

Research Paper

# Centromere Protein N Participates in Cellular Proliferation of Human Oral Cancer by Cell-Cycle Enhancement

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## Abstract

Centromere protein N (CENP-N), an important member of the centromere protein family, is essential for kinetochore assembly and chromosome segregation; however, the relevance of CENP-N in cancers remains unknown. The aim of this study was to investigate CENP-N expression and its functional mechanisms in oral squamous cell carcinoma (OSCC). CENP-N expression was up-regulated significantly *in vitro* and *in vivo* in OSCCs. Overexpressed CENP-N was closely ( $p < 0.05$ ) correlated with tumor growth using quantitative reverse transcriptase-polymerase chain reaction, immunoblot analysis, and immunohistochemistry. CENP-N knockdown (shCENP-N) cells showed depressed cellular proliferation by cell-cycle arrest at the G1 phase with up-regulation of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> and down-regulation of cyclin D1, CDK2, and CDK4. Interestingly, we newly discovered that calcitriol (1, 25-dihydroxyvitamin D3) controlled the CENP-N expression level, leading to inhibition of tumor growth similar to shCENP-N cells. These results suggested that CENP-N plays a critical role in determining proliferation of OSCCs and that calcitriol might be a novel therapeutic drug for OSCCs by regulating CENP-N.

Key words: Calcitriol, Cell-cycle arrest at G1 phase, Cellular proliferation, Centromere protein N, Oral squamous cell carcinoma

## Introduction

A centromere, an intersection of the short and long arms of the chromosome, forms kinetochore on centromeric chromatin in mitosis. During cellular division, kinetochore proteins regulate DNA separation by spindle microtubules [1]. Therefore, the centromere is extremely important in cell-cycle regulation and chromosomal segregation [2]. Centromere proteins (CENPs), which comprise 18 subtypes, are related dynamical to association and dissociation during mitosis with microtubule regulation. Among the CENPs, CENP-A, a centromere-specific nucleosome, acts as a foundation for centromere assembly. CENP-A integrates

centromeric chromatin instead of histone H3 for maintenance of centromeric chromatin [1,3].

CENP-N binds directly to the centromere-targeting domain of CENP-A [4,5]. CENP-N depletion causes down-regulation of several CENPs and is considered essential for making a new centromere [6]. Other functions of CENP-N, including in cancer research, are unclear, whereas centromere, mainly the CENP complex, is well elucidated.

The current study found that CENP-N is overexpressed in oral squamous cell carcinoma (OSCC) *in vitro* and *in vivo*. We also identified a novel drug for tumor growth suppression through CENP-N

down-regulation.

## Materials and methods

### Ethics statement

The ethics committee of Chiba University approved the protocol of the current research project (protocol number, 680). All subjects provided written informed consent for inclusion in this study.

### OSCC cells and OSCC tissue samples

We purchased 10 OSCC cell lines from RIKEN BioResource Center (Tsukuba, Ibaraki, Japan) and the Japanese Collection of Research Bioresources Cell Bank (Ibaraki, Osaka, Japan). We harvested human normal oral keratinocytes (HNOKs) from healthy patients as normal control cells and cultured them as described previously [7–9]. OSCC samples were resected at the Department of Oral-Maxillofacial Surgery, Chiba University Hospital. The pathologists at Department of Pathology of Chiba University Hospital diagnosed these samples as OSCC as mentioned previously [10–13].

### Gene expression analysis

Quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) was performed as described previously [7,8]. The sequences of the gene-specific primers were as follows: *CENP-N* (5'-CAGACTCCG TACGCCTTAC-3' and 5'-GGTCCATTTTCAATC TGATG-3') and *glyceraldehyde-3-phosphate dehydrogenase* (5'-AGCCACATCGCTCAGACAC-3' and 5'-GCCCAATACGACCAAATCC-3') (Universal Probe-Library, Roche, Tokyo, Japan).

### Immunoblot analysis

Protein extraction from OSCCs and HNOKs and immunoblot analysis were performed as described previously [13,14]. The antibodies were anti-CENP-N (rabbit, 16751-1-AP, Proteintech, Rosemont, IL, USA), anti- $\alpha$ -tubulin (mouse, sc-5286, Santa Cruz Biotechnology, Shanghai, China), anti-CDK2 (rabbit, #2546), anti-CDK4 (rabbit, #12790), anti-p21<sup>Cip1</sup> (rabbit, #2947), anti-p27<sup>Kip1</sup> (rabbit, #3686), and anti-cyclin D1 (rabbit, #2978) (Cell Signaling Technology Japan, Tokyo, Japan).

