YY1 as a controlling factor for the Peg3 and Gnas imprinted domains

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Abstract

Imprinting control regions (ICRs) often harbor tandem arrays of transcription factor binding sites, as demonstrated by the identification of multiple YY1 binding sites within the ICRs of Peg3, Nespas, and Xist/Tsix domains. In the current study, we have sought to characterize possible roles for YY1 in transcriptional control and epigenetic modification of these imprinted domains. RNA interference-based knockdown experiments in Neuro2A cells resulted in overall transcriptional up-regulation of most of the imprinted genes within the Peg3 domain and also, concomitantly, caused significant loss in the DNA methylation of the Peg3 differentially methylated region. A similar overall and coordinated expression change was also observed for the imprinted genes of the Gnas domain: up-regulation of Nespas and down-regulation of Nesn and Gnasxl. YY1 knockdown also resulted in changes in the expression levels of Xist and Snrpn. These results support the idea that YY1 plays a major role, as a trans factor, in the control of these imprinted domains.

Keywords: Genomic imprinting; ICRs; YY1

A small number of mammalian genes are subject to an unusual dosage control, called genomic imprinting, in which one of two alleles of the genes is repressed in a parental-origin-specific manner. The imprinted genes are clustered in specific regions of chromosomes, and each imprinted domain is typically controlled by small genomic regions, termed imprinting control regions (ICRs) [1–3]. These ICRs are usually located in CpG-rich regions near the promoters of imprinted genes and methylated differentially between the two parental alleles. These regions often show tandem repeat sequence structure [4,5] and the core sequences of these tandem repeats have been shown in several cases to correspond to transcription factor binding sites. Known transcription factors binding to repeat regions include CTCF for the ICR of the H19/Igf2 imprinted domain and YY1 for the differentially methylated region (DMR) of the Peg3, Nespas, and Xist/Tsix imprinted domains [6–9]. In the H19/Igf2 imprinted domain, CTCF functions as an enhancer-blocker for controlling allele-specific expression of H19 and Igf2 [10,11]. However, the in vivo functions of YY1 for the Peg3 and other imprinted domains require further investigation.

The mammalian transcription factor YY1 is a ubiquitously expressed, multifunctional protein that can function as an activator, repressor, or initiator binding protein depending upon the sequence context of YY1 binding sites with respect to other regulator elements (reviewed in [12–14]). The protein has a DNA binding domain at the C-terminus and other modulating domains at the N-terminus displaying repression, activation, and protein–protein interaction activities. YY1 interacts with several key components of general Pol II transcription machineries, including TBP, TAFs, and TFIIB, as well as histone-modifying enzymes, including p300, HDACs, and PRMT1 (reviewed in [12–14]). YY1 is evolutionarily well conserved throughout all vertebrate lineages and at least two genes similar to vertebrate YY1 are found in fly genomes. One of these YY1 homologues is involved in the Polycomb complex-mediated repression mechanism [15]. Recent studies also support a similar role for YY1 in this heritable silencing mechanism of vertebrates [16,17]. We have previously identified an unusual tandem array of multiple YY1 binding sites located within the Peg3 DMR [8] and later confirmed the presence of similar clustered YY1 binding sites within the ICRs of Xist, Tsix, and Nespas [9]. The localization of these multiple YY1 binding sites within imprinting control regions is very
unusual and suggests a potential role for YY1 in mammalian genomic imprinting.

In the current study, we have lowered the YY1 protein levels through RNA interference techniques and subsequently analyzed the short- and long-term effects of this YY1 knockdown on the transcription and DNA methylation of the Peg3 DMR and other YY1-associated genomic regions. Our results indicate that YY1 may function as a controlling factor for the Peg3 and Gnas imprinted domains and also that YY1 may be involved in maintaining the proper methylation status of these differentially methylated, imprinting control regions.

