Reciprocal imprinting of human GRB10 in placental trophoblast and brain: evolutionary conservation of reversed allelic expression

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Genomic imprinting may have evolved not only to regulate fetal growth and development, but also behaviour. The mouse Grb10 gene provides a remarkable model to explore this idea because it shows paternal expression in brain, whereas in the placenta and most other embryonic tissues, expression is from the maternal allele. To assess the biological relevance of this reciprocal pattern of imprinting, we explored its conservation in humans. As in mice, we find the human GRB10 gene to be paternally expressed in brain. Maternal allele-specific expression is conserved only in the placental villous trophoblasts, an essential part of the placenta involved in nutrient transfer. All other fetal tissues tested showed equal expression from both alleles. These data suggest that the maternal GRB10 expression in placenta is evolutionarily important, presumably in the control of fetal growth. As in the mouse, the maternal transcripts originate from several kilobases upstream of the imprinting control region (ICR) of the domain, from a promoter region at which we find no allelic chromatin differences. The brain-specific paternal expression from the ICR shows mechanistic similarities with the mouse as well. This conserved CpG island is DNA-methylated on the maternal allele and is marked on the paternal allele by developmentally regulated bivalent chromatin, with the presence of both H3 lysine-4 and H3 lysine-27 methylation. The strong conservation of the opposite allelic expression in placenta versus brain supports the hypothesis that GRB10 imprinting evolved to mediate diverse roles in mammalian growth and behaviour.

INTRODUCTION

Genomic imprinting is a form of non-Mendelian inheritance in eutherian mammals affecting around 80 genes in the mouse and 40 in human (1). It is a mechanism of transcriptional control that results in gene expression from only one allele according to its parental origin. As a consequence, the maternally and paternally inherited genomes have different effects on embryonic growth and development. The evolutionary reason for this parent-of-origin specific gene expression is thought to reflect potential conflict between the parental genomes over nutritional demands (2). Maternally expressed imprinted genes would suppress growth, whereas those that are paternally expressed would promote growth (3). Deregulation of genomic imprinting can give rise to abnormal development. The parent-of-origin specific nature of expression means that for some regions, (epi)mutations result in reciprocal phenotypes depending on which parental allele is affected, as seen in the fetal overgrowth syndrome Beckwith–Wiedemann syndrome and the growth restriction of Silver–Russell syndrome (SRS) (1,4).

The allelic differences in transcriptional activity originate from patterns of DNA methylation established in the male
and female gametes. These differential patterns of methylation are maintained throughout somatic development (5). Differentially methylated regions (DMRs), which acquire their allelic methylation in the germ line and are essential for imprinted gene expression, are referred to as imprinting control regions (ICRs) (6). The presence of a single ICR within an imprint domain implies a role as a cis-acting control element, co-ordinating regulation of several genes (7). ICRs are associated with differential chromatin modifications. Methylation of ICRs are coupled with repressive chromatin modifications such as histone H3 lysine-9 di/trimethylation (H3K9me2/3) and H4K20me3. Unmethylated alleles are associated with permissive chromatin modifications including histone acetylation and H3K4me2/3 (8,9).

The murine growth factor receptor-binding protein 10, Grb10, is a potent growth inhibitor (10). It is an adaptor protein that regulates IGF-1 receptor and insulin receptor signalling through E3 ubiquitin-protein ligase Nedd4 degradation of these receptors (11). In most mouse tissues, Grb10 is expressed from the maternal allele, except in brain, where it is expressed predominantly from the paternal allele (12,13). This reciprocal expression is due to alternative promoter usage, with the maternal expression arising from an unmethylated CpG island (13). We and others showed that human GRB10 is biallelically expressed in most tissues, except in brain, which shows preferential paternal expression (14–17). The human orthologue has been considered a strong candidate gene for SRS, since maternal uniparental disomy (mUPD7) and small duplications of 7p12 encompassing the GRB10 gene have been reported in 10% of cases of this growth restriction syndrome. However, no coding or epimutations have been identified in GRB10 in SRS patients (4,13–15,18,19). Recently, the DMR of the mouse Grb10 gene was found to be essential for imprinting within this locus (20) and was shown to be marked by mono-allelic bivalent chromatin, with enrichment of both H3K4me2 and H3K27me3 on the paternal allele (21). This DMR is the promoter for the paternally derived, brain-specific transcripts, and expression only occurs when the H3K27me3 is resolved, leaving the permissive H3K4me2 on the paternal allele (21).

