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ENSA expression correlates with attenuated tumor propagation in liver cancer

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ABSTRACT

Endosulfine alpha (ENSA) is an endogenous ligand of sulfonylurea receptor that was reported to be associated with an ATP-dependent potassium channel that controls insulin release and the onset of type 2 diabetes. ENSA also interacts with microtubule-associated serine/threonine-protein kinase-like (MASTL) to regulate the cell cycle. Previously, we identified *ENSA* as a possible bivalent gene in mesenchymal stem cells (MSCs) and hypothesized its methylation might determine cellular differentiation and transformation. Because there was no link between aberrant *ENSA* expression and tumorigenesis, we aimed to determine if *ENSA* is abnormally regulated in liver cancer and plays a role in liver cancer propagation. The epigenetic states of the *ENSA* promoter were evaluated in different cancer cell lines and patient samples. *ENSA* was overexpressed in a liver cancer cell line, and its interaction with MASTL and possible tumor suppression capabilities were also determined in cultured cells and mice. Distinct *ENSA* promoter methylation was observed in liver cancer (n = 100 pairs) and breast cancer (n = 100 pairs). *ENSA* was predominantly hypomethylated in liver cancer but was hypermethylated in breast cancer. Overexpressed ENSA interacts with MASTL and suppresses hepatic tumor growth. We also found that *ENSA* is hypermethylated in CD90-expressing ($CD90^+$) cells compared to CD90 non-expressing ($CD90^-$) liver cancer cells. These data reveal *ENSA* methylation changes during hepatic tumor evolution. Overexpressed ENSA suppresses tumor growth in an established hepatic cell line whereas hypermethylated *ENSA* might help maintain liver cancer initiating cells.

1. Introduction

Endosulfine alpha (ENSA; NM_207042) was initially identified as an endogenous ligand of sulfonylurea receptor associated with ATP-dependent potassium channels in pancreatic beta cells. Thus, ENSA is considered a regulator of insulin secretion [1–3]. ENSA was also found to collaborate with microtubule-associated serine/threonine-protein kinase-like (MASTL; NM_001172303) to regulate entrance into the cell cycle during mitosis [4–6]. Through a phosphorylation cascade, MASTL is also involved in checkpoint recovery, which is critical for cell cycle progression after genomic damage [5,6]. Because dysregulation of the cell cycle, particularly after cell damage, is the initial and critical step in tumorigenesis, it is reasonable to hypothesize that loss of ENSA/MASTL function could lead to cancer. Additionally, because the role of ENSA is diversified and might be cell-type specific, how ENSA dysregulation is associated with tumorigenesis remains to be solved.

DNA methylation is a dominant silencing epigenetic mark in the cellular genome [7,8]. Normal DNA methylation regulates cell fate, whereas abnormal DNA methylation transforms normal cells [9]. A genetic hypomorphic model indicated that global hypomethylation could lead to the loss of genome stability and accelerate cellular transformation [10,11]. In contrast, increased DNA methylation within tumor suppressor genes such as *HIC1* and *RassF1A* is sufficient to transform a somatic stem cell into cancer stem cell-like (CSC-like) cells [12]. Taken together, these data suggest that

Abbreviations: ENSA, endosulfine alpha; MSC, mesenchymal stem cell; 5-Aza, 5-aza-2′-deoxycytidine; H3K27me3, histone 3 lysine 27 trimethylation.

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associated abnormal DNA methylation indicates dysregulated gene function, which might be associated with tumorigenesis.

Previously, we found that *ENSA* is a bivalent locus in somatic stem cells, suggesting it could have critical role in cell fate determination [13,14]. We also found that the *ENSA* methylation state changed in breast cancer cell lines after environmental signals such as estrogen was changed [15]. With its cell cycle altering capacity, we hypothesized that epigenetic changes of the *ENSA* promoter could result in expression changes and link the ENSA/MASTL pathway with tumorigenesis.

In this report, we explore the epigenetic state of the *ENSA* promoter in breast and liver patient samples. To determine whether the expression of *ENSA* is regulated by DNA methylation, we treated the cancer cells with a methylation inhibitor, 5-aza-2′-deoxycytidine (5-Aza) [16], and found elevated *ENSA* expression. We found that elevated ENSA interacts with MASTL and suppresses the hepatic tumor growth in cultured cells and xenograft mice. Furthermore, the *ENSA* promoter is hypermethylated in CD90-expressing HepG2 cells [17] but is hypomethylated in CD90 non-expressing HepG2 cells. We speculate that silenced ENSA function might be essential for liver cancer initiating cells but is not necessary for tumor expansion in liver cancer.

