

# Expression and regulatory effects on cancer cell behavior of NELL1 and NELL2 in human renal cell carcinoma

Ritsuko Nakamura,<sup>1</sup> Takeru Oyama,<sup>1</sup> Ryosuke Tajiri,<sup>1</sup> Atsushi Mizokami,<sup>2</sup> Mikio Namiki,<sup>2</sup> Masaru Nakamoto<sup>3</sup> and Akishi Ooi<sup>1</sup>

<sup>1</sup>Department of Molecular and Cellular Pathology, Kanazawa University Graduate School of Medical Science, Kanazawa; <sup>2</sup>Integrative Cancer Therapy and Urology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan; <sup>3</sup>Aberdeen Developmental Biology Group, School of Medical Sciences, University of Aberdeen, Aberdeen, UK

## Key words

Cell migration, methylation, NELL1, NELL2, renal cell carcinoma

## Correspondence

Ritsuko Nakamura, Department of Molecular and Cellular Pathology, Kanazawa University Graduate School of Medical Science, 13-1 Takaramachi, Kanazawa 920-8640, Japan.

Tel: +81-76-265-2193; Fax: +81-76-234-4228;

E-mail: [rnakamura@staff.kanazawa-u.ac.jp](mailto:rnakamura@staff.kanazawa-u.ac.jp)

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Neural epidermal growth factor-like like (NELL) 1 and 2 constitute a family of multimeric and multimodular extracellular glycoproteins. Although the osteogenic effects of NELL1 and functions of NELL2 in neural development have been reported, their expression and functions in cancer are largely unknown. In this study, we examined expression of NELL1 and NELL2 in renal cell carcinoma (RCC) using clinical specimens and cell lines. We show that, whereas NELL1 and NELL2 proteins are strongly expressed in renal tubules in non-cancerous areas of RCC specimens, their expression is significantly downregulated in cancerous areas. Silencing of *NELL1* and *NELL2* mRNA expression was also detected in RCC cell lines. Analysis of *NELL1/2* promoter methylation status indicated that the CpG islands in the *NELL1* and *NELL2* genes are hypermethylated in RCC cell lines. NELL1 and NELL2 bind to RCC cells, suggesting that these cells express a receptor for NELL1 and NELL2 that can transduce signals. Furthermore, we found that both NELL1 and NELL2 inhibit RCC cell migration, and NELL1 further inhibits RCC cell adhesion. These results suggest that silencing of NELL gene expression by promoter hypermethylation plays roles in RCC progression by affecting cancer cell behavior.

**K**idney cancers, including renal cell carcinoma (RCC) and urothelial carcinoma of the renal pelvis and ureter, account for approximately 3% of all cancer incidences. Although mortality rates of kidney cancers are stabilizing, the number of new cases is increasing in the USA.<sup>(1)</sup> Kidney cancers are classified according to their histological type and origin. Cancers originating from kidney epithelia are called RCC. The most common subtype is clear cell RCC (CCRCC), which derives its histological appearance from intracellular accumulations of glycogen and lipid. The risk factors and molecular mechanisms of renal carcinogenesis have been extensively studied. Loss of heterozygosity or mutations of the von Hippel-Lindau (*VHL*) tumor suppressor gene and the mesenchymal–epithelial transition (*c-MET*) proto-oncogene, which are responsible for inherited tumor syndromes involving the kidney, have been found in sporadic RCC.<sup>(2,3)</sup> Epigenetic inactivation by DNA methylation was shown to cause downregulation of the mRNA expression of RAS-associated domain family-1A (*RASSF1A*), a tumor suppressor gene, in RCC.<sup>(4,5)</sup> However, the mechanisms of RCC development and progression remain unclear.

Neural epidermal growth factor-like (Nel) is a multimeric and multimodular extracellular glycoprotein with significant structural similarity to thrombospondin-1. Whereas Nel was

originally identified in chickens,<sup>(6)</sup> two related genes, *Nel*-like1 (*NELL1*) and *Nel*-like2 (*NELL2*), were subsequently described in mammals, including humans.<sup>(7)</sup> Based on sequence similarities, mammalian *NELL2* appears to be the ortholog of chicken *Nel*. Human *NELL1* and *NELL2* have approximately 55% homology at the amino acid level. The human *NELL1* and *NELL2* genes were mapped to chromosomes 11p15.1 and 12q12, respectively.

Previous studies showed that NELL1 plays important roles in osteogenic differentiation.<sup>(8,9)</sup> NELL1 is overexpressed in patients with unilateral coronal synostosis,<sup>(8)</sup> which is a common form of craniosynostosis. NELL1 also induces bone regeneration in calvarial defects<sup>(10)</sup> and in a rat femoral distraction model.<sup>(11)</sup>

*Nel*-like2 is predominantly expressed in the nervous system and has been implicated in neural development.<sup>(7,12–14)</sup> Recently, we showed that chicken *Nel* (*NELL2*) regulates retinal axon guidance<sup>(15)</sup> and production of retinal ganglion cells during development.<sup>(16)</sup>

Expression of *NELL1* and *NELL2* genes has been studied in human lymphoma, in cancers of the central nervous system, prostate, and bladder, and in cancer cell lines.<sup>(17–20)</sup> Furthermore, *NELL1* and *NELL2* expression was reported in adult and embryonic normal kidney.<sup>(6,7,17)</sup> However, little is known

about the expression and functions of NELL1 and NELL2 in RCC. Here we show that, whereas NELL1 and NELL2 are strongly expressed in non-cancerous renal tubules, their expression is downregulated in cancerous areas of CCRCC specimens and in RCC cell lines. In addition, the CpG islands in the *NELL1* and *NELL2* promoter regions are hypermethylated in RCC cells. *In vitro*, NELL1 suppresses the migration and adhesion of RCC cells, and NELL2 suppresses RCC cell migration. These results suggest that NELL protein expression is downregulated in RCC, presumably by promoter hypermethylation, and that lack of NELL expression may contribute to RCC progression by altered regulation of cancer cell behavior.

