Overexpression of *Hr* links excessive induction of Wnt signaling to Marie Unna hereditary hypotrichosis

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Marie Unna hereditary hypotrichosis (MUHH) is a rare autosomal dominant hair disorder. Through the study of a mouse model, we identified a mutation in the 5'-untranslated region of the *hairless (HR)* gene in patients with MUHH in a Caucasian family. The corresponding mutation, named ‘hairpoor’, was found in mutant mice that were generated through N-ethyl-N-nitrosourea mutagenesis. Hairpoor mouse mutants display partial hair loss at an early age and progress to near alopecia, which resembles the MUHH phenotype. This mutation conferred overexpression of HR through translational derepression and, in turn, decreased the expression of Sfrp2, an inhibitor of the Wnt signaling pathway. This study indicates that the gain in function of HR also results in alopecia, as seen with the loss of function of HR, via abnormal upregulation of the Wnt signaling pathway.

INTRODUCTION

Autosomal dominant Marie Unna hereditary hypotrichosis (MUHH; MIM 146550) is characterized by abnormal hair density on the scalp, eyebrows, eyelashes or body. Affected individuals are born with sparse hair that is characteristically coarse, wiry and twisted in early childhood, and progress to complete baldness (1–3). Although genetic heterogeneity has been reported in Dutch, Belgian, British, French, German and Chinese families, the majority of MUHH pedigrees have been linked to chromosome 8p21 near the *hairless* (*HR*) gene (4–9).

The HR protein is localized in the nucleus, has a zinc-finger domain and acts as a transcriptional corepressor interacting with nuclear receptors such as the thyroid hormone receptor, the vitamin D receptor and the retinoic acid-related orphan receptor–α (10–13). Multiple mutations of *HR* have been reported to cause a hair loss phenotype in rodents and humans. The human *HR* gene has been found to harbor mutations responsible for hair defect disorders including alopecia universalis congenita (MIM 203655), atrichia with papular lesions (MIM 209500) and most recently MUHH. With the exception of MUHH, these disorders are inherited in a recessive inheritance mode and are caused by loss of function of *HR* because of missense, nonsense or deletion mutations (14–19).

Mouse models with various mutations in *Hr*, such as *Hr*br, *Hr*rb and *Hr*m1Enu, have been actively studied to understand the pathophysiology of human hair loss disorders (20–23). Homozygous mutant mice with autosomal recessive mutations in *Hr* display a distinctive phenotype with a strikingly similar pattern of hair loss to each other. These mutant mice grow hair normally in postnatal stages; however, they shed hair progressively from the eyelids to the hind limbs and subsequently

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show an absence of hair follicles (HFs) and the formation of abnormal structures such as uricles and dermal cysts in the skin (21–25). In $Hr^{hr}$ mice, apoptotic cell death in HFs was observed in the transition stage from catagen to anagen, suggesting that $Hr$ may play a role in regulating the HF growth cycle (26). Additionally, targeted disruption of $Hr$ revealed that dysregulation of the Wnt signaling pathway underlies the loss of function of $Hr$ resulting in hair loss, which was restored by the expression of $Hr$ in keratinocytes (27,28).

Recently, 13 distinct mutations have been identified in patients with MUHH. Interestingly, these mutations were present in the 5' untranslated region (UTR) of $HR$ (29), in contrast to mutations present in the coding sequences of $HR$ that result in loss of function. In addition to the characteristic sparse hair phenotype at birth, MUHH is different from other $HR$ defect-associated disorders because it is inherited in a dominant manner, whereas the other disorders are inherited in a recessive mode. Although mutations responsible for MUHH have been reported, it is not known how these mutations cause the MUHH phenotype and no animal model is currently available.

In the present study, using an animal model of MUHH, namely 'hairpool' ($Hr^{hp}$) mice, we show that an MUHH-causing mutation resulted in the overexpression of $HR$ and induced proliferation and differentiation of the skin. In addition, we documented abnormally excessive induction of Wnt signaling in this mouse model, indicating the involvement of Wnt signaling in the manifestation of the MUHH phenotype.