Given the predominant maternal expression of murine Grb10, we performed a detailed investigation of the human transcripts, focusing on previously unexamined tissues that could account for the clinical features of SRS. We find robust maternal expression for all splice variants in cytokeratin-7-positive villous trophoblast cells of the placenta. Surprisingly, these maternal transcripts, and the biallelically expressed transcripts found elsewhere, originate from the same promoter, which is associated with an unmethylated CpG island region, called CGI-1. As in the mouse, the brain-specific paternally derived transcripts initiate from the DMR of the locus. To investigate whether allelic histone modifications could account for this complex imprinting pattern, we compared the distribution of various histone modifications across a 15 kb interval encompassing the unmethylated CGI-1 promoter region and the DMR region (a CpG island called CGI-2) in lymphoblasts, brain and placenta. This comparative analysis revealed additional mechanistic similarities in GRB10 imprinting between humans and mice.

RESULTS

Imprinting of GRB10 splice variants

The human GRB10 gene is subject to alternative splicing of the 5’ exons, many of which are non-coding. To date, there have been 13 different splice variants described in the literature or identified in EST databases. Previous reports have suggested that these splice variants may be subject to differential allelic expression, since the γ1 splice form is expressed from the maternal allele in muscle, whereas in other tissues, this and all other transcripts originating from the UN1 exon, adjacent to the unmethylated CGI-1, are biallelic (14). The paternal expression observed specifically in brain has been observed for all isoforms except γ2. However, the precise promoters utilized for each of these splice forms are unknown (14,16,17).

To further characterize the imprinting of the various GRB10 splice variants, we assessed the allelic expression in fetal tissues, including first trimester and term placenta. Splice form-specific RT–PCR primers were designed between exons, spanning alternative splice sites, therefore only allowing the amplification of a single specific transcript (17) (Supplementary Material, Fig. S1). Using these primer sets, we were able to confirm that in the majority of tissues, GRB10 is not imprinted, showing equal expression from both parental alleles (Supplementary Material, Table S1). Using published primers that simultaneously amplify all isoforms, and those that distinguish between β and γ1 splice variants (16,17), we found that GRB10 is expressed preferentially from the paternal allele in brain and spinal cord (Fig. 1, Supplementary Material, Fig. S1 and Table S1).

Robust maternal expression in villous trophoblasts

In addition to first trimester and term placenta tissue, we also assessed the imprinting status of GRB10 in villous trophoblast cells isolated from the same placenta samples. These cells were subjected to negative immunoselection using a monoclonal antibody against HLA class 1, which resulted in a population of cells that were >98% cytokeratin 7-positive, a marker specific for villous trophoblast cells. We found that all splice variants in these cells were expressed from the maternal allele, whereas this allelic expression pattern is masked in whole placenta tissues, where GRB10 is largely biallelic in its expression (Fig. 1, Supplementary Material, Fig. S1 and Table S1).

GRB10 promoter-specific imprinting

Although many GRB10 splice variants have been described, their tissue-specific promoters have not been reported. To find the transcriptional start sites for each splice variant, we performed 5’-RACE on placenta- and brain-derived RNA. Using anchored primers in exons 2 or 3, we identified many additional placental splice variants, including transcripts comprising the three novel exons UN1A, UN3.1 and UN3.2 (UN1A 52956-53065, UN3.1 39229-39146 and UN3.2 38800-38772 of AC004920, respectively) (Fig. 2A). With the exception of the σ variant, which has a truncated UN4 first exon (14), the remaining transcripts had either UN1 or
UN1A as their initiating exon. UN1A is a novel first exon mapping 230 bp upstream of UN1, and 700 bp from the unmethylated CGI-1. We also isolated a brain-specific transcript that originates from exon UN2, which is part of the 5' end of two previously identified ESTs (AJ271366 and AK289511). This confirms previous reports that the UN2 exon could be a brain-specific promoter (15) and that UN2 is not included in transcripts that initiate from either UN1 or UN1A.

