Correction of aberrant imprinting of *IGF2* in human tumors by nuclear transfer-induced epigenetic reprogramming

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Loss of genomic imprinting of insulin-like growth factor II (*IGF2*) is a hallmark of many human neoplasms. We attempted to correct this aberrant epigenotype by transferring nuclei from human tumor cells that showed loss of *IGF2* imprinting into enucleated mouse and human fibroblasts that had maintained normal *IGF2* imprinting. After nuclear transfer, the abnormal biallelic expression of *IGF2* in tumor nuclei transiently converted to normal monoallelic imprinted expression in the reconstructed diploid cells. In tetraploid hybrid cells, however, normal *IGF2* imprinting was permanently restored in the tumor genome. Inhibition of the synthesis of putative *trans* imprinting factors with cycloheximide led to loss of *IGF2* imprinting in normal cultured fibroblasts, suggesting that normal cells produce proteins that act in *trans* to induce or maintain genomic imprinting. These data demonstrate that an abnormal tumor epigenotype can be corrected by *in vitro* reprogramming, and suggest that loss of imprinting is associated with the loss of activity of non-CTCF *trans* imprinting factor(s) that are either inactivated or mutated in tumors.

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Introduction

Insulin-like growth factor II (*IGF2*) is an imprinted gene that is expressed only from the paternal allele in a tissue-specific, promoter-specific, and development-specific manner (DeChiara *et al*, 1990; Hu *et al*, 1995; Szabo and Mann, 1995). However, *IGF2* imprinting is lost in a host of human neoplasms, resulting in biallelic *IGF2* expression (Feinberg, 1993; Ogawa *et al*, 1993; Rainier *et al*, 1993) and abnormally high *IGF2* production. Biallelic *IGF2* expression is as an early event in tumorigenesis both in animal models (Christofori *et al*, 1994) and in human studies (Okamoto *et al*, 1997; Cui *et al*, 1998). Development of β -cell carcinomas in transgenic mice carrying the insulin regulatory region/ST-40 large T-antigen requires the abnormal activation of the maternal *Igf2* allele as the second signal in the tumor pathway (Christofori *et al*, 1994). Biallelic activation of *Igf2* coincides with the switch to the hyperproliferative stage in preneoplastic foci and tumors (Christofori *et al*, 1995), while homologous deletion of the *Igf2* gene significantly reduces tumor burden (Christofori *et al*, 1994; Haddad and Held, 1997). We demonstrated that transcriptional suppression of *IGF2* by targeted methylated hairpin oligonucleotides reduced the growth of implanted human hepatocarcinomas in mice and prolonged lifespan in those animals (Yao *et al*, 2003). Feinberg and co-workers have shown that loss of *IGF2* imprinting in peripheral blood leukocytes may provide a potential biomarker in diagnosing individuals with high risk of colorectal cancer (Cui *et al*, 2003), and that tumor phenotype in *Apc* + /*Min* mice can be modified simply by altering *IGF2* epigenotype (Sakatani *et al*, 2005), reinforcing the concept that *IGF2* plays a role in cancer. While loss of *IGF2* imprinting is a hallmark of tumorigenesis in a variety of human malignancies, including hepatoma, lung cancer, breast cancer, colorectal cancer, leiomyosarcoma, osteosarcoma, leukemia, and Wilms' tumor, the molecular mechanisms underlying this epigenetic change have not been elucidated.

In the mouse, *Igf2* imprinting is regulated by parent-specific epigenetic modifications in the differentially methylated regions (DMRs) of the *Igf2/H19* imprinting domain located on chromosome 7 (Razin and Cedar, 1994; Reik *et al*, 2000; Arney, 2003). An imprinting control region (ICR) that is located between the *Igf2* and *H19* contains four CTCF binding sites that are differentially methylated according to their parental origin (Bell and Felsenfeld, 2000; Hark *et al*, 2000). The methylated paternal allele blocks the binding of CTCF and allows access of two enhancers located downstream of *H19* to the *Igf2* promoters, permitting transcription of *Igf2* and resulting in the silencing of *H19* from the paternal allele. The unmethylated maternal allele, however, binds CTCF, which then insulates the enhancers from interacting with *Igf2*. As a result, *Igf2* is suppressed and *H19* is transcribed from the maternal allele. When this ICR DMR is deleted or mutated, the normally suppressed maternal *Igf2* allele is expressed, leading to biallelic expression.

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In human tumors, however, the regulation of *IGF2* appears to be much more complicated, as loss of *IGF2* imprinting (LOI) is not necessarily linked to, and may be independent of, epigenetic marks in the various DMRs, including the ICR. In some cases, *IGF2* LOI is accompanied by alterations of DNA methylation at CTCF binding sites; however, in other tumors, *IGF2* loss of imprinting persists even when the ICR maintains its normally differentially methylated state. In some tumors, persistent *IGF2* imprinting is accompanied by abnormal epigenetic modifications, for example, hypomethylation or hypermethylation, at CTCF binding sites. Furthermore, aberrant imprinting of *IGF2* is not necessarily linked with *H19* allelic expression. In some cases, the genes are coordinately expressed with maternal *IGF2* and paternal *H19* imprinting remaining intact, while in other tumors, *IGF2* can be biallelically or monoallelically expressed, independent of the imprinting status of *H19* (Cui *et al*, 2002; Ulaner *et al*, 2003a). These data suggest that other mechanisms in addition to epigenetic modifications of the CTCF binding sites in the ICR are involved in the control of *IGF2* imprinting in tumors.

In this communication, we tested an innovative concept that dysregulated *IGF2* imprinting in tumor cells can be corrected or normalized by 'epigenetic reprogramming' following nuclear transfer, a technique that has been successfully used to reprogram somatic cells in animal cloning. We further attempted to examine whether aberrant *IGF2* imprinting in tumors was corrected by supplementation of the missing *trans* imprinting factor(s) in tumors and/or of the repair of abnormal epigenetic modifications of *cis* elements in ICRs.