RESULTS

A mutation in the 5'-UTR of $HR$ as identified in patients with MUHH

We have been studying the mutant mouse, $Hr^{hp}$, whose phenotype and inheritance mode resembles that of MUHH in humans, and identified a mutation in the 5'-UTR of $Hr$ that cosegregated with this phenotype (30). In an effort to identify the corresponding mutation in MUHH, we first confirmed the presence of a longer 5'-UTR in the human $HR$ gene by analyzing the sequences of $HR$ cDNA from several cell lines, skin and brain tissues. A comparison of mouse, rat and human $HR$ sequences (Supplementary Material, Fig. S1A) revealed that the 5'-UTR sequence of human $HR$ has 90% homology to the 5'-UTR sequence of mouse $Hr$. These 5'-UTRs contained highly conserved sequences with four putative upstream open reading frames (uORFs), of which the second uORF was the longest. The length and interval of the uORFs was conserved among the 5'-UTRs from mouse, rat and human, suggesting functional conservation (Supplementary Material, Fig. S1B).

Mutational analysis was performed on four affected and three unaffected individuals from a Caucasian family with a reported history of MUHH (individuals #28, 29, 13, 30, 14, 15 and 16 in Fig. 1A) (4). Sequence analysis revealed a T-to-C transition substitution at position −320 from the translation start site of $HR$ (NM_005144). All affected individuals were heterozygotes carrying one mutant allele ($C$) and one normal allele ($T$), which was confirmed by subcloning and sequencing each allele (Fig. 1B). In contrast, unaffected individuals were homozygous for the normal allele. This T-320C substitution was not detected in 100 Caucasian individuals from a human variation panel, suggesting that it was a mutation and not a polymorphism. The T-320C mutation of $HR$ was confirmed by restriction fragment-length polymorphism (RFLP) analysis (Fig. 1C). As has been previously reported (29), the T-320C mutation changed the upstream ATG (uATG) start codon of the second uORF to ACG, presumably abolishing the second uORF (Fig. 1D).

Proliferation and differentiation of skin cells was increased in hairpool mice

The $Hr^{hp}$ mouse was derived by N-ethyl-N-nitrosourea (ENU) mutagenesis (31) and its phenotype was inherited in a semidominant manner, displaying a more severe phenotype in homozygotes (Fig. 2A). The heterozygous mice had shorter and sparser hair than wild-type mice by 7 days after birth and gradually advanced to baldness as they aged, a progression that was similar to what is observed in patients with MUHH (Fig. 2B). Sequence analysis of $Hr$ in homozygotes revealed a T-to-A transversion substitution at position 403 in the second uORF (NM_021877) (30), which corresponded to the T-320 position in human $HR$ (Supplementary Material, Fig. S1A).

Histological evaluation of skin revealed that at 5 weeks of age wild-type mice had normal long HFs that grew deep into the bottom of the subcutis layer. In contrast, $Hr^{hp}$ heterozygous mice had much shorter HFs that reached into the end of the dermis or the upper region of the subcutis layer, and a reduced number of HFs compared with age-matched wild-type mice (Fig. 2C, D, F and G). Remarkably, the homozygous mice had much shorter HFs that reached into the end of the subcutis layer (Fig. 2A). The heterozygous mice had shorter and sparser hair than wild-type mice by 7 days after birth and gradually advanced to baldness as they aged, a progression that was similar to what is observed in patients with MUHH (Fig. 2B). Sequence analysis of $Hr$ in homozygotes revealed a T-to-A transversion substitution at position 403 in the second uORF (NM_021877) (30), which corresponded to the T-320 position in human $HR$ (Supplementary Material, Fig. S1A).

Epidermal hyperplasia led us to examine cell proliferation by monitoring the expression of Ki67 in the skin cells of 3-week-old wild-type and $Hr^{hp}$ mice. At this telogen phase of the hair cycle, few proliferating cells were detected in wild-type mice. However, a large number of proliferating cells were observed in follicular cysts and the basal cell layer of the interfollicular epidermis in $Hr^{hp}$ mice (Fig. 3A). An investigation of keratin markers revealed that the proliferating cells were K14-positive (Fig. 3B). The detection of keratinocyte differentiation markers indicated that the epidermal hyperplasia was associated with an expansion of differentiated compartments.

