SUPPLEMENTARY MATERIALS AND METHODS

SAMPLE PREPARATION, ITRAQ LABELING, AND LC-MS/MS ANALYSIS

The total protein were extracted from 3 groups (WB, WB7, WB11), and protein samples were then reduced, alkylated, digested, and labeled with iTRAQ reagents as previously described. Protein labeled with the iTRAQ tags as follows: WB-115 isobaric tag, WB7–116 isobaric tag and WB11–117 isobaric tag. The LC-MS/MS analysis was performed as described previously. iTRAQ-labeled peptides fragmented to produce reporter ions at 115.1, 116.1 and 117.1, and fragment ions of the peptides were simultaneously produced, resulting in sequencing of the labeled peptides and identification of the corresponding proteins. The ratios of the peak areas of the three iTRAQ reporter ions reflected the relative abundances of the peptides and the proteins in the samples.

IMMUNOHISTOCHEMISTRY ANALYSIS ON TISSUE MICROARRAYS

Tissue array containing 75 HCC samples (Shanghai OUTDO Biotech, China), and mouse anti-HRG monoclonal antibody (R&D Systems, Inc. US) were used in this study.

The process of immunohistochemistry was according to the protocol of HRG antibody. Briefly, paraffin sections were first deparaffinized and then hydrated. After microwave antigen retrieval, as required, endogenous peroxidase activity was blocked with incubation of the slides in 3% H2O2, and non-specific binding sites were blocked with 10% rabbit serum. After serial incubation with primary antibody (1:400) and secondary antibody, the sections were developed in diaminobenzidine solution under a microscope and counterstained with hematoxylin. Negative control slides omitting the primary antibodies were included in all assays.

SIRNA TRANSFECTION

Three pairs of predesigned siRNA oligonucleotide against HRG were purchased from Biomics. The sequences of siRNA were listed in Supporting Table 1. Three pairs of siRNA for each target were mixed respectively and transfected into Huh7 cells and MHCC-97H cells at the final concentration of 50 nM using Lipofectamine 2000 (Invitrogen) as manufacturer’s instruction. The culturing medium was changed to complete medium after 12 h of transfection and the cells were cultured for another 48 h until detection of gene knockdown.

SOFT AGAR COLONIZATION

For clone formation assay, 1ml of sterilized 0.6% low melting point (LMP) agarose (Sigma) in complete medium was added to one well of 6-well plate first. After the medium became solid gel, 1ml of 0.4% LMP agarose in complete medium with 1000 Huh7 cells and MHCC-97H cells was added on top of the base gel. After culturing for 14 days, clone (>50 cells) numbers were assessed microscopically. All experiments were performed in triplicate.

WESTERN BLOT ANALYSIS

Equal amounts of total proteins (20 μg) were separated by 10% SDS-PAGE and transferred onto PVDF membrane using a Bio-Rad SemiDry apparatus. The membrane was blocked by 5% milk or 2% BSA at room temperature for 1 h. Then, the membrane was incubated with specific primary antibody with suitable dilution at 4°C overnight. The used primary antibodies and their dilution information were listed in Supporting Table 2. After 3 times of 10 min washing by TBST, the membrane was further incubated with HRP-conjugated secondary antibodies (Bio-Rad) at room temperature for 1 h, and then washed again by TBST for 3 times of 10 min. ECL prime Western Blotting Detection Reagents (GE) and ChemiDoc XRS+ system (Bio-Rad) were used to visualize the bands on membrane.

IMMUNOFLUORESCENCE MICROSCOPY

For Immunofluorescence staining, cells grown on glass coverslip were fixed in 4% paraformaldehyde and permeabiliized using 0.5% Triton X-100. Then cells were incubated with the primary antibody overnight at 4°C. After thorough washing, cells were then incubated with Alexa-Fluor 555 anti-mouse IgG or anti-rabbit IgG (1:100 dilution, Cell Signaling Technology, Danvers, MA). Finally, cells were washed and stained with DAPI. Images were captured using a Leica fluorescence microscope.

QUANTITATIVE RT-PCR

Total RNA was extracted from cultured cells using TRizol Reagent (Invitrogen) according to the manufacturer’s instruction. 2 μg of total RNA was reversed transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kits (Thermo). cDNA were prepared for subsequent quantitative PCR amplification with
SYBR Premix Ex Taq (TAKARA) using IQ5 (Bio-Rad). The experimental Ct (cycle threshold) was calibrated against that of beta-actin control product. The used paired primers for each gene were listed in Supporting Table 3.