Results

The short-term effects of YY1 knockdown on the transcription of the Peg3 domain

Of three siRNA constructs designed to knock down YY1, we found one construct that consistently lowered the YY1 protein level in transiently transfected cells of both Neuro2A (Fig. 1A) and NIH3T3 lines (data not shown). Western blot analyses indicated up to 90% reduction in the YY1 protein level in the cells transiently transfected with this YY1-siRNA construct, while control cells with no transfection (NT) and with transfection using another siRNA construct containing a scrambled sequence (Scr) showed no change in the YY1 protein level. Two independent Western blots using β-actin and p53 antibodies also confirmed the target-specific knockdown of YY1 by this siRNA construct.

We analyzed the short-term (transient) effects of the YY1 knockdown on the transcription of the endogenous loci that are known to be associated with YY1 binding sites [9]. For this series of tests, total RNA was first isolated from two different pools of cells that had each been transiently transfected with the Scr- or the YY1-siRNA construct and used to generate cDNA for real-time quantitative RT-PCR. In this qRT-PCR scheme, the relative abundance of a given mRNA between two types of cells was measured by the difference in the arbitrary Ct (threshold cycle) values. As shown in Fig. 1B, the set of 19 genes analyzed in this assay showed a broad range of averaged Ct values, indicating a wide range of expression levels among the individual genes in Neuro2A cells. The internal control genes that were used for the normalization of two different amounts of cDNA templates showed higher expression levels (β-actin, GAPDH, 28S). In contrast, the three genes that were included to monitor interferon response showed much lower expression levels (Oas2, Mx1, IFMT1), confirming no interferon response in our siRNA transfection experiments [18,19]. The imprinted genes in the Peg3 domain (Fig. 2C; Peg3, Usp29, Zim1, Zim2, Zim3, Zfp264) also showed a...
wide range of expression levels in Neuro2A cells. Peg3, also known as Pw1 [20], showed the highest expression level, whereas Zim3 expression was not detectable at all. Comparison of Ct values of these genes revealed up-regulation of Peg3, Zim1, and Zim2 in the YY1-siRNA-transfected cells. By contrast, the Ct value difference for Usp29 and Zfp264 may not be significant since two Ct values overlap within error ranges. We also tested the effects of YY1 knockdown on the expression levels of other YY1-associated genes, such as the genes in the Gnas and Xist/Tsix domains as well as other nonimprinted genes, including Sp1 and GR (glucocorticoid receptor). Except for slight up-regulation in Sp1, most genes were not affected by transient YY1 knockdown. Overall, our transient knockdown experiments hinted at one possibility that lowering the YY1 protein level may have an immediate impact, specifically, up-regulation, on the transcription of several genes in the Peg3 imprinted domain.

The long-term effects of YY1 knockdown on the transcription of imprinted domains

Using an RNAi strategy similar to that described above, we also analyzed the long-term effects of YY1 knockdown on the transcription of the genes located within the Peg3- and other YY1-associated imprinted domains. For this analysis, an inverted DNA sequence derived from mouse YY1 was incorporated into the 3′-UTR of the β-galactosidase gene in the pcDNA3.1/His/lacZ vector (Invitrogen). This scheme allowed us to monitor easily the expression of the YY1-targeting RNA portion by in situ β-galactosidase staining. This construct along with a control pcDNA3.1/His/lacZ vector without the YY1-targeting portion (EV; empty vector) was transfected individually into Neuro2A cells to derive stable cell lines using G418 selection. Of six stable single cell lines isolated, two cell lines (Nos. 6-2 and 6-4) showed the lowest levels of the YY1 protein (Fig. 2A) and thus were selected for our analyses. Total RNA was first isolated from four different types of cells: two control cells, NT and EV, and two YY1 knockdown cells, 6-2 and 6-4. These isolated RNAs were used to compare the expression levels of a given gene among the different cells using (1) RT-PCR with fixed numbers of cycles, 30 to 35 (Fig. 2B), and (2) quantitative RT-PCR (Figs. 2C and 2D).