### Immunohistochemistry (IHC)

IHC of OSCCs and normal tissues (100 samples) was performed as described previously [13], with appropriate antibodies such as anti-CENP-N (Proteintech). The OSCCs and normal tissues were obtained from the primary OSCC patients from 2007 to 2016, excluding distant metastasis. The IHC scores were calculated from the staining intensity and proportion of the cells stained for CENP-N. Regarding

the intensity of the stained cells, we classified four levels (0, 1, 2, 3) as described previously [14]. The median score of all OSCCs was 153.99. CENP-N was classified as positive and negative using the median score of all OSCCs as the cut-off points [15,16]. Cases with a score over the median score were considered CENP-N-positive.

### Transfection

Transfection with shRNA vector was performed as described previously [10,11,17–19]. We transfected control shRNA (shMock) (sc-37007, SCB) and CENP-N shRNA (shCENP-N) (sc-76640, SCB) into Ca9-22 and SAS cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). RT-qPCR and immunoblot analysis were performed to investigate the efficiency of CENP-N knockdown.

### Cellular proliferation

To investigate the effectiveness of CENP-N knockdown on cellular growth, we performed a cellular proliferation assay as mentioned previously [20–24]. For details, shCENP-N and shMock cells (Ca9-22 and SAS) were disseminated in six-well plates (1×10<sup>4</sup> viable cells/well). Every 24 h, the cells were trypsinized and counted in triplicate using a hemocytometer (up to 168 h).

### Cell-cycle analysis

shCENP-N and shMock cells (Ca9-22 and SAS) were synchronized in the G2/M phase using 200 ng/ml nocodazole (Sigma-Aldrich Japan, Tokyo, Japan) for 16 h as reported previously [10,14,20–24]. After treatment, these cells were analyzed using the BD Accuri™ C6 Flow Cytometer (Becton-Dickinson, Tokyo, Japan) with the CycleTEST Plus DNA reagent kit (Becton-Dickinson).

### Calcitriol treatment

To determine the optimal concentrations of calcitriol (1 $\alpha$ , 25-dihydroxyvitamin D3) (Cayman Chemical, Ann Arbor, MI, USA) for CENP-N expression in OSCC cells, we performed RT-qPCR and immunoblot analysis after treatment with calcitriol. We then evaluated whether calcitriol affected the cellular proliferation and cell cycle by treating the OSCC cells with calcitriol for 72 h. In the cellular proliferation assay, the OSCC cells were cultured with calcitriol. Dimethylsulfoxide was used as the experimental control.

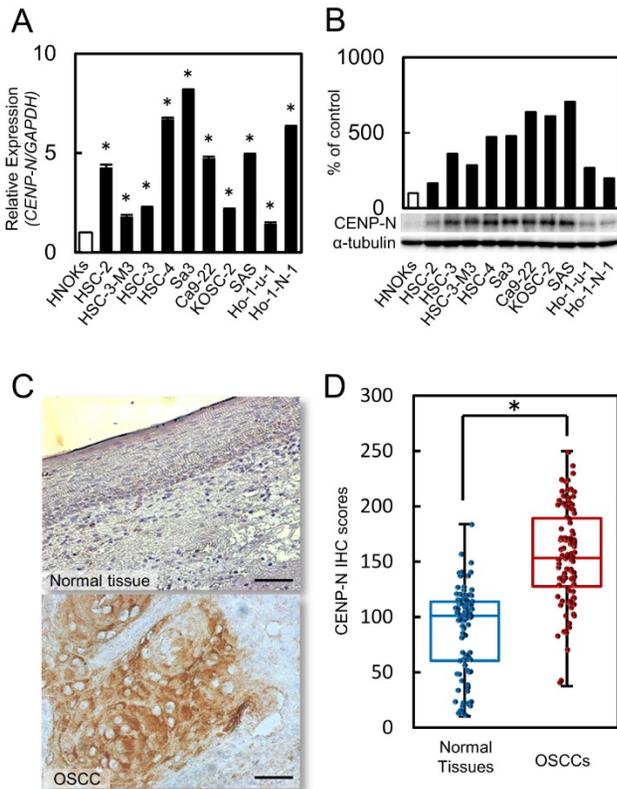
### Statistical analysis

Fisher's exact test and Student's *t*-test were used to analyze the statistical differences. *p* < 0.05 indicates statistical significance. All data are expressed as the mean  $\pm$  standard error of the mean.

