

# Nucleoplasmic calcium regulates cell proliferation through legumain

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**Background & Aims:** Nucleoplasmic Ca<sup>2+</sup> regulates cell growth in the liver, but the proteins through which this occurs are unknown.

**Methods:** We used Rapid Subtraction Hybridization (RaSH) to subtract genes in SKHep1 liver cells expressing the Ca<sup>2+</sup> buffer protein parvalbumin (PV) targeted to the nucleus, from genes in cells expressing a mutated form of nuclear-targeted PV which has one of two Ca<sup>2+</sup>-binding sites inactivated. The subtraction permitted the selection of genes whose expression was affected by a small alteration in nuclear Ca<sup>2+</sup> concentration.

**Results:** The asparaginyl endopeptidase legumain (LGMN) was identified in this screening. When Ca<sup>2+</sup> was buffered in the nucleus of SKHep1 cells, LGMN mRNA was decreased by 97%, in part by a transcriptional mechanism, and decreased expression at the protein level was observed by immunoblot and immunofluorescence. Treatment with hepatocyte growth factor increased LGMN expression. Knockdown of LGMN by siRNA decreased proliferation of SKHep1 cells by ~50% as measured both by BrdU uptake and mitotic index, although an inhibitor of LGMN activity did not affect BrdU incorporation. A significant reduction in the fraction of cells in G2/M phase was seen as well. This was associated with increases in the expression of cyclins A and E. Furthermore, LGMN expression was increased in hepatocellular carcinoma cells relative to normal hepatocytes in the same specimens.

**Conclusions:** These findings suggest a new role for LGMN and provide evidence that nuclear Ca<sup>2+</sup> signals regulate cell proliferation in part through the modulation of LGMN expression. Increased expression of LGMN may be involved in liver carcinogenesis.

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## Introduction

Ca<sup>2+</sup> regulates a wide range of activities in the liver, including bile secretion [1,2], canalicular contraction [3], metabolism [4], gene transcription [5,6], apoptosis [7,8], and growth of liver tumors [9]. The spatial patterns of Ca<sup>2+</sup> signals determine the specificity of these signals and which responses are activated [10,11]. Increases in nucleoplasmic Ca<sup>2+</sup> have specific biological effects that differ from the effects of increases in cytosolic Ca<sup>2+</sup>. These effects include activation of distinct genes and transcription factors [5,6,12,13], activation of intranuclear kinases [14,15] and regulation of cell proliferation [9]. The nucleus contains the machinery required for local formation of Ca<sup>2+</sup> signals, including PIP2, PLC, and InsP<sub>3</sub>R-gated Ca<sup>2+</sup> stores [16–18], and several mechanisms permit selective activation of Ca<sup>2+</sup> signaling pathways within the nucleus. This includes direct coupling between integrin receptors and the nucleus [19] and translocation of receptor tyrosine kinases such as c-met and the insulin receptor to the nucleus [17,18]. However, the specific targets of Ca<sup>2+</sup> signals in the nucleus that are responsible for regulating cell proliferation are not clear.

Legumain (LGMN) is an endopeptidase that was first identified in plants [20] and later in humans and mice [21], and parasites [22] and helminthes [23]. LGMN hydrolyzes peptides and proteins on the carboxyl side of asparaginyl residues. This enzyme is predominantly localized in late endosomes and lysosomes [24] and has been implicated in antigen processing [25,26], regulation of biosynthesis of lysosomal proteins [27], and extracellular matrix turnover [28]. LGMN also is present in the tumor microenvironment where it is expressed by macrophages and contributes to metastatic behavior by promoting cell migration and tissue invasion [29]. Increased expression of LGMN is associated with poorer differentiation of tumors and a higher degree of necrosis and apoptosis. LGMN co-localizes with integrins at the invading front of tumors and expression of this enzyme is associated with increased invasiveness [30,31]. Thus, until now the effects of LGMN have been related to its actions as an endopeptidase that modulates the invasive and metastatic potential of cells. Here we identified LGMN as one target of nuclear Ca<sup>2+</sup> and characterize the role of this gene in regulating cell proliferation.

**Keywords:** Nuclear calcium; Legumain; Hepatocellular carcinoma.

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Abbreviations: RaSH, Rapid Subtraction Hybridization; PV, parvalbumin; LGMN, legumain; BrdU, 5-bromo-2'-deoxyuridine; HCC, hepatocellular carcinoma.



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**Materials and methods**

*Materials, reagents and cell lines*

The SKHep1 liver cell line was from ATCC (Manassas, VA). Cells were grown at 37 °C with 5% CO<sub>2</sub> in DMEM supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum (Gibco, Grand Island, NY). SuperScript First-Strand Synthesis System for RT-PCR, TRIzol reagent and lipofectamine 2000 were from Invitrogen (Eugene, OR). Anti-LGMN polyclonal antibody was from ABCAM (Cambridge, MA). Enhanced chemiluminescence reagent (ECL plus) and horseradish peroxidase (HRP) antibodies were from GE Biosciences (Buckinghamshire, UK). Mouse monoclonal antibodies to  $\alpha$ -tubulin,  $\beta$ -actin, and anti-gamma tubulin were purchased from Sigma–Aldrich (St. Louis, MO).

*PV-DsRed adenovirus constructs and infection conditions*

Ca<sup>2+</sup> was selectively buffered within the nucleoplasm by expressing parvalbumin (PV) with a nuclear localization sequence fused to DsRed [6]. One of the two Ca<sup>2+</sup> binding sites of PV is disrupted in the CD mutant, which was used as well. Recombinant adenoviruses pAd-PV-NLS-DsRed and pAd-PV-NLS-CD-DsRed [9] were used for delivery to SKHep1 cells, which were infected at a multiplicity of infection (MOI) of 100. Cells were used 48 h after infection. It has previously been demonstrated that PV-NLS effectively and selectively buffers Ca<sup>2+</sup> in the nucleus of transfected cells [6].

*Rapid Subtraction Hybridization (RaSH)*

RaSH was performed using a modification of the method previously described [32]. Total RNA (25  $\mu$ g), isolated from cells infected with pAd-PV-NLS-DsRed or pAd-PV-NLS-CD-DsRed (control adenovirus), was used for cDNA synthesis. cDNA then was digested with *Mbol* (Fermentas, Glen Burnie, MD) followed by ligation to adapters XDPN-14 (5'-CTGATCACTCGAGA-3') and XDPN-12 (5'-GATCTCTC-GAGT-3') at 4 °C overnight. Mixtures were diluted in 100  $\mu$ l of 10 mM Tris/1 mM EDTA, pH 7 and used for PCR amplification with 10  $\mu$ M XDPN-18 (5'-CTGATCACTCGAGAGATC-3'). To obtain TESTER samples, 3  $\mu$ g of the PCR products (DRIVER) were digested with *XhoI*. The TESTER cDNA (3  $\mu$ g) was mixed with 10  $\mu$ g of the DRIVER cDNA in 10  $\mu$ l of a hybridization solution [0.5 M NaCl/50 mM Tris, pH 7.5/0.2% SDS/40% (vol/vol) formamide] and boiled for 5 min, then incubated at 42 °C for 48 h. Part of the mixture (3  $\mu$ l) was ligated with 1  $\mu$ g of *XhoI*-digested pZerO-1 (Invitrogen, Carlsbad, CA) plasmids and transformed into DH-10B bacteria. The colonies with positive clones were subjected to DNA plasmid extraction and submitted for automated sequencing. Sequences were confirmed by BLASTN.