Results

Nuclear transfer-induced correction of aberrant *IGF2* imprinting in tumor cells

We first tested whether the abnormal *IGF2* epigenotype in tumor cells could be corrected by *in vitro* epigenetic reprogramming. We reasoned that the loss of imprinting in tumors

could be reversed when nuclei from tumor cells were transferred into a cellular environment where *IGF2* imprinting was normally maintained. The intact *IGF2* imprinting system in normal cytoplasm would correct aberrant imprinting either by providing *trans* imprinting factors that were missing in tumor cells or by altering the existing epigenetic modifications in ICRs (Figure 1).

We employed nuclear transfer techniques that have been successfully used to reprogram the tumor epigenome (Tada *et al*, 1997; Li *et al*, 2003; Hochedlinger *et al*, 2004) in order to examine whether normal fibroblast cytoplasm could correct aberrant *IGF2* imprinting in a series of human tumor cell lines. We were particularly interested in examining whether restoration of *IGF2* imprinting would be accompanied by changes in epigenetic signals in these tumor cells, as has been reported in a mouse tumor model (Bell and Felsenfeld, 2000; Hark *et al*, 2000).

Two normal fibroblast cell lines, HBF1 (human) and MBW2 (mouse), that demonstrated maintenance of *IGF2* imprinting (*IGF2* MOI), served as host cell lines in which to reprogram the tumor nuclei. Four human tumor cell lines that showed loss of *IGF2* imprinting (*IGF2* LOI) with distinct DNA methylation patterns at the CTCF binding region and at DMR₀ (Table I) were used to obtain nuclei for transfer into the normal fibroblasts. The human *IGF2/H19* ICR contains seven differentially methylated binding sites that are normally methylated only on the maternal allele, while DMR₀ is normally methylated on the paternal allele. In the first group of cell lines (WTCL and SKNEP), methylation in the ICR is the same as is seen in normal fibroblasts (HBF1 and MBW2), while DMR₀ is hypomethylated compared to normal cells. In the H522 cell line, both the ICR and DMR₀ sites are hypomethylated. Finally, HRT18 cells maintain normal methylation at the ICR DMR, but they are hypermethylated at DMR₀. The fact that the epigenetic configuration of these cell lines varies despite the common epigenetic read-out of *IGF2* LOI indicates that site-specific DNA methylation alone cannot be the sole regulator of imprinting. Comparison of the

Figure 1 Schematic diagram of correction of aberrant *IGF2* imprinting by nuclear transfer-induced epigenetic reprogramming. The nuclei of tumor cells with loss of *IGF2* imprinting (*IGF2* LOI) were transferred into enucleated HBF1 and MBW2 fibroblasts that had maintenance of *IGF2* imprinting (MOI). After epigenetic reprogramming, aberrant *IGF2* imprinting in the transferred tumor nuclei was corrected.

Table 1 Genomic imprinting and DNA methylation of *IGF2* in selected human cell lines

Cell lines	<i>IGF2</i> imprinting	DNA methylation		Nuclear transfer
		ICR	DMR ₀	
WTCL (Wilms' tumor)	LOI	+/-	-/-	Nuclear donor
SKNEP (Wilms' tumor)	LOI	+/-	-/-	Nuclear donor
H522 (lung cancer)	LOI	-/-	-/-	Nuclear donor
HRT18 (colorectal cancer)	LOI	+/-	+/+	Nuclear donor
HBF1 (human fibroblast)	MOI	+/-	+/-	Acceptor
MBW2 (mouse fibroblast)	MOI	+/-	+/-	Acceptor
ASPC cells (pancreatic cancer)	MOI	+/-	-/-	CHX-treated

LOI: loss of *IGF2* imprinting (bi-allelic expression).

MOI: maintenance of *IGF2* imprinting (mono-allelic expression).

+/-: two parental alleles were differentially methylated.

+/+: two parental alleles were fully methylated.

-/-: two parental alleles were unmethylated.

CHX: cycloheximide.

multiple epigenetic modifications in these tumor cells before and after nuclear transfer should therefore provide important information to guide our understanding of the mechanisms underlying *IGF2* LOI in tumors.

Nuclei from the four tumor cell lines were isolated as karyoplasts, and cells from the two normal fibroblast cell lines were enucleated by Ficoll gradient centrifugation (Volloch *et al*, 1987). Tumor nuclei were then introduced into the enucleated fibroblasts by chemical fusion (Kishi *et al*, 2003) or electroporation (Peura and Vajta, 2003). Reconstructed cells, called cybrids to distinguish them from hybrids that contain the genomes of two cells, were collected for analyses of *IGF2* imprinting and epigenetic modifications.

Prior to nuclear transfer into the mouse MBW2 fibroblasts, the four human tumor cell lines (WTCL, SKNEP, HRT18, and H522) expressed *IGF2* biallelically (Figure 2A and B, lane 3, A and B alleles). In the reconstructed cybrid clones, *IGF2* expression was relatively low (see the following section for *IGF2* quantitation by real-time PCR). Nevertheless, in every reconstructed cybrid clone we observed monoallelic expression of *IGF2* (Figure 2A, lanes 4–7 and Figure 2B, lanes 4–11). Control cells, in which tumor nuclei were transferred into enucleated tumor cells, continued to show biallelic *IGF2* expression (Figure 2A, lane 8–9 and Figure 2B, lane 12). Imprinting of *H19* was also restored in H522 cells (Figure 2B, bottom panel, lanes 4–11), the only cell line that contained an informative heterozygous *RsaI* polymorphic restriction enzyme site in the *H19* coding region. Similar data were also obtained when tumor nuclei were transferred into enucleated human HBF1 fibroblasts (data not shown). Thus, the cytoplasm of normal fibroblasts is able to reset *IGF2* epigenetic imprinting in nuclei derived from tumor cells that show biallelic expression of *IGF2*. It was also of interest to note that both mouse and human cytoplasts are able to induce imprinting in the human tumor nuclei. We preferred to use mouse fibroblasts as the host because we were able to track the introduced human nuclei in an unambiguous fashion in reconstructed cybrids simply by using human *IGF2*-specific primers.