## Materials and Methods

**Clinical samples.** Formalin-fixed paraffin-embedded CCRCC tissues were obtained from surgical pathology files (between 2010 and 2012) of the Pathology Section of Kanazawa University Hospital (Kanazawa, Japan). This project was approved by the research ethics committee on genetic analysis of Kanazawa University (approval no. 320).

**Immunohistochemistry.** Four-micron sections of CCRCC samples were analyzed by immunohistochemistry (IHC) using anti-NELL1 and anti-NELL2 antibodies (HPA051535, 1/100 and HPA035715, 1/300 dilutions; Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol, except that antigen retrieval was carried out at 121°C for 15 min. The LSAB2 kit Universal (Dako, Glostrup, Denmark) was used for detection. NELL1 and NELL2 expression was scored for both cancerous and non-cancerous areas on a graded 0 (no expression) to +3 (very strong expression) scale. Immunohistochemistry scores were calculated by subtracting the scores of cancerous areas from those of adjacent non-cancerous areas. Therefore, positive IHC scores indicated downregulation of NELL expression in cancer cells, whereas negative scores indicated upregulation.

**Cell lines.** Renal cell carcinoma (OS-RC-2, TUHR14TKB, and VMRC-RCW) and HEK293T cell lines were obtained from the Riken Cell Bank (Tsukuba, Japan). Renal cell carcinoma and HEK293T cells were cultured in RPMI and DMEM, respectively, containing 10% FBS and penicillin/streptomycin.

**Quantitative RT-PCR, methylation-specific PCR, and bisulfite sequencing PCR.** Quantitative RT-PCR (qPCR) was carried out with first-strand cDNAs of the cell lines and SYBR PremixEx TaqII (Takara Bio, Otsu, Japan) using the Viia7 system (Life Technologies, Carlsbad, CA, USA). Unmethylated cytosines in the genomic DNAs from the cell lines were converted to uracil

by bisulfite reactions using the EZ DNA Methylation-Gold Kit (ZYMO Research, Irvine, CA, USA). Methylation-specific PCR (MSP) was carried out using the TaKaRa EpiScope MSP Kit. Bisulfite sequencing PCR (BSP) was carried out using the bisulfite-treated genomic DNAs and EpiTaq HS (Takara). The PCR products were inserted into the pGEM-T easy vector (Promega KK, Tokyo, Japan) and transformed into DH5 $\alpha$ , and plasmids isolated from 10 colonies of each sample were sequenced. The primer sequences and PCR conditions used are shown in Table 1.

**5-Azacytidine treatment of RCC cell lines.** The OS-RC-2, VMRC-RCW, and TUHR14TKB cells were plated in 100-mm plates on day 0. The cells were treated with 5  $\mu$ M 5-azacytidine (Sigma-Aldrich) for 5 days. Total RNA extraction, cDNA synthesis and qPCR were carried out as described above.

**Luciferase assay.** *NELL1* and *NELL2* putative promoter regions were ligated into pCpGL-basic<sup>(21)</sup> (kindly provided by Prof. Michael Rehli, University Hospital Regensburg, Regensburg, Germany). *NELL1* and *NELL2* transcriptional factors, RUNX2<sup>(22)</sup> and E2F1<sup>(23)</sup> were kindly provided by Prof. Yoshiaki Ito (National University of Singapore, Singapore) (pEF-BOS-RUNX2)<sup>(24)</sup> and Prof. Kristian Helin (University of Copenhagen, BRIC, Copenhagen, Denmark, E2F1/pCMV-HA)<sup>(25)</sup> Addgene plasmid #24225). *RUNX2* was amplified from pEF-BOS-RUNX2 and inserted into pCMV-HA-N (Takara Bio). After incubation with/without methyltransferases, NELL1/pCpGL and NELL2/pCpGL were transfected with their transcriptional factors and pGL 4.74 into HEK293T cells. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega KK).

**Preparation of alkaline phosphatase fusion proteins.** NELL1 protein fused to an alkaline phosphatase (AP) tag (NELL1-AP), NELL2-AP, and AP proteins were prepared and purified as previously described.<sup>(26)</sup>

**Cell migration assay.** The cell migration assay was carried out basically as previously described.<sup>(27)</sup> The lower side of the Transwell membrane inserts (product #3422; Corning, Corning, NY, USA) was coated with 40 nM or 400 nM AP, NELL1-AP, or NELL2-AP protein at 4°C overnight, blocked with 20 mg/mL BSA in PBS for 3 h at 4°C, and then washed with RPMI-1640. OS-RC-2 or VMRC-RCW cells ( $1 \times 10^5$  cells) were dissociated with 1 mM EDTA and were seeded inside the Transwell. The cells were then allowed to migrate to the lower side of the membrane in RPMI-1640 with 1% FBS at 37°C overnight, fixed with 4% paraformaldehyde, and then stained with hematoxylin. The upper side of the membrane was wiped with a cotton swab, and the cells that had migrated to the lower side of the membrane were quantified. For each Transwell membrane, at least three

**Table 1.** Primer sequences and PCR conditions

Gene	PCR	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Annealing temperature	PCR cycles
<i>NELL1</i>	Quantitative RT-PCR	ATGGCCAGGTGTGGACCTTG	TCTGGTGTACATTCTGGGC	60	40
<i>NELL2</i>	Quantitative RT-PCR	CCACTTAAGTCGGCTCTTGC	ATGGTCTGGTCTTGCCTC	60	40
<i>GAPDH</i>	Quantitative RT-PCR	GTCTCCTCTGACTTCAACAGCG	ACCACCTGTGCTGTAGCC	60	40
<i>NELL1</i>	MSP-Methyl.	GATTCGATTTCGGTGTTC	CTACGTTCCGTAAAAACCCG	60	35
<i>NELL2</i>	MSP-Methyl.	CGTTTTATTGTTGGTAGGGGGC	TTCCTACCGAAAAACGAAA	60	35
<i>NELL1</i>	MSP-Unmethyl.	TGGGATTTTGATTTTGGTGTTC	ACTACATTCATCTAAAAACCCAAAC	55	40
<i>NELL2</i>	MSP-Unmethyl.	TTATGTTTTATTGTTGGTAGGGGGT	AATTCCTACCAAAAAACAAAAC	63	35
<i>NELL1</i>	Bisulfite sequencing	TTATAGGGGTTAATAGGAGAGAGG	CCTAACTTAAACCR*CCAACTTACTAA	55	35
<i>NELL2</i>	Bisulfite sequencing	TGTTTTTGTGTATGTTGGTTT	AAAAAAAACCTCCCAAC	55	35

\*R(A+G). Methyl., methylated; MSP, methylation-specific PCR; Unmethyl., unmethylated.

high-power fields were examined under a microscope. Each experiment was repeated three times.