To assess the relative abundance of transcripts originating from UN1, UN1A and UN2, northern blots containing polyA RNAs of fetal tissue were hybridized with specific anti-sense riboprobes. This revealed that UN1 and UN1A have similar expression profiles, both in tissue-specificity and abundance, presumably because they are in close proximity and therefore within the same chromatin domain (Fig. 2B). Using the same approach, we could not detect expression of the brain-specific transcript using an UN2 anti-sense riboprobe. This suggests that most of the GRB10 expression in fetal brain originates from the unmethylated CGI-1 region. qRT-PCR confirmed that 25–30% of brain expression originated from UN2 and is presumably below the detection sensitivity of northern hybridization (Supplementary Material, Fig. S2). The high CG content of exon UN2 also makes this an undesirable...
probe. Expression from all three promoters was detectable in adult brain, but at ~20% of fetal brain levels (Supplementary Material, Fig. S2).

To assess the parent-of-origin of expression for all transcripts arising from UN1, UN1A and UN2, we performed RT–PCR with specific forward primers in conjunction with the common exon 4 reverse primer. The resulting RT–PCR products reflect alternative splicing, but as no splicing event occurred after the polymorphism in exon 3, allelic expression could be determined by sequencing with the reverse primer. All transcripts from UN1 and UN1A were biallelically expressed in brain, spinal cord, lung, limb, tongue, umbilical cord, skin, kidney and adrenals (Supplementary Material, Table S1). However, all trophoblast samples exhibited robust maternal expression. Placental samples were either biallelic or expressed preferentially from the maternal allele. RT–PCR products from UN2 could only be detected in brain and were also mono-allelically expressed, but from the paternal allele, in all informative cases (Fig. 3).

DNA methylation and allelic histone modifications at the GRB10 promoter regions

Both the human and mouse GRB10/Grb10 promoter regions are associated with CpG islands. We have previously shown that CGI-1 is unmethylated on both parental alleles, whereas CGI-2 is a maternally methylated DMR (13,18). We found no tissue-specific differences in DNA methylation between villous trophoblast, brain and placenta, even though their allelic expression profiles are strikingly different (Supplementary Material, Fig. S3; data not shown) (13,18).

With no discrepancy in DNA methylation between imprinted and non-imprinted tissues, we next investigated whether allelic histone modifications could account for the tissue-specificity of the allelic expression. First, we determined the abundance of various histone modifications in maternal uniparental disomy (mUPD7) and paternal uniparental disomy (pUPD7) lymphoblastoid cell lines using both chromatin immunoprecipitation (ChIP) and quantitative PCR at seven sites throughout the 5' region of GRB10. Our analysis focused on trimethylation of Lys-27 on histone H3 (H3K27me3), di- and trimethylation of Lys-9 on histone H3 (H3K9me2/3) and trimethylation of Lys-20 on histone H4 (H4K20me3) as markers of repressive chromatin, and acetylation at Lys-9 on histone H3 (H3K9ac) and dimethylation of Lys-4 on histone H3 (H3K4me2) as markers of active chromatin. We confirmed the quality of the precipitated fractions by assessing the allelic enrichment at the PEG10 and MEST DMRs, both of which also map to human chromosome 7, and for which the allelic-histone profiles had previously been reported (22). With the exception of the CGI-2 DMR, there was no consistent difference between the mUPD7 and
pUPD7 cell lines in the 5′ region of GRB10, suggesting a general lack of allelic enrichment for the histone modifications analysed (data not shown). No allelic enrichment for permissive or repressive histone modifications was observed at exons UN1 or UN1A, which correlates with the absence of expression for GRB10 in lymphoblast cells. Within the CGI-2 DMR region, we observed allelic-specific enrichment of H3K4me2 and H3K27me3 in pUPD7 cells, suggesting that the repressive H3K27me3 and permissive H3K4me2 co-existed on the same parental allele, reminiscent of a bivalent chromatin domain. In addition, we also observed enrichment of H4K20me3 in mUPD7 cell, which was associated with the DNA-methylated allele (data not shown).

