Cinacalcet attenuates the renal endothelial-to-mesenchymal transition in rats with adenine-induced renal failure

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SECONDARY HYPERPARATHYROIDISM frequently contributes to the natural progression of chronic kidney disease (CKD) (6, 13). Although the effect of elevated parathyroid hormone (PTH) levels on mineral metabolism has been well recognized, recent evidence has demonstrated the involvement of PTH in fibrotic disorders (5, 8, 14, 35). The beneficial effects of strategies lowering PTH, such as vitamin D analog treatment, on the progression of renal fibrosis have also been documented (37). As a therapeutic alternative, the calcimimetic compound cinacalcet (CINA) has been reported to effectively suppress serum PTH levels in CKD (2, 22). Recently, in vivo studies (17, 30) have further suggested the antifibrotic role of CINA treatment could attenuate renal fibrosis via suppression of endothelial-to-mesenchymal transition (EndMT) has been shown to be an important mechanism involved in renal fibrosis. The present study aimed to investigate whether CINA treatment attenuated renal EndMT in rats with adenine-induced chronic renal failure (CRF). Compared with the control group, serum PTH was significantly higher in the CRF group and was suppressed after CINA treatment. Serum calcium, phosphorus, and calcium × phosphorus product levels were similar in the CRF group and CINA-treated CRF group. Renal collagen accumulation was significantly increased in the CRF group, which was markedly ameliorated by CINA treatment. Expression of the endothelial marker CD31 was significantly downregulated in rats with CRF, whereas expression of the mesenchymal markers fibroblast specific-protein 1 and α-smooth muscle actin was markedly upregulated. These changes were inhibited by CINA treatment. The protein levels of these EndMT-related markers were strongly correlated with serum PTH concentrations. Furthermore, in the in vitro study showed that PTH could significantly increase the expression of fibroblast specific-protein 1 and α-smooth muscle actin and decrease CD31 in mRNA and protein levels in a concentration- and time-dependent manner. In conclusion, our study suggests that reducing serum PTH by CINA treatment could attenuate renal fibrosis via suppression of EndMT in the adenine-induced CRF rat model.

CINACalcet; parathyroid hormone; endothelial-to-mesenchymal transition; chronic kidney disease; renal fibrosis

Animals. Study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Southeast University (Nanjing, China). A rat model of chronic renal failure (CRF) and secondary hyperparathyroidism was established as previously described (11). In brief, 8-wk-old male Wistar rats (SLAC Laboratory Animal) were randomly divided into the following three groups: a control group (CTL group; n = 6), a vehicle-treated CRF group (CRF group; n = 8), and a CINA-treated CRF group (CINA + CRF group; n = 8). CRF was induced by feeding rats a 0.75% adenine diet for 4 wk. Rats fed the diet without adenine served as controls. After adenine withdrawal, all animals were maintained on a 1.03% phosphorus diet for the next 8 wk. All diets were provided by XieTong Biotechnology. At the initiation of the adenine diet, rats in the CRF + CINA group were administered CINA (sc-207438, Santa Cruz Biotechnology) orally once daily (10 mg/kg) until the time of euthanization. Based on exposure levels, the equivalent dose to 10 mg/kg is 60 mg, which was used clinically (15).

Cell culture. Primary human renal glomerular endothelial cells (ECs) were purchased from ScienCell Research Laboratories (Carlsbad, CA) and cultured as previously described. Briefly, cells were grown in endothelial culture medium (ScienCell), 1% EC growth supplement (ScienCell), and 1% penicillin-streptomycin solution (ScienCell) in 5% FBS and 80% confluence, the culture medium was changed to serum-free solution for 24 h before the use of cells in all experiments. Human recombinant PTH fragment 1–34 [PTH (1–34); P3796, Sigma] was then added to the serum-free culture medium.

Serum and urine biochemistry. Serum creatinine, blood urea nitrogen, and total calcium and phosphorus concentrations were measured at the Southeast University-affiliated Zhongda Hospital using an autoanalyzer system (Hitachi). Serum PTH determinations were performed with ELISA (Lengton). Urinary protein levels were measured with a protein assay kit using the modified Bradford method (KeyGen).