2. Materials and methods

2.1. Cell culture

The colon cancer cell lines HCT116 and HT29 were cultured in McCoy's 5A media. HepG2 cells, a liver cancer cell line, were cultured in MEM. The breast cancer cell lines MCF7 and ZR75 were cultured in MEM and RPMI-1640, respectively. All media were supplemented with 10% fetal bovine serum (Invitrogen), 100 mg/ml penicillin/streptomycin (Invitrogen), and 2 mM L-glutamine (Invitrogen). The cells were cultured in incubators supplied with 5% CO_2 at 37 °C.

2.2. Isolation of CD90-expressing HepG2 cells

HepG2 cells (1×10^7) were harvested and incubated with the FITC-coupled CD90 antibody (Millipore, CBL415F). After further coupling with FITC microbeads (Miltenyi Biotech), the side population of cells were separated using AutoMACs (Miltenyi Biotech) with the double positive program.

2.3. 5-Aza treatment

Cells were treated with 5 μM 5-Aza or an equal final volume of DMSO for 5 consecutive days.

2.4. Transfection and isolation of ENSA-expressing HepG2 cells

Cloned *ENSA* (in *pEGFP*; the physical map and validated sequence are shown in Fig. 1) was transfected using DMRIE-C (Invitrogen) into 5×10^5 cells/well (0.4 μg/well) of a six-well plate according to the manufacturer's instructions. Transfected cells were grown in 0.5 mg/ml G418 to select for stable clones.

2.5. Chromatin immunoprecipitation (ChIP) assays

Semi-quantitative ChIP-polymerase chain reaction (qChIP-PCR) was performed as described previously [18,19]. All antibodies were from Upstate Biotechnology. PCR primers are listed in Table 1.

2.6. Semi-quantitative real-time methylation-specific PCR (qMSP)

qMSP experiments were conducted and products were quantified according to the protocol described by Yan et al. [20]. Briefly, bisulfite converted genomic DNAs (0.5 μg) were subjected to real-time PCR with methylation specific primers (Table 1). The qMSP reactions were performed using the SYBR Green I PCR kit (Roche) in an iQ5 Real-Time PCR instrument (Bio-Rad). Melting analyses were performed, followed by PCR reactions to ensure a specific amplicon was generated. Col2A1 (NM_033150) was used to construct a standard curve and as a loading control. Serial dilutions of Col2A1 amplified bisulfite-converted DNA were used to generate the standard curve. The methylation percentage was calculated as: [Mean of target gene]/[Mean of Col2A1]; fold change was calculated as: [Tumor (or treatment) methylation percentage]/[Control (or mock-treated) methylation percentage].

2.7. Semi-quantitative RT-PCR and qRT-PCR

Total RNA isolation, first-strand cDNA synthesis, and detection of transcripts were performed as described [15,21]. Briefly, total RNA (2 μg) was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen). qRT-PCR was performed using the SYBR Green I PCR kit (Roche) and an iQ5 Real-Time PCR instrument (Bio-Rad). Serial dilutions of *GADPH* (NM_002046) amplified cDNA were used as a control to generate standard curves and *GAPDH* from each sample was used as a loading control. The primers are listed in Table 1.

2.8. Immunoprecipitation (IP)

Protein IP was performed using the Catch and Release 2 kit (Millipore, 17–500) and an antibody against ENSA (Santa Cruz, SC81883) following the manufacturer's protocol. The immunoprecipitated proteins were separated on 8% SDS–PAGE gels and blotted onto PVDF membranes. Antibodies against ENSA (Santa Cruz, SC81883) and MASTL (Abcam, Ab86387) were used for detection.

2.9. Soft agar assays

Cells were plated in soft agar at a density of 5×10^4 cells per well. After 1 week in culture, cells were stained with 0.01% crystal violet and the number of spheres (>50 cells) from each dish was counted.

