Maelstrom Promotes Hepatocellular Carcinoma Metastasis by Inducing Epithelial-Mesenchymal Transition by Way of Akt/GSK-3β/Snail Signaling

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Amplification of 1q is one of the most frequent chromosomal alterations in human hepatocellular carcinoma (HCC). In this study we identified and characterized a novel oncogene, Maelstrom (MAEL), at 1q24. Amplification and overexpression of MAEL was frequently detected in HCCs and significantly associated with HCC recurrence (P = 0.031) and poor outcome (P = 0.001). Functional study demonstrated that MAEL promoted cell growth, cell migration, and tumor formation in nude mice, all of which were effectively inhibited when MAEL was silenced with short hairpin RNA (shRNAs). Further study found that MAEL enhanced AKT activity with subsequent GSK-3β phosphorylation and Snail stabilization, finally inducing epithelial-mesenchymal transition (EMT) and promoting tumor invasion and metastasis. In addition, MAEL up-regulated various stemness-related genes, multidrug resistance genes, and cancer stem cell (CSC) surface markers at the messenger RNA (mRNA) level. Functional study demonstrated that overexpression of MAEL increased self-renewal, chemoresistance, and tumor metastasis.

Conclusion: MAEL is an oncogene that plays an important role in the development and progression of HCC by inducing EMT and enhancing the stemness of HCC. (HEPATOLOGY 2014;59:531-543)

Hepatocellular carcinoma (HCC) is the fifth most common cancer and ranks as the third leading cause of cancer-related deaths in the world.1 It is believed that HCC pathogenesis is a long-term progressive process involving the accumulation of multiple genetic and epigenetic alterations.2 Amplification of the long arm of chromosome 1 has been detected in 58%-78% of primary HCC cases, suggesting that one or more oncogenes within the amplified region play a critical role in HCC development.3,4 Our previous work had demonstrated that CHD1L at 1q21 plays a critical oncogenic role in the development and progression of HCC. Overexpression of CHD1L promotes cell proliferation,5 induces tumor metastasis by way of epithelial-to-mesenchymal transition (EMT),6 sustains cell survival, and increases drug resistance by inhibiting Nur77-mediated apoptosis.7 Recently, we performed an integrative RNA sequencing (RNA-Seq) to identify differentially expressed genes between three pairs of clinical samples of HCC and their adjacent nontumor tissues.8 Overexpression of Maelstrom (MAEL) at 1q24 was observed in all three HCC tumor tissues compared with their matched nontumor counterparts.

The MAEL gene was initially identified in Drosophila9 and is required for spermatogenesis and meiosis.10 The human MAEL protein contains a high mobility group (HMG) domain in its N-terminal segment that is known to mediate DNA binding and a novel MAEL-specific domain in the C-terminal segment.11

Abbreviations: CHD1L, chromodomain helicase/ATPase DNA binding protein 1-like gene; CSCs, cancer stem cells; DAB, 3,5-diaminobenzidine; DDP, cisplatin; EMT, epithelial-mesenchymal transition; FISH, fluorescence in situ hybridization; HCC, hepatocellular carcinoma; HMG, high mobility group; IF, immunofluorescence; IHC, immunohistochemistry; MAEL, maelstrom; miR-7, microRNA-7; piRNA, piwi-interacting RNA; qRT-PCR, quantitative reverse transcription PCR; shRNA, short hairpin RNA; TCTB, translationally controlled tumor protein; TICs, tumor-initiating cells; TMA, tissue microarray.

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The localization of MAEL is dynamic, shuttling between the nuage and the nucleus. In mouse, MAEL has been found to interact with the chromatin-remodeling factor SNF5 and chromatin-associated protein SIN3B, implying that MAEL may have a nuclear function in controlling gene expression. In normal human tissue, expression of MAEL has been detected only in the testis, whereas its abnormal expression has been observed in various cancer cell lines.