In a second group of controls, we cross-transferred *IGF2* LOI tumor nuclei into other enucleated LOI tumor cytoplasts, for example, WTCL-SKNEP, WTCL-H522 and WTCL-HRT18. *IGF2* was biallelically expressed in these cross-transferred cells (data not shown). These data suggest that the various

Figure 2 Epigenetic correction of aberrant *IGF2* imprinting in human tumor cells. (A) Restoration of *IGF2* imprinting in three tumor cell/fibroblast cybrids. Lane 1: 100 bp marker, lane 2: tumor genomic DNA (gDNA); lane 3: tumor cDNA, lanes 4–7: fusion cells, lanes 8–9: self-transferred control cells. In lanes 4–7, the nuclei (karyoplasts) of tumor cells were transferred into enucleated normal mouse MBW2 fibroblasts. In lanes 8–9, the nuclei of tumor cells were transferred into enucleated tumor cells of the identical cell line. The parental alleles of *IGF2* were distinguished after treatment with *HhaI*. (B) Correction of *IGF2* and *H19* imprinting in H522 tumor cells. Lane 1: 100 bp marker, lanes 2: H522 genomic DNA (gDNA); lane 3: H522 cDNA (bi-allelic expression of *IGF2*), lanes 4–11: H522/MBW2 reconstructed cells; lanes 12: H522/H522 control reconstructed cells. In lanes 4–11, nuclei of H522 tumor cells were transferred into enucleated normal mouse MBW2 fibroblasts. Lane 12 shows controls, in which nuclei of H522 tumor cells were transferred into enucleated H522 cells. The parental alleles of *IGF2* were separated by *ApaI* and the parental alleles of *H19* were distinguished by *RsaI* polymorphic restriction enzymes.

tumor cell lines, each of which has distinct DNA methylation patterns at the DMRs, cannot 'complement' each other and repair *IGF2* imprinting.

Alteration of *IGF2* imprinting is independent of DNA methylation at known DMRs

To determine potential mechanisms underlying the restoration of imprinting, we examined DNA methylation at two critical DMR sites, the *IGF2/H19* ICR (CTCF binding sites) and DMR₀, which contains an *IGF2* silencer (Constancia *et al*, 2000; Eden *et al*, 2001). Genomic DNA that was extracted from tumor cells that demonstrated *IGF2* LOI and from reconstructed cybrids that expressed *IGF2* monoallelically was treated with sodium bisulfite to convert unmethylated cytosines into uracil residues, and the DNA was then subjected to amplification with specific primers covering DMRs (Arney, 2003). No differences in DNA methylation at these loci were observed between the imprinted and LOI cells (Figure 3A). For example, the H522/MBW2 cybrids showed hypomethylation at both ICR and DMR₀ sites, as did the native H522 cells (Figure 3A, lanes 3 and 4). Both WTCL/

MBW2 and SKNEP/MBW2 cybrids were differentially methylated at the ICR and were hypomethylated at DMR₀ (Figure 3A, lanes 6, 8 and 9), and HRT18/MBW2 cybrids were differentially methylated at the ICR and hypermethylated at DMR₀; these patterns were identical to those found in the wild-type tumor cells. Similarly, we did not find a correlation between the restoration of *IGF2* imprinting and DNA methylation in the recently discovered kvDMR₁ located near the *KCNQ1* gene (Smilnich *et al*, 1999; Engel *et al*, 2000) (data not shown).

We also confirmed the status of DNA methylation in H522 cybrids by other restriction enzymes (Supplementary Figure S1) and by the full bisulfite sequencing method. DNA sequences covering the sixth CTCF binding site in the *IGF2/H19* ICR and DMR₀ were amplified with PCR, cloned into TA vector, and were sequenced for the 'T' or 'C' residues at the CpG sites to determine the status of DNA methylation.

Figure 3 DNA methylation at DMRs in the *IGF2/H19* imprinting domain. (A) DNA methylation by COBRA method (CTCF binding site, top panel; DMR₀, bottom panel). Wt: wild-type tumor cells; Cy: nuclear transfer reconstructed cybrids. Lane 1: 100bp marker, lanes 2, 5, 7 and 10: control tumor cells; lanes 3–4, 6, 8–9 and 11–12: tumor/MBW2 reconstructed cells. Genomic DNA was treated with sodium bisulfite. After amplification with primers specific for CTCF binding region and DMR₀, PCR products were digested with *Mlu*I (CTCF binding region site) and *Hha*I (DMR₀) to separate methylated and unmethylated DNA. (B) Results of bisulfite sequencing in H522 tumor cybrids. After sodium bisulfite treatment, genomic DNA was amplified with PCR, cloned into TA vector, and sequenced for the 'C' or 'T' at CpG sites. Locations of PCR primers are indicated by numbered arrows. A total of 15 CpG dinucleotides at the sixth CTCF binding site of the *IGF2/H19* ICR are numbered from GenBank Accession No. AF087017. Each line represents a single sequenced PCR molecule. Black squares represent methylated CpG dinucleotides, and while squares represent unmethylated CpG dinucleotides. Left panel: control cybrids derived from transferring H522 nuclei into enucleated H522 tumor cells, which expressed *IGF2* biallelically; right panel: tumor cybrids derived from transferring H522 nuclei into enucleated normal fibroblasts, which had correction of *IGF2* imprinting and expressed *IGF2* monoallelically.