2.10. In vivo tumorigenesis and monitoring tumor growth

Six-week-old nude mice (Narl: ICR-Foxn1nu) were inoculated subcutaneously with 1×10^5 of *ENSA*-overexpressing or control HepG2 cells. Tumor growth was monitored two months after inoculation by FMT4000 using AngioSense tracking dye. The tumors were surgically removed and subjected to immunohistochemical exams.

2.11. Immunohistochemistry

Tumor masses that were surgically removed from inoculated nude mice were embedded in optimum cutting temperature media and sectioned on a cryostat (Leica) into 12 mm sections. Sections were stained with the indicated antibodies, and detection was performed with Fluorescein– or Texas red–conjugated anti-mouse or rabbit IgG (Vector Labs), followed by DAPI staining. Sections were also stained with hematoxylin and eosin (H&E; Vector Labs) for pathologic exams.

2.12. Statistical analyses

Paired student's *T*-test was used to test the methylation difference between tumors and non-tumors. Spearman's coefficients were deduced from sorted methylation differences from both cancers.

2.13. Human subjects and animal care

Tumor samples were collected from Chang-Hua Christian Hospital following their Institutional Review Board regulations. All mice were maintained, treated, and sacrificed in accordance with the protocols and regulations of the Chung Cheng University Institutional Animal Care and Use Committee.

3. Results

3.1. Epigenetic status of the ENSA promoter

We first examined if the *ENSA* promoter was regulated by epigenetic marks, particularly DNA methylation. Trimethylated histone 3 at lysine 27 (H3K27me3) is the subtract of Polycomb group proteins [22–25] and the maintenance of this epigenetic mark is critical for maintaining the "stemness" of stem cells [26–29]. Strikingly, a previous report indicated that these marks are more alike between cancer and embryonic stem cells than between normal and stem cells [30]. Although H3K27me3 represents silencing marks in their associated genes, they were found to co-localize with active histone marks, and these loci were "bivalent" in nature [31]. Their abilities to be further activated or silenced suggest they could be further manipulated in terms of expression and cell-fate determining function. Previously, we determined that the *ENSA* promoter is bivalent in somatic stem cells (mesenchymal stem cells, MSC) and suggested it could be further modified to alter cell physiology [18]. We probed the *ENSA* epigenetic states and determined that both acetylated histone 3 (active) and H3K27me3 (silencing) marks were enriched within its promoter (Fig. 1A).

The appearance of DNA methylation within the bivalent loci often indicates further changes in associated gene expression and cell physiology. We sought to determine if the *ENSA* promoter could be further methylated in cancer cells, and if the methylation could determine *ENSA* expression. As shown in Fig. 1B, after treatment with 5-aza, a methylation inhibitor, *ENSA* expression was reactivated in HCT116, MCF7, and ZR75 cancer cells accompanied by decreased *ENSA* methylation. This result indicates that methylation is sufficient to regulate *ENSA* expression, and the different methylation states in different cancer cell types suggest that they might be distinctly regulated in different cancers.

3.2. Abnormal ENSA methylation within patient samples

To validate if dysregulation of DNA methylation within the *ENSA* promoter occurs *in vivo*, qMSP was used to detect the *ENSA* methylation states within different cancer samples (Fig. 2). *ENSA* was significantly hypomethylated in liver cancer samples compared to their paired adjacent normal ($n = 100$, Fig. 2A). However, in breast cancer samples, *ENSA* was significantly hypermethylated (representative ones are shown, $n = 100$, Fig. 2B). These data indicate that dysregulation of DNA methylation might occur during tumorigenesis and the occurrence might be specific to the tissue or individual (Fig. 2C).

3.3. ENSA interacts with MASTL and suppresses the hepatic tumor growth

Dysregulated ENSA might interfere with cell cycle progression via its interaction with MASTL and thus affect tumor progression.