*Real time PCR*

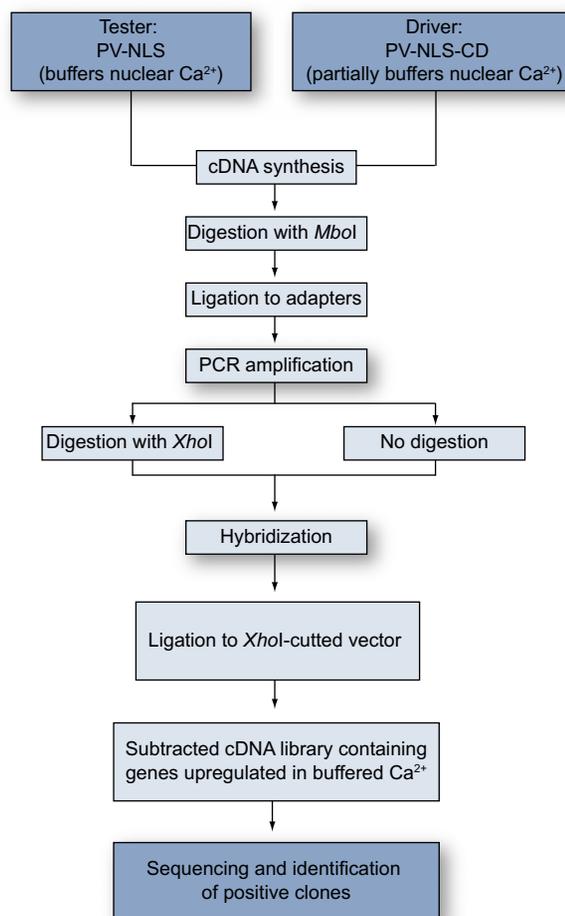
Total RNA was isolated from cells using TRIzol and reverse-transcribed using SuperScript II (Invitrogen). Sense and antisense primer sequences for PCR amplification of  $\beta$ -actin were: 5'-GACGGCCAGGTCATCCTATTG-3' and 5'-AGGAAGGCTG-GAAAAGAGCC-3' and 5'-GCATAGGATCCGGCAAAGTC-3' and 5'-TCCAGTAGATCCA TGCTCAGTGA-3' for *LGMN*. DNA templates were amplified by real time PCR using the Sybr Green Method.  $\beta$ -Actin was used as an internal control. Experiments were performed in triplicate. After amplification, 10  $\mu$ l of reaction mixture were subjected to electrophoresis on agarose gels and visualized after ethidium bromide staining.

*Western blot and CDK2 kinase assay*

Standard methods were used for immunoblots [33]. Cells were lysed at 4 °C and 50  $\mu$ g of total cellular protein was separated by SDS–PAGE. Membranes were blocked and then incubated with an affinity-purified polyclonal antibody against human *LGMN* (1:1000). Membranes were then incubated with peroxidase-conjugated secondary antibody (1:5000). Bands were revealed by enhanced chemiluminescence. For experiments involving HGF stimulation, cells were starved overnight and treated with HGF (100 ng/ml, at 37 °C) for the indicated time points prior to protein extraction. CDK2 activity was evaluated by a modified in vitro Histone H1 phosphorylation assay [34].

*Immunofluorescence*

Confocal immunofluorescence was performed as described [7]. SKHep1 cells were fixed with 4% paraformaldehyde and incubated with anti-*LGMN* antibody (1:200) for 2 h at room temperature followed by incubation with goat anti-rabbit second-



**Fig. 1. Schematic outline of the RaSH protocol.** Tester and driver libraries were constructed, followed by digestion with *XhoI* of the tester library only. After hybridization, differentially expressed sequences were cloned into *XhoI*-digested vectors, resulting in a subtracted cDNA library enriched in genes displaying differential expression. By using the PV-NLS library as the tester and the PV-NLS-CD library as the driver, RaSH was used to produce a subtracted cDNA library enriched in genes up-regulated when nuclear Ca<sup>2+</sup> is buffered (adapted from [32]). Down-regulated genes can be isolated using the PV-NLS-CD library as the tester and the PV-NLS library as the driver.

ary antibody conjugated with Alexa 488 (1:500), for 1 h. Images were obtained using a Zeiss LSM 510 confocal microscope (Thornwood, NY) and mean fluorescence was quantified with ImageJ software (NIH, Bethesda, MD).

*Transfection of siRNA*

Silencer select siRNA sequences specific for *LGMN* or scrambled control sequences were acquired from Ambion (Austin, TX). Cell cultures were treated with 20 nM of siRNA using lipofectamine 2000 (Invitrogen). Cells were incubated at 37 °C in an atmosphere of 5% CO<sub>2</sub> for 72 h prior to use.

*Measurement of BrdU incorporation*

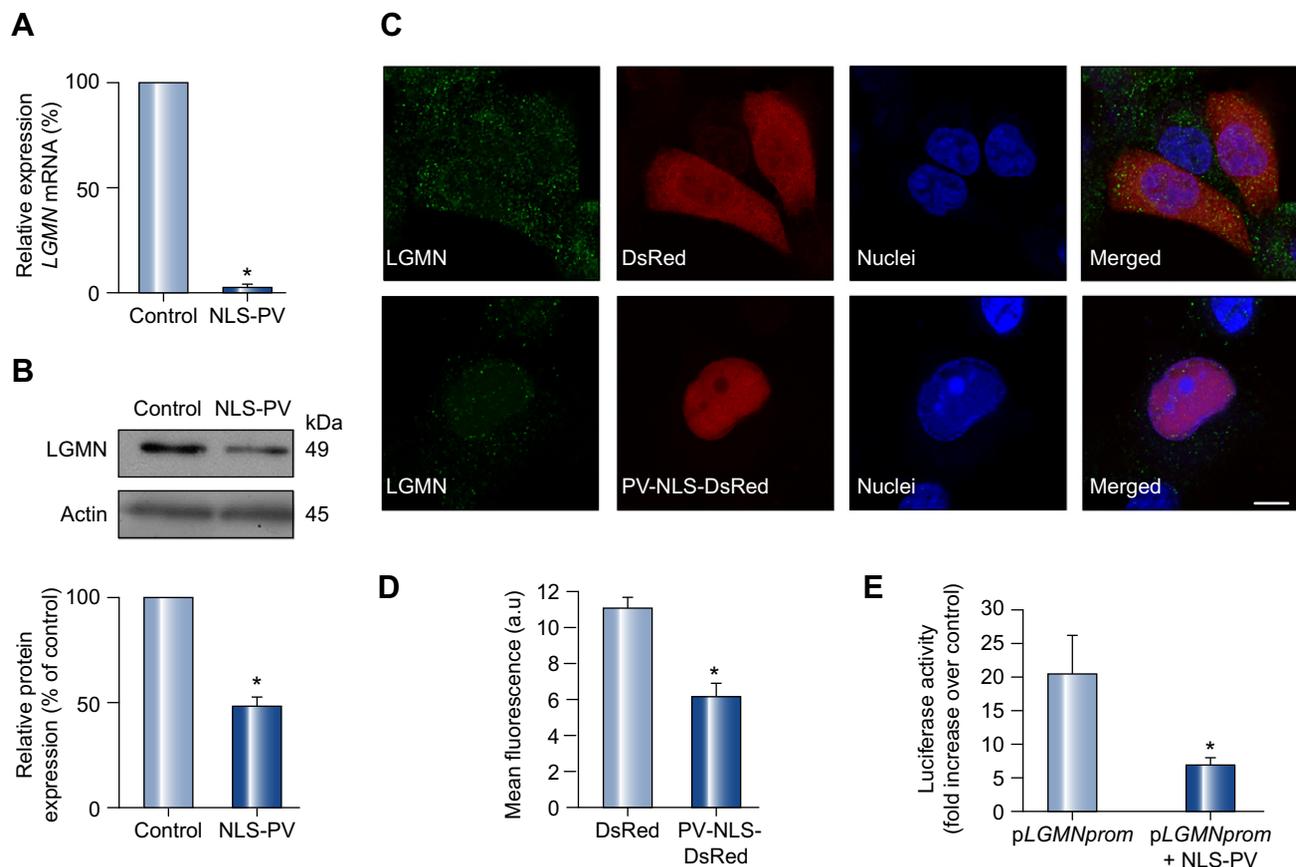
Cell proliferation was measured by BrdU incorporation using ELISA (Roche Applied Science). SKHep1 cells were plated in 96-well culture plates, starved for 24 h and transfected with siRNA. Seventy hours afterward, cells were treated for 2 h with BrdU labeling solution. Cells were then fixed, and anti-BrdU antibody was added. BrdU incorporation was measured colorimetrically. For experiments employing the legumain inhibitor MV026630, cells were treated for 24 h with the inhibitor at 25 or 50  $\mu$ M. Control cells were treated with DMSO alone.

