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Lipocalin 2 negatively regulates cell proliferation and epithelial to mesenchymal transition through changing metabolic gene expression in colorectal cancer

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Key words

Colorectal cancer, epithelial–mesenchymal transition, glucose metabolism, lipocalin 2, proliferation, Warburg effect

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Lipocalin 2 (LCN2), a member of the lipocalin superfamily, plays an important role in oncogenesis and progression in various types of cancer. However, the expression pattern and functional role of LCN2 in colorectal cancer (CRC) is still poorly understood. The purpose of the present study was to investigate whether LCN2 is associated with proliferation and the epithelial–mesenchymal transition (EMT) in CRC and to elucidate the underlying signaling pathways. LCN2 was preferentially expressed in CRC cells compared to normal tissues. However, LCN2 expression was significantly lower in metastatic or advanced-stage CRC than in non-metastatic or early stage CRC. Knockdown of LCN2 using small interfering RNA (siRNA) in CRC cells expressing a high level of LCN2 induced cell proliferation and a morphological switch from an epithelial to mesenchymal state. Furthermore, downregulation of LCN2 in CRC cells increased cell migration and invasion involved in the regulation of EMT markers. Knockdown of LCN2 also induced glucose consumption and lactate production, accompanied by an increase in energy metabolism-related genes. Taken together, our findings indicated that LCN2 negatively modulated proliferation, EMT and energy metabolism in CRC cells. Accordingly, LCN2 may be a candidate metastasis suppressor and potential therapeutic target in CRC.

Colorectal cancer (CRC) is one of the most common malignancies worldwide and CRC metastases are the leading cause of poor prognostic outcomes.[1] Although several advances have improved CRC prognosis, the appearance of metastases is still common despite proper CRC resection and new adjuvant treatments.[2] Much current CRC research focuses on discovering new molecules that can regulate CRC development, progression and metastases.

Lipocalins (LCN) are a family of proteins with several functions, including regulation of immune responses, modulation of cell growth and metabolism, iron transport and prostaglandin synthesis.[3] One of the most studied LCN, LCN2 (neutrophil gelatinase associated lipocalin, NGAL), is a 25-kDa glycoprotein that was initially purified from neutrophil granules.[4] LCN2 exists as a 25-kDa monomer and a 46-kDa homodimer, and in a covalent complex with neutrophil gelatinase.[5] LCN2 expression is regulated in human epithelial cells during cancer progression.[6] However, the expression pattern for LCN2 shows significant differences between tumor tissue and relative normal counterparts for each cancer type. In breast, colorectal, liver, pancreatic, oral and ovarian carcinomas, higher levels of LCN2 are observed.[6–9] Conversely, lower levels of LCN2 are observed in esophageal cancer in head and neck cancer, and in hematological malignancies.[6,9] In addition, different functional roles have been identified for LCN depending on the kind of cancer. LCN2 has an oncogenic role in promoting tumorigenesis through enhancing tumor cell proliferation and metastatic potential.[10,11] Mechanistic studies have reported that LCN2 regulates tumor development through the combined expression of LCN2 and matrix metalloproteinase 9 (MMP9), which sustains a high gelatinolytic action and promotes the epithelial to mesenchymal transition (EMT).[12–14] In contrast to its oncogenic function, some studies have shown that LCN2 acts as a tumor suppressor gene. LCN2 suppresses proliferation and negatively modulates tumor invasive ability by regulating the EMT process.[8,15] Moreover, microarray data indicate that the level of LCN2 in metastatic tissues is significantly lower than in primary tumors of several cancer types.[6] Nonetheless, the expression pattern and functional roles of LCN2 in cancer progression remain highly controversial.

LCN2 has consistently been suggested to play a role in the progression of CRC. Higher expression of LCN2 in CRC neoplastic tissue than in normal tissue has been identified with immunohistochemistry and microarrays.[6] However, an association between advanced cancer stage or metastasis and LCN2 expression has not been established. Furthermore, a functional role for LCN2 in CRC progression and the metastatic process remains to be examined.

The purpose of this study was to evaluate the expression of LCN2 in relation to clinicopathologic features using human CRC specimens and to explore the role of LCN2 in CRC progression and its underlying molecular mechanisms *in vitro*.

Materials and Methods

Cell culture. Human colorectal cancer cell lines HT-29, DLD-1, SW480, HCT116 and SW620 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 units of penicillin and 100 units of streptomycin in a humidified 5% CO_2 environment at 37°C.

Patients and tissue specimens. Eighty CRC and paired normal tissues were obtained through the Biobank of Chonbuk National University Hospital, a member of the National Biobank of Korea. All patients had a pathological diagnosis of CRC, and each paired sample was classified according to the TNM Classification of Malignant Tumors (TNM), frozen in liquid nitrogen and stored at −80°C. Patient characteristics are shown in Table 1. The present study consisted of 32 (40%) women and 48 (60%) men with a mean age of 63.1 years. The study protocol was approved by the Institutional Review Boards of Chonbuk National University Hospital (IRB no. 2016-04-018-002).

RNA isolation and real-time quantitative PCR. Total RNA from cells or human normal tissue/matched tumor samples was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), according to the manufacturer's protocol. Real-time quantitative PCR (RTQ-PCR) was performed using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). In brief, 20 μL of master mix was prepared on ice with 10 μL 2 × SYBR, 1 μL primers, 2 μL DNA and 7 μL nuclease-free water. The master mix was initially denatured at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 30 s. The geometric average Ct value was used to calculate the relative expression of LCN2 using the 2-ΔΔCT method, which was normalized to beta-2-microglobulin (B2M). Primers used in this experiment were: 5′-TCACCTCCGTCCTGTTTAGG-3′ (forward) and 5′-CGAAGTCAGCTCCTTGGTTC-3′ (reverse) for LCN2, and 5′-CCTGAATTGCTATGTGTCTGGG-3′ (forward) and 5′-TGATGCTGCTTACATGTCTCGA-3′ (reverse) for B2M. Relative expression of LCN2 was also calculated as an average of normal tissue expression value for comparisons. The LCN2 expression level was subdivided into a low and high group according to the median of the ratio, which was 12.8.