In the Peg3 imprinted domain, the expression of most of the resident genes except for Zim3 was detectable in the Neuro2A-derived stable cells, and also the expression levels of Peg3, Usp29, and Zim1 differed in two YY1 knockdown cells relative to the control cell lines. Another series of independent qRT-PCR analyses further confirmed that the expression levels of these three genes in the YY1 knockdown cell lines were higher than those of the control cell set, ranging from three- to fivefold (Fig. 2C). This increase in expression levels in Peg3, Usp29, and Zim1 is consistent with the slight up-regulation of Peg3, Zim1, and Zim2 that was observed from the transient YY1 knockdown experiments (Fig. 1B). It is interesting that, in both cases, more than one gene was affected similarly by YY1 knockdown.

In the Gnas domain, however, stable YY1 knockdown had opposite effects among the resident genes: whereas Nesnas expression was increased (threefold), decreased expression of Nesp (three- to fourfold) and Gnasx1 (fivefold) was observed in the YY1 knockdown cell lines (Figs. 2B and 2C). Given the frequent detection of coregulation between sense and antisense gene pairs in imprinted domains, the up- and down-regulation of Nesnas/Nesp may represent two connected outcomes. By contrast, the expression levels of an alternative transcript, Exon1A, were relatively low with no obvious difference among the cells except for the slight increase observed in the 6-4 cell.

In contrast to genes in the Peg3 and Gnas imprinted domains, the knockdown effects in stable cells were somewhat less obvious for other YY1-related genes, such as Xist and Surprn, the latter of which is another YY1-involved imprinted gene identified independently by others [21]. Despite the fact that we detected no obvious difference by RT-PCR, qRT-PCR analyses revealed that Xist expression levels were slightly increased by 1.5- to 2-fold in the YY1 knockdown cells (Fig. 2B).
(A) 

![Image of YY1 and Actin Western Blots]

(B) 

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(C) 

![Genetic Diagram with expression levels for Zfp264, Zim3, Usp29, Peg3, Zim1, and Zim2]

![Expression Levels for Nesp, Nespas, Gnasxl, and Exon 1A]
Like wise, the expression levels of Snrpn were also increased by 3-fold, which agrees well with the independent result derived from the studies of a YY1-deleted mouse [22]. The expression change for GR in YY1 knockdown cells is readily noticeable by both RT-PCR and qRT-PCR, but other YY1-related, nonimprinted genes did not show any major change. Expression levels of many other genes were not affected by YY1 knockdown, including several DNA methyltransferases, CTCF, and macroH2A1 (data not shown). Taken together, long-term YY1 knockdown resulted in changed expression levels for the imprinted genes in the Peg3 and Gnas domains in a somewhat coordinated manner. The observed coordinated response may be an indication that YY1 is involved in the overall regulation of these imprinted domains, possibly through ICRs.

The long-term effects of YY1 knockdown on DNA methylation of imprinted domains

We have also investigated the DNA methylation status of Peg3, Nespas, and Xist DMRs to analyze potential long-term effects caused by YY1 knockdown. As shown in Fig. 3A, genomic DNAs from four different Neuro2A cells were digested first with BamHI (lane B) and later individually with methylation-insensitive MspI (lane B+M) and methylation-sensitive HpaII (lane B+H). Hybridizations with the two probes covering the promoter and YY1 binding regions of the Peg3 DMR showed different band patterns between the control cells and the YY1 knockdown cells. In YY1 knockdown cells, the sizes of methylated DNA fragments become smaller (lane B+H), indicating DNA methylation loss in these cells. These initial results were further analyzed by performing bisulfite sequencing (Fig. 3B). We have performed independently another Southern blot using the restriction enzyme NarI, the recognition site of which overlaps with the sequence of YY1 binding sites (GGGCCCATCTT) that are located within the Peg3 DMR. These results indicated that the CpG sites of the YY1 binding sites also lost DNA methylation in the YY1 knockdown Neuro2A cells (data not shown). These results again confirm the loss of methylation in the Peg3 DMR. These results are also consistent with the up-regulation of Peg3 and Usp29 that we have observed in stable YY1 knockdown experiments (Fig. 2C).