To confirm these observations in tissues, we compared brain and placenta material, using qualitative ChIP, followed by PCR and enzymatic digestion using HOT-STOP PCR. This allowed us to evaluate the relative abundance of the maternal and paternal alleles for each of the bound and unbound fractions. We assessed five regions corresponding to heterozygous SNPs: 1.5 kb downstream of CGI-1 (rs12718925); 300 bp from the UN1A transcription start site (rs10261151); 1.5 kb from UN1 transcription start site within intron 1 (rs6593185); mid-intron 1 (rs7791286), and within the CGI-2 DMR (rs23019226 or 2301927). We did not observe any allelic enrichment at any region in placenta or brain, except at the CGI-2 DMR, complementing our previous observation in UPD lymphoblasts (Supplementary Material, Fig. S4; Fig. 4).

We did not observe any allelic enrichment in the region surrounding rs10261151, 300 bp downstream of CGI-1, in placenta, in spite of the observed maternal expression in villous trophoblasts (Supplementary Material, Fig. S4). This may be because this region is located within an SINE element, where high-copy repeats have recently been shown to be associated with defined DNA- and histone methylation patterns (23). Indeed, assessing the DNA methylation profile of this SINE in genomic DNA, which was heterozygous for rs10261151, we observed methylation on both the parental alleles (Supplementary Material, Fig. S3). This is presumably coupled with biallelic enrichment of repressive histone modifications.

**Figure 4.** Allele-specific histone modifications at GRB10 CGI-2. (A) DNA extracted from antibody bound (B) and unbound (U) chromatin fractions were PCR amplified. For each bound fraction, we determined the ratio between the parental alleles, after correction against the allelic ratio of the input chromatin. Asterisks indicate the lanes where the allelic ratios were >2. A bivalent chromatin domain was observed in placenta, with paternal allele enrichment for H3K4me2 of 3.1-fold, and 2.7-fold for H3K27me3. (B) Resolution of the CGI-2 bivalent domain in brain, with paternal allele enrichment of the permissive histone modification H3K4me2 and H3K9ac with corrected allelic enrichment >2. The levels of H3K27me3 on the paternal allele precipitation decrease, whereas H3K9ac increases. The allelic enrichment of SNRP N ICR is shown as an example of a ChIP enrichment control.
In placenta, we observed mono-allelic bivalent chromatin at the DMR with both H3K4me2 and H3K27me3 precipitating on the paternal allele (Fig. 4A; Supplementary Material, Fig. S4). In addition, we also observed H4K20me3 on the maternal allele, confirming our observations in UPD lymphoblastoid cell lines (Supplementary Material, Fig. S4). In maternal allele, confirming our observations in UPD lymphoblastoid cell lines (Supplementary Material, Fig. S4). In fetal brain, the bivalent chromatin configuration had become resolved, with a lack of H3K27me3 on the paternal allele, but with the continued presence of H3K4me2. In addition, we observed H3K9ac on the unmethylated paternal allele (Fig. 4B). This H3K4me2 enrichment, with the addition of H3K9ac, presumably allows for the paternal expression from UN2 that we observed in fetal brain.

DISCUSSION

The imprinting of human GRB10 is complex, involving many splice-variants combined with tissue-specific expression signature, but also demonstrate for the first time maternal GRB10 expression exclusively in villous trophoblasts of the placenta. This maternal expression originates from the major promoter region, UN1 or UN1A, whereas the brain-specific paternal expression arises from UN2 embedded within the maternally methylated DMR of GRB10. The previously reported bias towards paternal preferential expression in brain, using RT–PCR to detect all isoforms (14–17), is explained by co-amplification of biallelic transcripts from UN1/UN1A and paternal expression from UN2. It remains to be determined whether the biallelic expression arising from UN1/UN1A is of glial cells origin, and the paternal expression from UN2 is exclusively found in neuronal cells, as reported in the mouse (24). Recently, a mouse transcript originating from an alternative first exon, in a similar position to human UN1A, was described. Oocyte-specific expression of this transcript has been proposed to be essential in establishing the germ line methylation at the Grb10 DMR (25). Therefore, transcripts from UN1/UN1A may also be involved in establishing the DNA-methylation imprint at the human CGI-2 region. The CGI-2 region shown also exhibits conserved chromatin features between humans and mice and represents the ICR of the locus (20). The DNA-methylated allele is associated with repressive histone mark H4K20me3, whereas the unmethylated paternal allele is enriched for the permissive modification, H3K4me2. In addition to these constitutive covalent histone modifications, we detect a mutually exclusive enrichment for the active and repressive marks H3K9ac and H3K27me3 on the paternal allele. H3K27me3 is detected on the paternal unmethylated allele of CGI-2 in all analysed tissues except brain, whereas paternal allele-specific enrichment of H3K9ac is only observed in brain, in the absence of H3K27me3. Recently, bivalent chromatin domains have been associated with numerous ‘poised’ lineage-specific genes in mouse and human ES cells (26). These genes are temporarily repressed until removal of H3K27me3 allowing subsequent gene expression, with the H3K4me2 status remaining unaffected (26,27). Studies have suggested that removal of H3K27me3 on its own is not always sufficient to bring about maximal transcription, and that in addition to loss of H3K27me3, increased histone acetylation is also required (28). This is the precise transcriptional activation process that occurs with both human and mouse CGI-2-associated brain paternal-specific transcripts, suggesting that bivalent chromatin constitutes the main mechanism involved in regulating the brain-specific expression of GRB10.