Table 1. Clinicopathologic correlations of LCN2 expression in patients with colorectal cancer

Parameter	Number (*n*)	Low (*n*, %)	High (*n*, %)	*P*-value
Age				
65<	42	24 (57.1%)	18 (42.9%)	0.189
65≥	38	16 (42.1%)	22 (57.9%)	
Gender				
Male	48	24 (50.0%)	24 (50.0%)	1.000
Female	32	16 (50.0%)	16 (50.0%)	
Differentiation				
Well	17	6 (35.3%)	11 (64.7%)	0.382
Moderately	55	30 (54.5%)	25 (45.5%)	
Poorly	8	4 (50%)	4 (50%)	
T stage				
T1/T2	40	13 (32.5%)	27 (67.5%)	0.002
T3/T4	40	27 (67.5%)	13 (32.5%)	
Lymph node				
No	52	21 (40.38%)	31 (59.62%)	0.021
Yes	28	19 (67.85%)	9 (32.14%)	
Distant metastasis				
No	77	37 (48.1%)	40 (51.9%)	0.240
Yes	3	3 (100%)	0 (0%)	
AJCC				
I/II	50	19 (38.0%)	31 (62.0%)	0.006
III/IV	30	21 (70%)	9 (30%)	

siRNA for inhibition of neutrophil gelatinase associated lipocalin expression. siRNA sequences used for targeted silencing of the LCN2 gene (NCBI Ref Seq NM_005564.4) were from Ambion (Austin, TX, USA). The sequences of sense and antisense of LCN2 siRNA were 5′-GCAUGCUAUGGUGUUCUUCTT-3′ (forward) and 5′-GAAGAACACCAUAGCAUGCTG-3′ (reverse). NGAL siRNA and scrambled siRNA for negative controls (Ambion, Austin, TX, USA) were transfected into CRC cells using TransiT- × 2 transfection reagent (Mirus Bio, Madison, WI, USA) according to the manufacturer's protocol.

RT-PCR. Total RNA was isolated from cultured cells using TRIzol Reagent and cDNA was synthesized with Super Script II reverse-transcriptase (Invitrogen) according to the manufacturer's protocol. The expression of GAPDH was used as an internal control. The following primer sequences were used: LCN2, 5′-TCACCTCCGTCCTGTTTAGG-3′ (forward) and 5′-CGAAGTCAGCTCCTTGGTTC-3′ (reverse), GLUT1, 5′-AAGCTGACGGGTCGCCTCATG-3′ (forward) and 5′-CTCTCCCCATAGCGGTGGACC-3′ (reverse), HK2, 5′-GAGCCACCACTCACCCTACT-3′ (forward) and 5′-ACCCAAAGCACACGGAAGTT-3′ (reverse), LDHA 5′-ATGGCAACTCTAAAGGATCA-3′ (forward) and 5′-GCAACTTGCAGTTCGGGC-3′ (reverse), MCT4, 5′-CCTGGGCTTCATTGACATCT-3′ (forward) and 5′-AGCAAAATCAGGGAGGAGGT-3′ (reverse). After initial denaturation at 95°C for 1 min, PCR was performed for various cycles (30 s at 94°C, 1 min at annealing temperature and 2 min at 72°C) using Taq polymerase. Reaction products (10 μL) were separated on 2% agarose gels and stained with Redsafe (Intron, Daejeon, Korea). DNA band intensity was analyzed by densitometry using an NαBI imager (Neogene Science, Suwon, Korea).

Cell viability, colony formation assay and morphological changes. DLD-1 and HT-29 cells were plated at a density of 1.0×10^4 cells per well in 24-well plates. After transfection with scrambled siRNA or LCN2 siRNA, the medium was removed and 200 μL of fresh medium plus 20 μL of 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT, 2.5 mg dissolved in 50 μL of DMSO, Sigma, St. Louis, MO, USA) were added to each well. After incubation for 4 h at 37°C, the culture medium containing MTT was removed and then 200 μL of DMSO was added, followed by shaking until the crystals were dissolved. Viable cells were detected by measuring absorbance at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

For the colony formation assay, cells (1×10^2 cells/well) were seeded into a six-well plate then transfected with scrambled siRNA or LCN2 siRNA. After 14 days of culture, the colonies were fixed with 3.8% formaldehyde for 20 min and stained with 0.1% crystal violet.

Morphological changes following LCN2 silencing were observed and images were captured using an inverted microscope (Olympus IX71, USA).

Protein extraction and western blotting. DLD-1 and HT-29 cells were harvested by resolving in RIPA buffer (50 mM Tris-HCl,150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and protease inhibitors) and were centrifuged at 16 300 *g* at 4°C for 30 min. After centrifugation, supernatants were used as whole cell extracts. The protein concentration in cell lysates or tissue lysates was measured using a Protein Quantification Kit from Bio-Rad. Either 50 or 30 μg of protein per lane was loaded onto SDS-polyacrylamide gels. After transferring and blocking, each PVDF membrane was probed with various antibodies (anti-LCN2, anti-E-cadherin, anti-Vimentin, anti-β-catenin, anti-Slug, anti-Snail, anti-MMP2, anti-ICAM-1, anti-Twist, anti-GLUT1, anti-GLUT3, anti-Hexokinase II, anti-LDHA, anti-LDHB, anti-MCT4 and anti-actin). Binding of antibody to antigen was detected using enhanced ECL Prime (GE Healthcare, NJ, USA), captured and analyzed by an Las-3000 Luminescent Image Analyzer (Fuji Film, Tokyo, Japan).

Wound healing assay. DLD-1 and HT-29 cells (1×10^5) were seeded in 6-cm culture plates and allowed to form a confluent monolayer. After transfection with scrambled siRNA or LCN2 siRNA, the monolayer was then scraped with a P200 pipette tip to generate a wound approximately 1-mm wide. Images of the wounds were captured at 0, 24, 48 and 72 h, and the wound area was determined using an inverted microscope (Olympus IX71). The ability of the cells to close the wound, as a measure of motility, was evaluated by determining the healed area.

***In vitro* migration and invasion assays.** A cell migration assay was performed using a Transwell system (24-wells, 8-μm pore size with poly-carbonate membrane; SPL, Gyeonggi-do Korea) according to the manufacturer's instructions. Briefly, post-transfected cells were trypsinized, and 1×10^5 cells were seeded into the upper chamber with serum-free opti-MEM media. The lower chamber was filled with 800 μL medium containing 10% FBS as a chemoattractant. After incubation for 24 h, cells on the lower side of the filter were fixed in 3.8% formaldehyde for 20 min and stained with 0.1% crystal violet solution. The numbers of moving cells on representative sections were counted using an inverted microscope (Olympus IX71) at 10 × magnification. Five fields were counted per filter in each group; the number of invaded cells for each experimental sample represented the average of triplicate wells repeated on three occasions.

For the invasion assay, the upper chamber was coated with extracellular matrix (BD Biosciences, Bedford, MA, USA), a soluble basement membrane matrix. The rest of the assay was performed as for the migration assay.