Figure 3B shows a typical example of the full DNA methylation sequencing of H522 tumor cells that had aberrant *IGF2* imprinting. In both original tumor cells and control cybrids, DNA was hypomethylated in the *IGF2/H19* ICR. After the correction of *IGF2* imprinting by *in vitro* reprogramming, we did not observe any changes in DNA methylation.

Depletion of putative *trans* imprinting factors as a potential mechanism underlying loss of *IGF2* imprinting in tumors

We then tested the possibility that the loss of *IGF2* imprinting in tumors was caused by an alternative mechanism, that is, inactivation or mutation of *trans*-imprinting factors in the tumor genome. After nuclear transfer, the normal fibroblasts would provide the missing imprinting factors. We first tested this hypothesis by taking advantage of the fact that the reconstructed cybrids did not contain the normal fibroblast genome that would be necessary to make the new putative *trans* imprinting factors. Thus, pre-existing imprinting factors would be present in the cybrids for a defined period of time before they underwent metabolic degradation. Moreover, as the cells divide, no new factors could be made and the existing factors would become systematically diluted. If this assumption were true, we would observe reversion to biallelic expression in reconstructed cybrids after repeated cell divisions as the pre-existing imprinting factors are depleted by dilution and catabolism.

We therefore tested the effect of prolonged culture on *IGF2* allelic expression. After 3–4 days of cell culture, the cybrids demonstrated normal *IGF2* imprinting (Figure 4A, lanes 5–10 and Figure 4B, lanes 4–9), but by 5 days after nuclear transfer, biallelic *IGF2* expression was again observed (Figure 4A, lanes 11–12 and Figure 4B, lanes 10–11). At the same time, *H19* imprinting was also lost in H522/MBW2 cybrids. Control cells showed no changes in *IGF2* imprinting (Figure 4A, lane 3). These data support the hypothesis that cytoplasmic *trans* imprinting factors are required for the maintenance of *IGF2* imprinting. In reconstructed cybrids, pre-existing imprinting factors remaining in enucleated fibroblast cytoplasm probably had a relatively short half-life (<4 days) and, in conjunction with dilution from cell division, these putative factors were probably depleted at day 5. As a result of the depletion of pre-existing imprinting factors, all cybrids ultimately reverted to biallelic *IGF2* expression starting from day 5.

Restoration of *IGF2* imprinting in the tumor genome in hybrid cells

To further assess the presence of putative *trans* imprinting factors, we directly fused normal mouse fibroblasts with each of the four human tumor cell lines. The resulting hybrid cells contained the nuclei of both the normal and the tumor cells. Because the mouse fibroblast genome should make a continuous supply of imprinting factors in the bi-nuclear hybrids, we predicted that we would observe a permanent conversion to *IGF2* imprinting in the tumor genome. To select hybrid clones, we transfected MBW2 fibroblasts with pQCXIN vector (BD Biosciences, CA) and tumor cells with pSM2c vector (Open Biosystems, AL). After cell fusion, hybrid cells were selected by both G418 and puromycin, and were used to analyze *IGF2* allelic expression using primers specific for human *IGF2*. As predicted, *IGF2* was expressed in a mono-

Figure 4 Time course of correction of *IGF2* imprinting in reconstructed tumor cybrids. (A) Temporary correction of *IGF2* imprinting in three reconstructed tumor cybrids. Lane 1: 100 bp marker; lanes 2: genomic DNA (gDNA); lane 3: cDNA (bi-allelic expression of *IGF2*); lane 4: self-transferred cells (control); lanes 5–12: reconstructed cells collected at different days following nuclear transfer into mouse fibroblasts. Two parental alleles of *IGF2* were separated by *Hha*I polymorphic restriction enzyme. (B) Temporary correction of *IGF2* and *H19* imprinting in H522 reconstructed tumor cybrids. Lane 1: 100 bp marker; lanes 2: genomic DNA (gDNA); lane 3: cDNA (bi-allelic expression of *IGF2*); lanes 4–9: reconstructed cells collected at different days following nuclear transfer into mouse fibroblasts. The parental alleles of *IGF2* were separated by *Apa*I and the parental alleles of *H19* were distinguished by *Rsa*I polymorphic restriction enzymes.

allelic manner in these hybrid cells (Figure 5, top panel, lanes 4–9), with no reversion to biallelic expression after at least 30 days of culture. These data strengthen the concept that the loss of *IGF2* imprinting in the tumor cells was related to *trans*-factors that are deleted or mutated during tumorigenesis.

It is interesting to note that allelic expression of the mouse fibroblast *Igf2* was not altered at all in the hybrids. While aberrant imprinting of the human *IGF2* was corrected (Figure 5, top panel, lanes 4–9), the mouse *Igf2* was still expressed monoallelically (Figure 5, bottom panel, lanes 4–9) as in MBW2 fibroblasts (lane 3). Similarly, we did not observe altered mouse *Igf2* imprinting in cybrids reconstructed from enucleated tumor cytoplasts and fibroblast nuclei (data not shown). These data indicated that *IGF2* imprinting is altered in a unidirectional manner in favor of the correction of tumor *IGF2* LOI. The unidirectional correction of *IGF2* imprinting thus suggests a ‘loss’, instead of ‘gain’ of function mechanism in tumor nuclei. It supports the hypothesis that *IGF2* LOI in tumor cells may be related to the inactivation of *trans* imprinting factors. Supplementation of imprinting factors from normal fibroblasts corrects and restores normal *IGF2* imprinting.

Figure 5 Correction of *IGF2* imprinting of the tumor genome in HRT18/MBW2 tetraploid hybrid cells. Lane 1: 100 bp marker; lanes 2: genomic DNA (gDNA); lane 3: cDNA (bi-allelic expression of *IGF2*); lanes 4–9: tetraploid fusion cells (permanent correction of *IGF2* imprinting). Two parental alleles of *IGF2* were separated by *Hha*I. PCR primers recognize human *IGF2* but do not recognize mouse *Igf2*.