Histology and immunohistochemical staining. Paraffin-embedded rat kidney sections (3 μm thick) were prepared by a routine procedure. Sections were stained with periodic acid-Schiff reagent according to a standard protocol. Kidney sections were also subjected to Masson trichrome staining to assess fibrotic lesions. Immunohistochemical staining was performed using the routine protocol. The antibodies used were collagen type I (ab-292, Abcam), fibroblast specific-protein...
1 (FSP1; ab-27957, Abcam), and α-smooth muscle actin (α-SMA; ab-5694, Abcam). For negative controls, the specific primary antibodies were replaced with PBS. For semiquantitative analysis, immunostaining intensity was computed as integrated optical density using Image-Pro Plus software as previously described (28).

**Immunofluorescence staining.** Cryosections (5 μm thick) or coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, and blocked with 10% BSA in PBS at room temperature. Slides were then immunostained with primary antibodies against CD31 (sc-1506) and α-SMA (ab-5694) at 4°C overnight. After an incubation with a mixture of the two secondary antibodies for 1 h in the dark at room temperature, images were captured using a laser scanning confocal microscope (LSM 510 META, Zeiss).

**Real-time PCR.** Total RNA was extracted using RNAiso Plus according to the manufacturer’s directions (TAKARA). RNA concentrations and purity were confirmed with a Nanodrop 2000 (Thermo). Samples with a relative absorbance ratio at 260/280 nm between 1.8 and 2.0 were used. All RNA samples were reverse transcribed (Applied Biosystems). The quantification of specific mRNAs was performed using an ABI Prism 7300 and a Sequence Detection System (Applied Biosystems) with the SYBR green Real-time PCR Kit (Applied Biosystems). The primer sequences are shown in Table 1. Relative mRNA values were normalized to β-actin and calculated using the comparative cycle threshold (ΔΔCt) method.

**Western blot analysis.** Equal amounts of protein obtained from each lysate were electrophoresed on a 4–20% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Pall) using electroblotting. Blots were incubated overnight with primary antibodies against CD31 (sc-1506), FSP1 (ab-27957), and collagen type I (ab-292) followed by horseradish peroxidase-labeled secondary IgG (Santa Cruz Biotechnology). Signals were detected by an enhanced chemiluminescence system (GE Healthcare), and β-actin was used as an internal control.

**Transmission electron microscopy.** To evaluate the morphology and ultrastructure of renal cells, kidney cortices were collected, fixed in 2.5% glutaraldehyde buffer (pH 7.4), and postfixed in 1% OsO4 and 1% glutaraldehyde buffer (pH 7.4), and postfixed in 1% OsO4 and 2.5% glutaraldehyde buffer (pH 7.4). Then, they were dehydrated in a series of ethanol and embedded in epoxy resin (JEOL). Ultrathin sections (60 nm) were cut on a microtome, placed on copper grids, stained with uranyl acetate and lead citrate, and examined using an electron microscope (JEM-1010, JOEL).

**Statistical analysis.** Data are expressed as means ± SD. Statistical analyses used one-way ANOVA followed by a Bonferroni correction using SPSS 16.0 statistical software. Pearson correlation analysis was used to evaluate the correlation between the serum PTH concentration and protein expression. P values of <0.05 were considered to be statistically significant.

**RESULTS**

**Physical and metabolic parameters of animals.** As shown in Table 2, despite a lower body weight, kidney weight indexes (left kidney weight/body weight) of CRF rats were significantly lower than those of control rats; these levels were reversed by CINA treatment. Compared with control rats, rats in the CRF group developed severe renal dysfunction, as evidenced by significant increases in their serum creatinine, blood urea nitrogen, and urinary protein levels. The administration of CINA largely prevented kidney dysfunction and proteinuria in this model. Serum calcium levels decreased, whereas levels of serum phosphorus levels and the calcium × phosphorus product increased in the CRF group compared with the control group. After CINA treatment, serum calcium, phosphorous, and calcium × phosphorus product levels tended to be maintained at low levels, but the differences were not statistically significant. Serum PTH concentrations were found to be markedly elevated in the CRF group compared with the control group. The administration of CINA significantly decreased serum PTH levels in rats with CRF.