## Results

### Increased CENP-N mRNA and protein expression levels in OSCCs

We evaluated the status of CENP-N expression by RT-qPCR and immunoblot analysis in 10 OSCC cell lines and HNOKs. Compared to the HNOKs, CENP-N mRNA expression status in the 10 OSCC cell lines was up-regulated significantly (Fig. 1A,  $p < 0.05$ ). Immunoblot analysis showed that CENP-N protein increased in the 10 OSCC cell lines compared to the HNOKs (Fig. 2B).



**Fig. 1.** Increased CENP-N mRNA and protein expression levels in OSCCs. (A) Significant ( $*p < 0.05$ , Student's *t*-test) up-regulation of CENP-N mRNA is seen in 10 OSCC cells compared with the HNOKs by RT-qPCR. The data are expressed as the mean  $\pm$  standard error of the mean (SEM) from three assays. (B) The expression of CENP-N protein is up-regulated in OSCC cells compared with the HNOKs by immunoblot analysis. (C) Representative IHC results for CENP-N protein in normal oral tissue and primary OSCC tissue. Scale bars = 50  $\mu$ m. (D) The status of CENP-N protein expression in primary OSCC (n = 100) and its normal counterparts by the IHC scoring system.

### CENP-N expression status in clinical specimens of primary OSCCs and correlation in clinical classifications

We performed IHC to investigate the CENP-N expression in primary OSCCs. High expression of CENP-N was observed in the nucleus of the OSCC specimens. However, the normal oral mucosa was not stained by the CENP-N antibody. Fig. 1C shows representative CENP-N data in normal oral tissue and primary OSCC using IHC. These IHC scores in

primary OSCCs were clearly greater in the mucosa than in normal oral tissues (Fig. 1D,  $p < 0.05$ ). The scores in the normal oral tissues ranged from 10.4 to 184 (median, 101.1) and in primary OSCC tissues from 37.6 to 249.8 (median, 153.9). Table 1 shows the correlation between the CENP-N protein expression status and clinicopathological classification of the patients with OSCC. Among the clinical classifications, the CENP-N expression level was correlated significantly ( $p < 0.05$ ) with the primary tumor size (Table 1).

**Table 1.** Correlation between CENP-N expression and clinical classification in OSCCs.

Clinical classification	Results of immunostaining			p-value
	Total	CENP-N negative	CENP-N positive	
Age at surgery (years)				
< 70	59	28	31	0.685*
$\geq 70$	41	22	19	
Gender				
Male	53	30	23	0.229*
Female	47	20	27	
T-primary tumor size				
T1+T2	57	35	22	0.015**
T3+T4	43	15	28	
N-regional lymph node metastasis				
Negative	61	32	29	0.682*
Positive	39	18	21	
TNM stage				
I+ II	43	25	18	0.225*
III+ IV	57	25	32	
Vascular invasion				
Negative	64	36	28	0.144*
Positive	36	14	22	
Histopathologic type				
Well	58	32	26	0.311*
Moderately+ poorly	42	18	24	

\*Fisher's exact test. \*\* $p < 0.05$ .

### shRNA knockdown cells of CENP-N in OSCC

Since overexpression of CENP-N was observed in OSCCs (Fig. 1), we established the shCENP-N and shMock cells from the OSCC cell lines (Ca9-22 and SAS). To investigate the efficiency of the transfection, we performed RT-qPCR and immunoblot analysis. The CENP-N mRNA expression in the shCENP-N cells was significantly ( $p < 0.05$ ) lower than that in the shMock cells (Fig. 2A). The CENP-N protein levels in the shCENP-N cells also decreased compared with the shMock cells (Fig. 2B).

### Cellular growth of shCENP-N and shMock cells

To evaluate the effect of the shCENP-N on cellular growth, a cellular proliferation assay was

performed. Compared with the shMock cells, we found a significant ( $p < 0.05$ ) decrease of cellular proliferation in the shCENP-N cells (Fig. 2C).