**Cell adhesion assay.** Ninety-six-well cell culture plates were coated with AP, NELL1-AP, or NELL2-AP protein (400 nM each) at 4°C overnight. After blocking with 10 mg/mL BSA, the wells were washed twice with PBS, and OS-RC-2 or VMRC-RCW cells were then plated at a density of  $2.5 \times 10^4$  cells/well. The cells were cultured at 37°C for 3 h in RPMI-1640 with 2% FBS, washed twice with PBS, and then fixed with 4% paraformaldehyde. Cell numbers in three high-power fields per well were counted using an inverted microscope. Experiments were repeated under the same conditions at least three times.

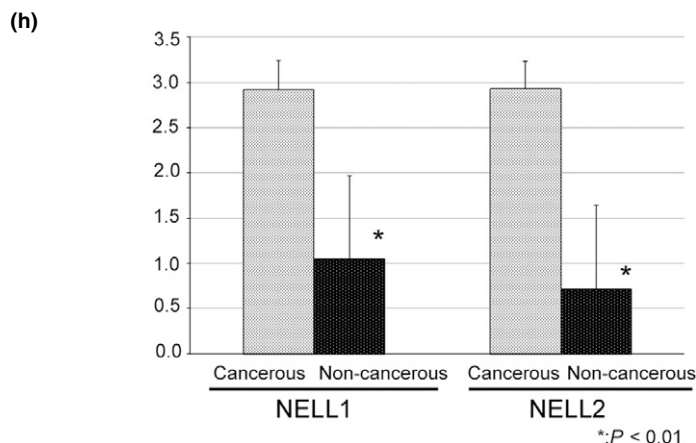
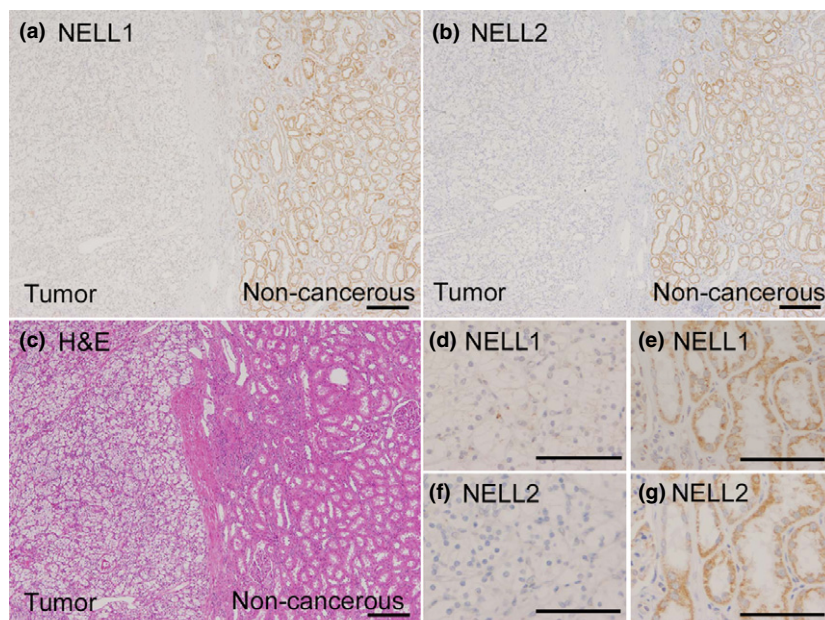
**Statistical analysis.** For statistical analysis, ANOVA (for IHC scores with more than three parameters) and Student's *t*-test (for IHC scores with two parameters, and cell migration and adhesion assays) were used. Supplementary information is available in Document S1.

## Results

**NELL1 and NELL2 protein expression is downregulated in CCRCC.** We first examined and compared NELL1 and NELL2

protein expression in cancerous regions and adjacent non-cancerous areas in tissue samples of 64 CCRCC patients. Both NELL1 and NELL2 proteins were abundantly expressed in renal tubules in non-cancerous regions (Fig. 1). At higher magnification (Fig. 1e,g), NELL1 and NELL2 proteins were observed to be localized in granular cytoplasmic patterns, consistent with the fact that NELL1 and NELL2 are secreted glycoproteins and thus processed in the secretory pathway. In contrast, NELL1 and NELL2 expression was significantly lower in cancerous areas (IHC scores, 1.88 (NELL1) and 2.17 (NELL2); expression grade of cancerous area/non-cancerous area, 1.05/2.09 (NELL1,  $P < 0.01$ ) and 0.72/2.93 (NELL2,  $P < 0.01$ ); Fig. 1h). No significant correlations were observed between the degree of NELL downregulation and clinicopathological parameters, including sex or age of patients, or vascular involvement or grade of tumors (Table 2).

**Downregulation of NELL1 and NELL2 expression in RCC cell lines.** We next examined and compared *NELL1* and *NELL2* mRNA and protein expression in RCC cell lines with that in the HEK293T cell line derived from human embryonic kidney using qRT-PCR and Western blot. We found by RT-PCR that *NELL1* and *NELL2* mRNA expression levels in the cancer cell lines were 10–1000 times lower than those in HEK293T cells



**Fig. 1.** Protein expression of neural epidermal growth factor-like like 1 (NELL1) and NELL2 in clear cell renal cell carcinoma (CCRCC) tissues. (a, b, d–g) Formalin-fixed paraffin-embedded sections of CCRCC tissues were immunostained with anti-NELL1 (a, d, e) or anti-NELL2 (b, f, g) antibody. Images (d, e) and (f, g) are higher magnification views of (a) and (b), respectively. Both NELL1 and NELL2 were detected in the cytoplasm of renal tubule cells in non-cancerous (a, b, right side [non-cancerous]), but not in cancerous areas (a, b, left side [tumor]). (c) H&E stain. Scale bar = 200  $\mu$ m (a–c), 100  $\mu$ m (d–g). (h) Average NELL1 and NELL2 expression grades in the cancerous and non-cancerous areas.