One recent study showed that MAEL is responsible for proper differentiation in the germline stem cell lineage by repressing microRNA-7 (miR-7). Current research on cancer biology suggests that tumor growth is driven by a subpopulation of cancer cells called cancer stem cells (CSCs) or tumor-initiating cells (TICs). These TICs possess a greater potential for self-renewal, enhanced tumorigenicity, and superior resistance to chemotherapeutic agents. EMT is a key process in cancer metastasis by which tumor cells acquire migratory characteristics, thereby disassociating from the primary tumor and migrating to distant sites. In the present study, amplification and expression of MAEL was investigated in primary HCC samples, and its clinical significance was also addressed. Both in vitro and in vivo assays demonstrated that MAEL had strong oncogenic function in the development and progression of HCC. Molecular study found that MAEL-transfected cells underwent EMT, had increased stemness, and expressed higher levels of several TICs-related genes.

Materials and Methods

HCC Samples and Cell Lines. A total of 91 paired specimens (tumor and adjacent nontumor tissues) were collected immediately following hepatectomy of HCC patients at the Sun Yat-Sen University Cancer Center (Guangzhou, China). Samples used in this study were approved by the Committees for Ethical Review of Research at Sun Yat-Sen University. All cell lines used (MIHA, SMMC7721, BEL7402, QGY7703, QGY7701, PLC8024, Huh7, H2P, and H2M) have been described in previous studies.

Plasmid Constructs and Transfection. Full-length human MAEL complementary DNA (cDNA) was amplified by polymerase chain reaction (PCR) and cloned into pcDNA3.1(+) expression vector (Invitrogen, Carlsbad, CA), and then transfected into QGY7701 and BEL7402 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells transfected with empty vector were used as controls. Stable MAEL-expressing clones were selected by Geneticin (Roche Diagnostics, Indianapolis, IN) at a concentration of 500 μg/mL.

Establishment of MAEL Knockdown Cells. Lentiviral containing short hairpin RNAs (shRNA) targeting MAEL was purchased from GeneCopoeia (Rockville, MD) and transfected into QGY7703 and PLC8024 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells transfected with empty vector were used as controls. Puromycin (final concentration: 2 μg/mL) was used to select stable clones.

Oncogenic Assays. For cell growth assay, cells were seeded in 96-well plates at a density of 1 × 10^3 cells per well, and cell growth rate was assessed with the XTT kit (Roche Diagnostics). For foci formation assay, 1 × 10^5 cells were seeded in 6-well plates, and after two weeks of culture cell colonies were counted by crystal violet staining. The results are expressed as the mean ± SD of three independent experiments. Xenograft tumor growth assay was established by subcutaneous injection of MAEL-expressing cells (4 × 10^6 cells for MAEL-7701, 2 × 10^6 cells for MAEL-7402, 5 × 10^6 cells for shMAEL-7703, 2 × 10^6 cells for shMAEL-8024) and empty vector-transfected control cells into the right and left dorsal flank of 4-week-old nude mice, respectively. Tumor formation in the nude mice was checked after 4 weeks. All animal experiments were conducted according to the institutional standard guidelines at Sun Yat-Sen University.

RNA Extraction and qRT-PCR. Total RNA was extracted using the TRIZOL Reagent (Invitrogen) and reverse transcription was performed using the Superscript III Reverse Transcriptase (Invitrogen). The cDNA was subjected to quantitative real-time PCR (qRT-PCR) using the SYBR Green PCR Kit (Applied Biosystems, Carlsbad, CA) and the assay was performed on an ABI PRISM 7900 Sequence Detector. 18S rRNA was used as an internal control. The relative levels of expression were quantified and analyzed.
using SDS 2.3 software (Applied Biosystems, Foster City, CA). The relative expression level (defined as fold change) of MAEL \((2^{ΔΔCt})\) was normalized to the endogenous 18S rRNA reference \((ΔCt)\) and related to the amount of target gene in control sample, which was defined as the calibrator at 1.0. Three independent experiments were performed to analyze the relative gene expression and each sample was tested in triplicate. The primer sequences are listed in the Supporting Methods.

**Fluorescence In Situ Hybridization (FISH).** The BAC clone (RP4-782G3) at 1q24.1 containing the MAEL gene probe and the chromosome 1 centromere probe were labeled with Spectrum-red and Spectrum-green (Vysis, Downers Grove, IL), respectively. FISH experiments were performed as described previously.\(^5\)

**Antibodies and Western Blotting.** Western blot analyses were performed according to the standard protocol. Information of the antibodies is listed in the Supporting Methods.