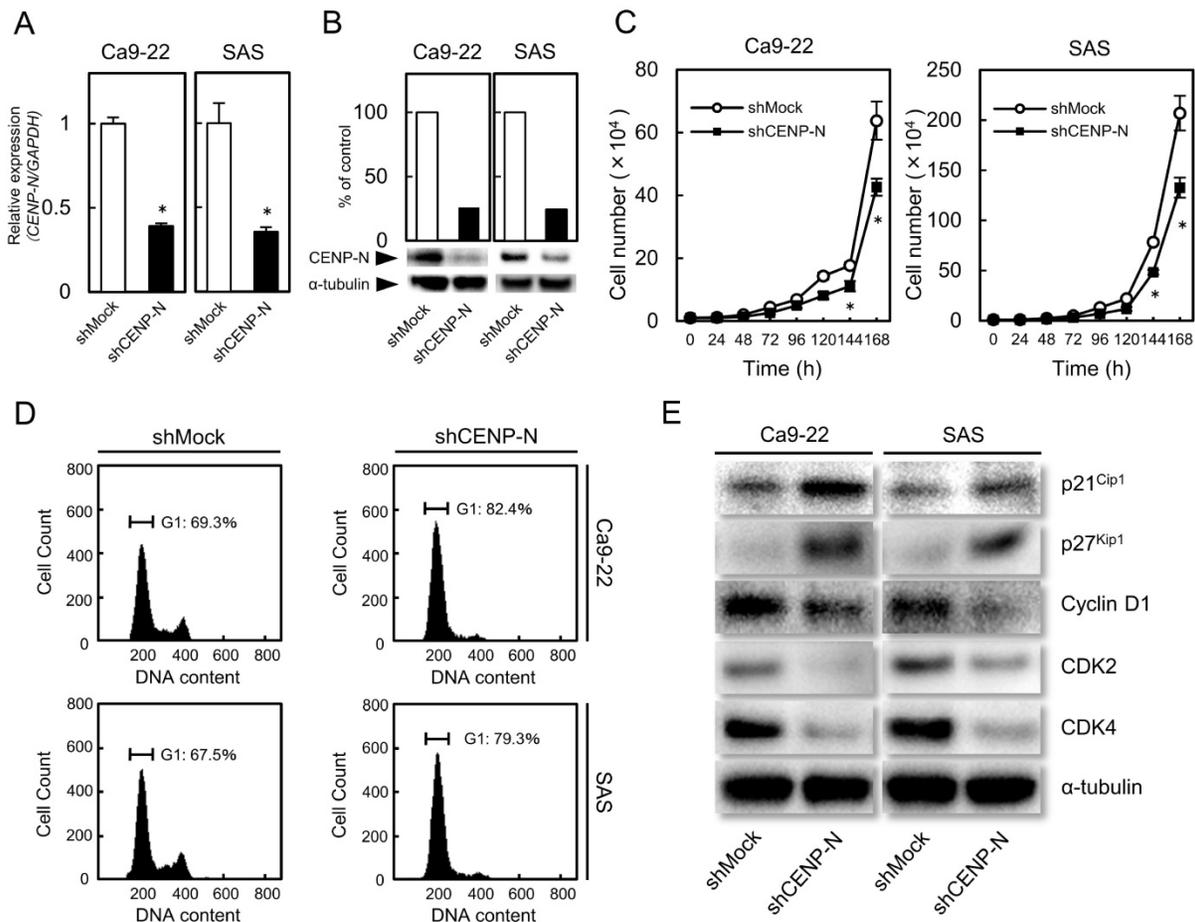
### Cell-cycle analysis of shCENP-N and shMock cells

Flow cytometric analysis showed that the proportion of the G1 phase in the shCENP-N cells was clearly ( $p < 0.05$ ) higher than that in the shMock cells (Fig. 2D). We also evaluated the protein levels of p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, cyclin D1, CDK2, and CDK4. As expected, p21<sup>Cip1</sup> and p27<sup>Kip1</sup> protein levels were up-regulated, and cyclin D1, CDK2, and CDK4 protein levels were down-regulated in the shCENP-N (Fig. 2E). These data indicated that the shCENP-N inhibited cellular proliferation by cell-cycle arrest at the G1 phase.