Alteration of *IGF2* imprinting by inhibiting the synthesis of *trans* imprinting factors

Finally, we inhibited protein synthesis in normal fibroblasts using cycloheximide (CHX) in order to deplete the cells of the putative imprinting factors, predicting that these cells would gradually lose *IGF2* imprinting, recapitulating the loss of imprinting in tumors. We treated human fibroblasts with various concentrations of CHX. In untreated control fibroblasts, *IGF2* imprinting was maintained and only the A allele was expressed (Figure 6A, lanes 3), but in fibroblasts treated with CHX (0.5–2.0 μ g/ml), loss of *IGF2* imprinting was apparent from day 5 of culture (Figure 6A, lanes 4–6). We also tested the effect of CHX treatment in a human pancreatic cancer cell (ASPC) that showed normal *IGF2* imprinting (MOI) and had normal DNA methylation in the ICR but DNA hypomethylation at DMR₀ (Table I). *IGF2* was monoallelically expressed from the B allele in ASPC, but was biallelically expressed when cells were treated with various concentrations of CHX, resulting in increased expression of *IGF2* in tumor cells (Figure 6B). CHX treatment, however, did not change the imprinting pattern in HRT18 and H522 tumor cells that already expressed *IGF2* from both alleles (data not shown).

We further tested whether the withdrawal of CHX would restore *IGF2* imprinting in treated cells. We first treated HFB1 cells with 0.5 μ g/ml CHX in six-well plates. At day 6, we collected half of the treated cells for *IGF2* imprinting analysis and the remaining cells were re-seeded and cultured for 2 more days in the absence of CHX. HFB1 fibroblasts lose *IGF2* imprinting at day 6 following the inhibition of protein synthesis, with both parental alleles detected in the treated cells (Figure 6A, lanes 7 and 8). Withdrawal of CHX led to restoration of *IGF2* imprinting by 48 h (Figure 6A, lanes 9 and 10).

Interestingly, we found that alterations of *IGF2* imprinting in CHX-treated human fibroblasts were not accompanied by changes in DNA methylation at CTCF binding sites in the ICR, DMR₀ and kvDMR₁ (Supplementary Figures S2–S5). Thus, these data also suggest that the maintenance of *IGF2* imprinting in these human fibroblasts may be primarily dependent upon *trans*-acting factors.

Alteration of *IGF2* imprinting is not associated with the abundance of CTCF protein

Our data thus reveal a previously unrecognized *trans*-acting mechanism that is required for maintaining normal *IGF2*

Figure 6 Alteration of *IGF2* imprinting in cells treated with CHX. (A) Human fibroblasts (HFB1). Lane 1: 100 bp marker, lanes 2: HFB1 genomic DNA (gDNA); lane 3: HFB1 cDNA (mono-allelic expression of *IGF2*); lanes 4–6: 1.0, 2.0, 3.0 μ g/ml CHX for 4 days; lanes 7–8: 0.5, 2.0 μ g/ml CHX for 5 days; lanes 9–10: 0.5, 2.0 μ g/ml CHX for 4 days, and followed by the withdrawal of CHX for 2 days. The parental alleles of *IGF2* were separated by *Apa*I and the parental alleles of *H19* were distinguished by *Rsa*I. (B) Pancreatic cancer cells (ASPC). Lane 1: 100 bp marker; lanes 2: ASPC genomic DNA; lanes 3: ASPC cDNA; lanes 4–6: 1.0, 2.0, 5.0 and 5.0 μ g/ml CHX for 5 days; lanes 8–10: 0.5, 5.0 and 5.0 μ g/ml CHX for 7 days. Two parental alleles of *IGF2* were separated by *Apa*I polymorphic restriction enzyme.

imprinting. As an insulator of transcription, CTCF is a good candidate to be one of the putative imprinting factors. When CTCF levels are diminished by RNA interference (RNAi) in mouse fibroblasts, *Igf2* imprinting is partially lost (Ling *et al*, 2006).

We thus used Western blotting to examine the abundance of CTCF in tumor cells that had loss of *IGF2* imprinting and in human fibroblasts collected before and after CHX treatment. The amount of CTCF protein did not decrease in CHX-treated human fibroblasts. On the contrary, the relative amount of CTCF was increased when protein synthesis was inhibited by CHX in fibroblasts (Figure 7, lanes 4) and in ASPC tumor cells (Figure 7, lane 2), where *IGF2* imprinting was lost after CHX treatment. CTCF protein was not reduced in tumors cells that expressed *IGF2* biallelically (Figure 7, lanes 5, 7 and 8). These data suggest that inactivation of non-CTCF *trans* imprinting factor(s) may be involved in the dysregulation of *IGF2* imprinting in human tumors.

The relation between genomic imprinting and *IGF2* expression

Dysregulation of genomic imprinting is one of the important factors that contribute to the increased expression of *IGF2* in tumors (Sakatani *et al*, 2005). We were interested in whether epigenetic correction of *IGF2* imprinting following nuclear transfer would alter expression of the growth factor in tumor cybrids. We thus quantitated *IGF2* mRNA transcripts in tumor cybrids using real-time PCR (Figure 8).

IGF2 was biallelically expressed in original tumor cells and self-fused control cybrids. After the correction of *IGF2* imprinting by transferring the tumor nuclei into normal

Figure 7 The abundance of CTCF protein does not correlate with *IGF2* imprinting in CHX-treated cells. CTCF protein was quantitated by Western blotting in CHX-treated cells. Lanes 1, 3, 5, 7 and 8: control cells; lanes 2, 4 and 6: CHX-treated cells. The same Western blot was stripped off and was stained for β -actin as the internal control.

