

# Upregulation of the Human Alkaline Ceramidase 1 and Acid Ceramidase Mediates Calcium-Induced Differentiation of Epidermal Keratinocytes

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Extracellular calcium ( $\text{Ca}_0^{2+}$ ) potently induces the growth arrest and differentiation of human epidermal keratinocytes (HEKs). We report that  $\text{Ca}_0^{2+}$  markedly upregulates the human alkaline ceramidase 1 (haCER1) in HEKs; and its upregulation mediates the  $\text{Ca}_0^{2+}$ -induced growth arrest and differentiation of HEKs. haCER1 is the human ortholog of mouse alkaline ceramidase 1 that we previously identified. haCER1 catalyzed the hydrolysis of very long-chain ceramides to generate sphingosine (SPH). This *in vitro* activity required  $\text{Ca}^{2+}$ . Ectopic expression of haCER1 in HEKs decreased the levels of D-e- $\text{C}_{24:1}$ -ceramide and D-e- $\text{C}_{24:0}$ -ceramide but elevated the levels of both SPH and its phosphate (S1P), whereas RNA interference-mediated knockdown of haCER1 caused the opposite effects on the levels of these sphingolipids in HEKs. Similar to haCER1 overexpression,  $\text{Ca}_0^{2+}$  increased the levels of SPH and S1P, and this was attenuated by haCER1 knockdown. haCER1 knockdown also inhibited the  $\text{Ca}_0^{2+}$ -induced growth arrest of HEKs and the  $\text{Ca}_0^{2+}$ -induced expression of keratin 1 and involucrin in HEKs. In addition, the acid ceramidase (AC) was also upregulated by  $\text{Ca}_0^{2+}$ ; and its knockdown attenuated the  $\text{Ca}_0^{2+}$ -induced expression of keratin 1 and involucrin in HEKs. These results strongly suggest that upregulation of haCER1 and AC mediates the  $\text{Ca}_0^{2+}$ -induced growth arrest and differentiation of HEKs by generating SPH and S1P.

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## INTRODUCTION

The mammalian epidermis is mainly composed of keratinocytes and has four distinct cell layers, the basal, spinous and granular layers, and stratum corneum (Watt, 1998). In the basal layer, stem cells continuously generate proliferative

keratinocytes, referred to as transit amplifying cells (Watt, 1998; Alonso and Fuchs, 2003; Christiano, 2004). Upon leaving the basal layer and migrating upwards into the suprabasal layers, transit-amplifying cells cease proliferation and initiate terminal differentiation (Watt, 1998).

Extracellular calcium ( $\text{Ca}_0^{2+}$ ) plays an important role in regulating the growth and differentiation of epidermal keratinocytes (Bikle *et al.*, 2004).  $\text{Ca}_0^{2+}$  binds to the calcium-sensing receptor, triggering an acute and then a sustained increase in intracellular  $\text{Ca}^{2+}$  ( $\text{Ca}_i^{2+}$ ), which, in turn, activates or upregulates genes negative for cell proliferation and those positive for differentiation (Tu *et al.*, 2004). In our previous study (Houben *et al.*, 2006), we demonstrated that in human epidermal keratinocytes (HEKs),  $\text{Ca}_0^{2+}$  upregulates the mRNA of the human ortholog (human alkaline ceramidase 1 (haCER1)) of the mouse alkaline ceramidase 1 (maCER1) that we recently cloned (Mao *et al.*, 2003). Similar to maCER1 mRNA, haCER1 mRNA is much more abundant in the skin than in other tissues (Houben *et al.*, 2006). maCER1 catalyzes the hydrolysis of ceramides to generate sphingosine (SPH), which, in turn, is phosphorylated to form sphingosine-1-phosphate (S1P), in cells (Mao *et al.*, 2003).

Ceramide, SPH, and S1P are bioactive lipids that mediate growth, differentiation, and apoptosis of various cell types (Hannun and Obeid, 2002; Futerman and Hannun, 2004).