**Table 2.** Correlation between neural epidermal growth factor-like like 1 (NELL1) and NELL2 subtraction scores (immunohistochemistry [IHC] score) and clinicopathological parameters

	No. of cases	Average of NELL1 IHC scores	P-value	Average of NELL2 IHC scores	P-value
Overall	64	1.88		2.17	
Sex					
Male	43	1.84	0.71†	2.16	0.93†
Female	21	1.95		2.19	
Age, years					
<60	26	1.65	0.19†	1.88	0.10†
≥60	38	2.03		2.37	
Grade					
G1	20	2.10	0.18‡	2.30	0.46‡
G2	43	1.74		2.09	
G3	1	3.00		3.00	
Vascular involvement					
v0	42	1.86	0.86†	2.19	0.86†
v1	22	1.90		2.14	
pT					
pT1	47	1.83	0.85§	2.11	0.69§
pT2	4	2.00		2.50	
pT3	13	2.00		2.31	
pT4	0				

†Student's *t*-test was carried out. ‡More than three parameters, however only two parameters had more than three samples. Student's *t*-test was carried out between G1 and G2. §ANOVA was carried out.

(Fig. 2). Consistent with this finding, the Western blot analysis showed that the protein expression of NELL1 and NELL2 was decreased in the RCC cells compared to that observed in HEK293 cells (Fig. S1). The combined results indicate that NELL1 and NELL2 expression is downregulated in RCC at both the mRNA and protein levels.

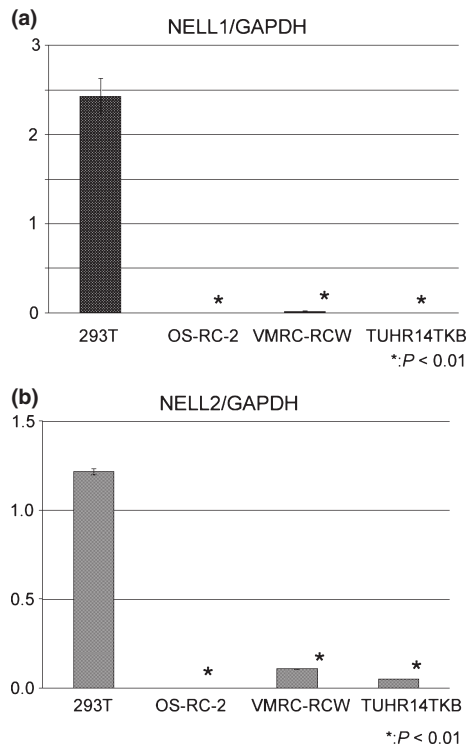
**Promoter hypermethylation of NELL1 and NELL2 genes in RCC.** Aberrant DNA hypermethylation of promoter CpG islands with associated gene silencing plays crucial roles in tumor progression. Because hypermethylation of the *NELL1* 5'-UTR has been reported in esophageal adenocarcinoma and colorectal cancer,<sup>(28,29)</sup> we speculated that the downregulation of the *NELL1* and *NELL2* genes observed in RCC may be caused by promoter hypermethylation in those genes. To test this hypothesis, the methylation status of putative promoter CpG islands of *NELL1* and *NELL2* genes in RCC cell lines was examined using MSP. *NELL1* gene promoter hypermethylation was detected in VMRC-RCW and TUHR14TKB cells. *NELL2* promoter hypermethylation was detected in VMRC-RCW cells, and weak hypermethylation was observed in OS-RC-2 cells. No significant methylation was detected in the *NELL1* or *NELL2* promoter regions in control HEK293 cells (Fig. 3).

To further investigate promoter methylation status, we carried out BSP of the CpG islands that included the putative promoter regions of *NELL1* (-690 to -100) and *NELL2* (-320 to +678) in RCC cell lines (Fig. 4). We numbered the CpG islands in these regions in the 5' to 3' direction (*NELL1*, Nos. 1-78; *NELL2*, Nos. 1-86). The analyses of the *NELL1* promoter detected CpG island hypermethylation throughout the analyzed region in VMRC-RCW cells, high methylation of CpG islands around No. 20 and No. 45 in OS-RC2 cells, and frequent methylation of CpG islands from No. 1 to No. 30 in TUHR14TKB cells (Fig. 4b). The analyses of the *NELL2* promoter region detected methylation of CpG islands around No. 10 in both OS-RC2 and VMRC-RCW cells (Fig. 4d). The

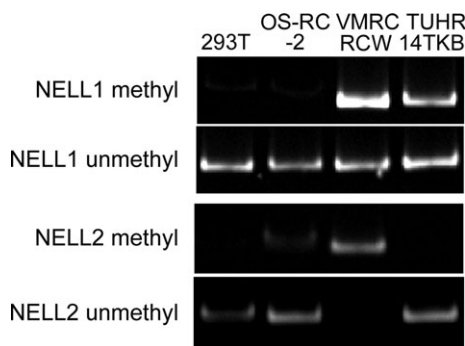
analyses of the *NELL2* promoter region showed methylation of CpG islands around No. 10 in both the OS-RC2 and VMRC-RCW cells and around No. 80 in the OS-RC-2 cells (Fig. 4d). No significant CpG island hypermethylation was detected at the *NELL1/2* promoter regions in the HEK293T cells.