Figure 8 Real-time PCR quantitation of *IGF2* mRNA transcripts. LOI = loss of *IGF2* imprinting. *IGF2* and the housekeeping ribosomal L7 protein gene were co-amplified in each cDNA synthesized from control and tumor cybrids (H522, WTCL, SKNEP and HRT18) and CHX-treated fibroblasts (HFB1). *IGF2* was quantitated in triplicate for each sample and was determined by a 'delta Ct and delta-delta Ct' calculation with reference to human L7 gene control. *IGF2* expression was normalized and presented as the percentage by using the *IGF2* level in controls as 100% (control: $n = 6$; treatment: $n = 12$).

fibroblasts, however, there was a trend toward reduced expression of *IGF2* mRNA in all tumor cybrids although the change was not statistically significant (Figure 8). Similarly, we also observed increased expression of *IGF2* in CHX-treated human fibroblasts (HFB1) that biallelically expressed *IGF2* (Figure 8).

Discussion

Resetting the epigenotype of a cell, a process called epigenetic reprogramming, plays a critical role in the de-differentiation of the terminally differentiated nucleus into a state equivalent to that of a pluripotent zygote. Unlike genetic changes, epigenetic modifications are reversible and do not alter the messages encoded in genomic DNA. Using this powerful nuclear transfer method, Jaenisch and his co-workers (Hochedlinger *et al*, 2004) have shown that nuclei from melanoma cells can be reprogrammed to direct normal development of a mouse embryo. Their pioneer work defini-

tively demonstrated that the removal of an abnormal epigenotype alone is enough to restore malignant cells to normal. Recently, Li *et al* (2003) confirmed this finding in medulloblastoma cells, finding that epigenetic reprogramming of medulloblastoma nuclei by somatic nuclear transfer abrogates the tumorigenic phenotype. In another study by Tada *et al* (1997), the nuclei of thymocytes were epigenetically reprogrammed when transferred into embryonic germ (EG) cells. After reprogramming, the epigenotype of the transferred somatic nucleus was altered and matched the epigenotype of pluripotent EG cells, resulting in demethylation of several imprinted genes including the paternally imprinted (maternally expressed) *H19*, *p57kip2*, *Igf2r* and the maternally imprinted *Igf2*, *Peg3* and *Peg1/Mest*. Although the phenomenon is now well-established, the mechanisms involved in the epigenetic reprogramming of somatic nuclei remain largely unknown.

In this communication, we have demonstrated that the aberrant epigenotype of *IGF2* can be corrected by transferring tumor nuclei into enucleated normal mouse or human fibroblasts. In every selected cybrid clone derived from four tumor cells (Figure 2) and from another colon cancer cell (HT29, data not shown), we observed correction of abnormal *IGF2* imprinting in tumor nuclei. Thus, the cytoplasm of normal fibroblasts, whether from the human or the mouse, contains sufficient imprinting factors needed to reset the *IGF2* imprint in nuclei derived from tumor cells.

It is especially interesting to note that aberrant *IGF2* imprinting is corrected in tumor cells that have distinct patterns of DNA methylation in DMRs, which have been shown to be critical in regulating the allelic expression of the mouse *Igf2*. At first, we predicted that correction of epigenetic modifications in DMRs, especially the CTCF binding region in the ICR, would be necessary for resetting the normal *IGF2* imprint in tumor nuclei. Following nuclear transfer, the imprinting machinery in the fibroblast cytoplasm would repair the altered DNA methylation in the DMRs, and this would lead to monoallelic expression of *IGF2*. Surprisingly, we did not observe any changes in DNA methylation at well-known DMRs (DMR₀, CTCF binding region, and kvDMR₁) in the cybrids that showed the correction of *IGF2* imprinting. Similarly, we induced biallelic expression of *IGF2* in normal human fibroblasts by the treatment of CHX (Figure 6A). Using both the COBRA method and bisulfite sequencing, we again did not find any changes in DNA methylation at these DMR sites. Thus, alterations of *IGF2* imprinting may not necessarily be accompanied by changes of DNA methylation in known ICRs.

It is possible that DNA methylation at these DMRs may play different roles in *IGF2* imprinting at different stages of the development. DNA methylation, as an imprinting signal, is critical to guide the establishment of *IGF2* imprinting during the early embryogenesis, as has been demonstrated by many animal studies, including knockout, deletion, mutation, and transgenic models. It is possible that after imprinting has been established, monoallelic expression of *IGF2* is faithfully maintained during cell replication by mechanisms that may not require or are less dependent upon DNA methylation. Thus, once imprinting is established, DNA methylation at DMRs may not be critical in controlling imprinting. This concept is supported by the lack of correlation between DNA methylation and the status of *IGF2*

imprinting as observed in this communication and in primary tumors reported by several groups (Moore *et al*, 1997; Sullivan *et al*, 1999; Cui *et al*, 2002).

Instead, data from this study strongly suggest a critical role of *trans* imprinting factor(s) in the maintenance of *IGF2* imprinting. As an insulator of transcription, CTCF was first suspected to be a candidate imprinting factor. However, quantitation of CTCF protein abundance did not detect significant differences between *IGF2* LOI and MOI tumors; CTCF levels did not change in human fibroblasts before and after the CHX treatment that alters *IGF2* imprinting (Figure 7). We have previously shown that in *IGF2* LOI tumor cells and in CNS where *IGF2* is biallelically expressed, CTCF binding sites in the ICR were normally semimethylated, and CTCF binds to the unmethylated allele (Yang *et al*, 2003a; Ulaner *et al*, 2003b). In this study, we also found that CHX treatment did not change DNA methylation in the *IGF2/H19* ICR (data not shown). Moreover, CHX-treated cells expressed *H19* mono-allelically even though *IGF2* was biallelically expressed (Figure 6A, bottom panel). Taken together, these data indicate that other factors in addition to CTCF may play an important role in determining *IGF2* allelic expression.