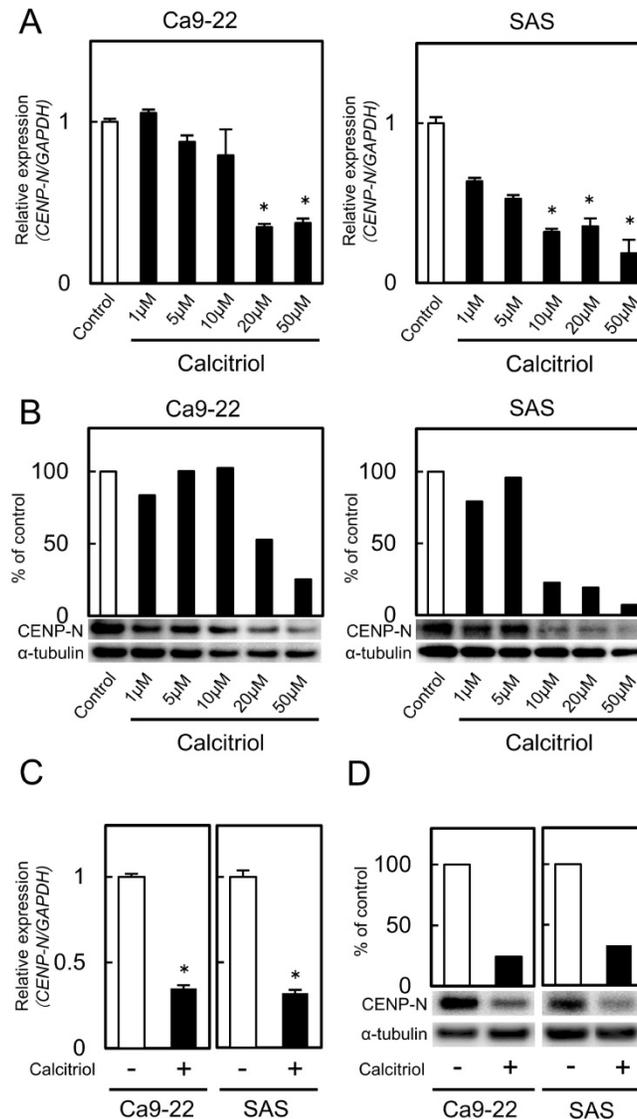
### Effect of calcitriol treatment

We treated the cells with calcitriol to determine the optimal concentrations for inhibiting CENP-N expression. Calcitriol inhibited *CENP-N* mRNA and

protein expression levels in a dose-dependent manner (Fig. 3A, B; optimal concentrations, 20  $\mu$ M with the Ca9-22 cell line and 10  $\mu$ M with the SAS cell line). *CENP-N* mRNA and protein levels in the cells treated with an optimal concentration of calcitriol decreased significantly ( $p < 0.05$ ) compared with control cells (Fig. 3C, D). To assess the effect of calcitriol on cellular growth, we performed a cellular proliferation assay. The cellular growth of the calcitriol-treated cells was significantly ( $p < 0.05$ ) lower than that of control cells (Fig. 4A). In the cell-cycle analysis, the percentage of the G1 phase in the calcitriol-treated cells was clearly ( $p < 0.05$ ) higher than that of control cells (Fig. 4B). We also evaluated the protein levels of p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, cyclin D1, CDK2, and CDK4. As expected, the p21<sup>Cip1</sup> and p27<sup>Kip1</sup> protein levels were up-regulated, and the cyclin D1, CDK2, and CDK4 protein levels were down-regulated in the cells treated with calcitriol (Fig. 4C). These data indicated that calcitriol treatment inhibited cellular proliferation by cell-cycle arrest at the G1 phase similar to CENP-N knockdown.



**Fig. 2.** shRNA knockdown cells of CENP-N in OSCC (Ca9-22 and SAS-derived transfectants). (A) *CENP-N* mRNA expression in shCENP-N cells is significantly ( $*p < 0.05$ , Student's *t*-test) lower than that in shMock cells. (B) Immunoblot analysis shows that the CENP-N protein levels in the shCENP-N cells are also markedly lower than that in shMock cells. (C) The shCENP-N cells and shMock cells were counted on 7 consecutive days. The results are expressed as the mean  $\pm$  standard error of the mean (SEM) of values from three assays. Cellular growth is inhibited significantly ( $*p < 0.05$ , Student's *t*-test) after 144 h culture in the shCENP-N cells. (D) Flow cytometric analysis shows that the percentage of the G1 phase in the shCENP-N cells is increased compared with the shMock cells. (E) Immunoblot analysis shows up-regulation of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> and down-regulation of cyclin D1, CDK2, and CDK4 in the shCENP-N cells compared with shMock cells.