In addition to being a brain-specific promoter region, CGI-2 in the mouse is the ICR of the domain and is involved in controlling maternal expression (20). In the mouse, the CGI-2 DMR is thought to behave as a methylation-sensitive insulator, similar to the ICR of the H19-Igf2 domain (29). The Grb10 CGI-2 region contains a 600 bp CG-rich repetitive sequence that binds the CTCF insulator protein in a methylation-sensitive fashion in vivo (15). Human CGI-2 does not have this CG-rich insulator repeat sequence, and this is thought to account for the lack of ubiquitous maternal expression in human. Our finding of maternal-specific expression in villous trophoblasts suggests that several epigenetic mechanisms may be involved in tissue-specific imprinting of human GRB10. One possible mechanism is that trophoblast-specific active histone modifications, such as those associated with the active chromatin configuration recently reported for highly expressed transcripts (30), mark the UN1/UN1A promoter region on the maternal allele. As whole placenta tissue was used for the ChIP, this active histone confirmation in villous trophoblasts is masked by other, biallelically expressing placenta cell types.

From the view of the kinship theory (also known as the parental-conflict theory) of genomic imprinting, the most important cell types in the placenta is the trophoblast lineage as these provide the main structural and functional components that bring the fetal and maternal blood systems into close proximity to allow nutrient exchange (2,31). The observation of robust maternal expression of GRB10 in villous trophoblasts may have important implications for the aetiology of SRS. Loss of methylation at the H19 ICR, and reduced Igf2 expression, is the only epi-mutation in SRS known to date, accounting for 30–50% of cases (4). However, 10% of patients have over-expression of GRB10 due mUPD7 and 7p12 duplication, and these cases would have a double dose of this maternally expressed growth inhibitor in villous trophoblasts. Such a scenario would be in keeping with the kinship theory (4,31) and could account for the observed IUGR in SRS patients.

Recently, the kinship theory was modified to include paternal care after weaning (33). This proposed biparental care model allows for the evolution of patterns of expression opposite to the ones originally predicted by the kinship theory, so that imprinted genes may be subject to reversion in their allelic expression after weaning. This is precisely what we observed for both murine and human GRB10, with maternal expression in trophoblast, and paternal expression in brain persisting into adulthood, due to alternative promoter usage. The biparental extension to the kinship theory was derived to explain the bi-phasic phenotype of Prader–Willi syndrome (PWS), where individuals are born growth restricted but subsequently develop insatiable appetite and obesity (33). However, the growth trajectory for probands with SRS is not bi-phasic. All probands remain growth restricted and do not
exhibit normal or catch-up growth (4), therefore opposing the biparental care model. Instead, our findings favour a model in which GRB10 regulates different pathways, possibly by interacting with different activated tyrosine kinase receptors in trophoblast and brain. Over-expression of maternally derived GRB10 in trophoblast would limit fetal growth, resulting in IUGR, whereas a lack of paternally derived growth-enhancing GRB10 in brain results in a weaning phenotype associated with poor suckling. In fitting with this hypothesis, all cases of mUPD7 have failure to thrive and feeding problems as confirmatory phenotypic features and, as a result, are fed via a gastrostomy tube (34). Therefore, a lack of paternally derived GRB10 expression in brain, for instance, due to non-resolution of the bivalent chromatin at the GRB10 DMR, could account for additional cases of non-syndromic post-natal growth restriction.