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Abbreviations: AC, acid ceramidase;  $\text{Ca}_i^{2+}$ , intracellular  $\text{Ca}^{2+}$ ;  $\text{Ca}_0^{2+}$ , extracellular calcium; D-KSFM, defined keratinocyte serum-free medium; EGF, epidermal growth factor; ER, endoplasmic reticulum; haCER1, human alkaline ceramidase 1; HEK, human epidermal keratinocytes; HKGS, human keratinocyte growth supplement; maCER1, mouse alkaline ceramidase 1; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PHS, phytosphingosine; qRT-PCR, quantitative RT-PCR; RT-PCR, reverse transcription-PCR; siCER1, haCER1-specific siRNA; siCON, control siRNA; siRNA, small interfering RNA; SPH, sphingosine; S1P, sphingosine-1-phosphate

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These bioactive lipids are also implicated in growth arrest or differentiation of HEKs. Ceramides have been shown to mediate apoptosis of HEKs in response to vitamin D (Bektas *et al.*, 2000), tumor necrosis factor- $\alpha$  (Kouba *et al.*, 2001), and UVB irradiation (Uchida *et al.*, 2003). An increased generation of dihydro-sphingosine, a natural analog of SPH, has been shown to induce apoptosis of keratinocytes (Tolleson *et al.*, 1999). Ceramide has also been implicated in the growth arrest of keratinocytes in response to vitamin D (Geilen *et al.*, 1996). Treatment with SPH (Wakita *et al.*, 1994) or S1P (Kim *et al.*, 2004) has been shown to inhibit the proliferation of keratinocytes. Treatment with phytosphingosine (PHS), another natural analog of SPH, induces not only growth arrest but also differentiation of keratinocytes (Kim *et al.*, 2006). These observations suggest that ceramide, sphingoid bases (SPH, dihydrosphingosine, and PHS), and their phosphates may play a role in growth arrest and/or differentiation of keratinocytes. As a calcium-regulated enzyme that controls the hydrolysis of ceramide to generate SPH and S1P, haCER1 may play a role in the calcium-induced growth arrest and differentiation of epidermal keratinocytes by regulating the levels of ceramide, SPH, and S1P.

In this study, we report that the cloning, characterization, and functional analysis of haCER1. We demonstrate that haCER1 is expressed mainly in HEKs. haCER1 upregulation increases the levels of SPH and S1P, whereas the RNA interference-mediated knockdown of its expression has the opposite effect. Similar to haCER1 upregulation, calcium also increases the generation of both SPH and S1P; and this is inhibited by haCER1 knockdown. haCER1 knockdown inhibits the calcium-induced growth arrest of HEKs as well as the calcium-induced expression of keratin 1 and involucrin. These results suggest that haCER1 (or maCER1) plays an important role in mediating the calcium regulation of growth and differentiation of epidermal keratinocytes by controlling the generation of SPH and S1P.

## RESULTS

### haCER1 is a *bona fide* alkaline ceramidase that preferentially hydrolyzes ceramides with a very long-chain unsaturated fatty acid

The coding sequence of haCER1 was cloned as described in Materials and Methods. Protein sequence alignment revealed that haCER1 is highly homologous to maCER1 (Figure 1a). To confirm that haCER1 is a *bona fide* ceramidase, we expressed the epitope-tagged haCER1 in a yeast mutant strain  $\Delta ypc1\Delta ydc1$ , which lacks any endogenous alkaline ceramidase activity due to the deletion of the yeast alkaline ceramidases (Mao *et al.*, 2000). Western blot analysis demonstrated that the Flag-tagged haCER1 was expressed in  $\Delta ypc1\Delta ydc1$  cells transformed with the haCER1 expression construct pYES2-Flag-haCER1 but not with the empty vector pYES2-Flag (Figure 1b). Microsomes prepared from yeast cells were measured for ceramidase activity at pH 8.0 using ceramides, dihydroceramides, and phytoceramides as substrates. Ceramidase activity was determined by the release of SPH, dihydrosphingosine, and PHS from ceramides, dihydroceramides, and phytoceramide, respectively. The results demonstrated that the microsomes from  $\Delta ypc1\Delta ydc1$  cells