**CINA treatment ameliorated renal fibrosis in CRF rat models.** Kidney histology clearly revealed visible nephropathy in CRF rat models (Fig. 1A). The area of renal fibrosis in the CRF group was significantly increased compared with the

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**Table 1. Primers sequence used for real-time PCR analysis**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Organism</th>
<th>Accession Number</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31</td>
<td>Rattus norvegicus</td>
<td>NM_031591.1</td>
<td>5'-TCTCCAAAGGCGAATGCACT-3'</td>
<td>5'-GATTTCCCTTTGGTCCAGA-3'</td>
</tr>
<tr>
<td>Fibroblast specific-protein 1</td>
<td>Rattus norvegicus</td>
<td>NM_013111.2</td>
<td>5'-AGCTACTGACCAAGCGAAGCT-3'</td>
<td>5'-GACAATGCGACAGCATCC-3'</td>
</tr>
<tr>
<td>α-Smooth muscle actin</td>
<td>Rattus norvegicus</td>
<td>NM_031004.2</td>
<td>5'-GACGGACATATTGGTTGACCT-3'</td>
<td>5'-TGGGGGATAGAGGGTTTTACGC-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Homo sapiens</td>
<td>NM_001419.4</td>
<td>5'-GACAGGAAGTCGACAGGAAGG-3'</td>
<td>5'-GATCGGTGACATCGAGAAGG-3'</td>
</tr>
<tr>
<td>CD31</td>
<td>Homo sapiens</td>
<td>NM_000044.2</td>
<td>5'-GATGCGACCCCCGCGAATCC-3'</td>
<td>5'-GATGGGATAGAGGGTTTTACGC-3'</td>
</tr>
<tr>
<td>Fibroblast specific-protein 1</td>
<td>Homo sapiens</td>
<td>NM_001961.2</td>
<td>5'-GTCACCTCCTCAGAAGATAC-3'</td>
<td>5'-TGACCAAATGGTGATCGAG-3'</td>
</tr>
<tr>
<td>α-Smooth muscle actin</td>
<td>Homo sapiens</td>
<td>NM_001419.4</td>
<td>5'-GACAGGAAGTCGACAGGAAGG-3'</td>
<td>5'-GATCGGTGACATCGAGAAGG-3'</td>
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<tr>
<td>β-Actin</td>
<td>Homo sapiens</td>
<td>NM_001101.3</td>
<td>5'-CTGGAAGGTGAGACAGGAAGG-3'</td>
<td>5'-GATCGGTGACATCGAGAAGG-3'</td>
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**Table 2. Physical and biochemical parameters of animal groups**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>CTL Group</th>
<th>CRF Group</th>
<th>CRF + CINA Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>523.54 ± 19.71</td>
<td>352.49 ± 48.45*</td>
<td>400.19 ± 38.19†</td>
</tr>
<tr>
<td>Kidney weight index, mg/g</td>
<td>3.66 ± 0.13</td>
<td>3.14 ± 0.29*</td>
<td>4.22 ± 0.47†</td>
</tr>
<tr>
<td>Serum sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatine, mmol/l</td>
<td>35.25 ± 4.72</td>
<td>366.6 ± 134.73*</td>
<td>126.1 ± 41.66†</td>
</tr>
<tr>
<td>Blood urea nitrogen, mmol/l</td>
<td>6.73 ± 0.53</td>
<td>94.7 ± 24.28†</td>
<td>27.99 ± 6.41†</td>
</tr>
<tr>
<td>Parathyroid hormone, pg/ml</td>
<td>78.46 ± 12.51</td>
<td>440.64 ± 43.25*</td>
<td>326.54 ± 47.86†</td>
</tr>
<tr>
<td>Phosphorus, mmol/l</td>
<td>2.40 ± 0.16</td>
<td>3.86 ± 0.50*</td>
<td>3.01 ± 0.94</td>
</tr>
<tr>
<td>Calcium, mmol/l</td>
<td>2.23 ± 0.04</td>
<td>1.86 ± 0.22*</td>
<td>1.81 ± 0.12</td>
</tr>
<tr>
<td>Calcium × Phosphorus product</td>
<td>5.35 ± 0.39</td>
<td>7.18 ± 0.92</td>
<td>5.45 ± 1.71</td>
</tr>
<tr>
<td>Urine sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteinuria, mg/24 h</td>
<td>10.52 ± 3.79</td>
<td>220.26 ± 23.07†</td>
<td>127.53 ± 45.53†</td>
</tr>
</tbody>
</table>