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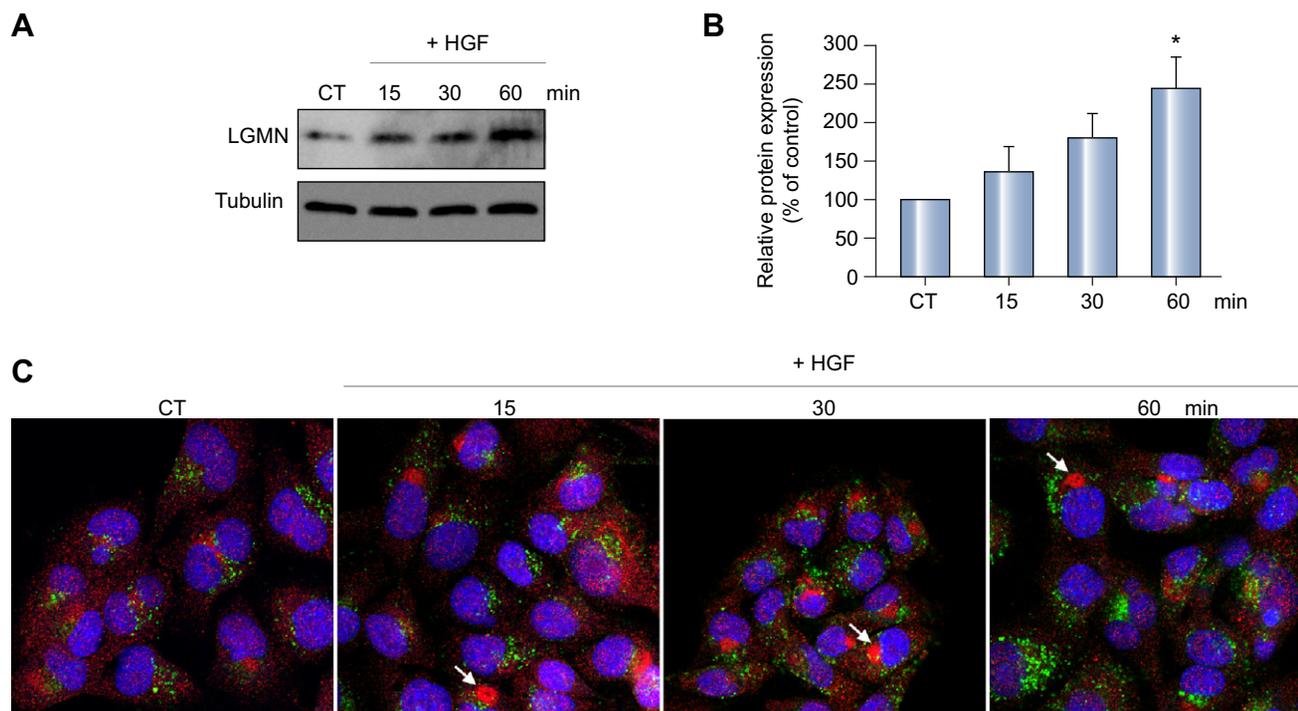
**Table 1. Sequences identified by RaSH.**

Clone GeneBank	Nomenclature	GeneBank Access	Change*
AT rich interactive domain 1A	<i>ARID 1A</i>	NM_006015.4	↑
Legumain	<i>LGMN</i>	NM_005606.5	↓
Transforming growth factor beta regulator 4	<i>TBRG4</i>	NM_030900.2	↓
Reticulon 4	<i>RTN4</i>	NM_207521.1	↑
Tubulin gamma	<i>TUB1</i>	NM_001070.3	↑
Ras homolog gene family member A	<i>RHOA</i>	NM_001664.2	↑
Mps One Binder kinase activator-like 1B	<i>MOB1</i>	NM_018221.3	↑
Similar to ribosomal protein L3	-	CX816870	↑
Splicing factor 3B	-	NM_012426.3	↑
Alanyl-tRNA synthetase	<i>AARS</i>	NM_001605.2	↑
Serpin peptidase inhibitor member 1	<i>SERPINE 1</i>	NM_000602.1	↑
Prohibitin 2	<i>PBHB2</i>	NM_007531.2	↓
Similar to ribosomal protein L27	-	CX816883	↑

\*Change means gene expression level compared to normal control. ↑means up-regulated; ↓means down-regulated when nuclear  $Ca^{2+}$  is buffered.



**Fig. 2. LGMN expression is decreased by buffering nuclear  $Ca^{2+}$ .** (A) Real time quantitative PCR was used to measure the relative expression of *LGMN* mRNA in the SKHep1 liver cell line. *LGMN* mRNA was decreased by  $97 \pm 2\%$  in cells transfected with PV-NLS, relative to non-transfected controls ( $p < 0.0001$ ).  $\beta$ -Actin gene was used to normalize expression in both groups. The data are expressed as mean  $\pm$  SEM of triplicate measurements and are representative of three separate experiments ( $p < 0.05$ ). (B) Immunoblot of whole-cell proteins from SKHep1 cells 48 h after infection shows that *LGMN* protein expression is decreased after buffering nuclear  $Ca^{2+}$ . Densitometric analysis confirms reduction of *LGMN* protein expression to  $52 \pm 9\%$  of controls ( $p < 0.01$ ). Expression of  $\beta$ -actin was used as a loading control. (C) Confocal immunofluorescence confirms decreased *LGMN* expression (green) in SKHep1 cells transfected with PV-NLS (red). Nuclei are identified by TO-PRO-3 staining (blue). Detection of the DsRed tag on PV-NLS confirms that it is localized to the nucleus. Results are representative of four independent experiments. Scale bar = 10  $\mu$ m. (D) Quantification of *LGMN* immunofluorescence shows that buffering nuclear  $Ca^{2+}$  reduces *LGMN* expression by 45% ( $11.1 \pm 0.4$  in DsRed group versus  $6.1 \pm 0.6$  a.u. in PV-NLS-DsRed cells;  $p < 0.001$ ). (E) Luciferase assay shows that *LGMN* promoter activity is inhibited when nuclear  $Ca^{2+}$  is buffered ( $20.6 \pm 5.3$  pLGMNprom versus  $6.9 \pm 0.9$  pLGMNprom + PV-NLS;  $p < 0.05$ ). Data are expressed as mean fold increase  $\pm$  SEM over empty vector in five separate experiments.