We also performed a similar set of methylation analyses on the DMR of Nespas, which is associated with multiple YY1 binding sites (Fig. 3C). In the Nespas DMR, one of the knockdown cells, 6-4, showed slightly different patterns compared to those of two control cells, but the significance of this difference is uncertain. Overall, the methylation levels of the Nespas DMR did not appear to be affected by YY1 knockdown in Neuro2A cells.

By contrast, the Xist locus appears to have reduced levels of DNA methylation in YY1 knockdown cells (Fig. 3D). In the two control cells, two different-sized DNA fragments representing methylated and unmethylated DNA fragments were detected at similar ratios in the BamHI/HpaII double digestion (lane B+H). These two bands are thought to represent the two different X chromosomes of the female-origin Neuro2A cells, active and inactive X chromosomes. In the two knockdown cells, the smaller sized DNA fragment derived from unmethylated DNAs is more dominant, indicating that this particular CpG site has lost its DNA methylation in both YY1 knockdown cell lines. This is consistent with the detection of slight up-regulation of Xist in the two YY1 knockdown cells (Fig. 2D). In sum, our DNA methylation analyses indicated that long-term YY1 knockdown results in hypomethylation in the DMRs of Peg3 and Xist, and this change is consistent with the transcriptional up-regulation observed in these two domains.

Discussion

RNAi-based YY1 knockdown experiments demonstrated that lowering YY1 protein levels caused global and somewhat coordinated changes in the expression levels of the genes located in the Peg3 and Gnas domains. The transcriptional up-regulation observed in the Peg3 domain was also accompanied by changes in the DNA methylation level of the Peg3 DMR, suggesting a possible role for YY1 in maintaining the proper methylation status of this DMR sequence.

Our recent identification of multiple YY1 binding sites within the ICRs of Peg3, Nespas, and Xist/Tsix suggests that YY1 plays a role for the imprinting control of these domains [9]. Given the colocalization of YY1 binding sites in these differentially methylated regions, it is likely that YY1 plays a role in maintaining and/or establishing the allele-specific
methylation of these regions. Consistently, our data showed that stable knockdown of YY1 resulted in hypomethylation in the DMRs of Peg3 and Xist (Fig. 3). Similar observations have been made in the studies of CTCF binding sites in the H19 ICR [10,11]. In this case, both ablation of CTCF binding sites within the H19 ICR and CTCF knockdown in mice resulted in

Fig. 3. Long-term effects on the DNA methylation status of the DMRs of (A) Peg3, (C) Nespas, and (D) Xist. The schematic diagram for each DMR is shown at the top, while the results derived from methylation analyses using Southern blot and bisulfite sequencing approaches are shown at the bottom. The schematic diagram shows the relative position of each DMR to BamHI sites (B) and two isoschizomer sites (MspI, methylation-insensitive; HpaII, methylation-sensitive). The diagram also indicates the genomic regions that have been used as probes for Southern blotting, which are marked by double-headed arrows with P1 through P4. The ovals in the diagram represent YY1 binding sites. For the methylation analyses, four different genomic DNAs were isolated from the cells of NT, EV, 6-2, and 6-4. These DNAs were first digested with BamHI (lane B) and later with MspI (lane B + M) or HpaII (lane B + H). Two different DNAs from NT and 6-4 were also analyzed with the bisulfite sequencing method for the methylation analysis of the promoter region of Peg3. Each row represents one individual DNA strand derived from this bisulfite conversion reaction, while the 24 circles in each row represent individual CpG sites located within this 430-bp promoter region. Ten different clones were successfully sequenced for the NT sample and 7 clones for the 6-4 sample.
hypermethylation of the H19 ICR, suggesting that CTCF may function as a protector for the unmethylated, maternal allele of the H19 ICR. This is somewhat opposite to the hypomethylation observed in YY1 knockdown stable cells in our experiments (Fig. 3). In the case of the Peg3 DMR, YY1 may be required for maintaining the methylated status of the inactive maternal allele. One likely scenario would be that YY1 recruits histone-modifying enzymes, such as HDACs, which are, in turn, required for DNA methylation (reviewed in [23]). This is plausible given the numerous interaction partners of YY1 that are involved in epigenetic modifications (reviewed in [13,14]).