Results are presented as means ± SD; n = 6 for the control (CTL) group, 8 for the chronic renal failure (CRF) group, and 8 for the cinacalcet (CINA)-treated CRF group. *P < 0.05 vs. the CTL group; †P < 0.05 vs. the CRF group.
CINACALCET ATTENUATES RENAL EndMT

A

CTL

CRF

CRF+CINA

B

CTL

CRF

CRF+CINA

C

Fibrotic area (%)

0

5

10

15

20

25

CTL

CRF

CRF+CINA

D

Collagen I

β-actin

CTL

CRF

CRF+CINA

1 2 3

1 2 3

1 2 3

-138kDa

-43kDa

E

Relative collagen I protein level

0

0.2

0.4

0.6

0.8

1

CTL

CRF

CRF+CINA

F

CTL

CRF

CRF+CINA
CINACALCET ATTENUATES RENAL EndMT

control group, and it was decreased by the administration of CINA (Fig. 1, B and C). A quantitative determination of kidney fibrotic lesions showed decreased fibrotic percentages in the CINA-treated CRF group compared with the CRF group (Fig. 1C). Expression of collagen type I was markedly upregulated in the CRF group compared with the control group; its expression was downregulated by CINA treatment (Fig. 1, D and E).

Based on transmission electron microscopy analysis, rats in the control group displayed normal structure in the kidney. In contrast, rats from the uremia group presented endothelial disseminated protrusions and extracellular matrix accumulations (Fig. 1F). These changes were attenuated by CINA treatment.

CINA treatment attenuates renal EndMT in CRF rat models. As determined by immunohistochemical analysis, FSP1 expression was significantly increased in the CRF group compared with the control group, and this induction was partially abolished by CINA treatment (Fig. 2). Renal expression of

Fig. 2. Fibroblast specific-protein 1 (FSP1) and α-smooth muscle actin (α-SMA) were detected by immunohistochemistry in the three groups. A: representative micrographs showing immunohistochemical staining for the renal expression of FSP1 and α-SMA. Original magnification: ×200. B: semiquantitative analysis for the immunochemical staining, as indicated. Data are presented as means ± SD; n = 6 rats/group. *P < 0.05 vs. the CTL group; #P < 0.05 vs. the CRF group.

Fig. 1. Morphological analysis of the following three groups: control (CTL), vehicle-treated chronic renal failure (CRF), and cinacalcet (CINA)-treated CRF (CRF + CINA). A: representative micrographs showing periodic acid-Schiff staining. Original magnification: ×200. B: representative micrographs showing Masson trichrome staining. Original magnification: ×200. C: semiquantitative analysis for the Masson trichrome-stained blue areas in different groups. Values are presented as mean ± SD; n = 6 rats/group. *P < 0.05 vs. the CTL group; #P < 0.05 vs. the CRF group. D and E: renal collagen type I expression was detected by Western blot analysis. Representative Western blots (D) and quantitative determinations of collagen type I protein levels (E) are shown. 1–3 denote each individual animal in a given group. Data are presented as means ± SD; n = 6 rats/group. *P < 0.05 vs. the CTL group; #P < 0.05 vs. the CRF group. F: representative micrographs showing the renal ultrastructure of the different groups, as indicated. Original magnification: ×10,000. The arrow indicates endothelial protrusions.
α-SMA was also dramatically increased in the CRF group, whereas treatment with CINA significantly attenuated its expression (Figs. 2 and 3A). Confocal microscopy revealed increased colocalization of CD31 and α-SMA (yellow) in renal tissue from the CRF group compared with the control group, and the administration of CINA abrogated this change (Fig. 3B). As shown in Fig. 4, compared with normal control rats, the endothelial marker CD31 was substantially downregulated in the kidneys of CRF rats, as illustrated by real-time PCR and Western blot analysis. Its expression was restored after CINA treatment. Although mRNA and protein levels of FSP1 and α-SMA were markedly upregulated in rats with CRF, the administration of CINA also suppressed FSP1 and α-SMA expression.