**Immunofluorescent (IF) Staining.** The IF staining was performed as described previously.\(^6\) Information of the antibodies is listed in the Supporting Methods.

**Wound-Healing and Transwell Invasion Assays.** For the wound-healing assay, MAEL- or empty vector-transfected cells were cultured on a 35 mm dish until confluence and then wounded with a 10 μL pipette tip. Migration photos were captured at 0, 24, and 48h hours after scratching. Invasion assay was performed with BD BioCoat Matrigel Invasion Chambers (Becton Dickinson Labware, Franklin Lakes, NJ) following the manufacturer’s instructions. The Matrigel membrane was stained with crystal violet and migrated cells were counted under a microscope. Both experiments were repeated in triplicate independently.

**Experimental Metastasis Assay.** Two groups of six mice each were given intravenous tail vein injections of \(1 \times 10^6\) Vec-7701 cells and MAEL-7701 cells, respectively. After 8 weeks the mice were sacrificed and the tumor nodules formed on the liver and lung surfaces were counted. Lungs and livers were excised and embedded in paraffin for further study.

**Drug Sensitivity Assays.** Cells were seeded in 96-well plates at a density of \(5 \times 10^3\) cells per well. After 48 hours treatment using the chemotherapeutic agent cisplatin (DDP) at different concentrations, cell sensitivity to DDP was determined by XTT Cell Proliferation Assay (Roche Diagnostics). The data represent three independent experiments.

**Sphere Assay.** Five hundred PLC8024 cells were seeded onto 24-well polyHEMA-coated plates (Sigma-Aldrich, St. Louis, MO) and cultured for 2 weeks in DMEM/F12 medium (Invitrogen) supplemented with 4 μg/mL insulin (BIOIND, Kibbutz Beit Haemek, Israel), B27 (1:50, GIBCO, Grand Island, NY), 20 ng/mL EGF (PeproTech, Rocky Hill, NJ) and 10 ng/mL basic FGF (PeproTech).

**Statistics.** SPSS 17.0 was used for all data analyses. The mRNA level of MAEL in HCC tumor tissues and adjacent nontumor tissues were compared using a paired Student t test. Clinical correlations were analyzed by Pearson chi-square test and survival analyses assessed by Kaplan-Meier plots and log-rank tests. Univariate and multivariate survival analyses were performed by Cox proportional hazards regression model. Results were considered statistically significant when \(P < 0.05\).

**Results**

**MAEL Was Frequently Amplified and Overexpressed in HCC.** Our RNA-seq data showed that MAEL was overexpressed in all three tested HCC tumor tissues. The expression pattern of MAEL was studied by qRT-PCR in 91 pairs of primary HCC tumor versus adjacent nontumor tissues. Overexpression of MAEL (defined as >2-fold increase) was detected in 47/91 (51.6%) of HCC tumor tissues compared with the corresponding nontumor tissues. The average fold change of MAEL expression in tumor tissues was significantly higher than that in the paired nontumor tissues (3.13- versus 1.0-fold change) \((P < 0.001,\) paired Student t test, Fig. 1A). Western blot analysis showed that MAEL was frequently overexpressed in HCC cell lines compared with the immortalized liver cell line MIHA (Fig. 1B). As the survival data were not available for the specimens used in the qRT-PCR, a tissue microarray containing 150 pairs of primary HCCs from another patient cohort was studied by IHC. Overexpression of MAEL was detected in 80/134 (59.7%) of informative HCC tumor tissues as compared with the corresponding nontumor tissues (Fig. 1C). Noninformative samples included lost samples and unrepresentative samples. Interestingly, stronger MAEL expression was often observed at the outer edges of tumor tissues and within the tumor cells.
invading the surrounding tissues (Fig. 1C, right panel).

Because amplification of 1q is frequently detected in HCC, the association of gene amplification with MAEL overexpression was investigated by FISH with a BAC clone containing the MAEL gene. FISH was performed on the previously described TMA and the results showed that MAEL amplification (≥4 copies) could be detected in 32/77 (41.6%) of informative HCCs (Fig. 1D). Non-informative samples included lost samples and unrepresentative samples. Amplification of MAEL could be detected in 22/39 (56.4%) of HCCs with MAEL overexpression, which was significantly (P = 0.011, χ² test) higher than that in HCCs without MAEL overexpression (10/38, 26.3%), suggesting that overexpression of MAEL was associated with MAEL amplification (Fig. 1E).