Detection of glucose uptake and lactate production. For detection of glucose and lactate concentration, DLD-1 and HT-29 cells (1×10^5) were seeded in six-well plates. After transfection, the culture medium was replaced by FBS-free RPMI1640. After 24 h, the supernatant of the culture medium was collected for measurement of glucose and the cell lysate was collected for measurement of lactate concentrations. The levels of glucose were determined using a Glucose Assay Kit (Sigma-Aldrich St.Louis, MO, USA) and the levels of lactate were determined using a Lactate Assay Kit (BioVision, Milpitas, CA, USA) under a microplate reader according to their respective manufacturer's protocols. At the same time, the number of cells in each well was counted. Glucose consumption and lactate production were normalized to cell number.

Statistical analyses. The association between LCN2 expression level and clinicopathologic factors in human specimens were analyzed using the χ^2-test or, when appropriate, the Fisher's exact test.

Data analysis for *in vitro* experiments, Student's *t*-test (for differences between two groups) or one-way ANOVA (for differences between multiple groups) were used. The data are presented as the mean ± SD of at least three independent experiments. All the data were entered into Microsoft Excel 5.0, and Graphpad Prism 5.0 was used. A probability (*P*) value less than 0.05 was considered statistically significant.

Results

LCN2 expression in human colorectal cancer specimens. To examine LCN2 expression patterns in patients with CRC, LCN2 protein and mRNA levels were analyzed using human CRC tissues and paired normal tissues. First, we analyzed the level of LCN2 protein in 36 paired tissues by western blotting and found that LCN2 expression in CRC was significantly higher than in the matched normal tissues ($P < 0.01$, Fig. 1a, Fig. S1). However, we did not observe the association between LCN2 protein level and T stage in CRC tissue. We also analyzed LCN2 expression in 80 frozen CRC tissues and paired normal colon tissues using RTQ-PCR. The mRNA level of LCN2 in CRC tissues was significantly higher than in paired normal tissues ($P < 0.01$, Fig. 1b).

Next, we statistically evaluated whether there were any correlations between LCN2 expression in CRC and clinicopathological variables. As shown in Table 1, the LCN2 expression level was not associated with age, gender, degree of differentiation or distant metastasis. Interestingly, LCN2 expression was negatively correlated with T stage and AJCC classification. Of the 80 patients with CRC, a high level of LCN2 was detected in 67.5% (27/80) and 32.5% (13/80) of patients classified as T1/T2 stage and T3/T4 stage, respectively. In AJCC classification, 62.0% (31/50) and 30% (9/30) of patients with grade I/II and III/IV showed a high level of LCN2, respectively, in each case. Based on these results, LCN2 levels were negatively associated with stage or grade of CRC in patients. Furthermore, a negative correlation was clearly indicated between LCN2 expression and lymph node metastasis. A high level of LCN2 was also observed in 59.62% (52/31) and 32.14% (9/28) of patients with lymph node metastasis and without metastasis, respectively. Taken together, these results suggest that LCN2 is predominantly involved in the early stages of tumorigenesis, and is negatively correlated with advanced stage and metastasis in CRC.

Increased proliferation with an epithelial–mesenchymal transition phenotype following LCN2 silencing. To select CRC cell lines suitable for LCN2 silencing, we examined expression levels of LCN2 in five CRC cell lines (HT-29, DLD-1 SW480, HCT116 and SW620) with western blotting and RT-PCR. As shown in Figure 2a, the protein level of LCN2 is much higher in HT-29 and DLD-1 cells, compared to SW480, HCT116 and SW620 cells. RT-PCR results were consistent with western blotting results. To further elucidate the biological function of LCN2 in CRC cells, we used a siRNA approach to inhibit endogenous LCN2 expression in the HT-29 and DLD-1 cell lines. Subsequent western blotting and RT-PCR showed a significant reduction in LCN2 expression (Fig. 2a) in these cell

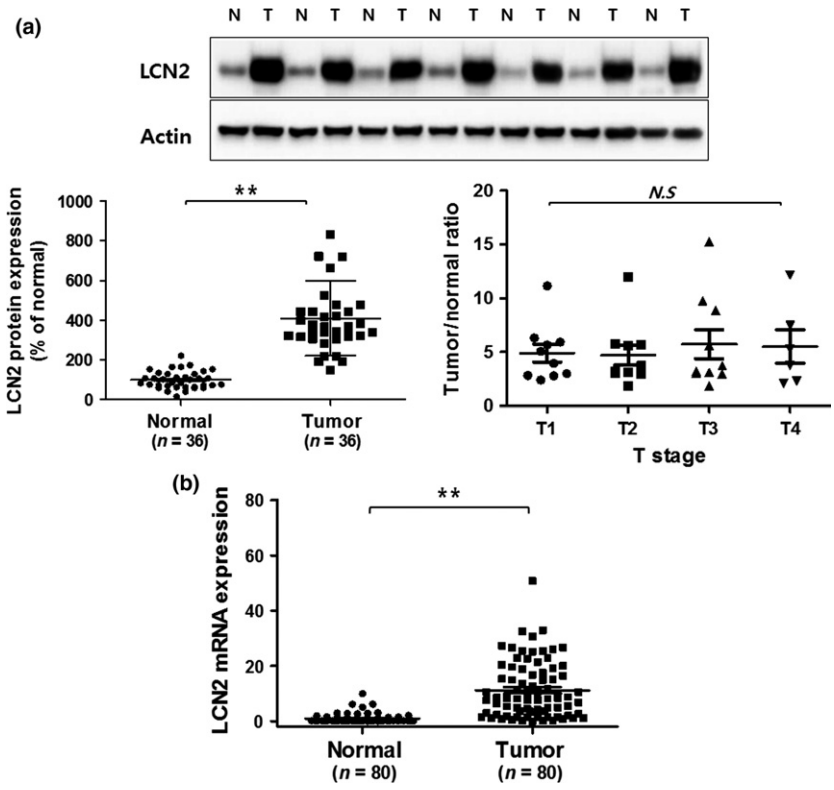

Fig. 1. LCN2 is upregulated in colorectal cancer (CRC) clinical specimens. (a) LCN2 protein levels were determined by western blotting in 36 CRC tissues compared with paired corresponding normal tissue, and the tumor-to-normal ratio was analyzed according to the pathological classification stage (T stage). (b) LCN2 mRNA levels were determined by real-time PCR in 80 CRC tissues compared with paired corresponding normal tissues. The data represent the mean ± SE of three independent experiments $^{**}P < 0.05$ *versus* normal. N.S., no significance.

lines, suggesting that LCN2 siRNA successfully downregulated LCN2 expression in HT-29 and DLD-1 cells.