If the *NELL1/2* downregulation observed in RCC cells is caused by promoter hypermethylation, the expression would be recovered by CpG demethylation. In order to test this hypothesis, we treated RCC cell lines with 5 μM 5-azacytidine and examined the effects on the *NELL1/2* mRNA expression. As shown in Figure 4(e), the *NELL1* expression was elevated in VMRC-RCW cells. The *NELL1* expression in TUHR14TKB cells was also significantly increased after 5-azacytidine treatment. The *NELL2* expression in OS-RC-2 cells was elevated by 5-azacytidine treatment (Fig. 4f). A slight increase in *NELL2* expression was observed in VMRC-RCW cells, although the difference was not statistically significant ( $P = 0.071$ ). Whereas the increases of *NELL1* and *NELL2* expression by 5-azacytidine were statistically significant in most of the above RCC cell lines, their expression levels after treatment were still lower than those in HEK293T (Figs 2,4e,f), suggesting that promoter methylation partially contributes to the silencing of *NELL* expression.

In order to confirm the effects of CpG methylation on *NELL1/2* expression, we carried out a luciferase assay using the putative promoter regions of *NELL1* and *NELL2*. Consequently, reporter constructs containing unmethylated *NELL1* and *NELL2* promoter regions showed high luciferase activity. In contrast, the luciferase activity was significantly reduced by treatment with M.SssI, which methylates all cytosine residues within the dinucleotide recognition sequence 5'-CpG-3'. Treatment with HpaII, which methylates the second cytosine residue in 5'-CCGG-3', or HhaI, which methylates the first cytosine residue in 5'-GCGC-3', resulted in partial reduction of the luciferase activity (Fig. 4g,h).



**Fig. 2.** mRNA expression of *NELL1* and *NELL2* in renal cell carcinoma cell lines. *NELL1* (a) and *NELL2* (b) mRNA expression levels in renal cell carcinoma cell lines OS-RC-2, VMRC-RCW, and TUHR14TKB and control HEK293T cells were analyzed using quantitative RT-PCR. Expression levels are quantified as *NELL1* or *NELL2*/*GAPDH* and plotted as means  $\pm$  SD.



**Fig. 3.** Methylation-specific PCR analysis of the *NELL* CpG islands in renal cell carcinoma cells. Genomic DNA was analyzed by PCR using specific primers for methylated (Methyl) or unmethylated (Unmethyl) CpG islands in *NELL1* or *NELL2* putative promoter regions (*NELL1* methylated, *NELL1* unmethylated, *NELL2* methylated, and *NELL2* unmethylated).

These data indicate that the CpG island of *NELL1* and *NELL2* promoter regions is hypermethylated in RCC, and suggest that downregulation of *NELL1* and *NELL2* expression in RCC was caused, at least in part, by promoter hypermethylation.

***NELL1* and *NELL2* bind to RCC cells.** The above data on *NELL1* and *NELL2* expression may suggest that downregulation of *NELL1* and *NELL2* gene expression is involved in the

development of RCC. If that is the case, it is likely that *NELL1* and *NELL2* proteins exert their functions by binding to a cell surface receptor and transducing intracellular signals. We therefore examined whether *NELL* proteins can bind to RCC cell lines using *NELL1*-AP or *NELL2*-AP as a probe (Fig. S2a). We incubated RCC and HEK293T cells with *NELL1*-AP or *NELL2*-AP, and binding was detected using the AP enzyme reaction.<sup>(30)</sup> Significant binding activity was detected in all cell lines tested, including HEK293T cells (Fig. S2b). These results indicate that *NELL1* and *NELL2* can bind to RCC cells, and suggest that *NELL1* and *NELL2* can be recognized through a specific receptor expressed on those cells.

**Effects of *NELL* proteins on RCC cell behavior.** We previously showed that chicken Nel (*NELL2*) can act as an inhibitory axon guidance molecule and regulate morphology of the growth cone.<sup>(15)</sup> As many axon guidance molecules also regulate behavior of the cell body, we examined whether *NELL* proteins can affect the migration and adhesion of RCC cells. We first tested the effects of *NELL1* and *NELL2* on cell migration by using a transfilter assay system. *NELL1*-AP significantly inhibited the migration of OS-RC-2 and VMRC-RCW cells in a dose-dependent manner. *NELL2*-AP also significantly inhibited the migration of OS-RC-2, but not that of VMRC-RCW cells (Fig. 5a–d).