**Fig. 3. HGF stimulation increases LGMN expression.** (A) Western blot analysis of total cell lysates prepared from control (non-stimulated) cells and cells stimulated with HGF (100 ng/ml) for the indicated time periods demonstrates a time-dependent increase in LGMN expression. (B) Bar graph shows the densitometric quantification of four separate experiments ( $p < 0.05$ , one-way ANOVA). (C) LGMN localization is altered after HGF treatment. SKHep1 cells were stimulated as above and examined by confocal immunofluorescence. LGMN (red) is present in punctate structures that do not co-localize with the lysosomal marker Lamp-1 (green), in either control or HGF-stimulated cells. Nuclei are labeled by TO-PRO-3 (blue). Upon HGF stimulation, LGMN accumulates near the nucleus (arrows).

*Mitotic index measurements*

SKHep1 cells treated with siRNA were labeled for phospho-histone-3 (Upstate Biotechnology, Chicago, IL) by immunofluorescence and then examined by confocal microscopy. Mitotic cells were defined by the presence of DNA condensation and phospho-histone-3 positivity [35]. Mitotic index was calculated as the percentage of mitotic cells per total cell number. At least 20 fields representing a total of >100 cells were visualized for each condition.

*Cell cycle analysis*

SKHep1 cells transfected with siRNA were trypsinized, washed in phosphate-buffered saline (PBS), fixed with 70% Ethanol at 4 °C and then washed with PBS. After centrifugation cells were supplemented with RNase (100 µg/ml Sigma) for 5 min and stained with propidium iodide (50 µg/ml). DNA content was determined using a FACSCalibur (BD Biosciences), and the data were analyzed using Flowjo software.

*Apoptosis assay*

Apoptosis was measured using a caspase-3 activity kit with colorimetric detection (BD Biosciences). Apoptosis was induced using 500 nM staurosporine (Sigma-Aldrich) as a positive control.

*Immunofluorescence in human tissue samples*

Paraffin-embedded sections of hepatocellular carcinoma (HCC) human liver were pretreated with 10 mmol/L Tris, 1 mmol/L EDTA buffer at 100 °C, incubated with anti-LGMN, and then labeled with Alexa 488-conjugated secondary antibodies [36]. Nuclei were stained with TO-PRO3. To ensure specificity of staining, images were obtained using machine settings at which no fluorescence was detectable in negative controls labeled with secondary antibodies alone. Specimens were observed by confocal microscopy and quantified with ImageJ. The diagnosis of hepatocellular carcinoma was made in each case using standard histopathologic

criteria [37]. These included the presence of a discreet liver mass composed of proliferation of hepatocytes exhibiting a variety of disordered growth patterns. Loss of lobular architecture, thickened hepatocellular cell plates, aberrant arteries, abnormal cytologic inclusions, and nuclear pleomorphism were observed to varying degrees in each case. Reticulin stains were used to highlight abnormal cellular arrangements.

