methylation of these CpG sites, indicating that the methylation is established during spermatogenesis, and consistent with the idea that these CpG sites may also be a component of the gametic imprint mark for the IGF2/H19 domain. Further supporting this notion was the finding that the IGF2 DMR is also highly methylated in the spermatozoa, in agreement with the methylation findings in sperm for this region from another study of infertility (Boissonnas et al., 2010). In human conceptual tissues, we found that the intron 3 region had mean methylation values ranging from 39.1% (placenta) to 66.1% (heart). These findings suggest that this region may maintain parent-of-origin-dependent methylation in somatic tissues.

The consensus CTCF sites at the human ICR each harbor four CpG dinucleotides, while the novel CTCF site in IGF2 intron 3 analyzed herein contains a single CpG dinucleotide. CTCF is known to bind to the ICR in a methylation-sensitive manner (Hark et al., 2000; Kanduri et al., 2000). CTCF binding induces a change in chromatin structure that results in insulator activity. Insulation at the ICR on the unmethylated maternal chromosome creates a blockade preventing interaction between the IGF2 promoters from enhancers downstream of H19, thus contributing to IGF2 repression on the maternal chromosome (Bell et al., 1999; Bell and Felsenfeld, 2000; Kanduri et al., 2000). This regulatory activity may also involve intrachromosomal interactions within the domain as well as interactions with matrix attachment regions (Kurukuti et al., 2006). The methylation status of the intron 3 CTCF consensus sequence and correlation with expression of IGF2 in ovarian cancer prompted us to hypothesize that methylation status may modulate binding of CTCF and that this in turn is influencing transcriptional activity of IGF2. Our results suggest that methylation of the single CpG dinucleotide within the IGF2 intron 3 CTCF binding motif may be inhibitory to CTCF binding.

The CTCF paralog, BORIS, has identical zinc fingers and DNA binding specificity as CTCF, but the sequences diverge outside the zinc fingers, conferring differences in regulatory capacities. BORIS was reported to not be detectable in most normal tissues due to exclusive expression of BORIS in the testis (Loukinov et al., 2002). More recently, however, others have reported that BORIS is
In ovarian cancer cells, the insulator activity of the IGF2 intron 3 CTCF binding motif was assessed using a luciferase reporter assay. The presence of the IGF2 intron 3 CTCF binding motif had no effect on luciferase transcription in HEY cells, while it was inhibitory to transcription in both OVCAR2 and CAOV2 cells. In contrast, HEY cells with virtually no expression of IGF2 mRNA and the lowest methylation of the intron 3 region showed enrichment of both CTCF and BORIS binding. Our results demonstrate that CTCF and BORIS are both able to bind the IGF2 intron 3 CTCF binding motif and that this binding is associated with differences in methylation and expression.

Results from a luciferase reporter assay showed that the presence of the IGF2 intron 3 CTCF binding motif had no effect on luciferase transcription in HEY cells, while it was inhibitory to transcription in both OVCAR2 and CAOV2 cells. Taken together, our results are perhaps most parsimonious with the idea that methylation of the intron 3 CTCF binding motif is inhibitory to CTCF/BORIS binding at the endogenous locus but permissive for binding in the context of the (presumed unmethylated) reporter construct. Furthermore, these results suggest that this region indeed has insulator function when unmethylated and bound to CTCF or BORIS, as is the case for the reporter construct in the CAOV2 and OVCAR2 cells, respectively. In the HEY cells, CTCF and BORIS may compete with each other for binding the intron 3 CTCF binding motif in the reporter construct with a net null outcome. These results contrast with those observed at the endogenous locus in these same cells, which differ at least in part by methylation status. The CAOV2 and OVCAR2 cells have high methylation at the intron 3 CTCF binding motif and express IGF2 at high levels, indicative of at least partial inhibition of CTCF or BORIS binding and low insulator activity function. In contrast, HEY cells have lower methylation, do not express IGF2 and exhibit binding of both CTCF and BORIS to the intron 3 CTCF binding motif. These results support the idea that lower levels of methylation are permissive for CTCF/BORIS binding, which leads to insulation of the IGF2 promoter(s) from enhancer activity. This is in agreement with the findings in vivo, where high levels of methylation at the intron 3 CTCF binding motif are correlated with higher levels of IGF2 transcription in both normal and malignant tissues. Indeed, mouse studies have shown that paternal methylation at an intragenic differentially methylated region of Igf2 (referred to as DMR2) is associated with increased transcription (Murrell et al., 2001). However, unlike the human IGF2 intron 3 CTCF binding motif analyzed here, the core Igf2 DMR2 region in the mouse is located within an exon and is densely populated with CpG dinucleotides.

A limitation of our study is that we did not measure the binding of CTCF or BORIS within the reporter construct, so we do not know if the results obtained are indeed consequent to binding of the endogenous proteins to the reporter construct. Furthermore, it was not possible to directly test how methylation of the CpG sites within and adjacent to the intron 3 CTCF motif
inserted into the reporter construct might have influenced protein binding and luciferase activity. It is possible that binding of these proteins at the endogenous locus in CAOV2 or OVCAR2 occurs only on the 10–15% of alleles within the cell population lacking methylation. Our results show enrichment of binding of CTCF or BORIS in CAOV2 and OVCAR2 cells, respectively, but do not address what proportion of the alleles are actually bound by these proteins.

In conclusion, elevated IGF2 expression in epithelial ovarian cancers is associated with increased methylation of a novel regulatory sequence motif that we have shown binds to the CTCF protein. The growth-stimulatory function of IGF2 likely confers a substantial advantage to malignant cells, given the ubiquity of IGF2 overexpression in ovarian cancer. Elucidation of the mechanisms that control IGF2 transcription is necessary if we hope to target this gene therapeutically. Positive correlation between methylation and IGF2 transcription in primary ovarian cancers suggests the potential for epigenetic therapies in controlling IGF2 overexpression in this disease.

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