transfected with pYES2-Flag-haCER1 exhibited high ceramidase activity towards D-e-C<sub>24:1</sub>-ceramide, slight activity on D-e-C<sub>24:0</sub>-ceramide and D-e-C<sub>18</sub>-ceramide, but no ceramidase activity on long- or medium-chain ceramides (D-e-C<sub>16</sub>-ceramide and D-e-C<sub>14</sub>-ceramide), dihydroceramides (D-e-C<sub>24:1</sub>-dihydroceramide, D-e-C<sub>24:0</sub>-ceramide, and D-e-C<sub>16</sub>-dihydroceramide), or phytoceramides (D-ribo-C<sub>24:1</sub>-phytoceramide, D-ribo-C<sub>24:0</sub>-phytoceramide, and D-ribo-C<sub>16</sub>-phytoceramide) (Figure 1c). As expected, the microsomes from the cells transfected with the vector pYES2-Flag exhibited no ceramidase activity on any of the ceramides, dihydroceramides, or phytoceramides (data not shown), suggesting that haCER1 is a *bona fide* ceramidase that prefers ceramides with a very long-chain unsaturated fatty acid as substrates.

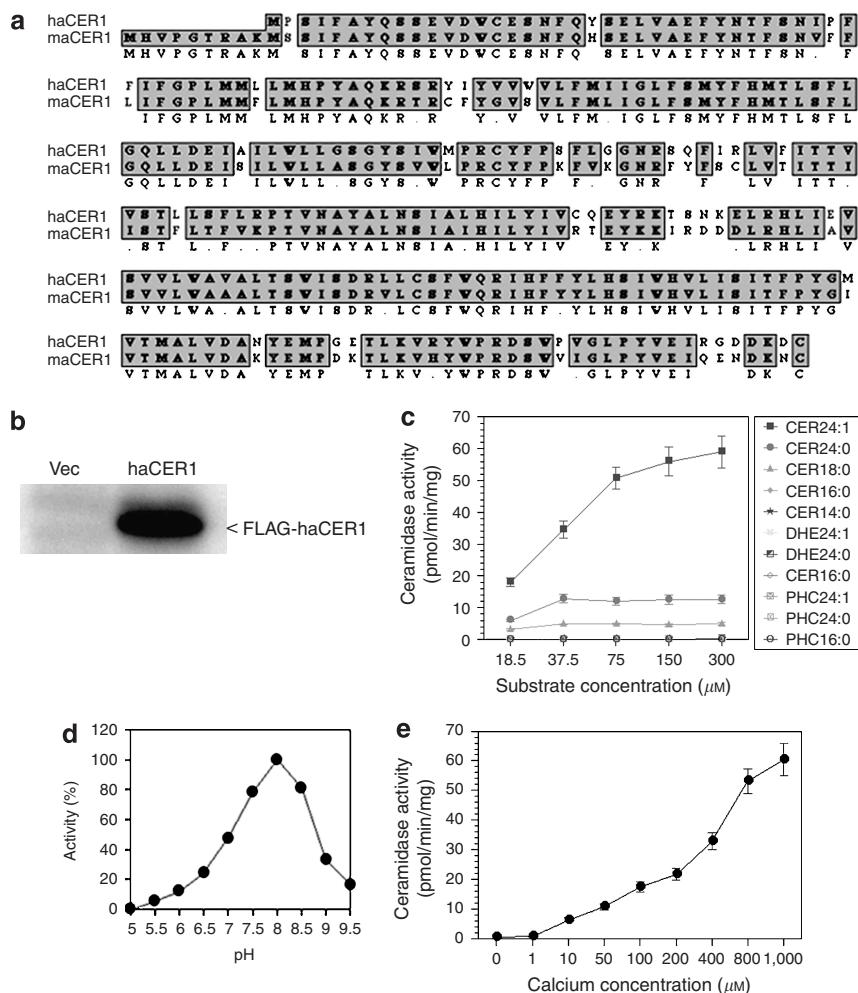
To determine the pH optimum of haCER1 for its *in vitro* activity, we measured ceramidase activity in the haCER1 microsomes at various pHs with D-e-C<sub>24:1</sub>-ceramide as substrate. The results showed that the microsomal ceramidase activity was the highest around pH 8 (Figure 1d), suggesting that haCER1 has an alkaline pH optimum.