In the YY1 knockdown cells, the Peg3 and Gnas domains showed somewhat global and coordinated responses against lowering the YY1 protein levels (Fig. 2C). In both domains, YY1 knockdown affected not a single gene but several genes in each domain. In the Peg3 domain, although the YY1 binding sites are located right next to the bidirectional promoter of Peg3 and Usp29, the impact of YY1 knockdown was also observed in Zim2 (transient experiments, Fig. 1) and Zim1 (stable experiments, Fig. 2). The transcription of all the affected genes in this domain was similarly up-regulated, and this up-regulation was also accompanied with hypomethylation in the Peg3 DMR (Fig. 3A). In the Gnas domain, multiple YY1 binding sites are located in the first intron of Nespas, but expression changes were detected in three genes, Nespas, Nesp, and Gnasx1 (Fig. 2C). In particular, the up- and down-regulation of Nespas and Nesp, respectively, is an expected outcome based on the antisense/sense relationship of the two genes, but only if YY1 is involved directly in the regulation of these two genes. The observed expression changes of the Nespas/Nesp pair, therefore, confirm that YY1 is indeed functionally involved in the regulation of the two genes. According to the results derived from mutant mice lacking the Nespas DMR, which coincides exactly with the multiple YY1 binding region [24], deletion of this DMR affected the transcription and imprinting of all the genes in this domain. In this case, the absence of Nespas expression in the mutant mice resulted in the up-regulation of Nesp. This situation appears to be opposite to that represented in YY1 knockdown cells, in which Nespas is up-regulated and Nesp is down-regulated. Nevertheless, the observed responses in both experiments provide a consistent outcome, a coordinated response between Nespas and Nesp. Along with the overall up-regulation observed from the Peg3 domain, this coordinated response supports the idea that YY1 indeed plays a major role in the regulation of these two imprinted domains.

Materials and methods

YY1 knockdown using RNA interference techniques

The sequences of the siRNA constructs used for this study are as follows: YY1 siRNA, sense strand, 5'-GATCCCCGAGAGAAACATCTCTGTGAATTACAACTCATCGAGGATTAAGAGAGAGGAGATCTGTTCATCCCCAGGAGGAA-3'; antisense strand, 5'-GATCCCCGAGAGAAACATCTCTGTGAATTACAACTCATCGAGGATTAAGAGAGAGGAGATCTGTTCATCCCCAGGAGGAA-3'; Scramble siRNA, sense strand, 5'-GATCCCCGAGAGAAATACGAGGAGATCTGTTCATCCCCAGGAGGAA-3'; antisense strand, 5'-GATCCCCGAGAGAAATACGAGGAGATCTGTTCATCCCCAGGAGGAA-3'. Duplex oligonucleotides were subcloned into the BglII and HpaII sites of siGaper vector (OligoEngine). To make the stable YY1 knockdown cell lines, we first generated one inverted DNA fragment using a DNA fragment derived from the transcribed region of mouse YY1 (GenBank Accession No. NM_009537, position 1222–1607), and later this inverted fragment was subcloned into the 3'-UTR of the pCDA3.1/His/αz vector (Invitrogen). These RNAi vectors were amplified in the Escherichia coli Sure 2 strain (Stratagene), which allows the accurate replication of inverted repeat-containing DNAs. These constructs were purified using the HiSpeed plasmid midikit (Qiagen) and transfected into cells using the GeneJuice transfection reagent according to the manufacturer's protocol (Novagen). For stable transfection experiments, transfected cells were selected by adding G418 (500 µg/ml; Calbiochem) to the culture medium.