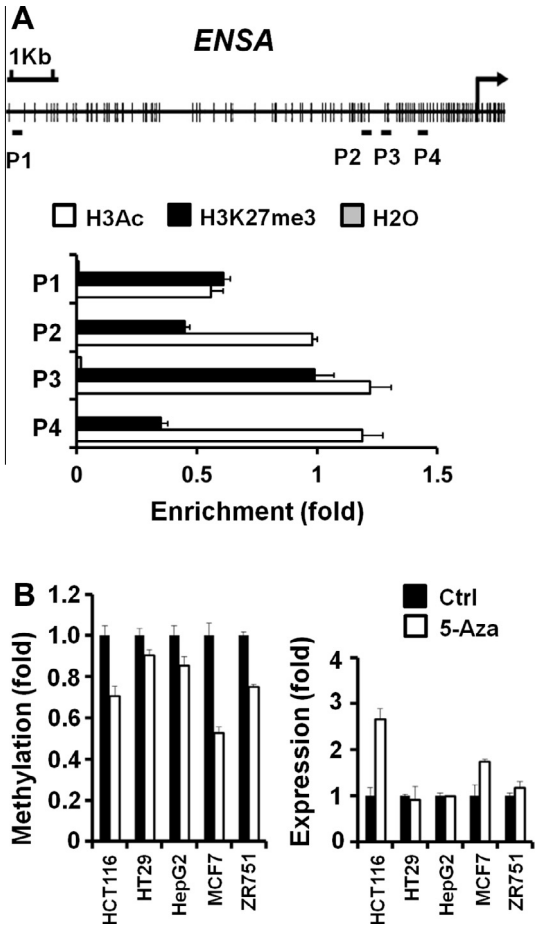

Fig. 1. Epigenetic status of the *ENSA* promoter. (A) The chromatin conformation of the *ENSA* promoter in HepG2 cells was detected using qChIP-PCR. The physical map of the *ENSA* promoter is illustrated in the top panel and vertical bars indicate the CpG sites. qChIP-PCR amplification/detection regions are marked by horizontal bars (P1–P4). The arrow indicates the transcriptional start site (TSS). Using qPCR, enrichments of acetylated histone 3 (H3Ac) or H3K27me3 were calculated compared with total input. Reaction without ChIP DNA was used as a negative control. (B) Methylation states of the *ENSA* promoter. qMSP and qRT-PCR were used to detected *ENSA* methylation (left) and expression (right), respectively, in the indicated cancer cell lines before (closed histogram) and after (open histogram) 5-aza treatment.

To link abnormal *ENSA* expression with tumor propagation, we overexpressed *ENSA* in different hepatic cancer cell clones (Fig. 3A) and evaluated whether tumor growth was suppressed. Despite overexpression of ENSA, expression of its interacting counterpart, MASTL, remained the same (Fig. 3A, lower panel). Overexpressed ENSA was identified in the nucleus and cytoplasm (Fig. 3B), and its interaction with MASTL was documented by immunoprecipitation followed by western blotting experiments (Fig. 3C). Soft agar experiments were performed to determine if anchorage-independent tumor growth was suppressed by ENSA expression. In Fig. 3D, the suppressed tumor growth indicates that overexpressed ENSA might act as a tumor suppressor in liver cancer.

3.4. Overexpressed ENSA attenuates liver cancer growth in vivo

To associate abnormal ENSA expression with tumor propagation *in vivo*, we inoculated ENSA-overexpressing clones into immune-deficient mice and observed the tumor growth. Although vector-only or mock-control HepG2 cells grew in nude mice, the

