

## Targeting L1 Cell Adhesion Molecule Using Lentivirus-Mediated Short Hairpin RNA Interference Reverses Aggressiveness of Oral Squamous Cell Carcinoma

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**Abstract:** The L1 cell adhesion molecule (L1CAM) has been implicated in tumor progression of many types of cancers, but its role in oral squamous cell carcinoma (OSCC) has not been investigated. In the present study, we demonstrated overexpression of L1CAM in OSCC cells, but not in normal keratinocytes, using both clinical specimens and cell lines. This overexpression demonstrated a strong correlation with less differentiation and a higher invasion potential of cancer cells, supporting the significance of L1CAM in human OSCC tumor progression. Targeting L1CAM gene expression in SCC4 cells overexpressing L1CAM using a lentivirus-mediated small hairpin RNA (shRNA) led to a significant reduction in cell proliferation *in vitro* via retardation of cell cycle at the G1 phase. In addition, shRNA knockdown of L1CAM strongly attenuated the migration and invasion of SCC4 cells, and this was also observed to parallel increased E-cadherin levels and decreased levels of vimentin, fibronectin, and Snail-family transcription factors, indicating that L1CAM expression was related to the epithelial-mesenchymal transition. Furthermore, while mice receiving orthotopically placed control SCC4 cells died within 40 days due to invasive tumor growth and regional lymph node metastasis, prolonged animal survival and complete suppression of tumor progression was observed in mice implanted with L1CAM-deficient SCC4 cells, further substantiating the fundamental importance of L1CAM in OSCC pathophysiology. Our findings suggested that L1CAM is a critical mediator of tumor progression in OSCC, and targeting L1CAM using lentivirus-mediated shRNA may be a useful molecular pharmaceutical approach for the treatment of advanced OSCC.

**Keywords:** L1 cell adhesion molecule; oral squamous cell carcinoma; epithelial-mesenchymal transition; RNA interference; targeted gene therapy

### Introduction

Oral cancer consistently ranks as one of the ten most frequently diagnosed cancers in the world.<sup>1</sup> Approximately

300,000 new cases of oral cancer occur each year, and nearly two-thirds of patients with this cancer will die of their disease.<sup>2,3</sup> The vast majority of malignant neoplasms in the oral cavity are squamous cell carcinomas, which account for 3–5% of all malignancies and tends to be highly invasive and spread rapidly.<sup>4</sup> Despite multidisciplinary treatment with surgery, chemotherapy, and radiation, more than 50% of patients with oral squamous cell carcinoma (OSCC) receiving

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therapy will eventually develop recurrent and metastatic disease, which carries a particularly poor prognosis.<sup>5</sup> Thus, pursuing novel therapeutic alternatives to standard therapy to improve the survival of patients with advanced OSCC is medically urgent. Molecular therapy that targets molecules and proteins selectively expressed by cancer cells is thought to offer a high therapeutic index for cancers.<sup>6</sup> Characterization of the specific molecular alterations associated with OSCC cell growth, invasion, and metastasis can advance our understanding of the molecular mechanisms underlying cancer induction and progression as well provide a basis for the development of new and effective therapeutic treatments for cancer patients.

It has become clear that adhesion molecules and adhesion processes are fundamentally involved, at various levels, in all steps of tumor progression, including detachment of tumor cells from the primary site, intravasation into the bloodstream, extravasation into distant target organs, and formation of secondary lesions.<sup>7</sup> The aberrant expression of adhesion-related molecules, such as integrins<sup>8–11</sup> and cadherins,<sup>12–14</sup> has been reported in several types of human cancers, and this is associated with the invasive and metastatic potential

of cancer cells as well as a poor prognosis, making these molecules potential candidates for antineoplastic targeted therapies.

The cell adhesion molecule L1 (L1CAM) has been recently identified as a key mediator of tumor progression due to its upregulation in certain human tumors.<sup>15</sup> Structurally, L1CAM belongs to the immunoglobulin superfamily, which is characterized by an extracellular region of multiple immunoglobulin-like domains and fibronectin type III repeats followed by a highly conserved cytoplasmic domain.<sup>16</sup> L1CAM was first described as a neural cell adhesion molecule based on a restricted distribution in the nervous system,<sup>17</sup> where it is involved in the control of neurite outgrowth, adhesion, fasciculation, migration, myelination, and axon guidance.<sup>18</sup> L1CAM promotes these cellular activities by interacting via its extracellular domain with a diverse group of cell adhesion molecules, extracellular matrix molecules, and signaling receptors.<sup>19</sup> In addition to its cell surface localization, the extracellular domain of L1CAM can be released from the cell surface by metalloproteinases, such as plasmin and ADAM10.<sup>20,21</sup> Non-neuronal expression of L1CAM has been observed in blood cells,<sup>22</sup> endothelial cells,

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epithelial cells, and reticular fibroblasts.<sup>23–25</sup> Recent studies have also demonstrated aberrant expression of L1CAM in several different types of human cancers, including melanoma, glioma, renal cancer, lung cancer, colon cancer, and ovarian cancer.<sup>26–30</sup> For some malignancies, the level L1CAM is also a significant indicator of subsequent metastasis and poor prognosis,<sup>26,31</sup> strongly suggesting a tumor-promoting function of L1CAM in advanced stages of these cancers. However, expression of L1CAM in OSCC has not been examined, and its role in oral cancer progression remains elusive.

In this study, we described for the first time the significant association of L1CAM protein expression with a more aggressive phenotype of OSCC in both clinical specimens and cell lines. L1CAM-targeting shRNA was effective at inhibiting OSCC cell growth with concomitant cell-cycle retardation at the G1 phase, which led to a nearly complete suppression of the tumorigenicity in an orthotopic OSCC animal model. Moreover, modulation of L1CAM expression in OSCC cell lines also caused changes in cell motility and gene expression that favored cancer cells undergoing epithelial–mesenchymal transition. Our results demonstrated the importance of L1CAM in OSCC tumor progression and

the therapeutic potential of L1CAM-targeting shRNAs for treatment of advanced oral cancer.

## Materials and Methods

**Cell Cultures.** Human oral squamous cell carcinoma cell lines SAS, SCC4, SCC9, and SCC25<sup>32</sup> were a gift from Dr. Kuo-Wei Chang at the Institute of Oral Biology, National Yang-Ming University, Taiwan. SAS cells were grown in DMEM medium with high glucose. SCC4, SCC9, and SCC25 cells were maintained in DMEM/F12 (1:1) medium (Invitrogen, Carlsbad, CA, USA) supplemented with 1% nonessential amino acids and 200 ng/mL hydrocortisone. All media described above were supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin–streptomycin (Hyclone, Logan, UT, USA). Primary human oral keratinocytes (HOKs) were purchased from ScienCell Research Laboratories (San Diego, CA, USA) and maintained in oral keratinocyte medium (ScienCell, San Diego, CA, USA) according to the manufacturer's instructions.