Fig. 1. Overexpression of MAEL in HCC. (A) Relative expression levels of MAEL detected by qRT-PCR in 91 pairs of HCC tissues. (B) Western blot analysis of MAEL expression in an immortalized liver cell line (MIHA) and eight HCC cell lines. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (C) Representative immunohistochemical stainings of MAEL expression in matched primary HCC sample and its corresponding nontumor tissue. In Case 2 the invading tumor cells are indicated by arrows (original magnification, 200×). (D) Representative images of MAEL copy number in two HCC cases. The BAC probe containing MAEL gene is indicated in red, and the centromere probe of chromosome 1 is shown in green (original magnification, 1,000×). (E) Statistical analysis showing the association of MAEL overexpression with gene amplification. MAEL(−), MAEL normal expression; MAEL(+), MAEL overexpression. (F) Kaplan-Meier analysis indicating the correlation of MAEL overexpression with poorer overall survival and disease-free survival rates of HCC patients. MAEL-NE, MAEL normal expression; MAEL-OE, MAEL overexpression.
**Table 1. Clinicopathological Correlation of MAEL Expression in HCC**

| Features                        | All   | R   | MAEL Normal | MAEL Upregulated | P  |
|---------------------------------|-------|-----|-------------|------------------|----|
| **Gender**                      |       |     |             |                  |    |
| Male                            | 125   | 62  | 63          |                  |    |
| Female                          | 9     | 2   | 7           |                  | 0.169|
| **Age**                         |       |     |             |                  |    |
| <60 years                       | 106   | 52  | 54          |                  |    |
| >60 years                       | 28    | 0.050 | 12     | 16               | 0.672|
| **Hepatitis B surface Ag**      |       |     |             |                  |    |
| Negative                        | 13    | 8   | 5           |                  |    |
| Positive                        | 115   | 0.093 | 53     | 62               | 0.383|
| **Serum AFP**                   |       |     |             |                  |    |
| <400                            | 75    | 36  | 39          |                  |    |
| ≥400                            | 53    | 0.008 | 25     | 28               | 1.000|
| **Tumor size**                  |       |     |             |                  |    |
| <5 cm                           | 36    | 23  | 13          |                  |    |
| ≥5 cm                           | 98    | 0.196 | 41     | 57               | 0.032|
| **Cirrhosis**                   |       |     |             |                  |    |
| Absent                          | 23    | 10  | 13          |                  |    |
| Present                         | 105   | 0.039 | 51     | 54               | 0.818|
| **Tumor encapsulation**         |       |     |             |                  |    |
| Absent                          | 81    | 41  | 40          |                  |    |
| Present                         | 47    | 0.078 | 20     | 27               | 0.463|
| **Microsatellite formation**    |       |     |             |                  |    |
| Absent                          | 94    | 45  | 49          |                  |    |
| Present                         | 30    | 0.018 | 15     | 15               | 1.000|
| **Adjacent Organ Invasion**     |       |     |             |                  |    |
| Absent                          | 85    | 48  | 37          |                  |    |
| Present                         | 34    | 0.245 | 10     | 24               | 0.009|
| **Thrombus**                    |       |     |             |                  |    |
| Absent                          | 105   | 53  | 52          |                  |    |
| Present                         | 24    | 0.101 | 9     | 15               | 0.268|
| **Recurrence**                  |       |     |             |                  |    |
| No                              | 66    | 38  | 28          |                  |    |
| Yes                             | 59    | 0.203 | 22     | 37               | 0.031|
| **Differentiation grade**       |       |     |             |                  |    |
| High grade                      | 80    | 37  | 43          |                  |    |
| Low grade                       | 54    | 0.037 | 27     | 27               | 0.726|

*Partial data not available; statistics based on available data.

†Adjacent organs include gallbladder (n = 12), diaphragm (n = 11), greater omentum (n = 5), peritoneum (n = 2), adrenal gland (n = 2) and intestine (n = 2).