CTCF binding to the ICR is critical in the establishment of *Igf2* imprinting in the mouse. Deletion of the locus containing the ICR led to biallelic expression of *Igf2* (Leighton *et al*, 1995; Thorvaldsen *et al*, 1998). Mutation of each CTCF binding site in the ICR also altered *IGF2* imprinting (Schoenherr *et al*, 2003). Using a transgenic RNAi-based approach to generate oocytes with reduced CTCF protein, Bartolomei and co-workers (Fedoriw *et al*, 2004) found that CTCF protected the ICR from *de novo* methylation during oocyte growth and was required for normal preimplantation development. However, this relatively straightforward regulation of mouse *Igf2* imprinting by CTCF is not always apparent in human tumors. One possible explanation for the discrepancies is that most mouse studies altered the ICR or CTCF in early development before *IGF2* imprinting was established. The maintenance of *IGF2* imprinting, while it may be closely linked to the initiating process, could require additional mechanisms, for example, putative *trans* imprinting factor(s). In tumors, loss of *IGF2* imprinting may be caused by dysfunction of a maintenance imprinting regulatory system. In addition, CTCF may require the presence of other partners to exert its function in regulating *IGF2* imprinting. Although CTCF and its binding to the ICR is normal, the absence or mutation of putative CTCF partners or non-CTCF factors may cause abnormal *IGF2* imprinting in tumors.

The putative imprinting factor(s) could be a tumor suppressor that controls the turnover of growth factors, like *IGF2*, in normal cells. Mutation or inactivation of the imprinting factors relaxes *IGF2* imprinting and thus provides a growth advantage to tumor cells. It will be of great interest to learn whether the correction of *IGF2* imprinting can alter tumor phenotypes in animal models, and if restoration to normal *IGF2* imprinting may provide a novel molecular target for anti-tumor therapies. Gene therapy to supply the missing imprinting factor may restore normal control of *IGF2* imprinting and may provide a therapeutic strategy to treat a variety of human malignancies with the abnormal *IGF2* imprinting.

Chromatin remodeling is a potentially powerful method for altering the biological properties of a cell. Recently, Collas and co-workers used extracts derived from another cell type

as an alternative strategy to reprogram a nucleus or a cell. For example, after exposure to the extracts of T cells, fibroblasts changed their gene expression pattern. This short *in vitro* reprogramming caused the upregulation of the hematopoietic cell-specific marker genes IL2, IL7, CD3, CD4 and RANTES, and the downregulation of the fibroblast-specific genes, like integrin $\beta 1$ (Hakelien *et al*, 2002). Similarly, when treated with extracts of undifferentiated NCCIT cells or mouse ES cells, epithelial 293T cells underwent genome-wide transcriptional programming and transitioned to a pluripotent cell phenotype involving a dynamic upregulation of hundreds of embryonic and stem cell markers (Taranger *et al*, 2005). It would be of interest to examine whether this simple strategy can be also used to replace the nuclear transfer method to reprogram tumor cells that have aberrant *IGF2* imprinting. By exposing the normal fibroblasts to extracts that contain the intact *trans*-imprinting factor(s), this strategy may be used to correct biallelic *IGF2* expression of tumor cells back to normal monoallelic expression.

In conclusion, in this study, we have demonstrated that aberrant *IGF2* imprinting in human tumor cells can be corrected to a normal imprinting pattern. Although the specific mechanisms underlying the nuclear transfer-mediated imprinting correction are not yet clear, the study strongly suggests that, in addition to epigenetic marks in the *IGF2/H19* DMRs, the absence of active non-CTCF *trans* imprinting factor(s) significantly contributed to the abnormal imprinting in tumors. We expect that this previously unappreciated mechanism may also be broadly applied to other imprinted genes. To date, nearly all of the relevant studies have examined alterations in *cis*-regulating elements at DMRs. Thus, in addition to searching for epigenetic modifications in known and other unidentified DMRs, we may have to switch our research focus to identify the *trans*-imprinting factors that are related to the maintenance of imprinting genes, including *IGF2* in tumors.

Materials and methods

Cell lines

Tumor cell lines H522, HRT18 and ASPC were purchased from ATCC (Rockville, MD). WTCL was a kind gift from Dr Benjamin Tycko, and SKNEP and G401 from Dr Herman Yeger. Mouse fibroblast MBW2 cells were cultured from an F1 newborn mouse derived from breeding a M. spretus male with a C57B/6 female. Human fibroblast cells HBF1 were cultured from the skin of a human fetus (Hu *et al*, 1997; Hu *et al*, 1998).

Nuclear transfer

Cells in which *IGF2* imprinting was maintained (HBF1 and MBW2) were used as the acceptor cells. They were enucleated by centrifugation through a Ficoll density gradient in the presence of cytochalasin B (Volloch *et al*, 1987). After centrifugation, the enucleated HBF1 and MBW2 cells (cytoplasts) were collected between the 15 and 18% Ficoll interfaces. The enucleated cells were stained with 0.4% Trypan blue to assess viability. Hematoxylin and eosin staining was used to determine the purity of separation.

Human tumor cell lines in which *IGF2* was biallelically expressed were used as donor cells. The nuclei were purified as karyoplasts, which contained the intact nucleus surrounded by a thin layer of cytoplasmic membrane. Karyoplasts were obtained from the bottom of the 30% region after Ficoll gradient centrifugation. After washing with PBS, the karyoplasts containing intact nuclei were collected by centrifugation at 2000 r.p.m. for 10 min and used for nuclear transfer. H&E staining was used to determine the purity of tumor nuclei.

Tumor nuclei were transferred into the enucleated fibroblasts by chemical cell fusion (Kishi *et al*, 2003) or electroporation using using BTX Electro Cell Manipulator 2001 (Peura and Vajta, 2003). Reconstructed cells were cultured in RPM 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, and were used for the analysis of genomic imprinting. To create hybrid cells containing both fibroblast and tumor cell genomes, we transfected MBW2 fibroblasts with pQCXIN vector (Clontech BD Biosciences, Palo Alto, CA) and tumor cells with pSM2c vector (Open Biosystems, Huntsville, AL). After cell fusion, hybrid cells were co-selected by G418 (200–400 µg/ml) and puromycin (1–2 µg/ml). Reconstructed cell clones were expanded and collected for the analyses of *IGF2* imprinting and DNA methylation.