To determine whether human LCN2 is involved in tumor cell proliferation or tumorigenicity in CRC cells, we performed MTT and colony formation assays. As shown in Figure 2c, the proliferation rate of DLD-1 and HT-29 cells silencing LCN2 was significantly higher than that of scrambled siRNA transfected cells. After transfection for 14 days, colony formation results were captured. We observed increased colonies in LCN2 knockdown DLD-1 and HT-29 cells compared to scrambled siRNA transfected cells (Fig. 2d). Moreover, LCN2 knockdown cells showed greater isolation and a more spindle-like morphology; these characteristics are typically associated with EMT. These data indicate that LCN2 negatively promoted proliferation with an EMT phenotype.

Association between endogenous level of LCN2 and epithelial–mesenchymal transition ability. LCN2 expression level was identified in five CRC cells (Fig. 2a). DLD-1 and HT-29 were employed as representatives of LCN2 highly expressed cells and SW620 was employed as a representative of LCN2 low expressed cells. After scrapping, the cells were incubated in serum-free media to minimize the growth factor effect in serum. As shown in Figure 3a, the expression level of endogenous LCN2 was negatively correlated with cell motility. LCN2 low expressed cells notably displayed increased cell migration compared with LCN2 highly expressed cells.

Wound healing assay results indicate that LCN2 may downregulated during the EMT process. Accordingly, we performed western blotting to demonstrate the association of endogenous expression level of LCN2 and EMT (Fig. 2b). The expression level of epithelial marker, E-cadherin was higher in LCN2 high expressed cells compared to low expressed cells. In contrast, we observed marked upregulation of mesenchymal markers (Vimentin, MMP2) in LCN2 low expressed cells (SW620). These results support a potential role of LCN2 in CRC progression and metastasis by reducing EMT ability.

Induced cell migration and invasion by LCN2 silencing through the epithelial–mesenchymal transition pathway. To verify that LCN2 regulated the EMT pathway, cell mobility was evaluated in wound healing and transwell migration and invasion assays. As shown in Figure 4a, more cells transfected with LCN2 siRNA migrated to the wounded area compared to scrambled siRNA. Quantification of the wounded area at 72 h revealed that LCN2 knockdown HT-29 cells increased their migratory rate to 49.9 ± 6.13%, which was significantly higher than for scrambled siRNA cells (26.6 ± 4.53%). The results from DLD-1 cells were consistent with those from HT-29 cells (at 72 h, LCN2 siRNA group; 68.06 ± 7.88%, scrambled siRNA group; 41.51 ± 6.13%). Trans-well migration and invasion assays also showed a significant increase following LCN2 silencing in CRC cell lines. In DLD-1 cells, LCN2 knockdown increased migration and invasion by 5.6 and 9.8-fold, respectively, compared to scrambled siRNA. The migration and invasion of HT-29 cells transfected with LCN2 siRNA was increased 3.6-fold and 4.2-fold, respectively (Fig. 4b,c).

To elucidate the mechanism by which LCN2 knockdown induced EMT, we examined the protein levels of EMT-associated markers in DLD-1 and HT-29 cells. The expression levels of mesenchymal markers, such as β-catenin, Vimentin, Slug Snail, MMP2, ICAM-I and Twist, were analyzed with western blotting. All mesenchymal markers were significantly increased by transfection with LCN2 siRNA, indicating that LCN2 regulated CRC cell migration and invasion through the EMT pathway (Fig. 4d).

Energy metabolism induced by LCN2 silencing. Energy metabolism, particularly increased glycolysis, leads cancer cells to undergo the EMT process, thus promoting cancer progression and metastasis.[16,17] To determine the alteration of energy