**Clinical Significance of MAEL Overexpression in HCC.** The association of MAEL overexpression with clinicopathological features in 134 HCC samples with informative IHC was statistically analyzed. The results found that overexpression of MAEL was significantly associated with tumor size (P = 0.032), adjacent organ invasion (P = 0.009), and recurrence (P = 0.031, Table 1). Furthermore, Kaplan-Meier analysis revealed that overexpression of MAEL was significantly associated with poorer overall survival (P = 0.001) and disease-free survival rates (P = 0.027) of HCC patients (Fig. 1F). Analyzed by univariate Cox regression, tumor size, adjacent organs invasion, alphafetoprotein (AFP), microsatellite formation, thrombus, and MAEL overexpression were all found to be significantly associated with overall survival (Supporting Table 1). Moreover, multivariate Cox proportional hazard regression analysis found AFP, microsatellite formation, thrombus, and MAEL overexpression to be independent prognostic factors for the overall survival of HCC patients (Supporting Table 1).

**MAEL Shows Strong Oncogenicity Function.** To determine whether MAEL has oncogenic function, two HCC cell lines, QGY-7701 and BEL-7402, were stably transfected with the MAEL construct, and ectopic expression of the MAEL in the cells was determined by RT-PCR and western blotting (Fig. 2A). Functional assays were used to characterize the tumorigenicity of MAEL. Cell growth assay showed that cell growth rates in MAEL-transfected cells were significantly higher than those in the control cells (P < 0.01, Fig. 2B). Foci formation assay yielded a higher number and larger colonies (P < 0.01) in the MAEL-transfected cells compared to the control cells (Fig. 2C). A xenograft tumor mouse model was established by subcutaneously injecting MAEL-7701 and MAEL-7402 cells into the right dorsal flanks of a group of 8 and a group of 11 nude mice, respectively. The same number of empty vector-transfected cells was injected into the left dorsal flank of each animal as control. After 4 weeks the mice were sacrificed and the size and weight of the xenograft tumors were measured. The results showed that tumors developed from MAEL-transfected cells were significantly larger and heavier (P = 0.0004 for MAEL-7701 cells, P = 0.0006 for MAEL-7402 cells) than tumors from control cells (Fig. 2D). Results from the IHC staining confirmed the expression of MAEL in xenograft tumors that developed from the MAEL-transfected cells (Fig. 2E).

**Overexpression of MAEL Increases Cell Migration and Tumor Metastasis.** Because MAEL overexpression was significantly associated with HCC invasion, the role of MAEL in tumor cell migration and invasion was investigated. The wound-healing assay showed that MAEL-transfected cells obtained quicker closure of the scratched “wound” compared with control cells (Fig. 3A). Transwell invasion assay further revealed significantly increased cell motility and invasion with MAEL expression (P < 0.05, Fig. 3B).

To evaluate the in vivo effects of MAEL on tumor metastasis, two groups of six mice each were injected intravenously in the tail vein with Vec-7701 or MAEL-7701 cells, respectively. After 8 weeks the mice were sacrificed and the metastatic nodules at the liver and lung surfaces were counted. A significantly larger number of metastatic nodules were induced at the surface.
of the livers and lungs of mice injected with the MAEL-7701 cells than those with the Vec-7701 cells ($P < 0.001$, independent Student $t$ test, Fig. 3C). Hematoxylin and eosin (H&E) staining confirmed that the nodules on the surfaces of mice livers and lungs were metastatic tumors (Fig. 3D).

**MAEL Silencing Inhibits Tumorigenicity and Cell Motility.** To further confirm the oncogenicity of MAEL, the MAEL gene was silenced by RNA interference (RNAi) using two targeted shRNAs (shRNA3 and shRNA4). The shRNAs were stably transfected into the HCC cell lines QGY7703 and PLC8024. A
scrambled shRNA was used as a negative control. RT-PCR and western blotting results indicated that the expression levels of MAEL in both mRNA and protein could be effectively silenced by shRNAs (sh3 and sh4) (Fig. 4A). Silencing of MAEL expression in the cells significantly inhibited the cell growth rate ($P < 0.05$, Fig. 4B) and frequency of foci formation ($P < 0.05$, Fig. 4C).