CELL PROLIFERATION ASSAYS

Huh7 cells and MHCC-97H cells (1000 cells/well) were dispensed in 100 μL aliquots into a 96-well plate. At the indicated time points, the 2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (CCK8, Cell Counting kit) was added to the cells for 1 h, and then the plate was read using an enzyme-linked immunosorbent assay plate reader at 450 nm.

TUMOR FORMATION ASSAYS

Male BALB/C nude mice (5–6 week old) were obtained from Shanghai Institute of Materia Medica (Chinese Academy of Sciences, Shanghai, China). The in vivo experiments were carried out strictly in accordance with a protocol approved by the Shanghai Medical Experimental Animal Care Committee (Permit Number: 2009–0082). 1×10⁷ cells were injected subcutaneously into the upper left flank region of nude mice. When the tumor reached 1 cm in diameter, they were cut into 2×2×2 mm³ sized pieces, and implanted into livers of nude mice. The mice were sacrificed at the 27th day after tumor implantation. The tumor size and weight were measured.
Supplementary Figure S1: Anchoring-independent growth and subcutaneous tumor formation was the important characteristic of transformed cells. The transformed cells could form colonies in soft agar, and the frequencies of colony formation were 0.02% and 2% in WB7 and WB11 cells, but WB cells could not grow in soft agar (Fig. 1A). The subcutaneous tumor formation in WB11 cells, and WB, WB7 cells could not form tumor (Fig. 1B). CCK8 assay displayed that transformed cells proliferated faster than WB cells (Fig. 1C). Cell migration showed that the migration ability was enhanced in WB7 and WB11, and WB11 cells has more migration ability than WB7 cells (Fig. 1D). These results strongly indicated that MNNG induced WB cells gained the characteristics of transformed cells. It implied that the WB7 cell may be in precancer status and WB11 cell, definitely in real cancer. Characteristics of transformed WB, WB7, and WB11 cells. A. Soft agar clone formation in WB7 and WB11. B. Subcutaneous tumor formation in WB7, WB11. C. The proliferation of WB, WB7, WB11 cells. D. The migration of WB, WB7, WB11 cells, *P < 0.05.
### Supplementary Table S1: Sequences of three pair siRNA

| Name   | Sequences (5’-3’)       |
|--------|-------------------------|
| SiRNA-1 | forward: CGGACAAUGUAAGGUAAUAdTdT<br>reverse: UAUUACCUCUACAUUGUCCGdTdT |
| SiRNA-2 | forward: CCGGUCUCUAUAGAUUUCUdTdT<br>reverse: AGAAAUCUAUGAGGACCGBdTdT |
| SiRNA-3 | forward: GCACCACAAACAUCCUCUAdTdT<br>reverse: UAGAGGAUGUUUGUGGUGcdTdT |
### Supplementary Table S2: List of primary antibodies used in this study

| Target protein | Company          | Dilution (Application)         |
|----------------|------------------|-------------------------------|
| HRG            | R&D              | 1:400 (IHC, IF), 1:1000 (WB)  |
| ERK1/2         | Cell Signaling   | 1:1000 (WB)                   |
| p-ERK1/2       | Cell Signaling   | 1:1000 (WB),                 |
| P38            | Sant Cruz        | 1:100 (WB)                    |
| p-P38          | Sant Cruz        | 1:100 (WB)                    |
| JNK            | Sant Cruz        | 1:100 (WB)                    |
| p-JNK          | Sant Cruz        | 1:100 (WB)                    |
| β-actin        | Kangchen biotech | 1:10000 (WB)                 |
| GAPDH          | Kangchen biotech | 1:10000 (WB)                 |
|                | forward     | reverse                                      |
|----------------|-------------|----------------------------------------------|
| HRG            | AGTGCTTCCAGCCATGAGG | TGGTGTTGATATATGCCGAAGAGAC                   |
| β-actin        | CCCGCAGTACAACCTTCTT  | AGGGTCAGGATGCCTCTCTT                        |
### Supplementary Table S4: Total 87 proteins were identified from transformation