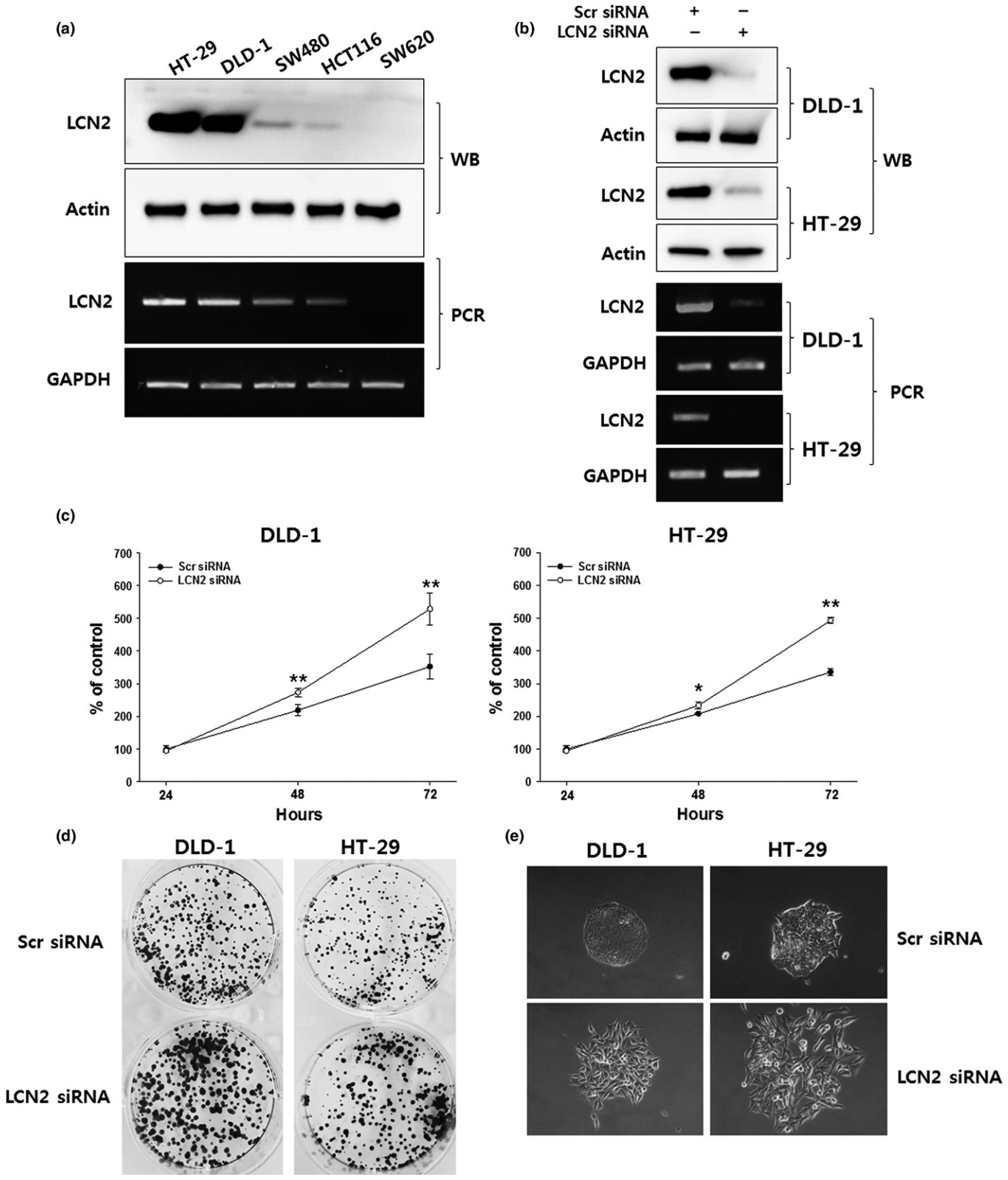

Fig. 2. Knockdown of LCN2 promoted cell proliferation and the epithelial–mesenchymal transition (EMT) phenotype. (a) Endogenous LCN2 levels were detected using western blot and RT-PCR analysis in human colorectal cancer (CRC) cell lines. (b) LCN2 expression was silenced by LCN2 siRNA in DLD-1 and HT-29 cells. Knockdown efficiency was analyzed with western blotting and RT-PCR. (c) Cell viability of DLD-1 and HT-29 cells transfected with scrambled or LCN2 siRNA was measured by MTT assay at 24-h intervals up to 72 h. Data represent the mean ± SE from triplicate samples and are representative of three independent experiments. $^{*}P < 0.01$ and $^{**}P < 0.05$. (d) Colony formation after transfection with scrambled or LCN2 siRNA was captured in DLD-1 and HT-29 cells. (e) Morphological changes of DLD-1 and HT-29 cells transfected with scrambled or LCN2 siRNA were captured using a phase contrast microscope (20 ×).

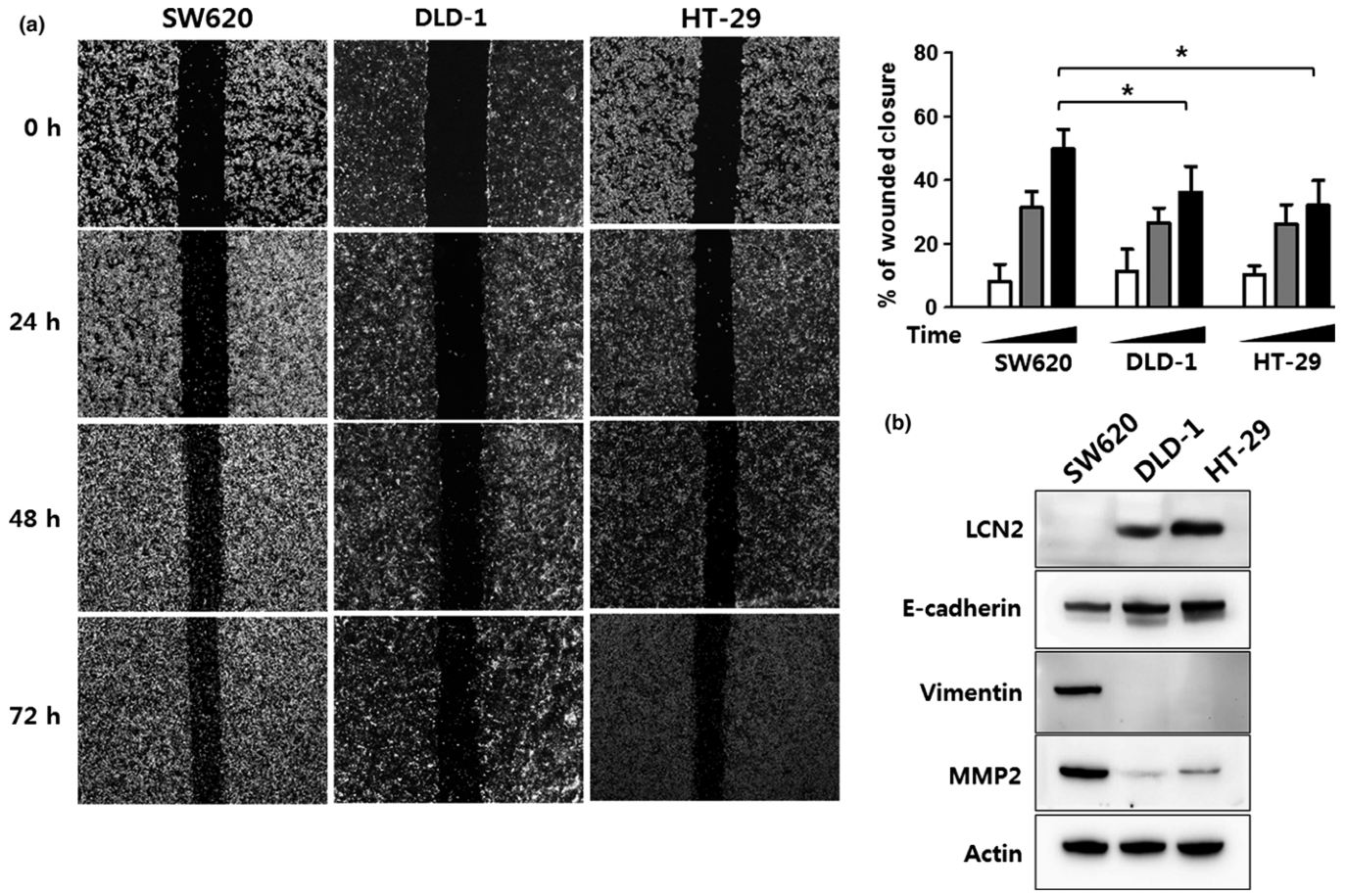

Fig. 3. Endogenous level of LCN2 affect to epithelial–mesenchymal transition (EMT) progress of colorectal cancer (CRC) cells. (a) Images of cell migration of different cell lines. Using the wound healing assay for the three cancer cell lines, wounds were compared at 0, 24, 48 and 72 h after scratching. Quantitative measurements of wound closure ability are shown. (b) Total cell lysates of SW620, DLD-1 and HT-29 cells were prepared for western blotting. The protein levels of EMT markers (E-cadherin, Vimentin and MMP2) were analyzed with the appropriate antibodies. Actin was used as a loading control.

metabolism through the EMT following LCN2 silencing, we first evaluated the glucose concentration in DLD-1 and HT-29 cells. As expected, glucose concentration was dramatically increased by transfection with LCN2 siRNA (Fig. 5a left). Moreover, conversion of pyruvate to lactate is a unique metabolic phenotype during tumorigenesis and cancer progression.[18] Therefore, the concentration of lactate was measured in cell lysates and showed significant elevation following LCN2 silencing in DLD-1 cells (Fig. 5a right). We also observed a slight increase in lactate production in LCN2 siRNA-transfected HT-29 cells, but the difference was not statistically significant.