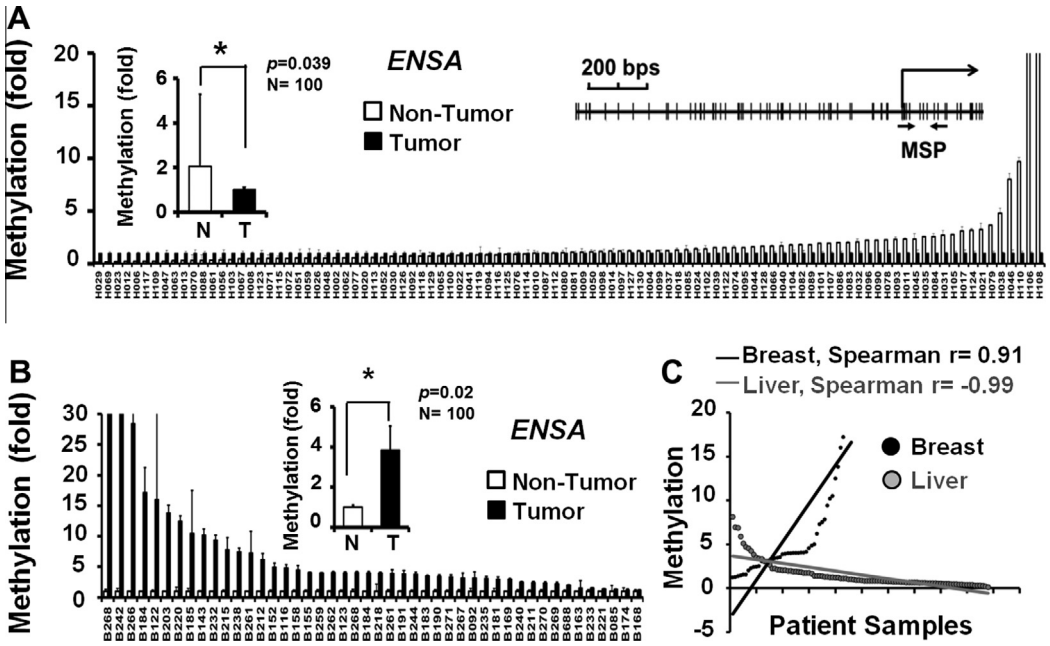

Fig. 2. *ENSA* methylation in patient samples. qMSP was used to detect the *ENSA* methylation state from different cancer specimens. (A) Methylation differences between paired adjacent normal (open bars) and tumor (closed) tissues from liver cancer patients. The majority became hypermethylated [$n = 100$, methylation fold = (tumor methylation)/(normal methylation)] as analyzed using paired student's *t*-tests. (B) Methylation difference between paired adjacent normal (open bars) and tumor (closed) tissues from breast cancer patients. Most samples became hypomethylated as determined using paired student's *t*-tests. (C) The methylation states in liver and breast cancers were inversely correlated because both regressed tendency lines had opposite Spearman's coefficients.