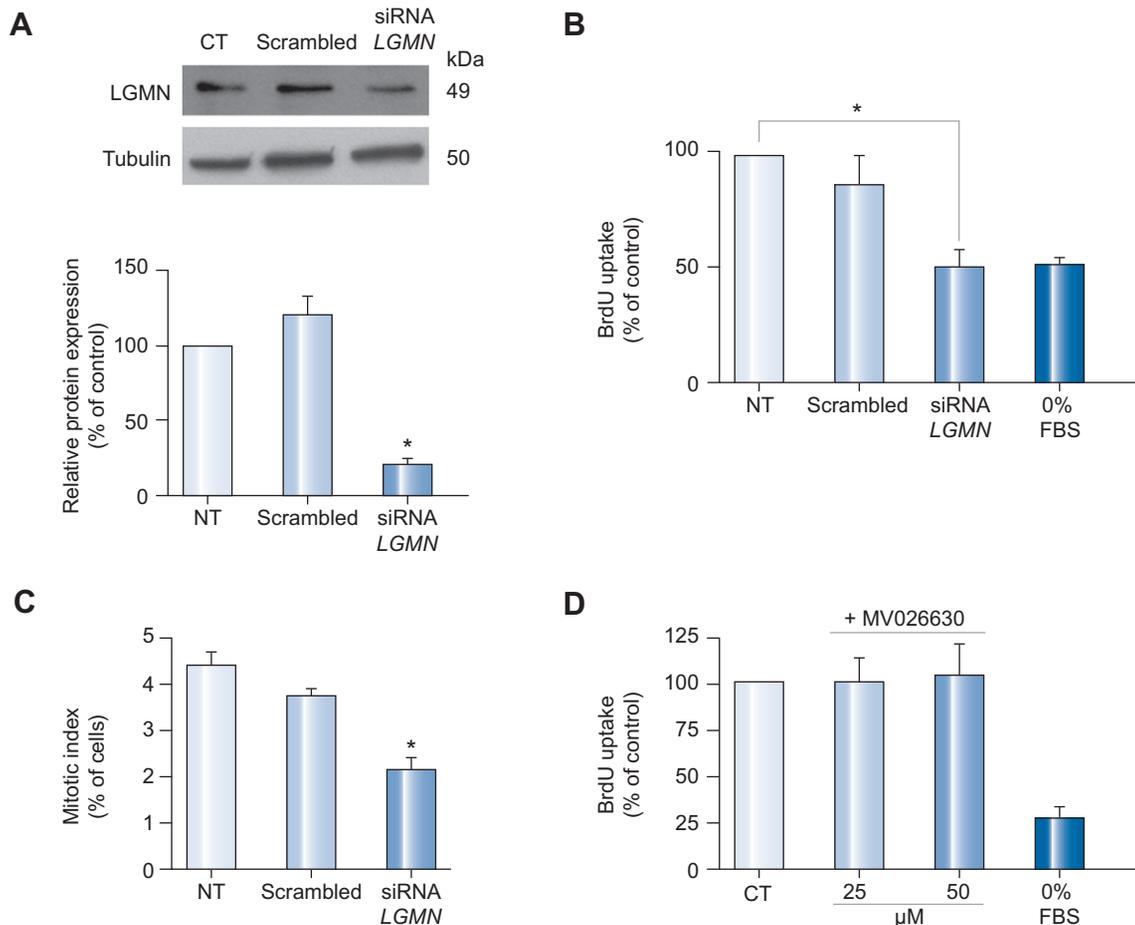
*Legumain promoter luciferase assay*

The plasmids pGL4.10[Luc2] and pRL-CMV, which provide constitutive expression of *Renilla* luciferase, were obtained from Promega (Madison, WI). For generation of the *LGMN* reporter vector, a 750 bp sequence upstream of the initiation codon of the *LGMN* gene was amplified by PCR from SKHep1 genomic DNA with the following primers: 5'-GGACCACCCAGAAACACC-3' and 5'-CTGCCTAAGGGCCACTG-3'. The product of this reaction was used as a template in a second round of amplification using primers: 5'-GCGCTCGAGTTGGCATTCTAAATAGGGAAGTTAA-3' and 5'-GCGCAGATCTGCGTGGCATCTGCCAAA-3', which introduced a 5' *XhoI* and 3' *BglIII* restriction site, respectively. The fragment was cloned into the pGL4.10[Luc2] to generate the pLGMNprom vector whose identity was confirmed by automated sequencing. Lipofectamine 2000 was used to transfect SKHep1 cells with 10 µg of pLGMNprom alone or in combination with 3 µg of pPV-NLS-DsRed, which expresses a nuclear targeted parvalbumin tagged with DsRed. Luciferase activity was measured with a Dual-Luciferase Reporter Assay (Promega, Madison, WI). Luciferase activity was normalized by *Renilla* luciferase activity. Each assay was performed in triplicate and the data were compared to empty vector controls.

*Statistical analysis*

Results are expressed as mean ± SEM. Prism software (GraphPad Software, San Diego, CA) was used for data analysis. Statistical significance was tested using Student's *t*-test or one-way ANOVA followed by Bonferroni post-tests, and  $p$  value <0.05 was taken to indicate statistical significance.

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**Fig. 4. Knock down of LGMN inhibits cell proliferation.** (A) Silencing of *LGMN*. SKHep1 cells were transfected with 20 nM siRNA for *LGMN*, and incubated for 72 h. Immunoblots demonstrate that *LGMN* but not scrambled siRNA knocks down *LGMN* expression.  $\alpha$ -Tubulin serves as a loading control. Densitometry confirms reduction of *LGMN* to  $21.5 \pm 3.2\%$  of non-transfected controls ( $p < 0.0001$ ). Results are representative of three separate experiments. (B) Knockdown of *LGMN* decreases BrdU incorporation in SKHep1 cells to  $50.7 \pm 8.1\%$  of controls ( $p < 0.0034$ ). (C) Mitotic SKHep1 cells were identified by confocal imaging of phospho-histone-3 labeling measured 72 h after knockdown of *LGMN*. The mitotic index is decreased to  $2.2 \pm 0.7\%$  of control in cells in which *LGMN* is silenced ( $p < 0.0001$ ). A total of 300 cells were examined in three separate experiments. (D) Inhibition of *LGMN* activity with MV026630 at either 25 or 50  $\mu$ M does not alter BrdU uptake in SKHep cells ( $p > 0.05$ ).

## Results

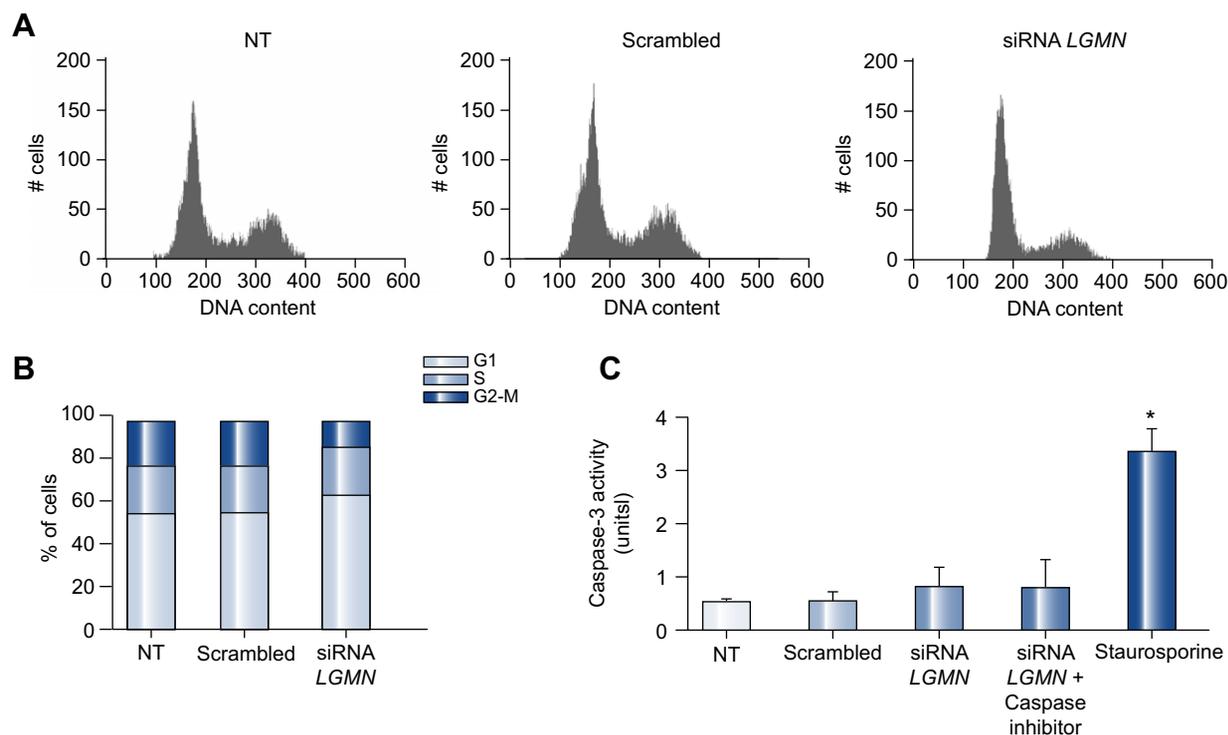
### Identification of *LGMN* as a protein that is sensitive to nuclear $Ca^{2+}$ by RaSH

To define the spectrum of gene expression changes occurring after buffering nuclear  $Ca^{2+}$ , a modified subtraction hybridization technique was used [32]. cDNAs libraries were obtained from SKHep1 cells treated with pAd-PV-NLS-DsRed or pAd-PV-NLS-CD-DsRed. These constructs have been used previously to selectively buffer nuclear  $Ca^{2+}$  in SKHep1 cells [6,9]. Two cDNA libraries were constructed, one with genes up-regulated and the second one with genes down-regulated when nuclear  $Ca^{2+}$  was buffered (Fig. 1). Colonies from the two subtractive libraries were isolated randomly and the PCR-amplified products were sequenced and compared with genes deposited in GenBank. One hundred and forty-five insert DNA fragments were amplified by PCR (data not shown). From this pool of positive clones, 13 differentially expressed clones were identified (Table 1). Among these 13 clones, expression was down-regulated in three and up-regulated in 10 when nuclear  $Ca^{2+}$  was buffered. Three of

these genes were chosen for validation by real time PCR: *LGMN*, *Transforming growth factor beta regulator 4*, and *Reticulon 4*. These three of the thirteen genes were studied further based on literature linking them to cell proliferation and tumor progression. However, additional experiments were only able to demonstrate that the expression of *LGMN* affected cell proliferation, so only that protein was studied in greater detail.