The thicknesses of the basal cell layers (K14), suprabasal layers (keratin 10, K10) and spinous layers (involucrin) of mutant mice were also increased (Fig. 3C). This epidermal
hyperplasia was not reported in Hr mutant mice including \( Hr^{hr}, Hr^{rh} \) and \( Hr^{m1ENU} \) (20–23). However, this phenotype was observed in Hr knockout mice with the induction of HR (27). In this transgenic mutant with Hr expression under control of K14 promoter, thicker epidermis was due to expansion of the undifferentiated compartment. In contrast, epidermal hyperplasia in the \( Hr^{Hp} \) mice is attributed to an expansion of the both undifferentiated and differentiated compartments. This discrepancy maybe caused by the difference in the promoter directing Hr expression. These findings suggest that HR overexpression induced the proliferation of epithelial cells and the terminal differentiation of epidermal keratinocytes, consequently resulting in epidermal hyperplasia and the formation of abnormally proliferating follicular cysts.

**T403A mutation conferred translational derepression of a reporter gene**

The T403A mutation changed the uATG of the second uORF to ACG (Fig. 1D), consequently abolishing the uORF. To investigate how the 5'-UTR regulated HR gene expression and to analyze the effect of the T403A mutation, we performed a reporter assay using constructs in which the wild-type or mutant 5'-UTR of Hr (695 bp) was inserted upstream of the green fluorescence protein (GFP) coding sequence (Fig. 4A). Remarkably, insertion of the mutant 5'-UTR sequence resulted in a 60% increase in GFP expression compared with wild-type 5'-UTR GFP expression; thus, the mutant 5'-UTR sequence showed a 6-fold higher expression than that of the wild-type construct (Fig. 4B and C). In contrast to GFP protein expression, GFP mRNA expression was not affected by the T403A mutation (Fig. 4D). This indicated that the \( Hr^{T403A} \) mutation, as well as its human counterpart, \( HR^{T-320C} \), confers derepression of HR expression and thus is a gain of function allele.

**Overexpression of Hr results in abnormal upregulation of Wnt signaling**

To determine the in vivo effects of the \( Hr^{T403A} \) mutation at the molecular level, we compared the expression levels of Hr in mutant and wild-type mice. As expected, expression of the

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**Figure 1.** The mutation was found in the 5'-UTR of HR responsible for MUHH. (A) The pedigree of the Caucasian family with MUHH used in this study (4). Black symbols depict affected individuals. The arrow indicates a proband. (B) The T-320C mutation is indicated by the red arrowhead. Affected individuals possess mutated (C) and normal (T) alleles. (C) The mutated allele was revealed by NcoI RFLP of a 792 bp PCR product amplified from genomic DNA. Upon NcoI digestion of the PCR product, the four affected individuals displayed 576 and 216 bp fragments from the mutated allele, and 368, 216 and 208 bp fragments from the normal allele. Numbers correspond to individuals in the pedigree (M; marker). (D) Schematic diagram of HR mRNA depicting the four uORFs in the 5'-UTR. The MUHH mutation is indicated by a red arrowhead in the second uORF.
HR protein was dramatically increased in the mutant mice at 3 and 5 weeks of age, which corresponds to the telogen and anagen growth stages of the hair cycle in wild-type mice (Fig. 5A). The HR protein gradually increased as the number of mutant alleles increased. The HR protein of HrHp/þ and HrHp/HrHp mutant mice was similar to that of wild-type mice at P14, the catagen stage. However, the difference in the amount of HR protein was striking when HR expression levels were low in wild-type mice. The amount of HR protein in HrHp/þ and HrHp/HrHp mutant mice was 2.1- and 6.4-fold greater than that in wild-type mice at P21, respectively. Similarly, the amount of HR protein was 3.9- and 12.5-fold higher in the skin of HrHp/þ and HrHp/HrHp mutant mice at P35 compared with that in age-matched wild-type mice.