**Vectors and RNA Interference.** RNA interference (RNAi) vectors, pLKO.1-shL1CAM (sh-L1 #1, TRCN0000063917, target sequence GCTAACCTGAAGGTTAAAGAT; sh-L1 #2, TRCN0000063914, target sequence, GCCAATGCCTA-CATCTACGTT) and a mammalian nontargeting shRNA control pLKO.1-shGFP (sh-NT, TRCN0000072178, target sequence, CAACAGCCACAACGTCTATAT) were obtained from the National RNAi Core Facility (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan). These RNAi vectors were constructed by inserting annealed oligonucleotides containing the shRNA sequence into *EcoRI* and *AgeI* sites from the downstream of U6 promoter in pLKO.1 vector. Recombinant lentivirus carrying a short-hairpin (sh) RNA were produced by cotransfecting 293FT cells with a mixture of plasmid DNA consisting of pCMV- $\psi$ R8.91(Gag/Pol/Rev), pMD.G (VSV-G envelope), and pLKO.1-shRNA vectors using TurboFect reagent (Fermentas, Glen Burnie, MA, USA) according to the manufacturer's instructions. After 2 days of transfection, virus-containing culture supernatant was collected and used to infect SCC4 cells in combination with 8  $\mu$ g/mL Polybrene (Sigma-Aldrich, St. Louis, MO, USA). Stable cell lines were selected with 2.5  $\mu$ g/mL puromycin (Calbiochem, La Jolla, CA, USA) for one week. For further establishing luciferase-expressing SCC4 cells that were pretransduced with shRNA, luciferase cDNA was removed from a pGL3-basic plasmid (Promega, Madison, WI, USA) and then cloned into the S2 bicistronic retroviral vector,<sup>33</sup> in which luciferase was driven by a retroviral long terminal repeat promoter. Retroviral produc-

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tion and infection were performed as described previously.<sup>34</sup> The resultant stable cell lines were selected with 0.8 mg/mL G418 (Calbiochem, La Jolla, CA, USA) for 10 days.

**Western Blot Analysis.** Protein extracts from cell lines were analyzed on SDS–polyacrylamide gels (15  $\mu$ g per lane) and transferred to Hybond ECL nitrocellulose membranes (GE Healthcare Life Science, Piscataway, NJ, USA). Blots were probed with 1:1000 diluted monoclonal mouse anti-human L1CAM (NeoMarker, Fremont, CA, USA), anti-E-cadherin (Cell Signaling Technology, Danvers, MA, USA), anti-vimentin (NeoMarker, Fremont, CA, USA) and anti-fibronectin (BD Biosciences, Bedford, MA, USA) according to the manufacturers' instructions. For loading control, blots were probed with an anti-EF1- $\alpha$  monoclonal antibody (1:10,000; R&D Systems, Minneapolis, MN, USA). After incubation with an HRP-conjugated secondary antibody (1:5000; GE Healthcare Life Sciences, Piscataway, NJ, USA), chemiluminescent signals were detected using an ECL Plus kit and exposed to Hyperfilm ECL (GE Healthcare Life Science, Piscataway, NJ, USA). Protein band quantification was carried out using ImageJ software.

**Cell Proliferation Assay.** Cells were seeded at a density of  $5 \times 10^3$  cells/well in a 96-well plate. Viable cells were measured using WST-1 reagent (Roche Applied Science, Mannheim, Germany) at each time point, followed by an additional incubation for 2 h at 37 °C. Absorbance was recorded at 450 nm using a Synergy 2 Multi-Mode microplate reader (BioTek, Winooski, VT, USA). All experiments were performed in triplicate.

**Boyden Chamber Invasion Assays.** The invasion of cancer cells was assessed using 24-well Transwell plates. Briefly,  $2 \times 10^5$  cells in 0.5% FBS-containing media were added to the upper chamber containing 8  $\mu$ m pore polycarbonate coated with 1 mg/mL of Matrigel; the lower chamber was filled with growth medium. After a 16 h incubation, the upper surface of the membrane was scrubbed with a cotton-tipped swab. The invading cells on the lower surface of the membrane were fixed and stained with 0.5% crystal violet. Random fields (5/membrane) were photographed at 40 $\times$  magnification and then quantified by measuring absorbance of dye extracts at 570 nm.

**Wound Healing Assay.** shRNA-expressing SCC4 cells resuspended in medium were seeded into 24-well plates. When cells reached 90% confluence, a single wound was created in the center of the cell monolayer by gentle removal of the attached cells with a sterile plastic pipet tip. The debris was removed by washing with serum free medium. Cells having migrated into the wounded area or those protruding from the border of the wound were visualized and photographed under a Zeiss Axioplan microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) with a 10 $\times$  objective at three preselected time points (0, 4, and 8 h). Each experiment was independently performed at least three times.