### Buffering nuclear $Ca^{2+}$ inhibits expression of *LGMN*

Real time PCR analysis showed that *LGMN* mRNA decreased by  $97 \pm 2\%$  ( $p < 0.0001$ ) when nuclear  $Ca^{2+}$  was buffered (Fig. 2A). A decrease in *LGMN* at the protein level was also observed 48 h after infection with the adenovirus PV-NLS. *LGMN* protein expression decreased by  $52 \pm 9\%$  ( $p < 0.01$ ) after nuclear  $Ca^{2+}$  was buffered (Fig. 2B). To confirm that expression of this gene was altered in SKHep1 cells expressing PV fused to DsRed within the nucleus, confocal immunofluorescence was performed. Staining for *LGMN* (Fig. 2C) indicated that the expression of this protein was lower in cells expressing DsRed-tagged PV within the nucleus when compared to control cells transfected with DsRed



**Fig. 5. Cell cycle kinetics after knockdown of LGMN.** (A) Representative FACS cell cycle profiles of non-transfected (NT) and siRNA-transfected SkHep cells 72 h after treatment with scrambled or LGMN siRNA. (B) In cells in which LGMN was silenced, there was a reduction in the fraction of cells in G<sub>2</sub>/M phase (7.4 ± 10.9% in LGMN siRNA versus 25.6 ± 3.5% in non-transfected; mean ± SD; *p* < 0.05), without a significant increase in the fraction of cells in G<sub>1</sub> or S phase. Cell cycle profiles were not changed in cells transfected with scrambled siRNA. Data are mean of three independent experiments. (C) Knockdown of LGMN does not induce apoptosis, as measured by caspase-3 activity. Staurosporine (500 nM) was used to induce apoptosis as a positive control for caspase-3, and a caspase-3 inhibitor was used as a negative control. Bar graph shows that caspase-3 activity was not increased in response to knockdown of LGMN (*p* > 0.05, by one-way ANOVA). Results are representative of four independent experiments (\**p* < 0.001).

alone (quantified in Fig. 2D). Because buffering of nuclear Ca<sup>2+</sup> is associated with reduced LGMN mRNA levels, we investigated whether nuclear Ca<sup>2+</sup> transcriptionally regulates LGMN expression. A sequence encompassing 750 bp upstream of the LGMN start codon was used as a putative promoter in a dual-reporter luciferase assay. Luciferase activity driven by the LGMN promoter was significantly reduced when nuclear Ca<sup>2+</sup> was buffered by expression of parvalbumin in the nucleus (20.57 ± 5.34 pLGMN-prom alone as compared to 6.89 ± 0.88 pLGMN-prom + PV-NLS; *p* < 0.05; Fig. 2E). These data show that buffering nuclear Ca<sup>2+</sup> reduces LGMN expression, in part through transcription regulation. Next we examined whether hepatocyte growth factor (HGF), a potent mitogen for hepatocytes that also selectively increases nuclear Ca<sup>2+</sup> in SKHep1 cells [17], modulates LGMN expression. HGF (100 ng/ml) promoted an increase in LGMN expression in a time-dependent manner, reaching its maximum at 60 min (245.7 ± 37.9%, *p* < 0.05; Fig. 3A and B). This increase in expression was associated with partial redistribution of LGMN, which changed from a punctuate pattern observed in non-stimulated cells to the formation of a large peri-nuclear aggregate in cells treated with HGF (Fig. 3C).

**LGMN affects cell proliferation**

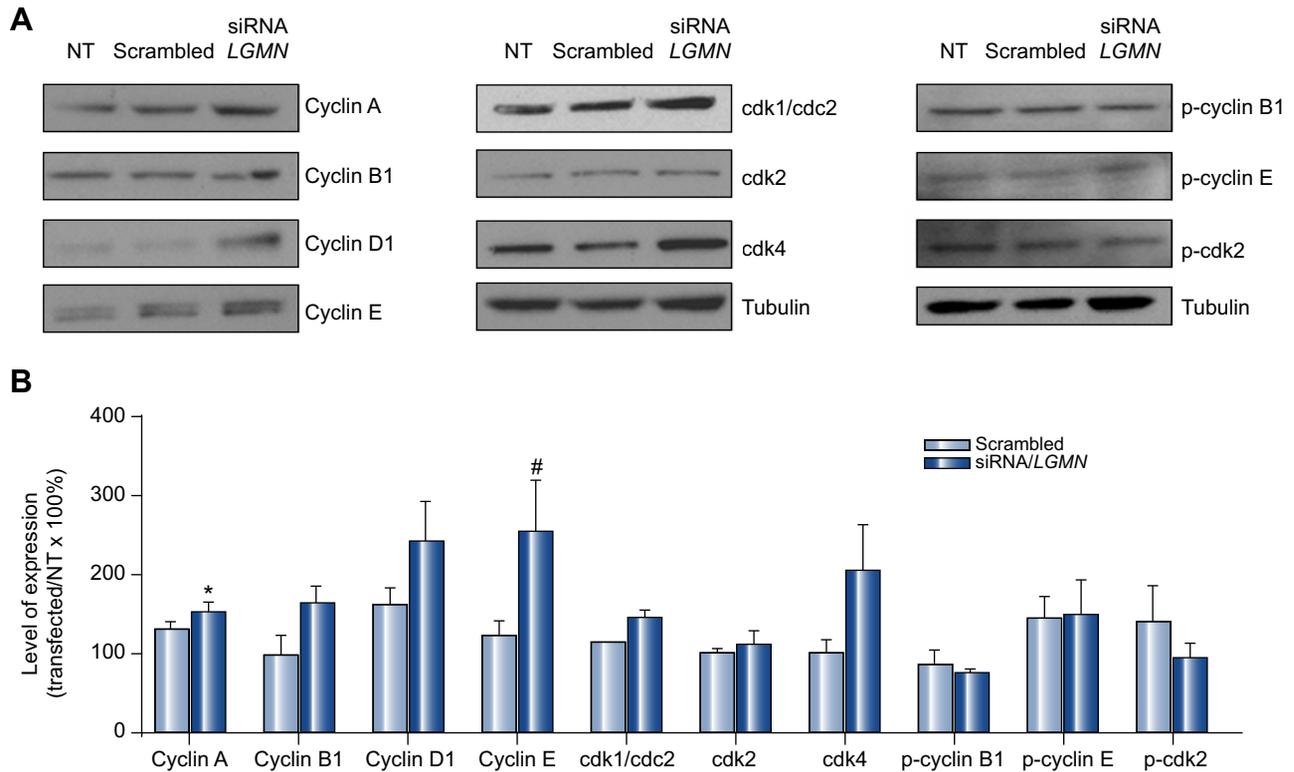
Nuclear Ca<sup>2+</sup> regulates proliferation of SKHep1 cells by controlling progression through the cell cycle [9]. Therefore, the relationship between LGMN expression and proliferation of these cells was investigated. RNA interference constructs decreased

LGMN expression by 78 ± 6% (*p* < 0.0001; Fig. 4A). Cell proliferation was assessed by BrdU incorporation in populations of cells in which LGMN was silenced. Knockdown of LGMN decreased BrdU incorporation by 49% (*p* < 0.005; Fig. 4B). The fraction of cells in mitosis also was quantified by calculation of the mitotic index. The fraction of cells in mitosis decreased when LGMN was silenced (Fig. 4C). Similarly, cell counting showed that proliferation of SKHep1 cells decreased after knockdown of LGMN (data not shown). Because LGMN's only known biological effect is its endopeptidase activity, BrdU uptake was further investigated in SKHep1 cells in the presence of the chemical inhibitor of LGMN, MV026630 [38]. Inhibition of LGMN enzymatic activity did not alter BrdU incorporation (*p* > 0.05 by one-way ANOVA; Fig. 4D), suggesting that LGMN's proliferative effect is independent of its endopeptidase activity. These results provide evidence that LGMN promotes cell proliferation and suggest that nuclear Ca<sup>2+</sup> regulates proliferation of SKHep1 cells in part through modulation of LGMN expression.