Fig. 3. MAEL promotes cell motility and HCC metastasis. (A) Wound-healing assay showing that MAEL promoted cell migration. Representative images were taken at 0, 24, and 48 hours after scratching. (B) Transwell invasion assay showing that MAEL promoted cell invasion. Representative images of invaded cells are shown in the left panel and the results are summarized in the right panel. The results are expressed as the mean ± SD of three independent experiments (***$P < 0.01$, independent Student t test). (C) Experimental metastasis model was used to evaluate the in vivo effects of MAEL on tumor metastasis by tail vein injections of cells. Representative images of livers and lungs derived from nude mice injected with MAEL- and empty vector-transfected QGY7701 cells are shown. Metastatic nodules at the surface of livers and lungs are indicated by arrows. Formation of metastatic nodules at the liver and lung surfaces are summarized in the right panel. (D) Representative images of H&E-stained sections derived from the liver and lung metastatic nodules. Sections of liver and lung derived from nude mice injected with empty vector-transfected QGY7701 cells were used as control (original magnification, 100×).
To demonstrate whether MAEL silencing could inhibit tumorigenicity in vivo, MAEL shRNA- and scrambled shRNA-transfected cells were subcutaneously injected into the left and right dorsal flanks of nude mice (n = 6), respectively. Tumors were observed in 6/6 mice injected with scrambled shRNA-transfected PLC8024 cells and in 3/6 mice with the MAEL shRNA-transfected PLC8024 cells. With the QGY7703 cells, tumors developed from MAEL shRNA-transfected cells were significantly smaller than tumors from sh-control cells (Fig. 4D). Further studies showed that knockdown of MAEL in QGY7703 and PLC8024 cells could inhibit cell migration (Fig. 4E) and cell invasion (P < 0.01, Fig. 4F).

**MAEL. Induces Epithelial-Mesenchymal Transition in HCC.** As EMT is one of the key events in tumor invasion and metastasis, the effect of MAEL on EMT was analyzed by investigating the expression levels of EMT markers and EMT-related transcription factors. Results from qRT-PCR showed decreased expression of three epithelial markers (E-cadherin, α-catenin, and β-catenin) with increased expression of two mesenchymal markers (vimentin and fibronectin) and two EMT-related transcription factors (Snail and Slug) in MAEL-transfected cells (Fig. 5A) compared with the control cells. An opposite expression pattern of these genes was observed in MAEL-silenced cells (Fig. 5B). The correlation between expressions of MAEL and Snail was further studied in the same cohort of HCC samples (only 86 specimens were available) by qRT-PCR. The results confirmed that expression of Snail was significantly correlated with MAEL expression (R = 0.537, P < 0.001, Fig. 5C). A recent report indicates that MAEL can repress the expression of miR-7, a tumor-suppressing miRNA targeting AKT. The correlation between expressions of MAEL and miR-7 was also studied in 64 miRNA available HCC specimens by qRT-PCR and the results showed that expression of MAEL was negatively correlated with miR-7 expression (R = 0.587, P < 0.001, Fig. 5C).

Western blot analysis further demonstrated that MAEL could down-regulate the levels of the epithelial markers α-catenin and β-catenin and up-regulate the mesenchymal markers vimentin and fibronectin (Fig. 5D). As the expression of E-cadherin in QGY-7701 and BEL-7402 cells was undetectable by western blot, we were unable to determine whether its expression was affected by MAEL. Results consistent with these findings were observed when MAEL was silenced in PLC8024 cells (Fig. 5D). Immunofluorescent staining confirmed that the increased expression of vimentin and decreased expression of β-catenin were also observed in the MAEL-7402 cells (Fig. 5F).

**MAEL Activates the Akt/GSK-3β/Snail Pathway to Induce EMT.** Activation of AKT plays an important role in inducing EMT by inhibiting GSK-3β, leading to the stabilization and nuclear localization of Snail, thereby triggering cell migration and EMT. We tested the expression of several proteins involved in the Akt/GSK-3β/Snail pathway by western blot analysis and observed that increased expression of phosphorylated Akt, phosphorylated GSK-3β (inactive form), Snail and Slug could be detected in MAEL-transfected cells (Fig. 5E). As expected, opposite expression patterns of these proteins were detected in MAEL-silenced cells (Fig. 5E).