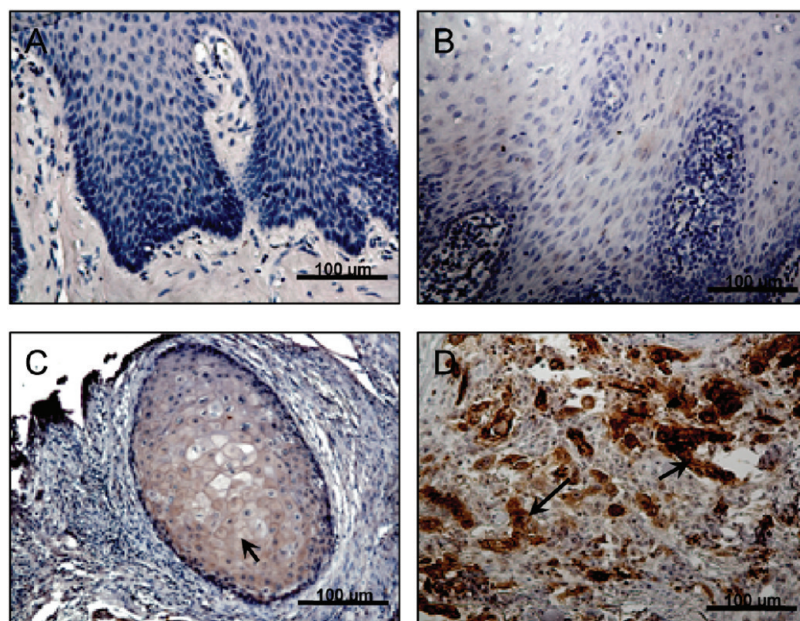
**Immunohistochemistry.** Archived paraffin-embedded oral cancer tissue specimens were collected with the approval of the Institutional Review Board. Written informed consent was obtained from all participants. Mouse tissues, including tongue and cervical lymph nodes, were collected at the end of the animal experiments (6 weeks after tumor implantation). Immunohistochemical analyses were performed as described previously<sup>35</sup> with the following modifications: Paraffin embedded sections were deparaffinized and rehydrated using xylene and decreasing concentrations of ethanol. Antigen retrieval was performed with a steamer by heating the slides in 10 mM sodium citrate (pH 6.0) for 10 min. Slides were incubated with mouse anti-human L1CAM monoclonal (1:40; Lab Vision, Fremont, CA, USA) and goat anti-firefly luciferase (1:10,000; Abcam, Cambridge, U.K.) antibodies at room temperature for 1 h, followed by incubation with HRP-polymer conjugate (Upstate, Lake Placid, NY, USA) under the same conditions. The chromogenic reaction was performed with diaminobenzidine (Sigma, St. Louis, MO, USA) for 5 min, and slides were counterstained with hematoxylin.

**Immunofluorescence Staining.** shRNA-expressing SCC4 cells grown on Nunc chamber slides (Thermo Fisher Scientific, Rockford, IL, USA) were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min. The slides were blocked with 2% bovine serum albumin and then incubated with rabbit anti-human E-cadherin antibodies (Cell Signaling Technology, Beverly, MA, USA) and rabbit anti-human vimentin antibodies (Lab Vision, Fremont, CA, USA) in a 1:100 dilution for overnight at 4 °C. The slides were washed and then incubated with Alexa Fluor 488 (for E-cadherin detection) and Alexa Fluor 546 (for vimentin detection) goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) in a 1:200 dilution for 1 h at room temperature. Slides were washed and mounted for confocal microscopy.

**RNA Extraction and Real-Time Quantitative PCR.** Total RNA was extracted from cell lines and first-strand cDNA was generated from 1  $\mu$ g of total RNA and random primers using Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in a total volume of 20  $\mu$ L. Quantitative RT-PCR was performed using a LightCycler 480 TaqMan Master kit with Universal ProbeLibrary probes (Roche Applied Science, Mannheim, Germany). The primers and probes were designed using a web-based Assay Design Center at [www.universalprobelibrary.com](http://www.universalprobelibrary.com), and sequences are shown in the Supporting Information (Table S1). The real-time PCR reaction was conducted according to the manufacturer's instructions, consisting of a denaturation step (10 min) and 55 cycles of amplification (95 °C for 10 s, 60 °C for 10 s, followed by single fluorescence acquisition at 72 °C for 10 s). The relative gene expression of a specific target in each group was represented as  $2^{-\Delta\text{CT}}$ ;  $\Delta\text{CT}$  was determined by subtracting

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**Figure 1.** Detection of L1CAM protein in OSCC patient tissues. Representative images (200 $\times$ ; scale bar =100  $\mu$ m) of immunohistochemical staining of L1CAM in OSCC tissues. (A) Normal oral epithelium exhibits negative L1CAM protein expression. (B) In squamous hyperplastic lesions, undetectable to very weak L1CAM immunoreactivity was present. (C) A well differentiated OSCC tumor stained positive with anti-L1CAM in the cytoplasm and at the cell surface of cancer cells, and (D) strong positive immunoreactivity for L1CAM was detected in poorly differentiated OSCC tumors. Arrows denote positive staining of L1CAM (C and D).

the average housekeeping gene (HSPCB) CT value from the average target gene value.

**Animals.** All animal work was performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Six-week-old male athymic nude (nu/nu) mice from BioLASCO Taiwan Co. (Taipei, Taiwan) were used for the experiments. To establish the orthotropic oral cancer model,  $2 \times 10^5$  luciferase-expressing cancer cells were resuspended in 20  $\mu$ L of PBS and directly injected submucosally into the anterior tongue of the mice, according to the published protocol.<sup>36</sup> Tumor growth and regional metastasis was noninvasively monitored using an IVIS bioluminescence imaging system (Caliper Life Sciences, Hopkinton, MA, USA) as described previously.<sup>37</sup>

**Statistical Analysis.** For the *in vitro* studies, all data are presented as the means  $\pm$  SD. Differences between groups were analyzed using the two-tailed, unpaired Student's *t* test. A *p* value of less than 0.05 was considered to be significant. In the mouse studies, Mann–Whitney rank-sum test was used for analysis.

## Results

**L1CAM Is Overexpressed in Cancerous Lesions of Patients with OSCC.** L1CAM expression has been detected in several types of cancers but has yet to be investigated in OSCC. To investigate whether L1CAM was involved in the progression of human OSCC, we first examined the expression levels of L1CAM in 25 oral tissues from patients with OSCC by immunohistochemical (IHC) staining (Figure 1). L1CAM expression was not observed in healthy oral epithelium (Figure 1A). Oral premalignant lesions, such as hyperparakeratosis and squamous hyperplasias (Figure 1B), had no or faint L1CAM immunoreactivity in almost all epithelium cells in the hyperplasia of stratum spinosum and were considered L1CAM-negative. In contrast, positive L1CAM immunoreactivity was seen in cancerous lesions of OSCC specimens. Compared to the low-grade (well differentiated) tumors (Figure 1C) that expressed L1CAM mainly in the cytoplasm and at the cell surface of the cells, significantly increased L1CAM expression was detected in late-stage (moderated to poorly differentiated) tumors (Figure 1D). The positive correlation between L1CAM expression levels and the stage of tumor differentiation indicated the significance of L1CAM expression in human OSCC tumor biology.