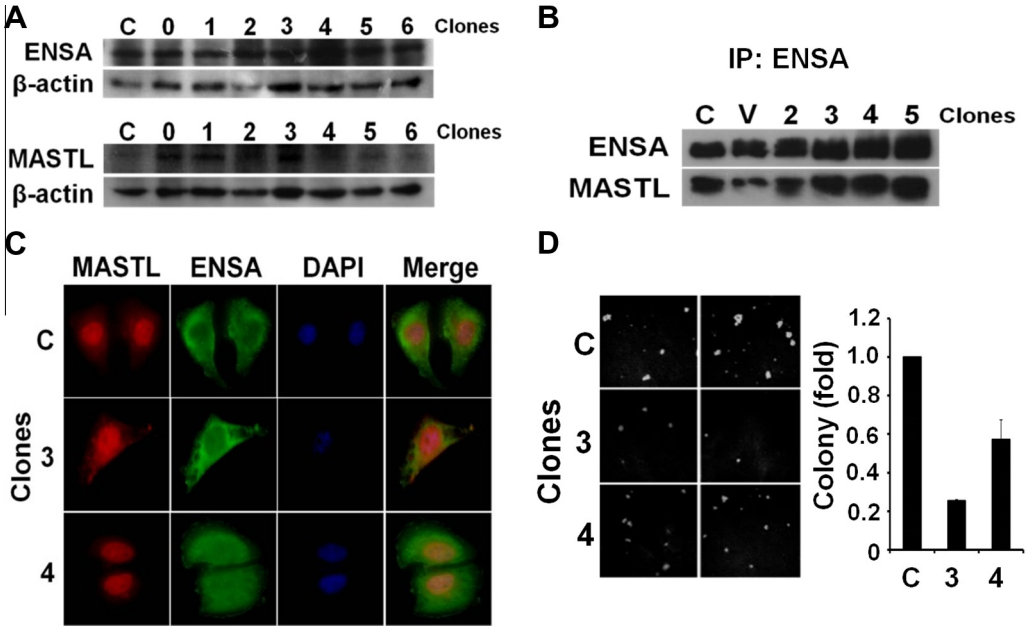

Fig. 3. *In vitro* characterization of ENSA-overexpressed HepG2 cells. (A) ENSA overexpression in HepG2 cells. ENSA was cloned into an expression vector, transfected into HepG2 cells, and stable clones (clones number 1–6) were isolated. Western blotting with ENSA and MASTL antibodies was used to confirm ENSA overexpression (upper panel) and normal expression of MASTL (lower panel) in the selected clones. Detection of β-actin was used as a loading control. (B) Immunostaining of the selected ENSA-overexpressing cells. Antibodies against ENSA (green fluorescence) and MASTL (Texas red) were used to examine the expression of both genes in selected clones and controls (designated as c). (C) ENSA and MASTL interaction. Total protein from control and ENSA-overexpressed HepG2 cell lysates were immunoprecipitated using an antibody against ENSA. The precipitated proteins were separated using SDS–PAGE and blotted with either ENSA or MASTL antibodies. (D) Soft agar analyses of the ENSA-expressing HepG2 clones. Controls and ENSA-overexpressing clones of HepG2 cells were plated in soft agar to evaluate anchorage-independent growth. Five days after plating, cell aggregates with greater than 50 cells (left) were counted and the number of formed aggregates from the clones were compared with the number from control (right). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ENSA-HepG2 clones did not grow or developed much smaller masses than the controls (Fig. 4A). These tumors are from clones of cells expressing both ENSA and MASTL, whereas other tumors were not (Fig. 4B). These data indicate that overexpressed ENSA attenuates tumor propagation in liver cancer. CD90-expressing ($CD90^+$) liver cancer cells were reported to be a potential tumor initiating side population [17]. Therefore, the *ENSA* methylation status was evaluated in these cells to associate the initiation