Western blot

For our Western blot analysis, the cells were lysed 48 h after transfection using lysis buffer (0.25 M Tris–HCl, pH 7.8, plus 0.1% NP-40) for 30 min at 4°C and protein cellular debris was removed by centrifugation for 10 min. Protein concentrations were determined by the Bradford assay kit (Pierce). Thirty micrograms of lysate was separated on 10% SDS–PAGE gels and transferred to the PVDF membrane (Hybond-P, Amersham) using a Mini Trans-Blot transfer cell (Bio-Rad). Membranes were blocked for 1 h in Tris-buffered saline containing 5% skim milk and 0.05% Tween 100 and incubated at 4°C overnight with anti-YY1 (sc-1703), anti-p53 (sc-6243), or anti-β-actin (sc-6115) antibodies (Santa Cruz Biotechnology). These blots were incubated for an additional 1 h with the secondary antibody linked to horseradish peroxidase (Sigma). The blots were developed using a Western blot detection system according to the manufacturer's protocol (Intron Biotech).

RT-PCR and quantitative PCR

Total RNAs were first purified from transfected cells using Trizol as described by the manufacturer (Invitrogen); second, first-strand cDNA was reverse transcribed using the SuperScript First-Strand Synthesis System (Invitrogen); and finally PCR amplifications were performed with a series of specific primer pairs using the Maxime PCR premix kit (Intron Biotech). Also, quantitative real-time PCR was performed with iQ SYBR Green Supermix (Bio-Rad) using the iycler IQ multicolor real-time detection system (Bio-Rad). All qPCRs were carried out for 40 cycles under standard PCR conditions. We analyzed the results of quantitative real-time PCR based on the threshold cycle value (ΔCt) was first calculated by subtracting the averaged Ct value of three internal controls from the averaged Ct value of a target gene, and later the ΔΔCt value was calculated by subtracting the ΔCt value for the targeted gene of a YY1 knockdown sample from the ΔCt value for that of the control. Fold differences were determined by raising 2 to the ΔΔCt power [25]. The primer sequences and PCR conditions are available upon request.

Southern blot and bisulfite sequencing

Genomic DNAs were purified using DNAzol (Invitrogen), and 5 µg of each genomic DNA was first digested with BamHI and later with either MspI or HpaII. These double-digested DNAs were separated on a 0.8% agarose gel and transferred by capillary blotting onto Hybond nylon membranes (Amersham). Membranes were hybridized with the 32P-labeled probes as indicated in the figures and analyzed by autoradiography. The probes used for this study are as follows: a 1.3-kb fragment corresponding to the Peg3 promoter, a 2.3-kb fragment for the Peg3 YY1 binding region, a 1.95-kb fragment for the Nespas YY1 binding region, and a 1.88-kb fragment of the Xir YY1 binding region. The methylation status of the promoter region of mouse Peg3 (GenBank Accession No. AC020961; 106803–107240) was also analyzed using the bisulfite sequencing method [26]. The DNAs isolated from NT and 6-4 were first digested with BglII, purified with phenol/chloroform extraction, and precipitated with ethanol. For the bisulfite conversion reaction, these DNAs were treated with the EZ DNA methylation kit (Zymo Research). The resultant single-stranded DNAs were used as templates for the PCR.
using specific primers that were designed for the C-to-T converted DNAs. The sequences for these primers are as follows: Peg3 Promoter F, 5′-AGAGGGTGTGTAGTGAAGTGTTAGGTG-3′, and Peg3 Promoter R, 5′-CATCCCTTCACCCACATCCATCC-3′. The PCR products were subeloned into the TOPO TA cloning vector (Invitrogen) for sequencing.

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