We previously demonstrated that maCER1, the mouse homolog of haCER1, is activated by calcium (Mao *et al.*, 2003). We thus investigated the effect of calcium on haCER1 activity. The haCER1 microsomes were assayed for ceramidase activity on D-e-C<sub>24:1</sub>-ceramide in the presence of different concentrations of calcium. The microsomes exhibited a slight ceramidase activity in the absence of calcium. The microsomal ceramidase activity was markedly increased in the presence of calcium, which exhibited the stimulatory effect at as low as 10  $\mu\text{M}$  and generated the maximal effect at 1,000  $\mu\text{M}$  (Figure 1e), suggesting that calcium also activates haCER1.

### haCER1 expression in epidermal keratinocytes is regulated by calcium and EGF

To better understand the physiological role of haCER1, we investigated tissue-specific expression of haCER1 mRNA. Northern blot analysis showed that a 1.5-kb haCER1 mRNA was highly expressed in skin, but was only slightly expressed or undetectable in other tissues (Figure 2a). Using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), we found that haCER1 mRNA was expressed in HEKs but not in dermal fibroblast cells (Figure 2b). Interestingly, haCER1 mRNA was substantially downregulated to an undetectable level in a spontaneously immortalized keratinocyte line, HaCaT cells, and an epidermoid carcinoma cell line A431 (Figure 2b). These results suggest that haCER1 mRNA is expressed in normal HEKs, but is downregulated in transformed or malignant keratinocytes.

We previously demonstrated that haCER1 mRNA is upregulated during keratinocyte differentiation (Houben *et al.*, 2006). Thus, we investigated whether haCER1 expression in HEKs is upregulated by Ca<sub>o</sub><sup>2+</sup>. HEKs at 80% confluence were treated for 24 hours with high-calcium (1.8 mM CaCl<sub>2</sub>) in defined keratinocyte serum-free medium (D-KSFM) with or without human keratinocyte growth supplement (HKGS). Quantitative RT-PCR (qRT-PCR) revealed that the expression of haCER1 mRNA was substantially upregulated by high calcium in the absence of HKGS, and to a lesser extent, in the



**Figure 1. haCER1 is a novel human alkaline ceramidase that prefers ceramide with a very long-chain unsaturated fatty acid as substrate.** (a) Protein sequence alignment between maCER1 and haCER1 was performed using the Clustal W method. Shaded were identical or similar amino acids between the two proteins. (b) Microsomes (20 μg proteins per lane) prepared from yeast cells transformed with pYES2-Flag (Vec) or pYES2-Flag-haCER1 (haCER1) were subjected to Western blot analysis using the anti-Flag antibody (Sigma Inc.). (c) The above microsomes were assayed for ceramidase activity at pH 8.0 using different ceramides. CER24:1, CER24:0, CER18:0, and CER14:0 represent D-e-C<sub>24:1</sub>, C<sub>24</sub>, C<sub>18</sub>, C<sub>16</sub>-ceramide, respectively. DHC24:1, DHC24:0, and DHC16:0 represent D-e-C<sub>24:1</sub>, C<sub>24</sub>, and C<sub>16</sub>-dihydroceramide, respectively. PHC24:1, PHC24:0, and PHC16:0 represent D-ribo-C<sub>24:1</sub>, C<sub>24</sub>, and C<sub>16</sub>-phytoceramide, respectively. (d) The microsomes isolated from the haCER1-expressing cells were measured for ceramidase activity on D-e-C<sub>24:1</sub>-ceramide in 25 mM sodium acetate (pH 5 or 6), Tris-HCl (pH 6.0–8.5), or glycine-NaOH (pH 8.5 and above) buffer. (e) The microsomes isolated from the haCER1-expressing cells were measured for ceramidase activity on D-e-C<sub>24:1</sub>-ceramide in the presence of various concentrations of CaCl<sub>2</sub>. The data are the means ± SD of three independent experiments performed in duplicate or triplicate.