**Fig. 3.** *CENP-N* mRNA and protein levels in calcitriol-treated cells. (A, B) Calcitriol significantly inhibits *CENP-N* mRNA and protein expression levels in the Ca9-22 and SAS cell lines in a dose-dependent manner (\* $p < 0.05$ , Student's *t*-test). (C) The expression of *CENP-N* mRNA after treatment with calcitriol (optimal concentration, 20  $\mu$ M in the Ca9-22 cell line and 10  $\mu$ M in the SAS cell line). The expression of *CENP-N* mRNA in the calcitriol-treated cells is lower than in the control cells (\* $p < 0.05$ , Student's *t*-test). (D) The expression of *CENP-N* protein in the calcitriol-treated cells is lower than in the control cells.

## Discussion

The current study showed that *CENP-N* was up-regulated in human OSCC and clinically positively correlated with tumor size. In the *CENP-N* knockdown models, cellular proliferation was regulated by arresting cell-cycle progression at the G1 phase, suggesting that *CENP-N* is highly correlated with the tumor size of the human OSCCs.

Of the CENPs, only *CENP-A* integrates centromeric chromatin instead of histone H3. *CENP-A* acts as an oncogene in the regulation of cellular growth in liver cancer [25]. *CENP-A* also is a reliable biomarker for tumor progression and prognosis in breast, ovarian, and lung cancers [26–30]. *CENP-N* binds the centromere-targeting domain of

*CENP-A*; however, the functions of *CENP-N* in cancer research have not been reported to date.

Similar to the previous data, which showed that knockdown of cyclin D1 and CDK4 shows negative regulation of *CENP-N* by microarray [31], we found that *CENP-N* knockdown cells not only down-regulated cyclin D1 and CDK4 but also up-regulated p21<sup>cip1</sup> and p27<sup>kip1</sup>. Thus, our data indicated for the first time that *CENP-N* plays a decisive role in tumor growth in OSCC by cell-cycle progression.

Calcitriol is the most biologically active metabolite of vitamin D with high affinity for the vitamin D receptor (VDR) and has been reported to have anticancer effects [32]. Calcitriol acts on bone and mineral homeostasis, inhibits proliferation, and