**L1CAM Protein Expression Is Associated with Aggressiveness in OSCC Cell Lines.** L1CAM protein levels were also examined in four independent OSCC cell lines: SAS, SCC4, SCC9, and SCC25 as well as normal oral keratinocytes (HOKs). Intact and cleaved L1CAM protein was

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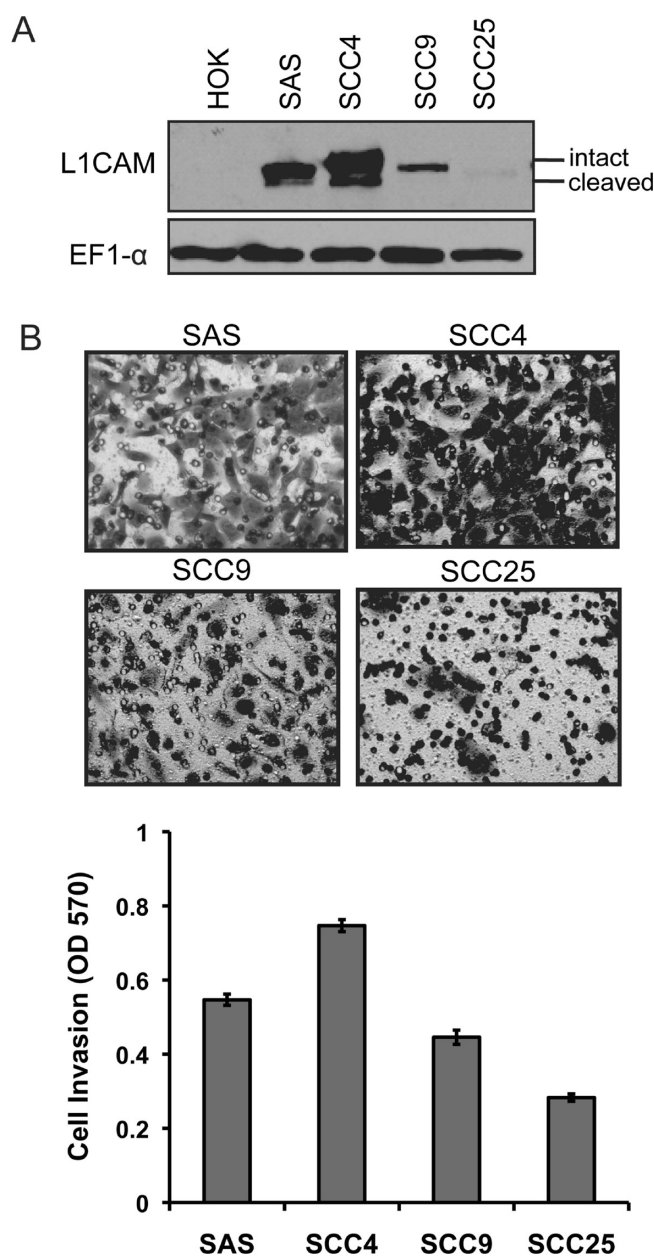
detected in cell lysates of all of the tested cancer cell lines. Based on Western blot analysis, a higher expression was observed in the SCC4 and SAS cell lines, and a lower expression was observed in the SCC9 and SCC25 cell lines; L1CAM expression was undetectable in HOKs (Figure 2A).

We next examine whether the L1CAM expression pattern in the OSCC cell lines was related to their metastatic potentials using Matrigel invasion chamber assays. The strongest invasion was noted in SCC4 cells, which expressed the highest levels of L1CAM, followed by SAS, SCC9, and then SCC25 (Figure 2B). The results obtained from these cell lines are in accordance with our clinical observations (Figure 1) indicating that L1CAM protein levels are associated with tumor malignancy.

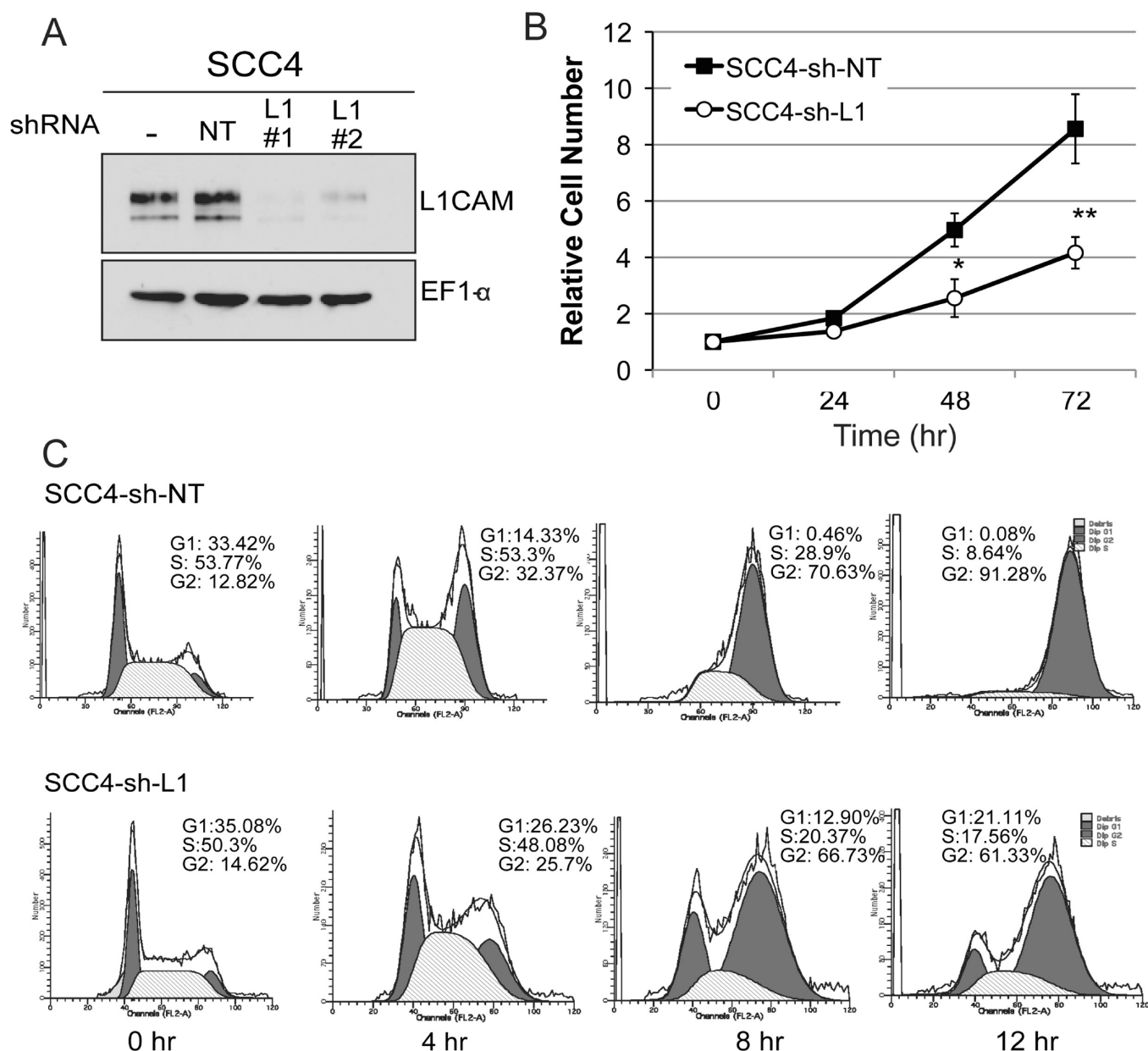
**Targeting L1CAM by Lentivirus-Mediated shRNA Inhibits OSCC Cell Growth and Cell Cycle Progression *in Vitro*.** To assess the functional significance of L1CAM expression during OSCC tumor progression, RNAi was used to target L1CAM gene expression. We stably expressed lentiviral vectors expressing a short hairpin (shRNA) sequence that targeted L1CAM (sh-L1) or a mammalian nontargeting sequence (sh-NT) in SCC4 cells, which had demonstrated high levels of L1CAM expression and invasive behavior. The two distinct shRNAs targeting L1CAM caused a dramatic reduction in L1CAM protein levels (96% and 85%, respectively) when compared with parental SCC4 cells. The sh-NT shRNA demonstrated no effects on L1CAM expression (Figure 3A). These results indicated the effectiveness of lentivirus-mediated shRNA in silencing L1CAM gene expression in OSCC cells.