To investigate how HR overexpression causes hair loss, we analyzed the association of Wnt signaling with HR because the abnormal regulation of Wnt signaling has been implicated in defective HF morphogenesis as well as the faulty cycling of HFs (32). We compared the expression of Wnt inhibitors and keratinocyte cells, including the Dickkopf (Dkk) family, sclerostin domain-containing protein 1 (Sostdc1), Wnt inhibitory factor 1 and the secreted frizzle-related protein (Sfrp) family (data not shown), in HrHp and wild-type mice. Remarkably, Sfrp2 expression was significantly reduced to 0.69- (± 0.43) and 0.31- (± 0.21) fold in HrHp heterozygotes and homozygotes, respectively, compared with wild-type mice (Fig. 5B). Consequently, the expression of the Wnt-responsive genes, myelocytomatosis oncogene (Myc) and cyclin D1 (Ccnld1), was significantly increased (Fig. 5C–E) in the mutant mice. To examine whether the reduction of Sfrp2 expression was directly associated with HR, we determined the expression level of Sfrp2 in the presence of HR of transient transfection experiment. The SFRP2 mRNA level in HR expressed cells was significantly and specifically reduced to 0.38- (± 0.08) folds compare with that of the mock-transfected control cells (Fig. 5F, right). In addition, overexpression of HR resulted in suppression of the Sfrp2 promoter activity (data not shown), suggesting that HR directly regulates the expression of Sfrp2 gene. These data suggest that overexpression of Hr may affect the formation of HFs and the epidermal layer through the modulation of Wnt signaling.

**DISCUSSION**

The complexity of the 5'-UTR of mRNA, such as GC content, uORFs and the presence of uATGs, often affects the
expression of a gene as a cis-regulatory factor at the translational level. In particular, uORFs are found in several viral and cellular genes and act as a barrier for ribosomal scanning in cap-dependent translation. The assembly and dissociation of ribosomes in uORFs causes ribosomes to fail in reaching the main ATG, thus suppressing the initiation of translation at the start codon of the main ORF (33–35). In some cases, short peptides from the uORF are expressed and regulate downstream gene expression in a sequence-specific manner (29,36). Recently, it was shown that mutations in the 5′-UTR cause human genetic diseases, such as hyperferritinemia-cataract syndrome (MIM 600886) and Charcot–Marie–Tooth disease, X-linked, type 1 (MIM 302800), through abnormal translational regulation (37,38).

The 5′-UTR of the Hr gene is 695 bp in length, which is composed of 67 bp of the first exon, 586 bp of the second exon and 42 bp of the third exon, and it has four uORFs. Because the mutation of the hairpoor mouse (T403A) and patients with MUHH (T-320C) results in removal of the second uORF, we suspected that the expression levels of the HR protein in hairpoor mice would be increased. Thus, using western blot analysis, we found that the expression levels of the HR protein in hairpoor mice was increased as the number of mutant alleles increased (Fig. 5A). This increase in HR expression resulted from derepression at the translational level by the mutation, as shown in reporter gene analysis (Fig. 4). We do not know at this point whether the mutation simply removed a translational barrier, thus allowing read-through by the ribosome to reach the main ORF or whether some other mechanism is at work to generate this HR overexpression. However, these results clearly indicate that the T403A mutation in Hr and the T-320C MUHH mutation result in a gain in Hr gene function, as suggested recently (29).

This leads to the interesting question: does this type of regulation of Hr expression exist in vivo under normal conditions, and, if it does, how does it relate to Hr function and the regulation of HF cycling? Interestingly, the HR protein is not expressed concurrently with Hr mRNA because the HR protein is not detected during the anagen stage of HF at which Hr mRNA is present (26,27). This suggests that delayed expression of the HR protein from an already existing mRNA occurs, which is regulated by a mechanism in which the 5′-UTR of Hr may play a critical role.

Another strain of hair defect mouse with an autosomal semi-dominant inheritance mode, HrN, has been described (39). Recently, we reported that HrN and HrHp mutants have substitution mutations in the neighboring nucleotides, A402G and T403A in the 5′-UTR of Hr, respectively (30). Although we have not performed a molecular dissection of the HrN mutation, it may result in the derepression of Hr gene translation as seen in the HrHp mutation.