To understand the molecular mechanisms of LCN2-regulated energy metabolism, we examined the expression level of mRNA and protein, which is critically linked to energy metabolism. We confirmed the expression level of glucose transporters (GLUT), hexokinases (HK), lactate dehydrogenases (LDH) and monocarboxylate transporters (MCT) with RT-PCR and western blotting (Fig. 5b,c). First, we examined the level of GLUT, which initiate the exportation of glucose through the plasma membrane. As shown in the second and third panel of Figure 5b, protein levels of GLUT1 and 3 were considerably higher in LCN2 siRNA-transfected cells compared to the scrambled siRNA group. However, LCN2 silencing did not regulate the expression level of GLUT12 (data not shown). Among HX, HK2 catalyzes the essentially irreversible first step of glycolysis by phosphorylating glucose to glucose-6-phosphate.[19] As expected, HK2 levels were much higher in LCN2 silencing cells than in scrambled siRNA-transfected cells. Among the glycolytic enzymes, LDH are necessary to maintain high glycolysis rates in the early stages of glucose metabolism.[20] Protein levels of LDHA and LDHB were also upregulated by LCN2 silencing in both cell lines. Lactate is transported across the cell membrane by MCT, so we confirmed alteration of MCT protein levels with LCN2 silencing. The protein level of MCT4 was elevated in LCN2 siRNA-transfected cells, but MCT2 was not detected (data not shown). In the same manner as for western blotting, we observed upregulation of the mRNA level of GLUT1, HK2, LDHA and MCT4 in LCN2 siRNA-transfected cells compared to the scrambled siRNA group. These findings indicated that LCN2 may be involved in EMT-associated energy metabolism.

Discussion

Colorectal cancer is the third leading cause of cancer in both men and women and accounts for 10% of all new cancer cases and cancer deaths. Upon diagnosis, 19% of CRC cases are metastatic, and while the overall 5-year survival rate for patients with CRC is 63%, the rate drops to 10% or less in

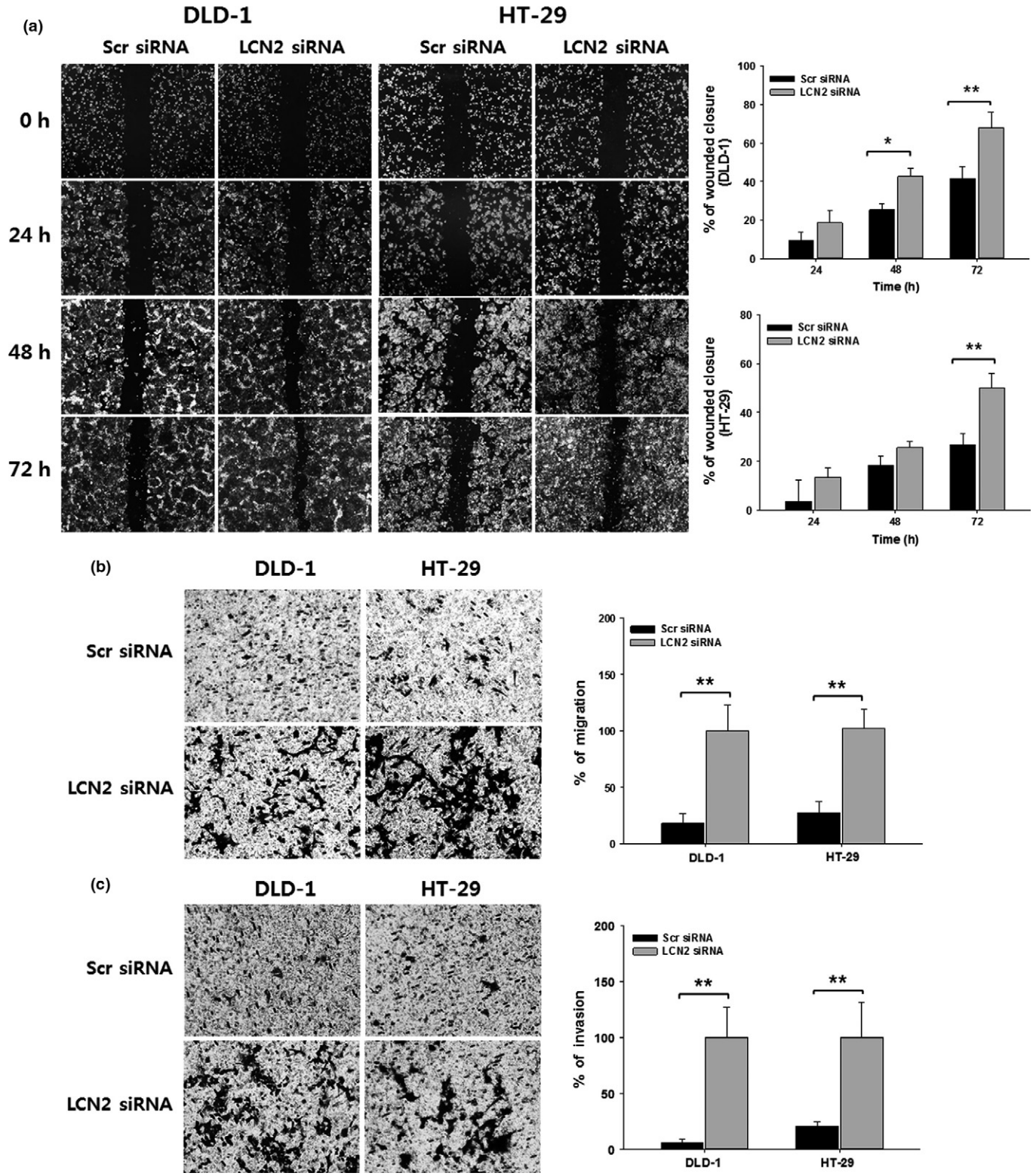

Fig. 4. LCN2 knockdown induced cell migration and invasion through the epithelial–mesenchymal transition (EMT) pathway. (a) Cell mobility as detected by a wound healing assay. After transfection, DLD-1 and HT-29 cells were scratched using a yellow tip and wound closure was evaluated at 0, 24, 48 and 72 h. Quantitative measurements of wound closure ability are shown. (b) Cell migration assay was performed after knockdown of LCN2 in DLD-1 and HT-29 cells using Transwell chambers. The number of cells traversing the filter to the lower chamber was counted to determine migration activity. (c) Cell invasion assays were performed after LCN2 knockdown in DLD-1 and HT-29 cells using Matrigel-coated transwell chambers. The number of cells traversing the filter to the lower chamber was counted to determine invasion activity. The histograms represent the mean ± SE from triplicate samples and are representative of three independent experiments. $*P < 0.01$ and $**P < 0.05$. (d) Total cell lysates of DLD-1 and HT-29 cells were prepared after transfection with scrambled or LCN2 siRNA for 72 h. The levels of LCN2, Vimentin, β-catenin, Slug, Snail, MMP2, ICAM-I, Twist and actin were then assessed with western blotting with the appropriate antibodies. Actin was used as a loading control.