We next determined whether the altered L1CAM expression in SCC4 cells could affect cell growth *in vitro*. A colorimetric WST-1 assay was used to quantify cell proliferation. We found that silencing L1CAM in SCC4 cells (SCC4-sh-L1) led to a 50% decrease in the number of cells after 48 and 72 h of culture ( $p < 0.05$  and  $p < 0.0001$ , respectively) when compared with the control SCC4 cells (SCC4-sh-NT) (Figure 3B). Noticeably, SCC4-sh-L1 and SCC4-sh-NT cells showed similar cell viability during the culture period, as measured by trypan blue dye exclusion assay (data not shown), suggesting that death-inducing pathways are not involved in the growth inhibition resulting from L1CAM silencing.

To understand the control mechanisms by which L1CAM regulated cell growth, we determined and compared the cell-cycle distribution of SCC4-sh-L1 and SCC4-sh-NT cells by measuring DNA content following treatment with nocodazole, a microtubule inhibitor, to arrest the cells during mitosis. As shown in Figure 3C, while the majority of the SCC4-sh-NT cells accumulated in a G2/M peak (70.63%) by 8 h after nocodazole treatment and completed one cell cycle over the next 4 h, SCC4-sh-L1 cells still retained 21.11% and 17.56% of their cell population in the G1 and S phases, respectively. Together, these results demonstrated that suppression of L1CAM expression by shRNA caused a G1 cell-cycle arrest and cell proliferation inhibition in SCC4 cells.



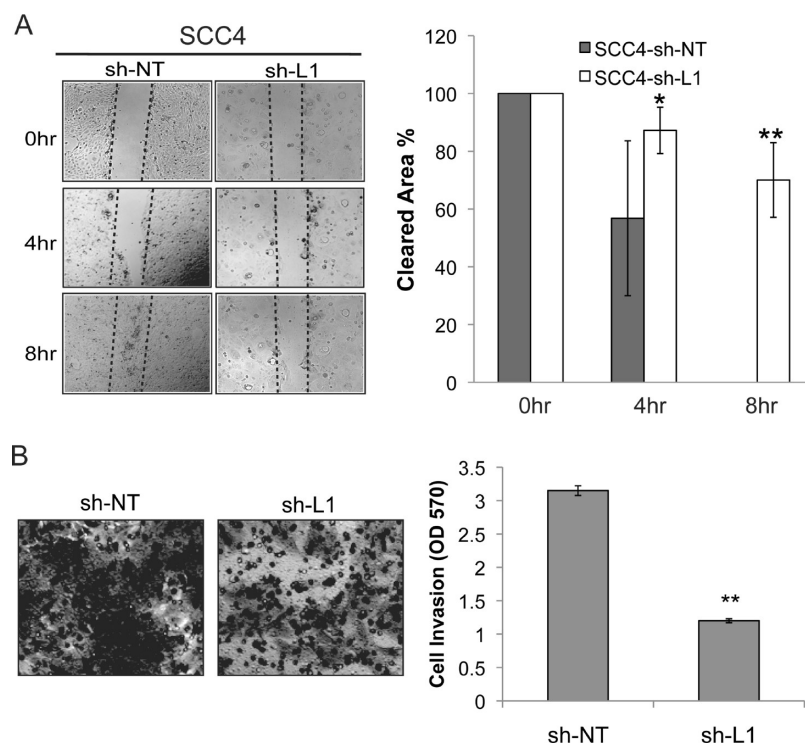
**Figure 2.** Correlation between L1CAM expression and the aggressiveness of OSCC cell lines. (A) Representative Western blot results indicating L1CAM expression in SCC25, SCC9, SCC4, and SAS human OSCC cell lines and normal human oral keratinocytes (HOK). EF1- $\alpha$  protein levels are shown to have varied loading quantities. (B) Characterization of the invasive ability of OSCC cell lines using a Matrigel chamber assay. Migrated cells through the Matrigel were stained with crystal violet 16 h after cell plating. The lower surface of the membrane was photographed (100 $\times$ ) and shown as a representative image from each cell line (upper panel). The number of invaded cells was quantified by measuring the absorbance of dye extract at 570 nm (lower panel). Results from one of three experiments are shown. Error bars indicate SD of triplicate measurements.



**Figure 3.** Effects of L1CAM gene silencing by shRNA on SCC4 cell growth and cell-cycle progression. (A) Western blot analysis of L1CAM expression in parental SCC4 and SCC4 cells infected with a lentiviral vector carrying a nontargeting shRNA (sh-NT) or shRNA targeting L1CAM (sh-L1). EF1- $\alpha$  protein levels are shown to vary in loading quantities. (B) Cell proliferation of shRNA-expressing SCC4 sublines was determined by WST-1 assay performed daily for 3 days. The relative cell number was assessed by absorbance at 450 nm and presented as the fold change relative to the day of plating (0 h). Error bars indicate SD of triplicate measurements. \* $p < 0.05$ , \*\* $p < 0.0001$  versus SCC4-sh-NT. (C) Representative FACS profiles of SCC4-sh-L1 and SCC4-sh-NT cells stained with propidium iodide for DNA content at the indicated time points after adding 100 ng/mL nocodazole to accumulate cells at the G2/M transition. Data was processed using the ModFit LT program, and the percentage of cells at each phase of cell cycle is indicated.