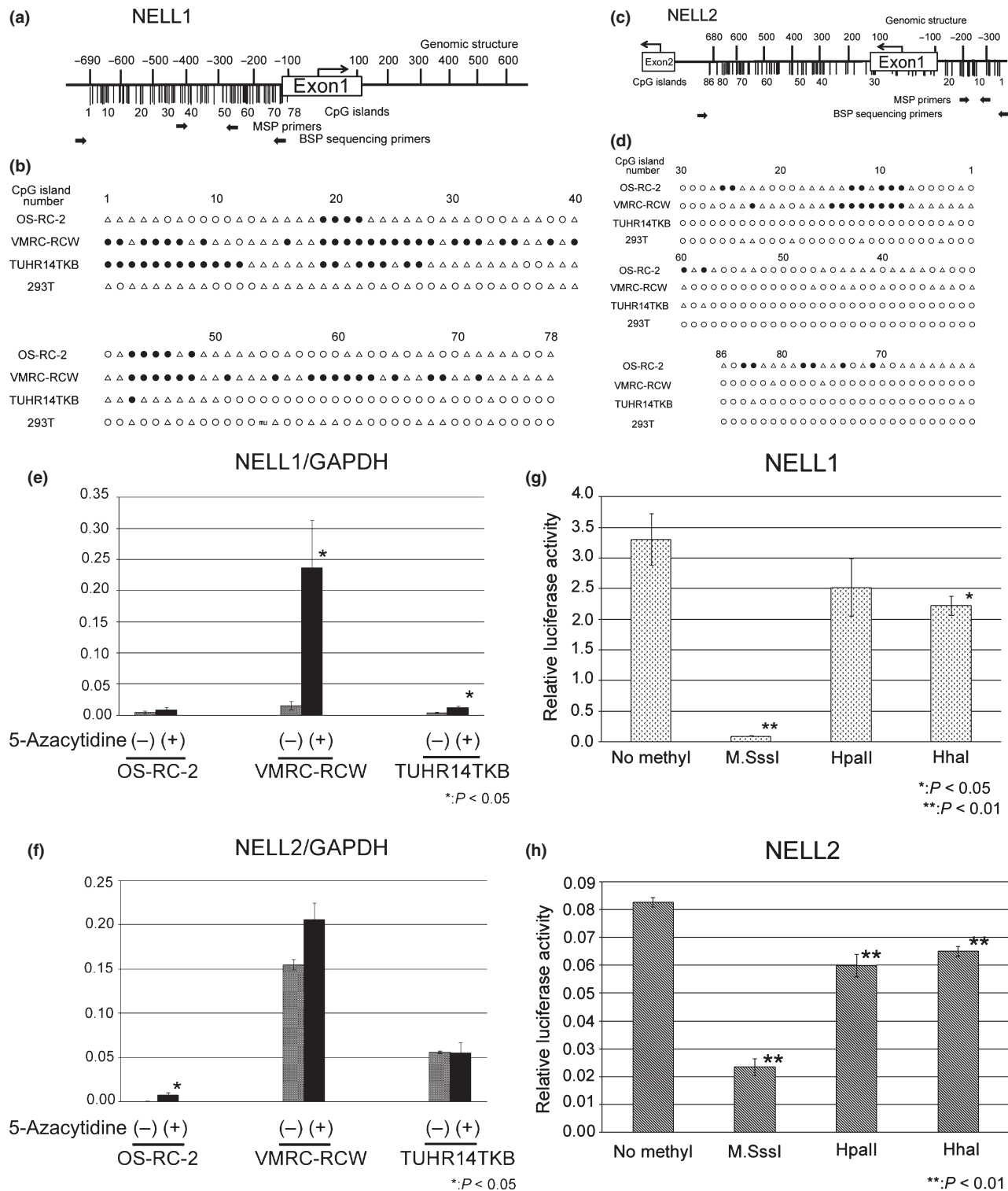
We next examined whether *NELL1* or *NELL2* proteins affect cell adhesion using *NELL*-AP protein-coated dishes. Significant numbers of OS-RC-2 and VMRC-RCW cells adhered to the AP-coated control dish. In contrast, the number of adherent cells on the *NELL1*-AP substratum was dramatically decreased. *NELL2*-AP did not affect adhesion of the RCC cells (Fig. 5e–l). The combined results indicate that *NELL1* and *NELL2* can regulate RCC cell behavior.

## Discussion

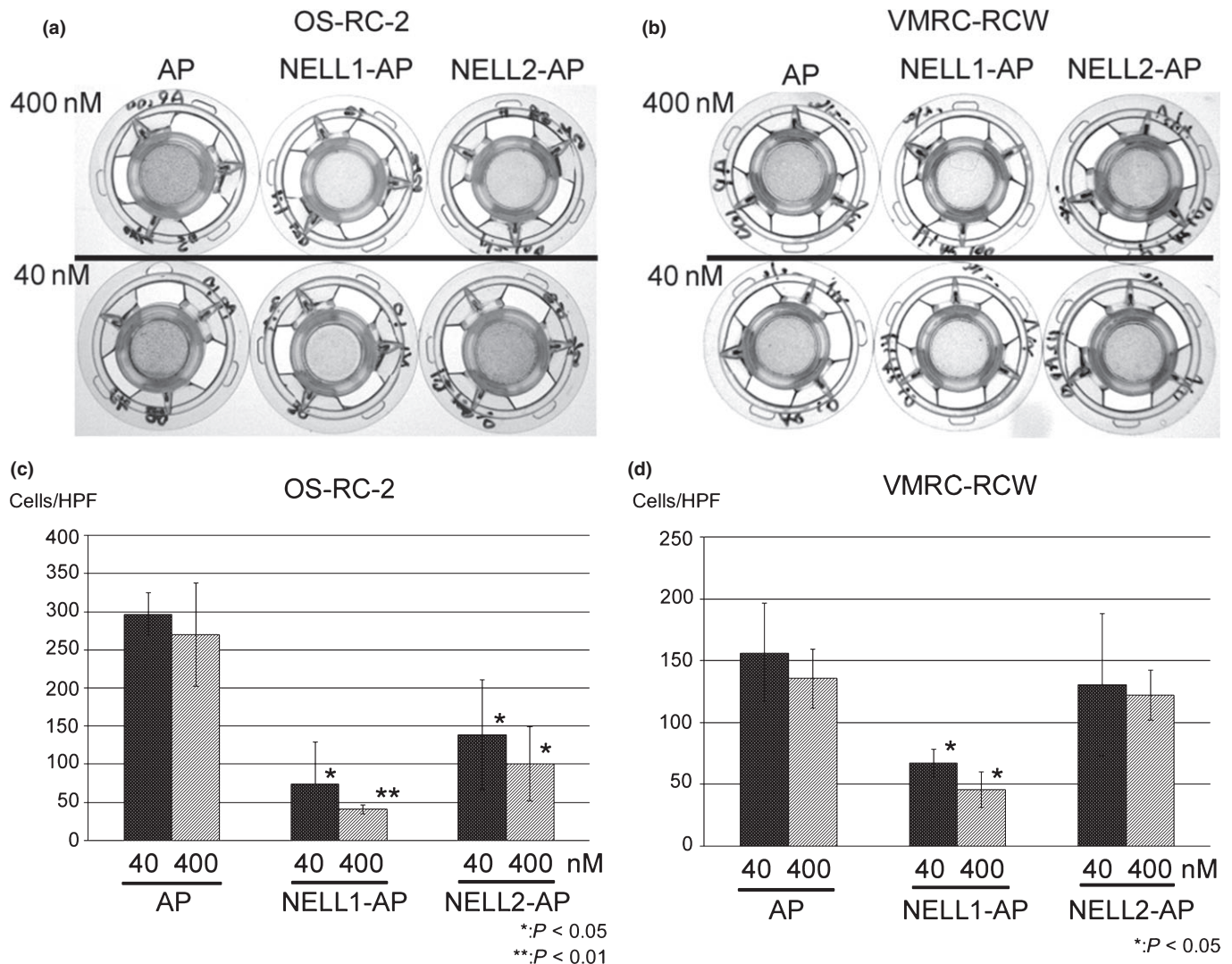
In the present study, we analyzed the expression, genomic DNA methylation, and functions of *NELL1* and *NELL2* in RCC. Immunohistochemical studies of clinical samples showed that, whereas *NELL1* and *NELL2* are strongly expressed in non-cancerous renal tubules, their expression is significantly downregulated in CCRCC areas (Fig. 1). Consistent with the results of clinical samples, *NELL1* and *NELL2* mRNA and protein expression was also reduced in RCC cell lines compared with that in HEK293T cells, which are derived from non-cancerous kidney (Figs 2, S1).