To confirm that the oncogenic effect of MAEL was by way of the activation of the Akt/GSK-3β/Snail pathway, the PI3K inhibitor LY294002 was used to investigate the correlation between the inhibition of PI3K and the increased cell motility induced by MAEL in MAEL-transfected 7701 and 7402 cells. The results showed that LY294002 could effectively decrease expression levels of phosphorylated Akt, phosphorylated GSK-3β, and Snail induced by MAEL in MAEL-transfectants (Fig. 6A). Transwell assay and wound-healing assay demonstrated that LY294002 could significantly decrease cell migration ability in MAEL-transfectants (P < 0.01, Fig. 6B; Supporting Fig. 1).

**MAEL Increases Stemness of HCC Cells.** We further examined if MAEL could increase stemness of HCC cells by determining expression levels of stemness-associated genes by qRT-PCR. The results showed that MAEL could up-regulate many stemness-associated genes (Naznog, Oct-4, Bmi-1, Notch-1, and Smo), multiple drug-resistant transporter genes (ABCC2, ABCG2) and surface antigens associated with cancer stem cells (CD24, CD44, CD133, CD105, and CD166) (Fig. 6C). We observed the reverse expression patterns of these genes in MAEL-silenced cells (Fig. 6C).

Functional assays were performed to determine whether MAEL could enhance the stemness of HCC cells. The sphere-forming ability of 8024-shMAEL cells in a sphere formation assay was significantly decreased (P < 0.05) compared with that of the 8024-shControl cells (Fig. 6D), suggesting that MAEL had self-renewal ability. The tumor formation assay was applied to test whether MAEL could increase in vivo tumorigenicity. The results showed that as few as 2 × 10^5 MAEL-7701 cells or 2 × 10^5 MAEL-7402 cells were sufficient to induce tumor formation in nude mice, whereas no tumor formation was observed with the same amount...
Fig. 4. Silencing MAEL by RNAi inhibits its tumorigenicity. (A) Two shRNAs against MAEL effectively decreased MAEL expression as detected by RT-PCR and western blotting. Scrambled shRNA (shControl) and GAPDH were used as negative and loading controls, respectively. Intensities of bands were analyzed by the Macintosh densitometry program ImageJ. Silencing MAEL expression could effectively inhibit cell growth (B), foci formation (C), tumor formation in nude mice (D), cell migration (E), and cell invasion (F). The results are expressed as the mean ± SD of three independent experiments (*P < 0.05; **P < 0.01, independent Student t test).
of control cells (Fig. 6E). To confirm that MAEL could confer chemoresistance, the XTT assay was used to compare the chemosensitivity of MAEL- and empty vector-transfected cells treated with DDP for 48 hours. The results showed that MAEL-transfected cells were more viable compared with control cells (Fig. 6F).
Fig. 6. MAEL increases the stemness of HCC cells. (A) Western blot analysis demonstrated that the Akt inhibitor LY294002 (LY) could effectively decrease expressions of p-AKT, p-GSK-3β, and Snail induced by MAEL. (B) Transwell migration assay showing that LY294002 could inhibit MAEL-induced cell migration. Representative images of migrated cells are shown in the left panel and the results are summarized in the right panel. The results are expressed as the mean ± SD of three independent experiments (**P < 0.01, independent Student t test). (C) Relative expression of stemness-associated genes (Nanog, Oct-4, Bmi-1, Notch-1, and Smo), multiple drug-resistant transporter genes (ABCC2, ABCG2), and surface antigens associated with cancer stem cells (CD24, CD44, CD133, CD105, and CD166) were compared by qPCR between MAEL-expressing and control cells or between MAEL-silenced and control cells. (D) Representative images showing decreased sphere forming ability in MAEL-silenced cells. The results are summarized as the mean ± SD of three independent experiments shown in the lower panel. (E) Tumor formation in nude mice shows increased in vivo tumorigenicity in MAEL-expressing cells compared with empty vector-transfected cells. (F) XTT assay shows stronger chemoresistance in MAEL-expressing cells compared with empty vector-transfected cells treated with the indicated concentration of cisplatin for 48 hours. The results are expressed as the mean ± SD of three independent experiments and shown in the lower panel (**P < 0.05, independent Student t test).
Discussion