| Uniprot-Accession | Protein Name                                      | Gene Name | 115 | 116 | 117 |
|-------------------|---------------------------------------------------|-----------|-----|-----|-----|
| P02454            | Collagen alpha-1(I) chain                         | Col1a1    | 1.72| 1.33|
| P02466            | Collagen alpha-2(I) chain                         | Col1a2    | 1.39| 1.79|
| P62630            | Elongation factor 1-alpha 1                       | Eef1a1    | 1.41| 1.22|
| Q99068            | Alpha-2-macroglobulin receptor-associated protein | Lrpap1    | 2.17| 2.09|
| P13383            | Nucleolin                                         | Ncl       | 1.57| 1.47|
| Q6IFW6            | Keratin, type I cytoskeletal 10                   | Krt10     | 7.73| 4.61|
| P62961            | Nuclease-sensitive element-binding protein 1       | Ybx1      | 1.24| 1.67|
| Q6IMF3            | Keratin, type II cytoskeletal 1                    | Krt1      | 2.63| 1.96|
| P00762            | Anionic trypsin-1                                 | Prss1     | 1.41| 1.31|
| P29457            | Serpin H1                                         | Serpinh1  | 1.42| 1.21|
| P13084            | Nucleophosmin                                     | Npm1      | 1.24| 1.28|
| Q4KMA2            | UV excision repair protein RAD23 homolog B         | Rad23b    | 1.56| 1.56|
| Q4FZU2            | Keratin, type II cytoskeletal 6A                   | Krt6a     | 6.79| 2.91|
| Q63945            | Protein SET                                       | Set       | 3.40| 2.33|
| P63029            | Translationally-controlled tumor protein           | Tpt1      | 1.41| 2.23|
| P24368            | Peptidyl-prolyl cis-trans isomerase B              | Ppib      | 1.85| 1.87|
| P04906            | Glutathione S-transferase P                       | Gstp1     | 2.47| 1.50|
| A0JPM9            | Eukaryotic translation initiation factor 3 subunit J| Eif3j   | 1.80| 2.40|
| P06302            | Prothymosin alpha                                 | Ptma      | 2.36| 2.86|
| Q6AYK6            | Calcyclin-binding protein                         | Cacybp    | 1.27| 1.58|
| P20059            | Hemopexin                                         | Hpx       | 2.83| 3.02|
| Q5RKG1            | Spindle and centriole-associated protein 1        | Spice1    | 3.40| 2.05|
| Q51034            | Uncharacterized protein C12orf43 homolog          |          | 1.31| 1.56|
| Q6IUR5            | Neudesin                                          | Nenf      | 1.96| 1.79|
| Q80Z30            | Protein phosphatase 1E                            | Ppm1e     | 1.41| 1.75|
| Q4V815            | ADP-ribosylation factor-like protein 6-interacting protein 4 | Arl6ip4 | 1.91| 1.51|
| P35434            | ATP synthase subunit delta, mitochondrial          | Atp5d     | 3.44| 1.82|
| Q00729            | Histone H2B type 1-A                              | Hist1 h2ba| 1.25| 1.32|
| Q7TQ84            | UAP56-interacting factor                          | Fytd1     | 1.33| 10.86|
| P85972            | Vinculin                                          | Vcl       | 0.33| 0.64|
| P04937            | Fibronectin                                       | Fn1       | 0.70| 0.71|
| P63018            | Heat shock cognate 71 kDa protein                 | Hspa8     | 0.62| 0.67|
| P45592            | Cofilin-1                                         | Cfl1      | 0.79| 0.68|
| Q9JI03            | Collagen alpha-1(V) chain                         | Col5a1    | 0.08| 0.30|
| Q9JI85            | Nucleobindin-2                                    | Nueb2     | 0.49| 0.68|