Serum PTH concentration was correlated with expression of EndMT markers. As shown in Fig. 5, a significant negative correlation was observed between serum PTH concentrations and renal CD31 protein levels ($r = 0.882, P < 0.01$). In contrast, expressions of FSP1 and α-SMA were positively correlated with serum PTH concentrations ($r = 0.889, P < 0.01$, for FSP1; $r = 0.853, P < 0.01$, for α-SMA).

PTH induced EndMT in cultured renal ECs. To further confirm the effect of PTH on renal EndMT, we performed in vitro experiments using cultured renal ECs. As PTH (1–34) is the shortest fragment with the same biological effect as intact PTH, commercially available PTH (1–34) was used to treat ECs (33, 34). The results of real-time PCR showed that PTH incubation led to a significant decrease in mRNA expression of CD31 and an increase in mRNA levels of FSP1 and α-SMA at 48 h posttreatment (Fig. 6A). In addition, renal ECs were treated with $10^{-10}$ mol/l PTH for various periods of time, and the results indicated that PTH caused a significant decrease in CD31 mRNA expression and an increase in FSP1 and α-SMA mRNA levels from 0 to 48 h posttreatment (Fig. 6D). As determined by Western blot analysis, PTH caused a significant decrease in the protein level of CD31 as well as an increase in the protein expression of FSP1 and α-SMA in a concentration- and time-dependent manner (Fig. 6, B, C, E, and F). Confocal microscopy detected that cells treated with $10^{-10}$ mol/l PTH for 48 h acquired α-SMA staining and lost CD31 staining compared with control cells (Fig. 6G).

DISCUSSION

CKD leading to end-stage kidney disease is associated with renal fibrosis, regardless of the initiating causes. Generally, renal fibrosis is considered to be a multifaceted process that is related to various pathological factors. Recently, increasing evidence has demonstrated a relationship between endothelial injury and renal disease. Ochodnicky et al. (29) identified that renal endothelial dysfunction preceded the development of kidney damage in a mouse model of spontaneous renal disease. In addition, the progression of renal fibrotic lesions was significantly accelerated in mice with endothelial dysfunction (18, 27, 42). Clinically, in renal biopsies of CKD patients, renal endothelial alterations were also strongly related with the...
parameters of renal injury and fibrosis (10, 19). Additionally, recent studies (20, 40) using endothelial lineage tracing have elucidated that renal ECs undergo EndMT, contributing to the initiation of renal fibrosis, which indicates that EndMT is an important mechanism in the progression of renal fibrotic disorders. Our previous work (24, 38) also demonstrated that high glucose, angiotensin II, inflammatory stress, and hyperlipemia-associated EndMT contribute to the progression of fibrosis. Meanwhile, inhibition of EndMT is able to ameliorate fibrotic disease (39). Thus, it has been proposed that inhibition of EndMT represents a potential therapeutic strategy against renal fibrosis. In the present study, our data showed that CINA treatment attenuated renal EndMT in the adenine-induced CRF rat model. These findings implied that CINA could prevent renal fibrosis by abrogating EndMT.