IGF2 imprinting

Total RNA was extracted from tissues by TRI-REAGENT (Sigma, St Louis, MO), according to the manufacturer's guide, and cDNA was synthesized with RNA reverse transcriptase. Genomic imprinting of *IGF2* was examined by PCR in cDNA samples as previously described (Hu *et al*, 1996) using primers specific for three polymorphic restriction enzymes (*Apa1*, *Alu1*, and *Hua1*) in the last exon of human *IGF2*. Allelic expression of *H19* was assessed by polymorphic restriction enzyme *Rsa1*.

PCR primers used to measure allelic expression of *IGF2* and *H19* included: *IGF2*, *Apa1*: #2505 (5'-primer)—CTTGGACTTTGAGTCA AATTGGCCT

#2506 (3'-primer)—GAGGAGCCAGTCTGGGTGTTGCTA;

IGF2, *Alu1*: #2949 (5'-primer)—GTCCCTCCTCTGCCATCACCTGA

#2950 (3'-primer)—GGATTTTGCCGAAATATTAGCGT;

IGF2, *Hua1*: #3302 (5'-primer)—GGCCAAACGTCATCGTCCCCTGAT

#2428 (3'-primer)—ACGTGGAACCGAGAGATTTTCGGG;

H19, *Rsa1*: #4237 (5'-primer)—GGAGTTGTGGAGACGGCCTTGAT

#4238 (3'-primer)—CCAGTCACCCGGCCAGATGGAG.

Measurement of DNA methylation

Total nucleic acids (TNA) extracted from tumor cells were used to examine DNA methylation patterns. As previously described (Hu *et al*, 1997; Ulaner *et al*, 2003a), TNA was treated with sodium bisulfite, and DNA in the *IGF2/H19* DMR was amplified with DNA methylation-specific primers designed for CTCF binding sites.

PCR primers used to quantitate DNA methylation at the CTCF binding site were #2035 (5'-primer): GATGGTAYGGAATTGGTTG TAGTTGTGG, and #2036 (5'-primer): TCCTATAAATATCCTATTC CAAATAACC. After PCR, methylated and unmethylated DNA was separated by *Mlu1*. PCR primers used to measure DNA methylation at DMR₀ were #2564 (5'-primer): GAGGTTTGGTAGAGAGGTTA TAGGT, and #2565 (3'-primer): CCAAATCCCACTATATAAC TAAATCCAC. After PCR, methylated and unmethylated DNA was separated by *Hha1*.

To examine the status of DNA methylation in every CpG site in DMRs, the amplified PCR DNAs were cloned into TA vector (Invitrogen, Carlsbad, CA) and were sequenced using the vector primer.

Cell treatment with CHX

Human fibroblasts cells (HBF1) were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 100 U/ml of penicillin and 100 µg/ml of streptomycin, and grown at 37°C with 5% CO₂. ASPC, a pancreatic cancer cell line keeping

normal *IGF2* imprinting in cell culture, was maintained in RPMI 1640 medium as recommended by ATCC. Cells were seeded in six-well plates at the density of 2×10^5 cells/well. Twenty-four hours following plating, cells were replaced with fresh medium and were treated with different concentrations (0.0, 0.5, 1.0, 2.0 and 5.0 µg/ml) of CHX. Tumor cells were collected after day 4 and analyzed for *IGF2* imprinting and DNA methylation.

In a separate study, fibroblasts were first treated with 0.5 or 1.0 µg/ml CHX. At day 5, cells were divided into two portions. Half the cells were collected to assess *IGF2* imprinting. The other half were seeded in a new plate supplemented with fresh DMEM without CHX. After two days, cells were collected to assess *IGF2* imprinting.

Western blotting of CTCF protein

Expression of CTCF protein was determined by Western blotting as previously described (Yao *et al*, 2003). Cells were lysed with boiling 1% SDS, 10 mM Tris-HCl, pH 7.4, sonicated for 30 s, and centrifuged at 15000 g for 5 min. Supernatant lysates with equal amounts of protein were used for immunoblotting of CTCF protein using the mouse monoclonal anti-human CTCF antibody (1:500, Santa Cruz Biotechnology, CA).

Q-PCR quantitation of IGF2 transcripts in tumor cybrids

IGF2 mRNA expression in tumor cybrids was examined by TaqMan Q-PCR as previously described (Yang *et al*, 2003b; Vu *et al*, 2004). Briefly, total RNA was extracted by TRI-REAGENT (Sigma, St Louis, MO). *IGF2* cDNA was synthesized with RNA reverse transcriptase and was quantitated by Q-PCR in triplicate using an ABI Prism 7900HT sequence detector (AB Applied Biosciences, CA) following the manufacturer's protocol. To quantitate PCR products, the housekeeping ribosomal L7 protein gene was co-amplified as the internal control. The primer sequences for assaying human *IGF2* transcripts were #4702 (5'-primer): GCCAAGTCCGAGAGG GACGTGTCG and #4703 (3'-primer): CAGGTGTCATATTGGAAG AACTTGC. The sequences for the L7 primers were #1266 (5'-primer): CGAAAGGCAAGGAGGAAGCTTATCT and #1267 (3'-primer): CGAATTTTCAGTTCTGTACATCTGCCT. After Q-PCR amplification, a 'melting curve analysis' was performed to confirm the homogeneity of all Q-PCR products. *IGF2* cDNA was determined by a 'delta Ct and delta-delta Ct' calculation as described in the manufacturer's protocol with reference to human L7 gene control.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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