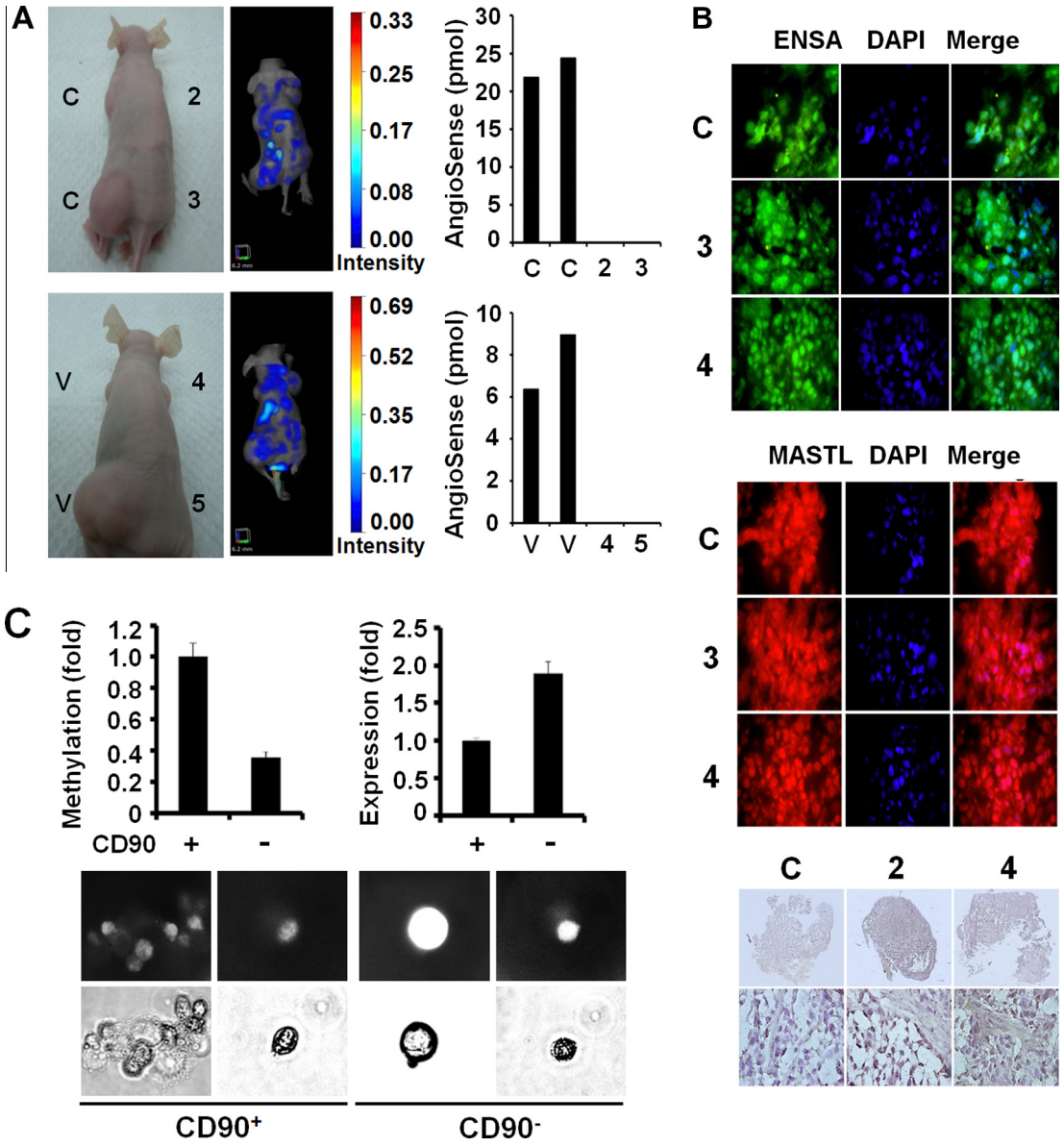

Fig. 4. *ENSA* expression *in vivo* and in CD90$^{+/-}$ side populations. (A) ENSA overexpression suppressed tumor growth in liver cancer. Control (C) and stable ENSA-overexpressed HepG2 cells were subcutaneously inoculated into immune-deficient mice. Tumor growth was monitored *in vivo* by tracking the tumor marker AngioSense in FMT 4000. Tumor heat images are shown on the left and the detected amount of AngioSense is summarized on the right. More examples from different overexpressed clones are shown in Fig. 2. (B) ENSA and MASTL expression in the inoculated liver tumors. ENSA and MASTL expression in the inoculated tumors shown in (A) were detected using immunohistochemistry and the indicated antibodies (more examples in Fig. 3). The bottom panel shows the H&E staining. (C) ENSA methylation and expression in CD90$^{+/-}$ side populations. The HepG2 CD90^{+} and CD90^{-} side populations were separated, and isolated. *ENSA* methylation states and expression were detected using qMSP (top left), qRT-PCR (top right), and immunostaining (bottom).

capability with ENSA expression. We found that *ENSA* is hypermethylated in CD90^{+} cells compared to CD90^{-} cells, and the expression was inversely correlated (Fig. 4C). This result indicates that *ENSA* hypermethylation might be required to silence the tumor suppressor function of ENSA at the initiation of liver cancer propagation, but might not be necessary for tumor propagation in later stages.

4. Discussion

DNA methylation is a dominant somatic inheritance that is a suitable biomarker and is associated with silenced genes [7,8]. It was hypothesized that environmental changes could be transmitted into the cells and memorized as DNA methylation, which further shapes gene expression in cells [32]. These lineage-inherited expression differences could be selected, and the cells will transform and evolve further [32]. If methylation occurs at the promoter of bivalent genes such as *ENSA* that affect cell cycle progression, specific cell fate changes might occur that accelerate tumorigenesis or tumor progression. Therefore, abnormal *ENSA* methylation could indicate that tumor propagation is initiated in specific cancers such as liver cancer.

In this report, we identified dysregulated *ENSA* methylation in liver and breast cancers. Their methylation states were opposite to each other (Fig. 2), indicating lineage-specific methylation changes. *ENSA* hypomethylation in liver cancer (Fig. 2A) usually indicates gain of ENSA function in tumorigenesis; however, the tumor suppression phenotypes in cultured cells (Fig. 3D) and *in vivo* (Fig. 4A) suggest that the function should be to suppress tumor

development. Considering that the ENSA/MASTL pathway is involved in checkpoint recovery during normal mitosis, abnormal ENSA expression might be required at the initial stage of tumor propagation. This rational was supported by the observation of ENSA hypermethylation in $CD90^+$ liver cancer initiating cells but not in the $CD90^-$ cells (Fig. 4C).

ENSA/NASTL has been reported to regulate the cell cycle [4–6,33,34] during mitosis and abnormal expression of ENSA has been associated with the onset and treatment of type 2 diabetes [2,35,36]. Although previous reports indicated that the sulfonylurea treatment of diabetes might correlate with tumor onset, there is no detection and correlation between cancer and abnormal ENSA expression Cell type-specific *ENSA* methylation anomalies (Fig. 2) suggest that the tumorigenesis effect of the ENSA/MASTL pathway might be tissue-specific.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.165.

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