**L1CAM shRNA Inhibits Migration and Invasion of OSCC Cells.** To elucidate the role of L1CAM in the process of OSCC metastasis, we determined the migration and invasive potential of SCC4-sh-L1 cells *in vitro*. We used a wound-healing assay to assess the role of L1CAM in cancer cell migration. As shown in Figure 4A, the movement of SCC4-sh-L1 cells toward the denuded areas was much slower

than SCC-sh-NT cells. Quantitative data showed that while the distance between the wound edges of SCC4-sh-L1 cells was reduced by 12.76% and 30% at 4 and 8 h, respectively, the gap width of the SCC4-sh-NT cells was closed by 43% at 4 h and completely filled at 8 h. In Matrigel chamber assays (Figure 4B), SCC4-sh-L1 cells exhibited a marked decrease ( $p < 0.001$ ) in the number of cells invading through



**Figure 4.** Effects of L1CAM gene silencing by shRNA on migration and invasion of SCC4 cells. (A) Wound healing assays using confluent cells gently wounded through the central axis. The wounded areas were examined under  $\times 100$  magnification, and images were taken at 0, 4, and 8 h. The dashed lines indicate the leading edge of the cut that was made across the monolayer (left). Cell migration was quantified by measuring the distance between the invading front of cells and plotted as the percentage relative to the zero time point (right). Error bars indicate SD of 10 random measurements. (B) Invasion of SCC4 cells was determined by Matrigel chamber assay at 16 h after incubation. Images are representative of the migration of SCC4-sh-L1 cells from three separate experiments performed in triplicate (left). The number of invaded cells was quantified by measuring the absorbance of dye extract of crystal violet at 570 nm. Results from one of three experiments are shown (right). Error bars indicate SD of triplicate measurements. \* $p < 0.05$ , \*\* $p < 0.0001$  versus SCC4-sh-NT (A and B).

the membrane after a 16 h incubation, demonstrating an approximate 65% decrease relative to that of the SCC4-sh-NT cells. These results suggested that L1CAM is important for the metastatic capacity and, thus, for the malignancy of OSCC cells.

**L1CAM Gene Silencing Causes Changes in the Expression of Epithelial and Mesenchymal Markers.** Epithelial–mesenchymal transition (EMT) is a critical biological process in epithelial tumor invasion, progression, and metastasis.<sup>38</sup> E-cadherin is a transmembrane protein that mediates the major cell–cell adhesions in epithelial cells, a function that has been linked to its role as a tumor suppressor.<sup>39</sup> Vimentin is an intermediate filament that participates in adhesion, migration, survival, and cell signaling processes normally occurring in mesenchymal cells, and it is also involved in pathological or physiological processes

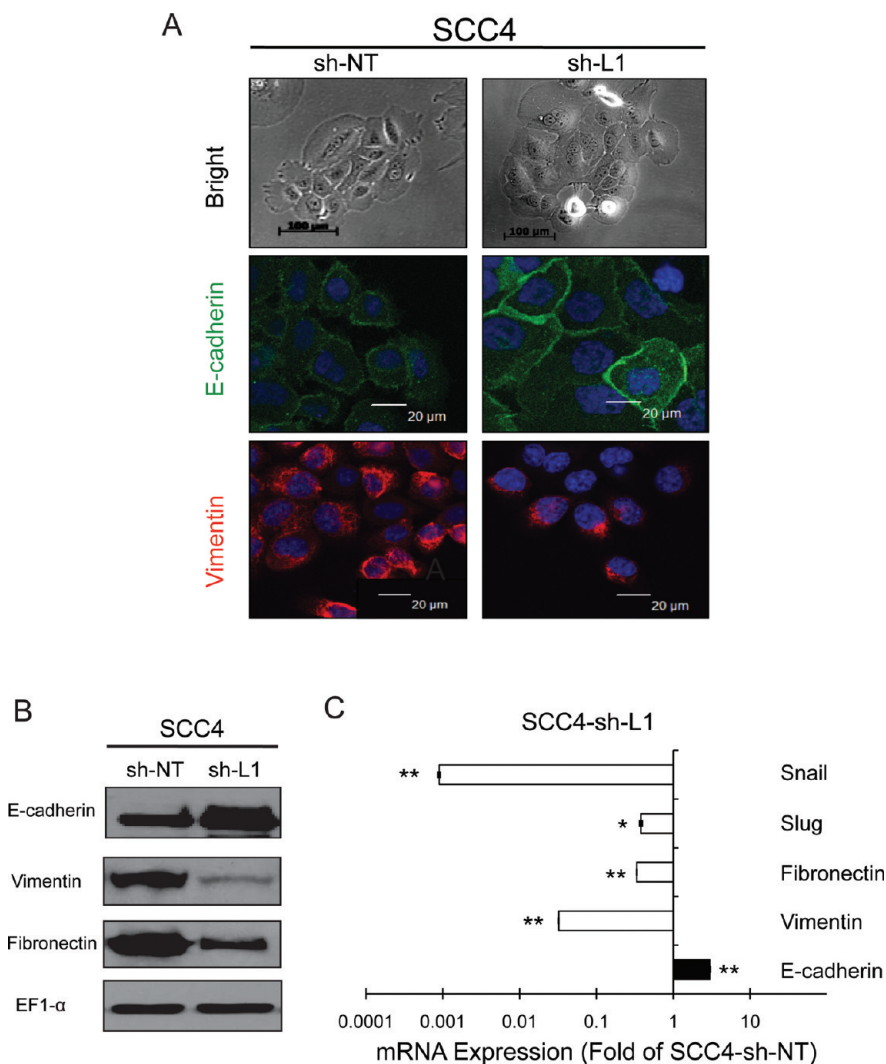
that require epithelial cell migration.<sup>40</sup> A defining feature of EMT is the loss of E-cadherin expression and gain of vimentin expression.<sup>41</sup> We next investigated if the influence L1CAM gene silencing on the migration and invasion of OSCC cells was related to EMT by determining the expression level of these EMT markers. As shown in Figure 5, L1CAM silencing markedly increased the protein levels of the epithelial marker E-cadherin and reduced the levels of the mesenchymal marker vimentin in SCC4 cells, as determined by immunocytochemical analyses (Figure 5A) and Western blotting (Figure 5B). Consistent with this protein profile, quantitative RT-PCR analysis (Figure 5C) showed a 3-fold increase in E-cadherin and a massive reduction ( $\sim 97\%$ ) in vimentin in SCC4-sh-L1 cells when compared with SCC4-sh-NT cells. Moreover, fibronectin, another mesenchymal marker, and known EMT-regulatory transcription factors, Slug and Snail, were also downregulated in SCC4-sh-L1 cells, further confirming that the altered gene