*NELL1* and *NELL2* expression has been previously reported in other cancers using clinical samples and cell lines. For example, *NELL1* gene loss was observed in more than 40% of Hodgkin's lymphoma patients.<sup>(20)</sup> *NELL2* mRNA expression is upregulated in benign prostate hyperplasia and prostate cancer.<sup>(18)</sup> *NELL2* is also considered as a candidate biomarker for bladder cancer.<sup>(19)</sup> Among nervous system tumors, *NELL1* and *NELL2* are predominantly expressed in neuroblastoma cell lines and are also expressed in medulloblastoma, central neurocytoma, and some astrocytic tumors.<sup>(17)</sup> A recent study showed that posterior fossa ependymoma is composed of two molecularly distinct groups, and that *NELL2* is the most significant marker for the group that represents less invasive and less metastatic tumors with better prognosis.<sup>(31)</sup> These results suggest that *NELL* may be involved in cancer development.

Our studies of DNA methylation status showed that the CpG islands in the *NELL1* and *NELL2* promoters are hypermethylated in RCC cells, whereas no significant *NELL1/2* promoter hypermethylation was detected in control HEK293 cells. The



**Fig. 4.** Hypermethylation of CpG islands in the putative promoter region of the *NELL1* and *NELL2* genes. (a, c) CpG islands (vertical bars) in the putative promoter regions of *NELL1* (a) and *NELL2* (c) genes are numbered in the 5' to 3' direction (*NELL1*, Nos. 1–78; *NELL2*, Nos. 1–86). Positions of primer sequences for methylation-specific PCR (MSP) and bisulfide sequencing PCR (BSP) are also shown. (b, d) Methylation status of CpG islands. ●, Methylated CpG islands (methylation detected in more than 8 of 10 clones); ○, unmethylated islands (methylation not detected in any of 10 clones); △, incompletely methylated CpG islands (methylation detected in one to seven clones). \*This nucleotide in HEK293T differed from the registered sequence in the database. (e, f) *NELL1* and *NELL2* mRNA expression levels in OS-RC-2, VMRC-RCW and TUHR14TKB cells with/without 5-azacytidine treatment. The *NELL1*, *NELL2* and *GAPDH* mRNA expression levels were measured using quantitative PCR. *NELL1* and *NELL2* mRNA expression levels were normalized by that of *GAPDH* and plotted as the mean ± SD. (g, h) Effects of promoter methylation on *NELL1* and *NELL2* expression. Firefly luciferase reporter constructs containing putative promoter regions of *NELL1* and *NELL2* genes (*NELL1*/pCpGL and *NELL2*/pCpGL) were treated with M.SssI, HpaII, or HhaI methyltransferases and then transfected into HEK293T cells with RUNX2/pCMVHA (for *NELL1*/pCpGL) or E2F1/pCMVHA (for *NELL2*/pCpGL) and the *Renilla* luciferase expression vector pGL 4.74. The ratio of the firefly/*Renilla* luciferase activity was plotted as the mean ± SD.



**Fig. 5.** Effect of neural epidermal growth factor-like like 1 (NELL1) and NELL2 on renal cell carcinoma cell migration and adhesion. (a–d) Transwell cell migration assay. The bottom side of the Transwell filter was coated with 40 nM or 400 nM alkaline phosphatase (AP), NELL1-AP or NELL2-AP. OS-RC-2 (a) or VMRC-RCW (b) cells were placed inside the Transwell chamber and allowed to migrate to the bottom side of the filter overnight. The cells that migrated to the bottom side of the filter were stained with hematoxylin and photographed. (c, d) Quantification of cell migration. The numbers of cells that migrated to the bottom side of the filter were plotted as mean  $\pm$  SD. (e–l) Cell adhesion assay. OS-RC-2 (left three wells of e, and f, h, j) and VMRC-RCW (right three wells of e, and g, i, k) cells were seeded on AP (e, f, g), NELL1-AP (e, h, i), or NELL2-AP (e, j, k) coated dishes (400 nM each). Low (e) and high (f–k) power views after cell adhesion assay. Adherent cells were stained with hematoxylin. Adherent cell numbers were quantified and plotted as means  $\pm$  SD (l). HPF, high-power field.

degree of promoter hypermethylation and effect of 5-azacytidine treatment varied between RCC cell lines. In the RCC cell lines in which promoter hypermethylation was particularly prominent (*NELL1* in VMRC-RCW and TUHR14TKB cells, *NELL2* in OS-RC-2 cells), the *NELL* mRNA expression was significantly recovered by 5-azacytidine treatment (Fig. 4e,f). In contrast, recovery of *NELL2* expression was not statistically significant in VMRC-RCW and TUHR14TKB cells. Interestingly, CpG islands around No. 80 in the *NELL2* promoter were methylated only in OS-RC-2, but not in VMRC-RCW or TUHR14TKB cells (Fig. 4d). In addition, *NELL2* expression levels without treatment were much lower in OS-RC-2 cells than those in VMRC-RCW or TUHR14TKB cells. These results suggest that CpG methylation around No. 80 may be important for *NELL2* downregulation, although it is

plausible that other factors are also involved in *NELL2* downregulation in OS-RC-2 cells, because the *NELL2* expression level restored by 5-azacytidine treatment was still lower than those in untreated VMRC-RCW, TUHR14TKB, and HEK293T cells.