Several mutant mice with Hr mutations display a distinctive pattern of hair loss (20–23). These mutant mice grow normal hair until the first hair cycle at which the hair loss starts from head to tail. By the third week after birth, baldness becomes...
complete and they do not regrow a normal hair coat. Although the molecular bases of these 
Hr mutations differ from each other, they all result in the loss of function of HR. Panteleyev
et al. (26) have shown that massive apoptosis occurs at the end of HF morphogenesis, which is followed by the failure of initiation of the HF cycle in 
Hrhr mice. This suggests that HR functions in the anagen initiation of HF cycling. The 
HrHp mice differed from the classical Hr mutants in that their trait was inherited in a semidominant mode and was caused by a gain of function rather than a loss of function of HR. Interestingly, heterozygous 
HrHp mice have shorter HFs compared with wild-type mice, and they show progressive hair loss, suggesting abnormal HF cycling. The Wnt signaling pathway plays a critical role in the cyclic regeneration of HF by regulation of anagen initiation in the adult hair cycle (27,40–42). Although we do not currently know the mechanism by which overexpression of HR causes hair loss, the progressive hair loss phenotype of 
HrHp may be caused by abnormal induction of Wnt signaling by suppression of 
Sfrp2 expression. Intriguingly, the regulation of another Wnt inhibitor, Sostdc1, by HR reportedly resulted in reinitiation of HF development in the postnatal hair cycle in 
Hr knockout mice (27). These findings suggest that HR modulates more than one regulator of the Wnt signaling pathway, and it appears crucial that the timing and level of HR protein expression is tightly regulated for normal HF development.

Meanwhile, Wnt signaling is also involved in the formation of hair placodes as well as the normal hair placode downgrowth by mediating interaction between epithelial and mesenchymal cells in the embryonic stage (32,42,43). Not only 
HrHp heterozygous mice have a sparse hair coat, but also homozygous 
HrHp mice do not grow any hair and have no functional HF, which clearly indicates a breakdown of normal HF morphogenesis. We reported that an increase in 
HR expression concurred with the activation of Wnt signaling through the inhibition of a Wnt inhibitor, 
Sfrp2, and the hair loss phenotype in 
HrHp mice. In addition, 
HrHp mice have increased proliferation and differentiation of skin cells compared with wild-type mice, which may be caused by excessive induction of Wnt signaling. The excessive induction of 
β-catenin, a key regulator of Wnt signaling, causes premature hair placodes to form and impairs HF down-growth in morphogenesis (42,44,45). It would be interesting to determine whether β-catenin is excessively induced in 
HrHp mice.

Finally, because HR acts as a transcriptional corepressor of several nuclear receptors including the thyroid hormone receptor, vitamin D receptor and retinoic acid-like orphan receptor-α, overexpression of HR may cause a series of their target genes to be expressed abnormally; this consequently results in the hairpoor phenotype. Thus, overexpressed HR may cause abnormal HF morphogenesis as well as faulty HF cycling by abnormal regulation of the transcription of many genes involved in these processes. On the basis of our results, we suggest that the Wnt signaling pathway, through 
Sfrp2, is involved in the manifestation of the 
HrHp phenotype. Clearly, further study should be conducted to understand this mechanism, to identify other genes involved and to determine their roles in these processes.
Here, we report an animal model for MUHH, HrHp mice, which overexpress the HR protein resulting in a hair defect phenotype in a dose-dependent manner. HrHp mice may serve as a model for identification of the role of the 5'-UTR of Hr during gene expression; however, these mice may also provide a new paradigm for understanding the modulation of HF morphogenesis and the regulation of HF cycle by HR protein. Further studies on the regulation of Hr expression during HF formation are required for a clearer understanding of the development and cycling of HFs. The HrHp mice offer an opportunity to elucidate the role of HR in diseases that cause HF defects, such as MUHH, and for the development of therapeutic treatments for some forms of hair loss.

MATERIALS AND METHODS

Mutational analysis of HR for MUHH

The affected family that took part in this study has been described previously (4). Genomic DNA was used as a template for PCR using primers listed in Supplementary Material, Table S1. All exons comprising 5'-UTR and the flanking introns of HR were screened by PCR followed by direct DNA sequencing. RFLP analysis was performed using Ncol restriction digestion, and DNA fragments were separated on a 12% polyacrylamide gel with 1 × TBE buffer. PCR was performed with the following primers: forward (5'-AATCAGCG GTTGTGTGGCC-3') and reverse (5'-TCGGGCTTCCT AACTTTCAGG-3') for RFLP. The human variation Caucasian panel of 100 was purchased from the Coriell Institute and genotyping was carried out using RFLP.