*Knockdown of LGMN alters the cell cycle profile rather than apoptosis*

In order to understand why reduced expression of LGMN inhibited cell proliferation, we investigated whether the decreased cell proliferation was due to apoptosis or changes in the cell cycle profile. Flow cytometry was used to examine whether reduction in the expression of LGMN alters the distribution of cells through the phases of cell cycle. Cells were synchronized in G<sub>0</sub> by serum

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**Fig. 6. Progression of cell cycle after knockdown of LGMN.** (A) Immunoblot of total protein from SKHeP1 cells tests the expression of various cell cycle regulatory proteins 72 h after knockdown of LGMN. (B) Densitometric analysis summarizes the results of the Western blots. There was a significant increase in the expression of Cyclin A (\* $p < 0.01$ ) and Cyclin E (\*\* $p < 0.05$ ) in cells transfected with LGMN siRNA relative to non-transfected. Expression of  $\alpha$ -tubulin was used as an internal control for protein loading. Data are mean of three independent experiments.

starvation for 24 h, and then released into the cell cycle by the addition of serum. siRNA was transfected and after 72 h the cells were fixed and stained with propidium iodide and submitted to FACS analysis (Fig. 5A). In cells in which LGMN was silenced, there was a significant reduction in the fraction of cells in G<sub>2</sub>/M (25 ± 2% of non-transfected cells, as compared to 7 ± 6% of siRNA-treated cells;  $p < 0.05$ ). Despite the reduction in the fraction of cells in G<sub>2</sub>/M phase, there was no significant increase in the fractions of cells in G<sub>1</sub> or S phase (Fig. 5B). No changes were observed in cells transfected with scrambled siRNA. Apoptosis was monitored by caspase-3 activation [39] and no increase was observed in cells treated with LGMN siRNA (Fig. 5C). Together, these findings suggest that absence of LGMN causes a decrease in SKHeP1 cell proliferation that is not due to an increase in apoptosis.

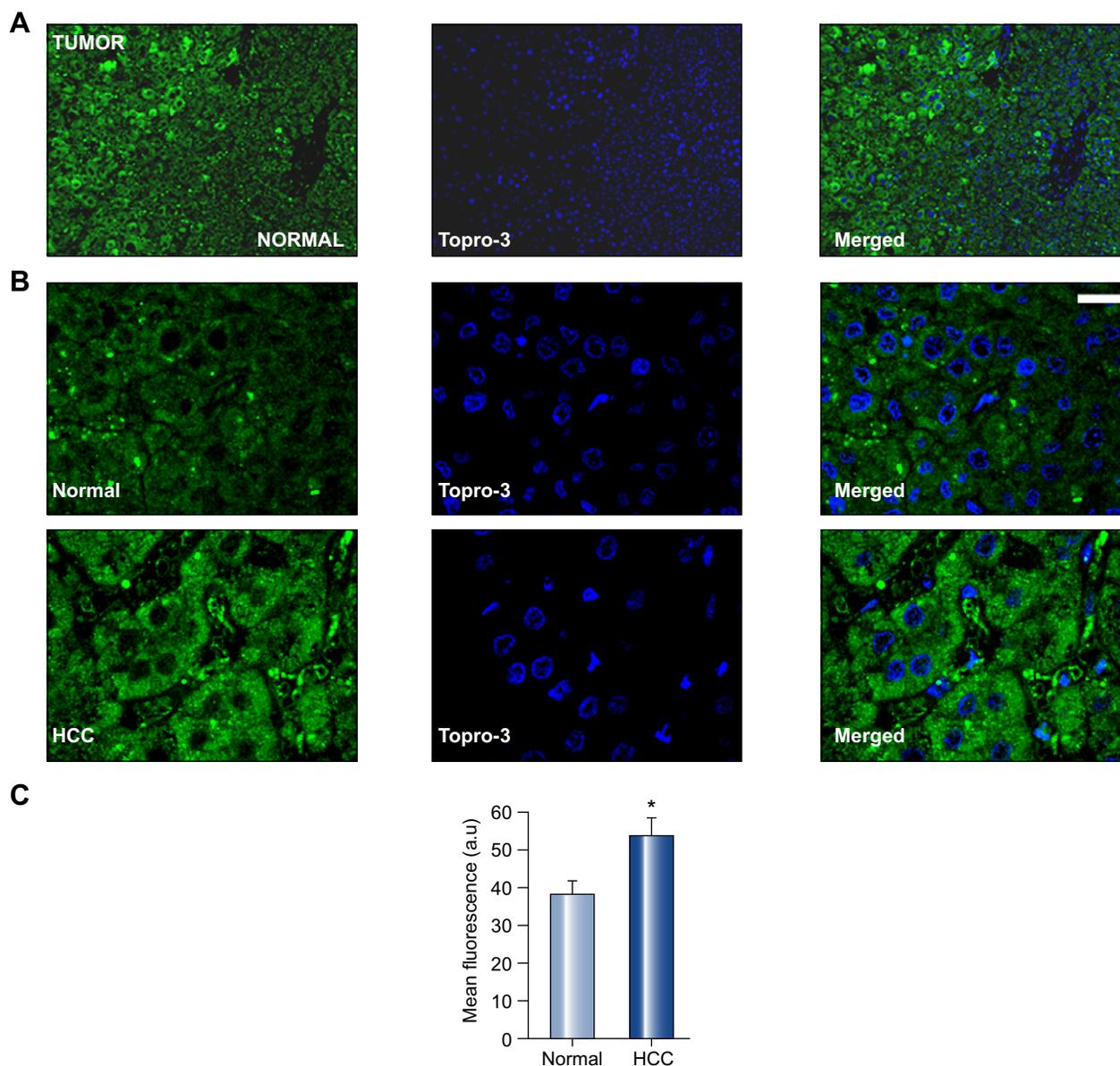
### Knockdown of LGMN modulates cell cycle proteins

The changes observed in the cell cycle profile after knock down of LGMN raises the question of whether the decrease in cell proliferation may be due to alterations in the expression of checkpoint proteins. To investigate this, we examined the expression of these proteins by Western blot (Fig. 6A). The expression of cyclin D1 and Cdk4, which form the complex that regulates the progression through G<sub>1</sub> phase, was unchanged, as was cyclin B1, which controls the G<sub>2</sub>/M transition (Fig. 6A). However, the checkpoint proteins of S phase showed increased expression when LGMN was silenced (Fig. 6A); both cyclins A and E expressions were signifi-

cantly increased ( $p < 0.05$ ; Fig. 6B). These increases in expression were not followed by an increase in phosphorylation or activity of CDK2 (Supplementary Fig. 1), nor were they associated with an increase in PCNA expression. Moreover, there was no change in either phosphorylation of cyclins B1 and E or expression of the Cdk inhibitors p21 and p27 (Fig. 6A and B and Supplementary Fig. 1A and B). Together, these findings suggest that LGMN has only minor effects on the expression profile of checkpoint proteins.