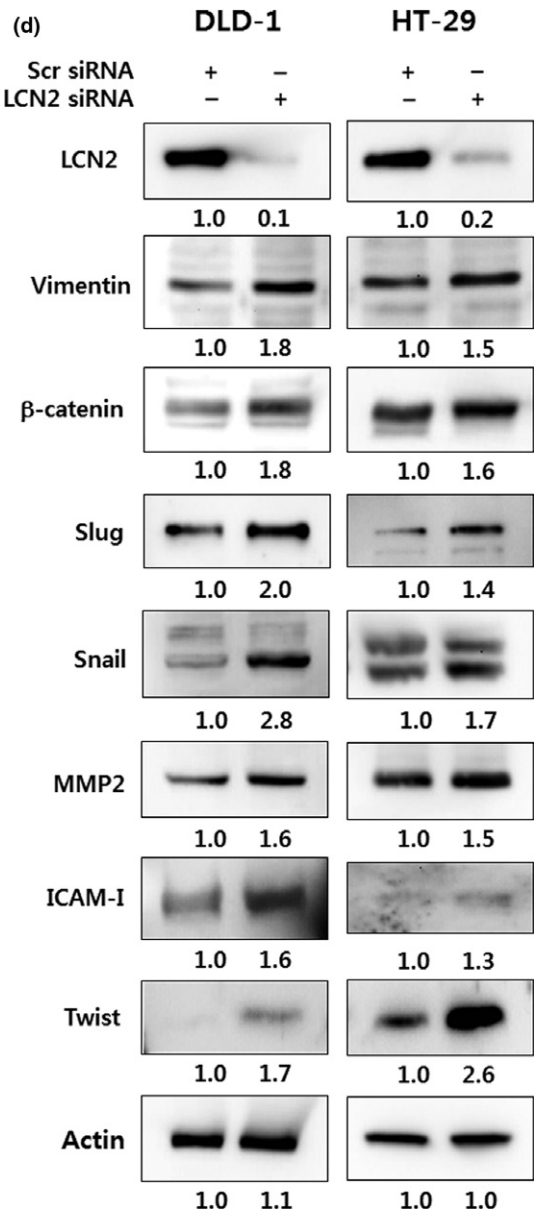

Fig. 4. Continued.

patients with metastatic CRC.[21] Although metastasis is the major factor for poor prognosis in CRC, the underlying processes remain elusive. Our findings using human CRC specimens reveal that LCN2 expression is upregulated in CRC tissues compared to normal tissues, and is inversely correlated with tumor stage and metastasis.

Previous studies have demonstrated the clinical impact of LCN2 on CRC progression and metastasis. In 1996, Nielsen *et al.* detected higher levels of LNC2 mRNA in epithelial cells in neoplastic lesions using *in situ* hybridization and showed a negative correlation with LCN2 expression and lymph node metastasis,[22] although they only used 11 CRC specimens. Among the CRC specimens, only 3 had lymph node metastasis, and the authors did not suggest that there were statistically significant differences between non-metastatic and metastatic tissues. In contrast, Marti *et al.* (2010 and 2013) report that LCN2 level is positively associated with neoplastic tissue volume, characteristics of neoplastic invasion, liver metastasis and recurrence, showing a prognostic utility in the serum of metastatic CRC patients.[23,24] Inversely, Fung *et al.* (2013) demonstrate that the LCN2 level in serum is not a suitable biomarker for CRC, finding no correlation with disease stage (Duke's stage and T stage). Therefore, the present study is the first to demonstrate the possibility of using LCN2 as a diagnostic and prognostic marker by classifying expression pattern by CRC stage and metastasis using quantitative analyses with sufficient specimens.

LCN2 has been implicated in cell proliferation because its expression is associated with a variety of proliferative cells.[25] However, reports of an associated functional role in tumorigenesis through regulation of cancer cell proliferation have varied. Several studies have shown that LCN2 expression promotes tumor growth and progression by inducing proliferation in breast cancer, prostate cancer and pancreatic ductal