Generation and maintenance of HrHp mice

Inbred BALB/c mice were used to produce ENU mutant mice (31). All mice were maintained as described previously (23). The hairpoor mouse strain was established in the Mutant Mouse Development Laboratory (NRL 2000), Laboratory of Toxicogenomics, Korea Institute of Toxicology, Taejon, Korea, as previously reported (31).

Tissue harvesting and immunostaining

Dorsal skin preparation, hematoxylin and eosin staining (H&E) and immunohistochemistry were performed by standard methods (24). For H&E staining and immunostaining, paraffin sections (10 μm) were fixed in 10% formalin. For all antibodies, antigen retrieval was carried out by treating slides with 10 mM sodium citrate buffer, pH 6.0. The following primary antibodies were used: K14 (rabbit polyclonal, 1:1000;
Covance); K10 (rabbit polyclonal, 1:500; Covance); involucrin (rabbit polyclonal, 1:1000; Covance) and Ki67 (rabbit monoclonal, 1:200; Thermo).

Immunoblotting

Western blot analysis was performed as described previously (23). Proteins were prepared from dorsal skins of mice in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Na2IO3, 0.1% SDS, 50 mM Tris–HCl, pH 8.0) and 30 μg of protein was separated by polyacrylamide gel electrophoresis. The rabbit polyclonal HR antibody was generated using a GST-fused HR polypeptide corresponding to the 1–1200 amino acid regions as antigen (Abfrontier). β-actin and β-tubulin (Sigma) were used as loading controls. The signal was visualized using an enhanced chemiluminescence detection kit (Amersham Bioscience). The relative expression level of HR was analyzed based on densitometry analysis.

RNA isolation and quantitative RT–PCR

Total RNA was extracted from the dorsal skin of mice and cells using TRIzol (Invitrogen), according to the manufacturer’s instructions. Total RNA for human brain and skin were purchased from Stratagene. Single-stranded cDNAs were synthesized in reverse transcription reactions using the SuperScript First-Strand Synthesis System (Invitrogen). Quantitative RT–PCR was carried out in a reaction mixture containing SYBR Premix Ex Taq (Takara) using a MX3000P PCR machine (Stratagene). Sequences of gene-specific primers are presented in Supplementary Material, Table S1. The relative levels of gene expression were determined by the comparative ΔΔCt method (46). The relative mRNA expression levels were determined using eight sets of +/+ and −/− samples for mouse experiments and three sets of control and test for transfection experiments. Statistical significance was determined by the Student t-test using Sigma plot.*P = 0.0539, **P = 0.0000018 for Sfrp2; *P = 0.018, **P = 0.0016 for Myc; **P = 0.00628 for Ccnd1; **P = 0.0002 for SFRP2.

Plasmid construction

The mouse Hr-5′-UTR (m_Hr-5′-UTR) of the T (Wt) and A (Mt) alleles were amplified by PCR using Pfu polymerase (Stratagene) and the following primers: forward (5′-GAGTGTTGCCAGGAGCAGG-3′) and reverse (5′-TCACGTCCGTTGACTTCATGG-3′) from cDNA of mouse skin from wild-type and mutant mice, respectively. PCR products corresponded to 1 – 695 bp of Hr mRNA (NM_021877). The products were subcloned into pEGFP-N2 and the resulting constructs were named pEGFP-N2/Hr_Wt_5′-UTR (Wt-GFP; Wt) and pEGFP-N2/Hr_Mt_5′-UTR (Mt-GFP; Mt). Mouse Hr full-length cDNA clone (BC049182) and pcDNA 3.1 (+) vector were purchased from Invitrogen.

Cell culture and reporter assay

The HEK293 was maintained in DMEM, supplemented with 10% fetal bovine serum at 37°C with 5% CO2. Transfection was performed using Lipofectamin (Invitrogen) according to the manufacturer’s instructions. The relative amounts of GFP and mRNA were determined by western blot and northern blot analyses, respectively, and compared by densitometry. β-actin and GAPDH were used as loading controls for western and northern blot analyses, respectively. Results are the average of three experiments performed in duplicate. The full-length Hr construct and pcDNA 3.1 (+) vector were transfected into HEK293 cells using polyethyleneimine (Sigma). β-Galactosidase activity was used to normalize transfection efficiency for all transfection experiments.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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