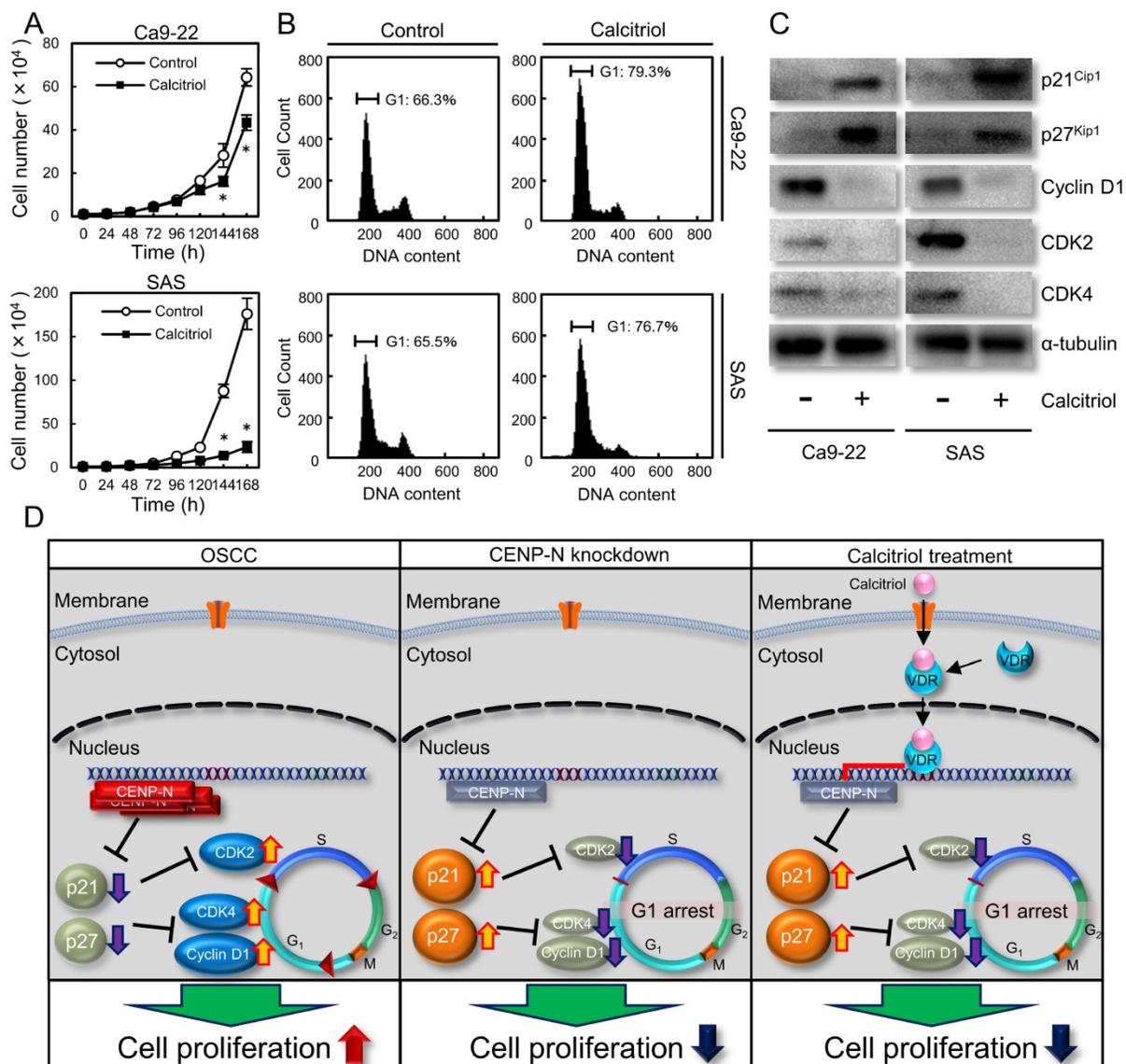
induces differentiation of various normal and malignant cells [33]. However, the exact molecular mechanism behind these anticancer effects is unknown. Calcitriol prescribed for patients with hypocalcemia, osteoporosis, and prevention of corticosteroid-induced osteoporosis, affects the cell cycle with accumulation of cells at the G1 phase of the cell cycle. In addition, calcitriol inhibits its downstream genes, including CENP-A [34,35]. Since CENP-N is a critical binding partner of CENP-A, we speculated that calcitriol also controls CENP-N expression. Consistent with our hypothesis, CENP-N expression decreased after treatment with calcitriol. In addition to our CENP-N knockdown experiments, calcitriol also had an anticancer effect on OSCCs by

cell-cycle arrest at the G1 phase with up-regulation of p21<sup>Cip1</sup> and p27<sup>Kip1</sup>, and down-regulation of cyclin D1, CDK2, and CDK4 (Fig. 4D). Therefore, these data suggested that CENP-N is an important target for cancer treatment using calcitriol.

In conclusion, CENP-N is a useful biomarker involved in the proliferation of OSCCs, and calcitriol might be a novel therapeutic drug for OSCCs through regulation of CENP-N.

### Abbreviations

CENP-N: Centromere protein N; OSCC: oral squamous cell carcinoma; CENP-A: centromere protein A; VDR: vitamin D receptor.



**Fig. 4.** Effect of calcitriol treatment. (A) To assess the effect of calcitriol on cellular proliferation, the calcitriol-treated cells and control cells were counted on 7 consecutive days. The results are expressed as the mean  $\pm$  standard error of the mean (SEM) of values from three assays. Cellular growth is inhibited significantly ( $*p < 0.05$ , Student's t-test) after 144 h culture in the calcitriol-treated cells. (B) Flow cytometric analysis shows the percentage of G1 phase in calcitriol-treated cells is increased compared with control cells. (C) Immunoblot analysis shows up-regulation of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> and down-regulation of cyclin D1, CDK2, and CDK4 in the calcitriol-treated cells compared with the control cells. (D) Schematic representation of the cellular proliferation pathway by CENP-N. CENP-N silencing and calcitriol-treatment are involved in G1 phase-related genes, leading to delay of cellular proliferation.

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## Competing Interests

The authors have declared that no competing interest exists.

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