DNA methylation of gene promoters is an important mechanism of transcriptional downregulation of gene expression. In cancer cells, many tumor suppressor genes have been shown to be silenced by promoter hypermethylation, including p16/CDKN2/MTS1,<sup>(32)</sup> retinoblastoma gene,<sup>(33)</sup> and EphA7.<sup>(34)</sup> In RCC, transcriptional silencing by promoter hypermethylation was reported for several tumor-suppressor genes, including *VHL*<sup>(35)</sup> and *RASSF1A*,<sup>(36)</sup> and differentially expressed in adenocarcinoma of the lung (*DAL-1/4.1B*).<sup>(37)</sup> DNA methylation profiling of 10 tumor suppressor genes in

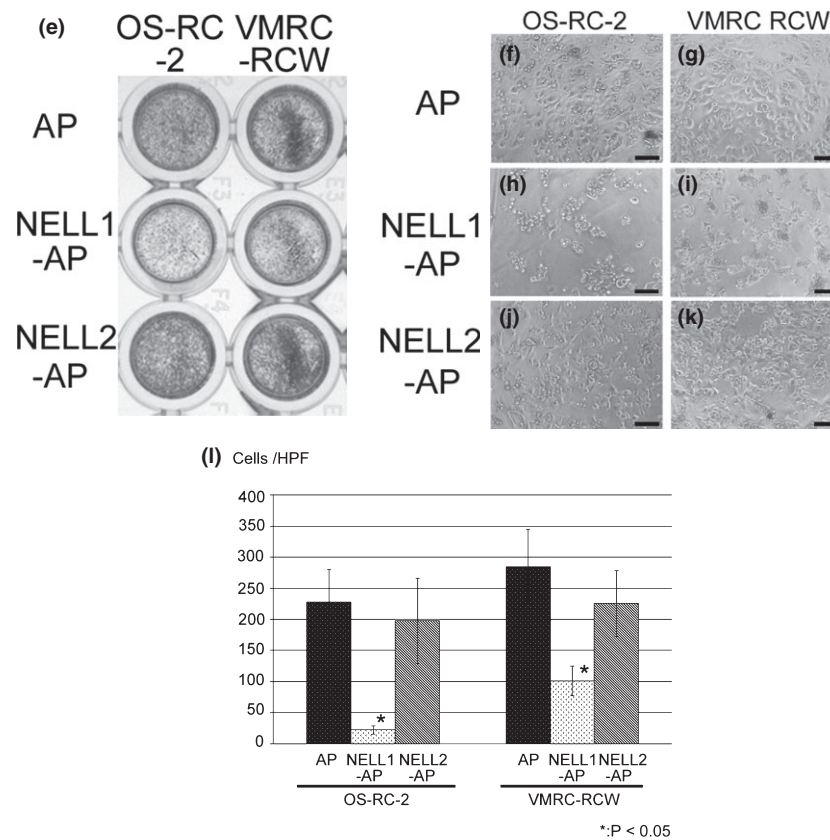


Fig. 5. continued.

100 kidney cancers showed hypermethylation of promoter DNA in 93% of kidney tumor cases, approximately two-thirds of which have two or more hypermethylated genes.<sup>(38)</sup>

Promoter hypermethylation and silencing of the *NELL1* gene were detected in approximately 44% of colorectal carcinomas.<sup>(29)</sup> In esophageal cancer, methylation levels of the *NELL1* gene are significantly higher in Barrett's metaplasia, dysplasia in Barrett's esophagus, and esophageal adenocarcinoma than those in normal esophagus, and inversely correlate with patient survival rates. These findings indicate that promoter hypermethylation of *NELL1* is a common event that occurs at early stages of Barrett's-associated esophageal cancer progression and is a potential biomarker of poor prognosis in early stage esophageal adenocarcinoma.<sup>(28)</sup>

*In vitro* assays of cell behavior in the present study indicated that both *NELL1* and *NELL2* inhibit RCC cell migration and that *NELL1* further inhibits RCC cell adhesion. As regulation of cell migration and adhesion plays crucial roles in cancer invasion and metastasis, these results raise the possibility that loss of *NELL* expression in RCC is involved in cancer progression. Although no significant correlation was found between *NELL* expression levels and the size or extent of the primary tumor (pT stages), our data show that hypermethylation and downregulation of the *NELL* genes already occurs at early stages of RCC progression. These findings are consistent with esophageal cancer data,<sup>(28)</sup> and suggest that decreased *NELL* expression plays roles in early oncogenesis.

Our results showing that *NELL1* and *NELL2* bind to RCC cells suggest that these molecules regulate the behavior of can-

cer cells by binding to a cell surface receptor and transducing intracellular signals. We also previously reported that chicken *Nel* (*NELL2*) can bind to and regulate the behavior of retinal axons.<sup>(15)</sup> Although no receptors have been identified for *NELL2*, recent studies have shown that integrins ( $\beta 1$ <sup>(39)</sup> and  $\alpha 3\beta 1$ <sup>(40)</sup>) act as cell surface receptors for *NELL1*. Binding of *NELL1* to these integrins promotes adhesion of several different types of cells, including bone marrow stromal cells, multipotential mesenchymal cells, chondroprogenitor cells, pre-osteoblasts, and human osteocarcinoma cells.<sup>(39,40)</sup> As it is likely that *NELL1* protein interacts with a variety of receptors through its different domains, the opposite effects of *NELL1* on cell adhesion may be mediated by different *NELL1* receptors that interact with different domains of *NELL1*. Further studies, especially regarding identification of additional receptors and signaling pathways, are required to fully understand *NELL* functions in cancer cell behavior regulation.

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### Disclosure Statement

The authors have no conflict of interest.



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## Supporting Information

Additional supporting information may be found in the online version of this article:

**Fig. S1.** Neural epidermal growth factor-like like 1 (NELL1) and NELL2 protein expression in renal cell carcinoma cell lines.

**Fig. S2.** Binding activity of neural epidermal growth factor-like like protein fused to an alkaline phosphatase tag (NELL-AP) to renal cell carcinoma cells.

**Doc. S1.** Supplementary materials and methods.