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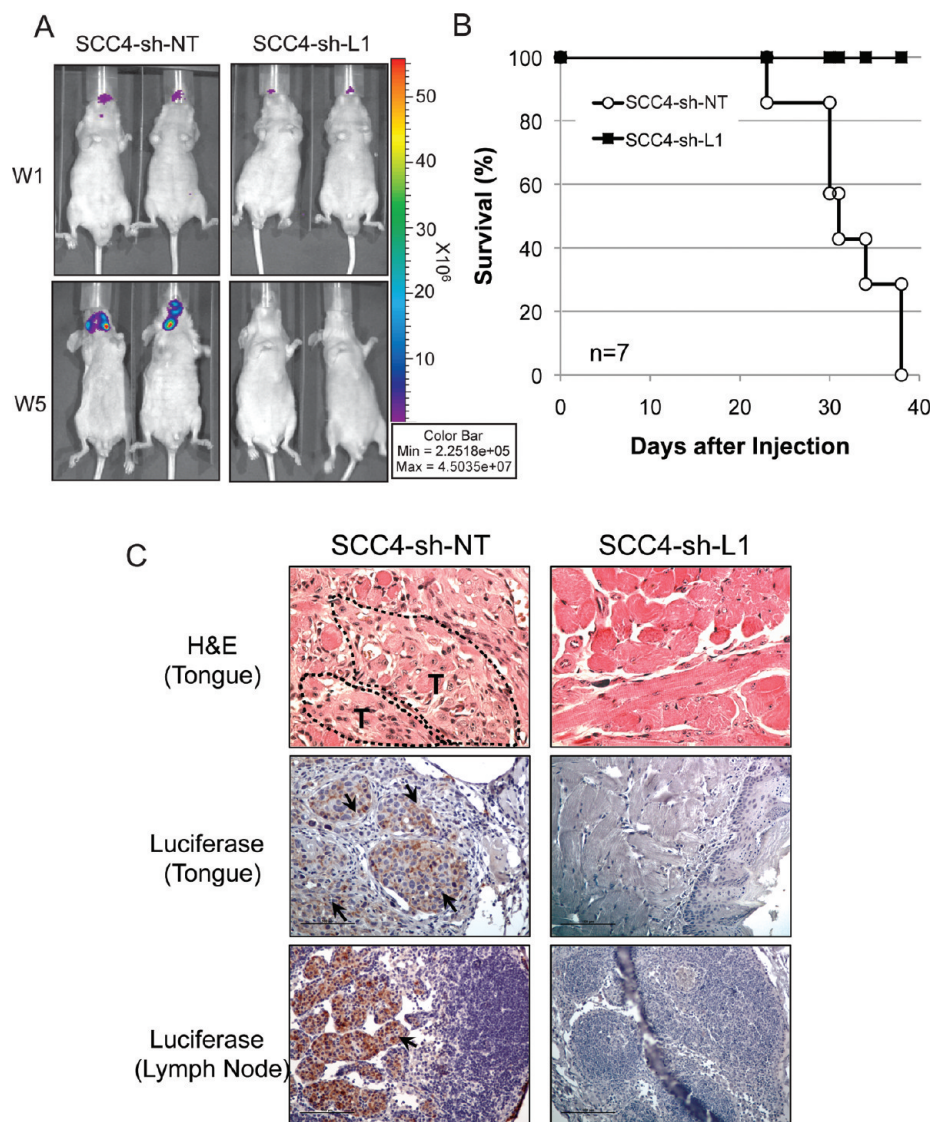


**Figure 5.** Effects of L1CAM gene silencing by shRNA on expression of epithelial–mesenchymal transition (EMT) markers. (A) Representative bright-field images of SCC4-sh-L1 and SCC4-sh-NT cells grown on a plastic surface (200 $\times$ ; scale bar = 100  $\mu$ m) and immunofluorescence staining for E-cadherin (green) and vimentin (red) expression in cells grown on cover glass under confocal microscopy ( $\times$ 400; scale bar = 20  $\mu$ m). Nuclei were counterstained with DAPI (blue). (B) Western blotting analysis of EMT markers. EF1- $\alpha$  is shown as the protein loading control. (C) Real-time RTPCR analysis of the above cells for epithelial (black bar) and mesenchymal markers (white bar). mRNA levels in SCC4-sh-L1 cells are displayed as the fold change relative to SCC4-sh-NT cells. Data are representative of three independent experiments and shown as the mean  $\pm$  SD. \* $p$  < 0.05, \*\* $p$  < 0.0001 versus SCC4-sh-NT.

expression caused by L1CAM knockdown was EMT-related. Taken together, these data suggested that targeting L1CAM expression in invasive OSCC cells reverses the EMT phenotype and, thus, attenuates the aggressiveness of these cancer cells.

**Targeting L1CAM Expression by shRNA Suppresses Tumor Growth and Lymph Node Metastasis of OSCC Cells in an Orthotopic Tumor Model.** To investigate of the role of L1CAM in OSCC tumorigenesis and metastasis *in vivo*, we used an orthotopic xenograft model of squamous cell carcinoma of the oral tongue. The model was generated by submucosal injection of human OSCC cells into the anterior tongue of nude mice, which has been reported to develop a high incidence of cervical lymph node metastases.<sup>36</sup> To monitor, in real time, the primary tumor growth

and distant metastasis noninvasively, SCC4-sh-L1 and SCC4-sh-NT cells were stably transduced with a luciferase reporter gene by retroviral infection.<sup>37</sup> We confirmed the equal bioactivity of the luciferase reporter between SCC4-sh-L1 and SCC4-sh-NT cells using an *in vitro* luciferase enzymatic assay (data not shown) prior to injection of cells into the mice. Mice that were orthotopically transplanted with luciferase-transduced SCC4-sh-L1 and SCC4-sh-NT cells were visualized by bioluminescence imaging at one-week intervals. One week after cell implantation, we observed bioluminescence at the site of the injection in all mice, regardless of the cell line used, demonstrating the success of the orthotopic transplantation (Figure 6A, week 1). While the control SCC4-sh-NT group ( $n$  = 7) showed a substantial increase of photon intensity in the primary site (mouth) and spreading into the