The development of HCC is a multistep process involving the accumulation of multiple genetic and epigenetic alterations that lead to the activation of oncogenes and inactivation of tumor suppressor genes. In this study we explored the oncogenicity of MAEL in the pathogenesis and progression of HCC. The MAEL gene is localized at the 1q24 region of the chromosome, which is one of the most frequently amplified regions in HCC. Overexpression of MAEL could be detected in 59.7% (n = 134) of HCCs with this overexpression being significantly associated with MAEL gene amplification (P = 0.011). Interestingly, the expression level of MAEL in the tumor tissues was not evenly distributed. Stronger MAEL expression was often observed at the edge of the tumor or within tumor cells that had invaded the surrounding tissues. Overexpression of MAEL was significantly associated with increased tumor invasion (P = 0.009), recurrence (P = 0.031), and poor outcome (P = 0.001). Taken together, these data strongly suggest MAEL as an oncogene that plays an important role in HCC progression.

Our functional studies demonstrated that MAEL had strong tumorigenicity, with overexpression of MAEL promoting cell growth, cell migration, and tumor formation in nude mice. These effects were shown to be effectively inhibited when MAEL was silenced with shRNAs. The role of MAEL in promoting tumor metastasis was further supported by the in vivo experimental metastasis assay. Because EMT plays a key role in tumor invasion and metastasis during tumor progression, we tested whether the effect of MAEL on cell motility was by way of induction of the EMT pathway. As expected, MAEL expression led to the decreased RNA and protein levels of many epithelial markers and increased expression of mesenchymal markers. In addition, the expression level of Snail, an important EMT transcription factor, was dramatically modulated by MAEL expression, increasing with MAEL overexpression and decreasing when MAEL was silenced.

Further study found that MAEL was able to induce EMT of HCC cells by way of the activation of the AKT-GSK3β-Snail signaling pathway. It has been reported that the activation of AKT is able to inhibit GSK3β activity, subsequently phosphorylating Snail and inducing its degradation and nuclear export, ultimately triggering cell EMT. Our data indicated that the expression of phosphorylated Akt (active form) increased or decreased accordingly when MAEL was overexpressed or silenced. This change positively correlated with the expression level of phosphorylated GSK-3β (inactive form) and Snail. Taken together, these results suggest that the role of MAEL in promoting EMT was through the activation of the AKT-GSK3β-Snail signaling pathway. The next question was how MAEL activated AKT. One recent study shows that a tumor suppressive microRNA-7 (miR-7) is frequently down-regulated in several cancers. Many predicted targets of miR-7 are involved in AKT and EGFR signaling pathways, including IGF1R, IRS1, IRS2, and PAK1. Interestingly, another study finds that MAEL can repress miR-7 expression by binding to the promoter of miR-7. In our study, we found that expression of miR-7 was negatively associated with MAEL expression in clinical samples, implying that MAEL might repress miR-7 expression and subsequently increase AKT activity.

As a Piwi-interacting RNA (piRNA) gene, MAEL is critical for Piwi-mediated gene expression at the transcriptional level in Drosophila. Piwi protein expression is for maintaining stem cells in the animal germline and is thus mostly restricted to germ cells and stem cells. In mouse, MAEL has been found to interact with the chromatin-remodeling factor SNF5 and chromatin-associated protein SIN3B, suggesting that MAEL might play a regulatory role in stemness-associated genes. To test this hypothesis, the regulatory effect of MAEL on various stemness-associated genes, multidrug resistance genes, and CSC surface markers was investigated in both MAEL overexpressed and silenced cells. The results indicated that MAEL could up-regulate these genes, leading to the increase of self-renewal, chemoresistance, and tumor metastatic abilities. Taken together, MAEL has been demonstrated to be a novel oncogene that plays an important role in the development and progression of HCC by inducing EMT by way of the AKT/GSK3β/snail pathway and enhancing the stemness of HCC through up-regulating many stemness-associated genes. Further characterization of MAEL may lead to the identification of new therapeutic targets for better clinical management of HCC.

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