CINA, as a calcimimetic agent, increases the sensitivity of the calcium-sensing receptor to extracellular calcium and suppresses the synthesis and secretion of PTH (26). A number of clinical trials have demonstrated that CINA efficiently reduced PTH levels in patients with stage 3–5 CKD (2, 4, 7, 22). Meanwhile, the significant decrease in PTH was accompanied by hypocalcaemia in some patients (4, 7, 31). This undesirable effect of CINA is believed to be a result of the reduced bone resorption, improved bone mineralization, and increased urinary calcium excretion (1, 3, 12, 36). In the present study, serum calcium levels were similar in the CRF group and CINA-treated CRF group. The exact mechanism is not clear, but it might be due to differences between animal and human models. As our data shown, PTH concentrations were markedly suppressed by CINA in rats with CRF. In addition, an in vitro study has shown that elevated PTH levels induced the expression of EndMT-related markers in a concentration- and

![Fig. 4. Effect of CINA on mRNA and protein expressions of CD31, FSP1, and α-SMA in the three groups. A: real-time PCR results showing the renal mRNA expression of CD31, FSP1, and α-SMA in the different groups of rats, as indicated. Data are expressed as means ± SD; n = 5 rats/group. B and C: Western blot analyses showing CD31, FSP1, and α-SMA protein expressions of the different groups of rats, as indicated. Representative Western blots (B) and quantitative determinations of protein levels (C) are shown. Data are expressed as means ± SD; n = 6 rats/group.](http://ajprenal.physiology.org/)

Meanwhile, inhibition of EndMT is able to ameliorate fibrotic disease (39). Thus, it has been proposed that inhibition of EndMT represents a potential therapeutic strategy against renal fibrosis. In the present study, our data showed that CINA treatment attenuated renal EndMT in the adenine-induced CRF rat model. These findings implied that CINA could prevent renal fibrosis by abrogating EndMT.

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![Fig. 5. Relationships between serum parathyroid hormone (PTH) concentrations and protein levels of CD31 (A), FSP1 (B), and α-SMA (C) in animal models.](http://ajprenal.physiology.org/)

Fig. 5. Relationships between serum parathyroid hormone (PTH) concentrations and protein levels of CD31 (A), FSP1 (B), and α-SMA (C) in animal models.
F144  CINACALCET ATTENUATES RENAL EndMT

A

B

C

D

E

F

G

CD31  a-SMA  DAPI  Merge

CTL

PTH

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time-dependent manner. Therefore, we propose that the beneficial efficacy of CINA in improving renal fibrosis and EndMT could most likely be attributed to decrease on PTH exposure. This result was consistent with the work by Jung et al. (17), which demonstrated that the effect of CINA on reducing myocardial and vascular fibrosis was attributed to its suppression of PTH levels.

Numerous studies have demonstrated that lowering PTH attenuates the progression of CKD. As a therapeutic alternative, calcimimetics have been shown to reduce albuminuria and attenuate glomerular and tubulointerstitial injury in subtotally nephrectomized rats (30, 32). The potential mediators were considered to be lowering of PTH, reversal of EC dysfunction, and reduction of profibrotic cytokines (30, 32). In the present study, we demonstrated that CINA treatment prevented renal fibrosis and endothelial damage. These results were also supported by previous clinical investigations (9, 23). Considering the involvement of endothelial alterations in the progression of renal fibrosis, CINA treatment might be beneficial for the improvement of renal outcomes in patients with CKD.

In conclusion, our study demonstrated that CINA could provide an antifibrotic effect in CRF rat models. The possible mechanism might be its efficacy in reducing serum PTH level and subsequent inhibitive effect on renal EndMT.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: M.W., R.-N.T., and B.-C.L. conception and design of research; M.W., M.X., and M.-M.P. performed experiments; M.W., R.-N.T., H.L., and B.-C.L. analyzed data; M.W., R.-N.T., H.L., and B.-C.L. interpreted results of experiments; M.W. prepared figures; M.W. drafted manuscript; M.W., R.-N.T., H.L., M.X., M.-M.P., and B.-C.L. approved final version of manuscript; R.-N.T., H.L., and B.-C.L. edited and revised manuscript.

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