MATERIALS AND METHODS

Collection of human material

A cohort comprising 65 fetal tissue sets (8–18 weeks) with corresponding maternal blood sample and 240 term placental samples was used in this study and is described elsewhere (35,36). An additional 31 human fetal brain samples were obtained from the HDBR Tissue Bank (Institute of Child Health). Adult brain (51 + 70 years) cDNA was purchased from Ambion. For some placenta, corresponding villous trophoblast cells were isolated by negative immunoselection (19). Immunostaining of first trimester placenta samples confirmed the specificity of the cytokeratin 7 antibody (Supplementary Material, Fig. S5). The mUPD7 and pUPD7 cells were cultured using identical conditions. DNA and RNA extraction and cDNA synthesis were carried out as described previously (19).

5′RACE

Brain and placenta RNA were used for 5′-RACE using the 5′ gene racer kit (Invitrogen). The PCR step was performed with the reverse primer located in either exon 2 or 3. The PCR products were subcloned into pGEM T-easy vector (Promega) and sequenced using an ABI prism 3100 DNA sequencer (Applied Biosystems).

Imprinting assays

For the imprinting analysis of GRB10 splice forms, we used previously published primers (16,17). Imprinting analysis of transcripts originating from the promoter regions identified by 5′-RACE used specific primers in the first exons in conjunction with a common GRB10 exon 4 reverse primer (see Supplementary Material, Table S2 for primer sequences). Each RT-PCR (amplified for 35–42 cycles, within log-phase of cycle profile; Supplementary Material, Fig. S6A) incorporated the Sst1 SNP located in exon 3 in the product and was subjected to both direct sequencing and restriction enzyme digestion (sequencing was determined to be a reliable and reproducible semi-quantitative method of determining allelic differences using spiked homozygous samples; Supplementary Material, Fig. S6B).

Northern blotting

To analyse relative expression levels of the GRB10 isoforms arising from each promoter, we used custom-made fetal northern blots containing 2 µg poly A+ RNA (Biochain). PCR products containing the first exon associated with each promoter region (UN1-D86962; UN1A-NM_001001555; UN2-AJ271366) were cloned into the pGEMT-easy vector (Promega). Single-stranded antisense probes were radiolabelled with α[32P]UTP using the T7/SP6 MAXiScript in vitro transcription kit (Ambion). Hybridizations were carried out overnight at 65°C and washed according to the manufacturer’s instructions. A β-ACTIN PCR probe was used to confirm equal loading of RNA in each lane.

Methylation analysis by bisulphite sequencing

Approximately 1 µg DNA was treated with sodium bisulphite as described previously (13). HotStar DNA polymerase (Qiagen) was used to amplify CpG island products using the following specific primers: upstream region F-AAGTGYGG GGGTGGA GTAGAGG and upstream region R-CACRC TCTATAAAAACAAATATTATC; CGI-1 F/R and CGI-2 F/R (18). PCR amplification, cloning and sequencing were performed as described previously (35).

Chromatin immunoprecipitation

Two fetal brain and three term human placenta samples were used for ChIP. ChIP was carried out as described previously (21,35) using the following Upstate Biotechnology antisera directed against H3K4me2 (07-030), H3K9me2 (07-441), H3K9me3 (06090459), H3K9ac (07-352), H3K27me3 (07-449) and H4K20me3 (07-463) (Upstate Biotechnology). ChIPed DNA was subjected to allele-specific PCR. Polymorphisms within the 15 kb interval between CGI-1 and CGI-2 were identified by interrogating SNP databases or genomic sequencing (see Supplementary Material, Table S2 for primer sequences and location). Only ChIP sample sets that showed enrichment for additional ICRs were used in the analysis. Relative band intensities of the maternal and paternal bands were determined using ImageMaster VDS software (Amersham Biosciences).

Precipitation levels in the mUPD7 and pUPD7 ChIP were determined by real-time PCR amplification, using SYBR Green PCR kit (Applied Biosystems). Each PCR was run in triplicate and results are presented as a percentage of immunoprecipitation, calculated by dividing the average value of immunoprecipitated DNA by the average value of the corresponding input chromatin, and normalized to the level of precipitation at the SNRPN ICR, a positive control from both active and repressive histone modifications not located on human chromosome 7.
SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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