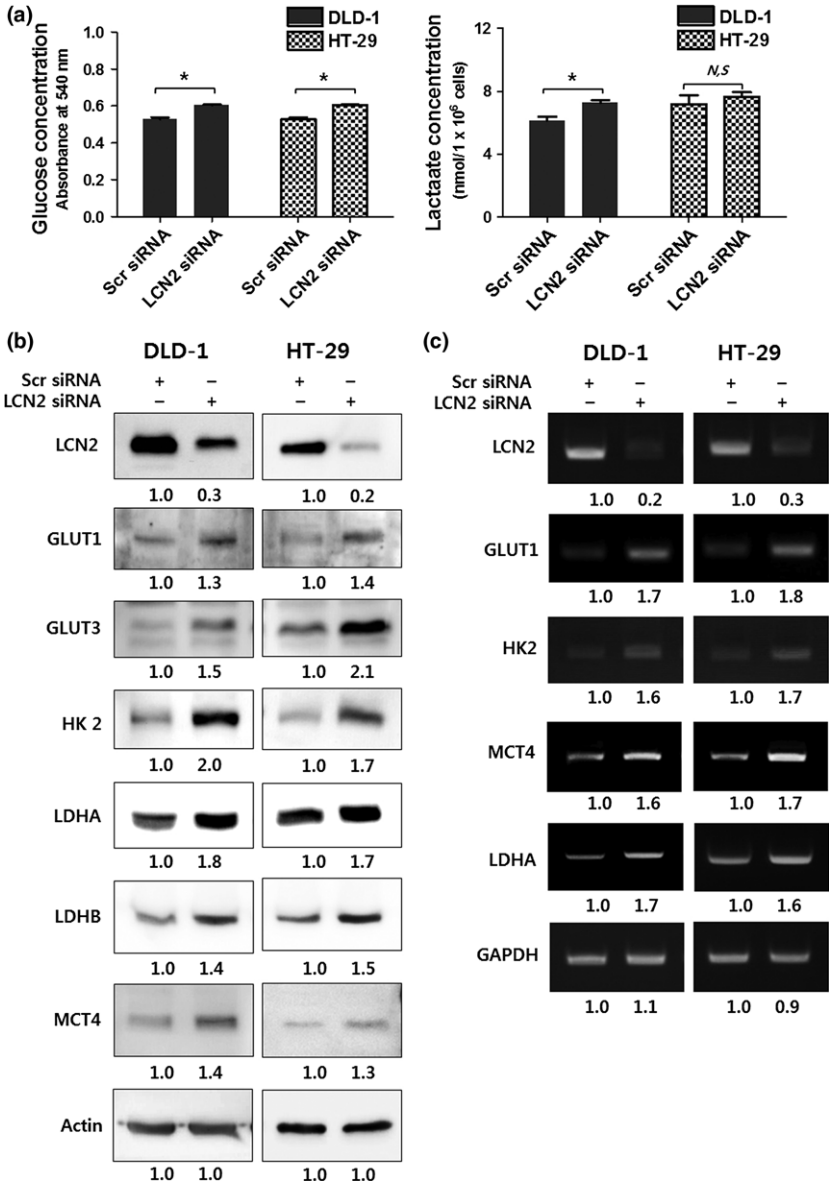

Fig. 5. Knockdown of LCN2-enhanced glucose metabolism in colorectal cancer (CRC) cells. (a) Glucose consumption and lactate production were measured in DLD-1 and HT-29 cells transfected with scrambled or LCN2 siRNA using a microplate reader. Histograms represent the mean ± SE from triplicate samples and are representative of three independent experiments. $^*P < 0.01$. (b) Total cell lysates of DLD-1 and HT-29 cells were prepared after transfection with scrambled or LCN2 siRNA for 72 h. The protein levels of glycolytic key enzymes (GLUT1, GLUT3, HK2, LDHA, LDHB and MCT4) were then assessed using western blotting with the appropriate antibodies. Actin was used as a loading control. (c) After transfection with scrambled or LCN2 siRNA for 48 h, RT-PCR was performed to determine the mRNA levels of LCN2, GLUT1, HK2, LDHA and MCT4. The mRNA level of GAPDH was used as a negative control. N.S., no significance.

adenocarcinoma.[7,26,27] In contrast, LCN2 expression and cell proliferation have a negative association with hepatocellular carcinoma cells.[8,28] In CRC cells, the correlation between LCN2 expression and cell proliferation has not been fully elucidated. In 2006, Lee *et al.* reported that LCN2 expression was upregulated in metastatic CRC cells but did not appear to induce CRC cell proliferation.[29] This finding is not consistent with our results. However, we observed that LCN2 silencing induced cell proliferation accompanied by high glycolysis. Accelerated glycolysis meets the demands of rapid cell proliferation and offers a favorable microenvironment for tumor progression.[30] Moreover, among the glucose translocation carriers, GLUT1 expression has been correlated with malignant features and poor prognosis in various cancers, including prostate, thyroid, CRC, liver and breast cancer.[31–35] Therefore, this study provides clear evidence that LCN2 negatively modulated CRC cell proliferation with reprogramming of energy metabolism, based on glucose consumption, lactate production and the expression level of key enzymes in glucose metabolism.

Induction of the EMT is thought to play a critical role in tumor aggressiveness and metastasis. The regulation of LCN2 in EMT and cancer metastasis has been associated in various cancer types. Of course, the functional role of LCN2 in EMT has also been differently reported by cancer type. In particular, conflicting results have been observed for the functional role of LCN2 in CRC cell migration/invasion and CRC metastasis. In 2006, Lee *et al.* showed that LCN2 overexpression decreased EGF-induced invasion and experimental liver metastasis in murine models.[29] However, in 2009, Hu *et al.* claimed that cells overexpressing LCN2 showed more invasive characteristics, including lower E-cadherin-mediated cell-to-cell adhesion.[36] The potential reasons for the opposing findings were not discussed adequately by the authors. Our findings are in agreement with Lee *et al.*, as we confirmed that LCN2 negatively regulated EMT in CRC cells as, shown through changes in cell morphology, migration/invasion and the expression level of mesenchymal markers (Vimentin, β-catenin, Slug, Snail, MMP2 ICAM-1 and Twist). Although pre-existing reports suggest the possibility that LCN2

participated in CRC cell EMT, the detailed underlying molecular mechanisms have not been identified. Therefore, our observations are the first evidence for the precise mechanism of LCN2 in CRC cell EMT. Furthermore, the negative correlation between LCN2 and CRC stage/metastasis observed in human CRC specimens can be explained by the results of *in vitro* experiments showing that LCN2 silencing promoted EMT processing in CRC cells.

High glycolysis is a common feature of cancer due to its high energy demand, defined as the "Warburg effect."[37] Glycolysis also plays critical roles during the EMT process, and provides metabolic advantages for EMT cells.[38] GLUT, initiators of glycolytic pathways, are highly likely to be involved in the EMT process. In particular, GLUT1 shows a strong association with invasive ability and contributes to tumor aggressiveness.[39,40] GLUT3 expression also influences the EMT process, because enhanced expression of GLUT3 is induced by ectopic expression of Zinc finger E-box binding homeobox 1 (ZEB1) or Snail in non-small lung cancer. At the same time, some EMT markers have been reported to be glycolytic switches for energy metabolism. Snail has been found to promote glucose metabolism by repressing fructose-1,6-biphosphatase in breast and prostate cancer.[16,41] Moreover, Yang L *et al.* report that Twist promoted glucose metabolism in Twist-positive breast cancer cells through activation of the β1-integrin/FAK/PI3K/AKT/mTOR pathway.[42] However, a correlation between energy metabolism and EMT/metastasis in CRC has seldom been reported. Because of the role of LCN2 in CRC cell proliferation and EMT, we questioned whether the impact was the result of energy metabolism reprogramming. As expected, knockdown of LCN2 increased glucose uptake, lactate production and expression of key enzymes underlying the Warburg effect. Therefore, the altered metabolism induced by LCN2 may be required for CRC cell growth, EMT and, by extension, CRC metastasis.

To our knowledge, this study is the first report to clarify the clinical and prognostic significance of LCN2 in CRC by classifying expression patterns according to CRC stage and metastasis. Using *in vitro* experiments, we observed that LCN2 negatively regulated cell proliferation and the EMT process through high glycolysis. Accordingly, we demonstrated a new role for LCN2 as a glycolytic switch in CRC cells. These findings provide new insight into the role of LCN2 and the underlying molecular mechanisms in CRC progression, suggesting LCN2 as a potential diagnostic marker and therapeutic target in CRC.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Western blot analysis of LCN2 protein isolated from paired normal (N) and colorectal cancer (CRC) tumor (T) tissue specimens in 36 colorectal cancer (CRC) patients.