(Continued)
| Uniprot-Accession | Protein Name                          | Gene Name | 115 | 116 | 117 |
|------------------|---------------------------------------|-----------|-----|-----|-----|
| Q07936           | Annexin A2                            | Anxa2     | 1   | 0.45| 0.46|
| P05964           | Protein S100-A6                        | S100a6    | 1   | 0.58| 0.44|
| Q63610           | Tropomyosin alpha-3 chain              | Tpm3      | 1   | 0.27| 0.31|
| P62260           | 14–3-3 protein epsilon                 | Ywhae     | 1   | 0.05| 0.10|
| Q9J19            | Na(+)/H(+) exchange regulatory cofactor NHE-RF1 | Slc9a3r1 | 1   | 0.38| 0.79|
| P02770           | Serum albumin                         | Alb       | 1   | 0.59| 0.34|
| P62982           | Ubiquitin-40S ribosomal protein S27a   | Rps27a    | 1   | 0.75| 0.59|
| P10111           | Peptidyl-prolyl cis-trans isomerase A  | Ppia      | 1   | 0.56| 0.22|
| P07632           | Superoxide dismutase [Cu-Zn]          | Sod1      | 1   | 0.37| 0.35|
| P63259           | Actin, cytoplasmic 2                  | Actg1     | 1   | 0.46| 0.39|
| P14668           | Annexin A5                            | Anxa5     | 1   | 0.05| 0.29|
| Q8VHK7           | Hepatoma-derived growth factor         | Hdgf      | 1   | 0.25| 0.18|
| P11980           | Pyruvate kinase isozymes M1/M2         | Pkm       | 1   | 0.76| 0.66|
| P09495           | Tropomyosin alpha-4 chain             | Tpm4      | 1   | 0.72| 0.37|
| P05943           | Protein S100-A10                      | S100a10   | 1   | 0.47| 0.34|
| P14669           | Annexin A3                            | Anxa3     | 1   | 0.76| 0.77|
| P04692           | Tropomyosin alpha-1 chain             | Tpm1      | 1   | 0.52| 0.37|
| Q63081           | Protein disulfide-isomerase A6        | Pdia6     | 1   | 0.59| 0.67|
| O35763           | Moesin                                | Msn       | 1   | 0.69| 0.48|
| O08629           | Transcription intermediary factor 1-beta | Trim28   | 1   | 0.79| 0.63|
| P48679           | Prelamin-A/C                          | Lmna      | 1   | 0.29| 0.71|
| P35704           | Peroxiredoxin-2                      | Prdx2     | 1   | 0.67| 0.76|
| Q63797           | Proteasome activator complex subunit 1 | Psm1     | 1   | 0.58| 0.20|
| P00507           | Aspartate aminotransferase, mitochondrial | Got2    | 1   | 0.73| 0.79|
| P61980           | Heterogeneous nuclear ribonucleoprotein K | Hnrnpk  | 1   | 0.06| 0.34|
| Q64119           | Myosin light polypeptide 6            | Myl6      | 1   | 0.69| 0.48|
| Q499N6           | UBX domain-containing protein 1       | Ubxn1     | 1   | 0.72| 0.70|
| P63100           | Calcineurin subunit B type 1          | Ppp3r1    | 1   | 0.77| 0.30|
| Q9WU49           | Calcium-regulated heat stable protein 1 | Carhsp1 | 1   | 0.68| 0.48|
| P0C0S7           | Histone H2A.Z                         | H2afz     | 1   | 0.46| 0.56|
| Q62764           | DNA-binding protein A                 | Csda      | 1   | 0.64| 0.79|
| P68511           | 14–3-3 protein eta                    | Ywhah     | 1   | 0.49| 0.47|
| Q510E7           | Transmembrane emp24 domain-containing protein 9 | Tmed9 | 1 | 0.74| 0.49|
| Q6AYH5           | Dynactin subunit 2                    | Dctn2     | 1   | 0.41| 0.79|
| P47875           | Cysteine and glycine-rich protein 1   | Csrp1     | 1   | 0.70| 0.40|

(Continued)
| Uniprot-Accession | Protein Name                                      | Gene Name | 115 | 116 | 117 |
|-------------------|--------------------------------------------------|-----------|-----|-----|-----|
| Q63041            | Alpha-1-macroglobulin                            | A1m       | 1   | 0.20| 0.52|
| Q63416            | Inter-alpha-trypsin inhibitor heavy chain H3     | Itih3     | 1   | 0.54| 0.22|
| Q99PS8            | Histidine-rich glycoprotein                      | Hrg       | 1   | 0.75| 0.59|
| P04785            | Protein disulfide-isomerase                      | P4 hb     | 1   | 1.22| 0.71|
| Q6IFU8            | Keratin, type I cytoskeletal 17                  | Krt17     | 1   | 3.31| 0.77|
| P31044            | Phosphatidylethanolamine-binding protein 1       | Pebp1     | 1   | 1.41| 0.72|
| P82995            | Heat shock protein HSP 90-alpha                  | Hsp90aa1  | 1   | 1.96| 0.61|
| Q9EPH2            | MARCKS-related protein                           | Marcksl1  | 1   | 1.34| 0.78|
| P05765            | 40S ribosomal protein S21                       | Rps21     | 1   | 1.85| 0.56|
| P84039            | Ectonucleotide pyrophosphatase/phosphodiesterase family member 5 | Enpp5 | 1 | 1.66 | 0.29 |
| P62859            | 40S ribosomal protein S28                       | Rps28     | 1   | 2.13| 0.25|
| P58775            | Tropomyosin beta chain                           | Tpm2      | 1   | 0.63| 1.31|
| P51886            | Lumican                                         | Lum       | 1   | 0.70| 1.46|
| P02401            | 60S acidic ribosomal protein P2                  | Riplp2    | 1   | 0.63| 1.33|
| P02600            | Myosin light chain 1/3, skeletal muscle isoform  | Myl1      | 1   | 0.48| 2.61|
| Q920J4            | Thioredoxin-like protein 1                      | Txnl1     | 1   | 0.23| 1.34|
| P21263            | Nestin                                          | Nes       | 1   | 0.32| 1.66|