**Figure 6.** Inhibition of SCC4 orthotopic tumor growth and lymph node metastasis by L1CAM gene silencing. SCC4-sh-L1 and SCC4-sh-NT cells also expressing the luciferase gene were injected into the anterior tongue of nude mice. Tumor growth and metastasis were monitored by bioluminescence imaging performed once a week. (A) Representative images taken at 1 and 5 weeks (W1 and W5) after injection to indicate tumor size and location are shown. (B) Mouse survival was monitored over the 6-week period of the experiment;  $n$  = number of mice used in each group. (C) Validation of regression of SCC4-sh-L1 xenografts by histopathological analysis (hematoxylin and eosin staining: H&E) and immunohistochemical staining of luciferase in tongue and adjacent lymph nodes (200 $\times$ ; scale bar = 100  $\mu$ m). Arrows denote positive staining.

neck area over time, the bioluminescence signal dropped and disappeared in all mice ( $n = 7$ ) in the SCC4-sh-L1 group (Figure 6A, week 5). A Kaplan–Meier survival curve showed that SCC4-sh-NT tumor-bearing mice were dead before 40 days. In contrast, mice implanted with SCC4-sh-L1 cells remained alive and healthy (Figure 6B) during the 6-week period of monitoring (42 days). Histopathological analysis of tumor tissue sections revealed invasive squamous cell carcinoma formation in the SCC4-sh-NT xenografts, whereas no tumor was seen in whole-mounted tongue tissues collected from SCC4-sh-L1-injected animals at the end of experiment (Figure 6C). In addition, abundant expression of luciferase was detected in both primary oral tumors and nodal metastases of SCC4-sh-NT mice but not in the SCC4-

sh-L1 tissues (Figure 6C), further confirming the complete regression of the SCC4-sh-L1 tumors.

## Discussion

L1CAM has been detected in many tumor types, but its expression has not been investigated in OSCC. Here we present the first comprehensive study of L1CAM expression in OSCC and its impact on OSCC tumor progression and metastasis. Our immunohistochemistry in human OSCC tissues representing different stages of disease progression demonstrated that L1CAM was absent in normal non-neoplastic tissues but expressed in OSCC where staining was significantly stronger and present in more cells with less differentiation. Thus, L1CAM may be useful as a tumor

biomarker for predicting the presence of OSCC in patients. Significantly, aside from a reduction in cell proliferation and migration/invasiveness *in vitro*, suppressed tumor growth *in vivo*, and increased survival of tumor-bearing animals by knockdown of L1CAM, targeting L1CAM expression in OSCC cells also resulted in cell-cycle arrest at the G1 phase and reversed EMT-like cellular events, further providing molecular insight into the role of L1CAM in triggering the transition from primary tumor formation to metastatic competence. In addition, as regional and distant metastasis is the main cause of death for patients with oral cancer,<sup>42</sup> our results suggested that L1CAM and its related signaling pathways could be targeted as novel treatment options.

Recently, EMT has gained significant clinical attention in OSCC due to microarray studies revealing upregulation of EMT-related genes in high-risk head and neck SCC tumors<sup>43</sup> and their metastases.<sup>44</sup> In this study, we described a new link between L1CAM and key regulators of EMT and OSCC tumor progression. Unlike breast carcinoma cells, where L1CAM had no effect on the total amount of E-cadherin but can induce the removal of E-cadherin from the adherens junctions,<sup>45</sup> we found that L1CAM knockdown increased the expression of E-cadherin protein in total cell lysates and of E-cadherin mRNA extracted from oral cancer cells, indicating that L1CAM-mediated E-cadherin downregulation occurred at the transcriptional level. The transcription factors of the Snail family, Snail (Snail) and Slug (Snail2), have the ability to bind to the promoter region of the E-cadherin gene.<sup>46,47</sup> L1CAM significantly affected Snail and Slug expression in SCC4 cells, strongly suggesting that the Snail family is a downstream target of L1CAM responsible for functional inhibition and consecutive suppression of E-

cadherin expression, leading to the induction of EMT. Moreover, we observed that downregulation of L1CAM significantly inhibited cell proliferation commensurate with an increased accumulation of cancer cells at the G1 phase and a corresponding reduction of cells in the S phase of the cell cycle *in vitro*. Furthermore, downregulation of L1CAM completely abolished tumorigenicity in an animal model. These results revealed a more versatile role of L1CAM in tumor promotion that was not merely limited to cell adhesion and migration, as evidenced by this and other studies,<sup>15</sup> but also includes a role as a potential oncogene in OSCC. While the mechanisms by which L1CAM modulates tumor progression are still under investigation, recent studies indicate that L1CAM affects the expression of Erk-dependent genes important for motility regulation.<sup>48</sup> In breast cancer cells, L1CAM overexpression also leads to increased  $\beta$ -catenin transcriptional activity, contributing to cell movement and colony scattering.<sup>45</sup> Several G1-phase positive regulators and transcriptional factors, such as cyclin D1 and c-Myc, are known target genes of the  $\beta$ -catenin/LEF-1 pathway,<sup>49,50</sup> and these have been reported to be overexpressed in both premalignant and cancerous lesions of oral tissues.<sup>51,52</sup> Our findings regarding L1CAM-mediated cell-cycle progression may open a new avenue for exploring the molecular mechanism of L1CAM function in the early events of carcinogenesis.

Targeted molecular therapy has been proven to significantly augment conventional therapy without increasing treatment-related toxicity. An antibody against the extracellular domain of L1CAM has been developed and shown to inhibit the growth of L1CAM-expressing ovarian carcinoma cells in nude mice.<sup>53</sup> The subcellular localization of L1CAM is not limited to the cell surface but is also reported in the

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nucleus as a cleavage product containing only the intracellular domain.<sup>54</sup> The transductional targeting approach described above, therefore, has the drawback of antagonizing the nuclear function of L1CAM. Recent studies have shown that RNA silencing is a specific and powerful technology for knockdown of oncogenic expression. In the present study, we successfully used lentivirus-mediated shRNA to inhibit the expression of L1CAM and its effects on tumor promotion, which led to complete tumor regression in an animal

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model. This work, based on a gene silencing approach, provided evidence that L1CAM is a promising mechanistic target for OSCC molecular targeted therapy.

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**Supporting Information Available:** List of primers and probes used in quantitative PCR. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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