### LGMN expression in normal human liver and hepatocellular carcinoma

Among the putative cDNAs identified by RaSH, LGMN is notable because this gene is highly expressed in several types of tumors. Its presence correlates with a poor prognosis and increased tumor invasiveness [29] and it can be used as a prognostic factor in breast cancer [30]. Expression of LGMN also may be involved in early development of colorectal cancer [40]. Therefore, we investigated LGMN expression in hepatocellular carcinoma (HCC). Liver resection specimens from patients diagnosed with HCC were examined by confocal immunofluorescence. Three samples which contain both normal and tumor tissue from each of five different patients were examined. LGMN expression was increased in tumor cells (53.9 ± 6.5 a.u.) when compared to normal hepatocytes (38.3 ± 4.3 a.u.) in the same tissue samples ( $p < 0.01$  by paired  $t$ -test, Fig. 7). These results suggest that LGMN expression is increased in hepatocellular carcinoma, similar to



**Fig. 7. Expression of LGMN is increased in hepatocellular carcinoma (HCC).** Confocal immunofluorescence images were obtained from paraffin-embedded surgical specimens of tumors from patients with HCC. Immunohistochemical staining was performed to determine the expression of LGMN (green) in tumor cells and in nearby normal hepatocytes. To-Pro-3 was used to identify cell nuclei (blue). (A) Low-power (10×) image of carcinoma cells and normal hepatocytes in the same field of view shows that LGMN staining is increased in the HCC. (B) Higher magnification (63×) images confirm increased the expression of LGMN and show that it is distributed throughout the cytoplasm in HCC (scale bar = 30 μm). Findings are representative of what was observed in three fields each of specimens from five separate patients. (C) Quantification of the average fluorescence in normal and HCC affected areas in the same specimen shows a significant increase in LGMN expression in the carcinoma cells (53.9 ± 4.8 a.u.) as compared to normal hepatocytes (38.3 ± 3.1 a.u.;  $p < 0.01$ , paired  $t$  test).

what has been observed in other solids tumors, and is consistent with the idea that the positive effects of LGMN on cell proliferation may promote carcinogenesis.

### Discussion

Intracellular  $Ca^{2+}$  signals regulate cell growth [41], and in hepatocytes, nuclear rather than cytoplasmic  $Ca^{2+}$  is responsible for this regulation [18]. The nucleus contains the machinery needed to

generate  $Ca^{2+}$  signals, and can form these signals independent of  $Ca^{2+}$  signals in the cytoplasm [15,33]. Hepatic mitogens such as insulin [18] and HGF [17] selectively activate this machinery. Buffering nuclear  $Ca^{2+}$  inhibits the growth of liver tumors in particular [9]. Specifically, liver tumors implanted in nude mice grew much more slowly when expressing parvalbumin in their nuclei, but not in their cytosol [9]. The proteins that link nuclear  $Ca^{2+}$  signals to cell proliferation have not been identified, although such  $Ca^{2+}$  signals have effects that would be expected to stimulate cell proliferation. For example, nuclear  $Ca^{2+}$  activates the

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transcription factors CREB [5] and Elk-1 [6] and stimulates the intranuclear activity of PKC [15] and CaMK-IV [14]. The current work identifies the expression of LGMN as a novel target of nuclear  $\text{Ca}^{2+}$ , and further shows that inhibition of LGMN expression impairs cell proliferation. Moreover, our findings show that the LGMN promoter is sensitive to nuclear  $\text{Ca}^{2+}$ , and a bioinformatics analysis identified a putative Elk-1 binding site on the promoter (not shown), so it is possible that nuclear  $\text{Ca}^{2+}$  regulates LGMN expression through Elk-1.

The findings that LGMN expression is increased in HCC or after treatment of SKHep1 cells with HGF suggest that LGMN may play a role in carcinogenesis in the liver. Although buffering nuclear  $\text{Ca}^{2+}$  and knockdown of LGMN each reduce cell proliferation, reduction of LGMN expression does not exactly mimic the effects of PV-NLS. For example, transfection of cells with PV-NLS increases the fraction of cells in  $G_2$ , and this is associated with a block in early prophase and an increase in the mitotic index [9]. In contrast, LGMN siRNA reduces both the fraction of cells in  $G_2$  and the mitotic index. Furthermore, PV-NLS reduces phospho-Cdk1 but does not alter cyclin expression, whereas LGMN siRNA increases expression of cyclins A and E. These results are counterintuitive to the classical model of cell cycle regulation [42] and findings in mouse hepatocytes during liver regeneration [43], both of which predict increased proliferation in cells with higher levels of cyclins due to prolonged CDK2 activation. However, the increased expression of cyclins A and E observed here was not followed by increased CDK2 activity [44]. This unexpected finding is supported by one report demonstrating that cyclin E overexpression promotes cell cycle arrest [45]. Alternatively, the increased expression of cyclin E observed here could be a compensatory up-regulation triggered by the decrease in cell proliferation after knock down of LGMN. Taken together, these results suggest that LGMN has only a minor effect on cell cycle kinetics, and so nuclear  $\text{Ca}^{2+}$  may act on cell proliferation only in part by regulating LGMN expression.

LGMN is an asparaginyl endopeptidase that hydrolyzes peptides and proteins on the carboxyl side of asparagine residues [24]. Studies in LGMN knockout mice have determined that LGMN is expressed predominantly in late endosomes and lysosomes, where it is involved in processing of cathepsins and lysosomal degradation [27]. LGMN is highly expressed in carcinomas of the breast, colon, and prostate, and in several central nervous system neoplasms, compared with the corresponding normal tissues, in which there is little or no LGMN expression [29]. These findings in other types of tumors are similar to what we report here in HCC. LGMN knockout mice have hepatomegaly, which is attributed to extramedullary hematopoiesis rather than hepatocyte proliferation because liver histology in these mice is normal [46]. However, the incidence of spontaneous HCC has not been studied in these animals, nor has LGMN expression been studied in xenograft models of liver tumors. LGMN is also involved in extracellular matrix remodeling, including fibronectin degradation, which is enhanced by LGMN over-expression [28]. LGMN-containing vesicles are localized to the invading edge of tumor cells, and tumor cells that over-express LGMN have increased invasive and migratory activity [29]. Collectively, these findings had been interpreted to suggest that LGMN promotes neoplasia by creating a tumor micro-environment that facilitates metastatic behavior. However, the current work suggests that LGMN may have direct effects on cell proliferation as well. Moreover, LGMN co-localizes with integrins [29], which also can directly

modulate cell growth. Further work will be needed to understand how LGMN enhances cell proliferation in the liver.

### Conflict of interest

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### Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jhep.2010.12.022](https://doi.org/10.1016/j.jhep.2010.12.022).

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