presence of HKGS (Figure 2c), suggesting that high calcium upregulates haCER1 expression in HEKs.

The results in Figure 2c indicate that HKGS inhibits the high-calcium-induced upregulation of haCER1 mRNA. We tested whether epidermal growth factor (EGF), a major growth factor in HKGS, inhibited the high-calcium-induced upregulation of haCER1 mRNA. HEKs were treated for 12 hours with EGF (20 ng/ml) in the presence of low calcium (0.06 mM CaCl<sub>2</sub>) or high calcium. qRT-PCR analysis demonstrated that EGF caused a decrease in the basal mRNA levels of haCER1 and markedly inhibited the high-calcium-induced upregulation of haCER1 mRNA (Figure 2c), suggesting that growth factors inhibit the high-calcium-induced haCER1 expression in HEKs.

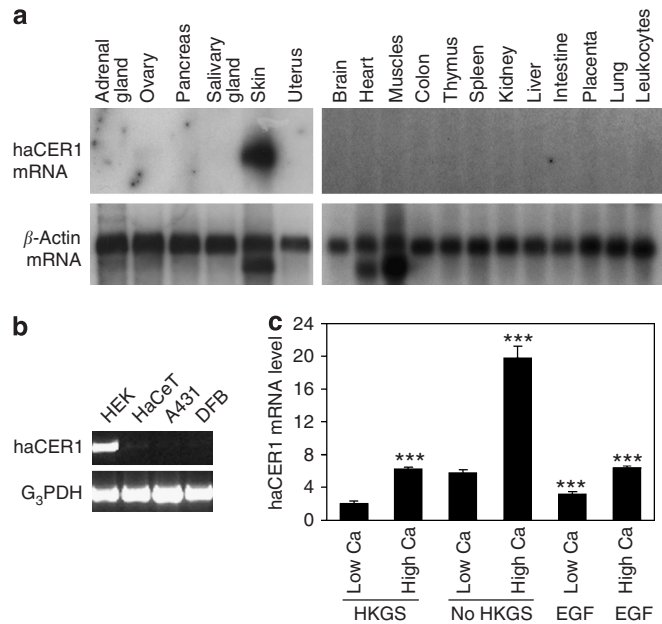
### haCER1 is localized to the endoplasmic reticulum

To better understand the role of haCER1 in regulating metabolism of ceramides and cellular responses in keratinocytes, we defined the cellular localization of haCER1 by indirect immunofluorescent staining. We previously demonstrated that maCER1 is localized to the endoplasmic reticulum (ER) (Mao *et al.*, 2003). To confirm whether haCER1 has the same cellular localization, HaCaT keratinocytes transfected with the haCER1 expression construct (pcDNA3-Flag-haCER1) were co-stained by antibodies against the Flag tag and calreticulin (an ER resident protein). Confocal microscopy revealed that haCER1 was colocalized with calreticulin to the perinuclear reticulum network (Figure 3), suggesting that haCER1 is indeed an ER protein.

**haCER1 overexpression increases SPH and S1P but decreases very-long chain ceramides in HEKs**

haCER1 catalyzes the hydrolysis of ceramides *in vitro*; thus, we determined the role of haCER1 in regulating the levels of ceramides and their derivatives. HEKs were transfected with

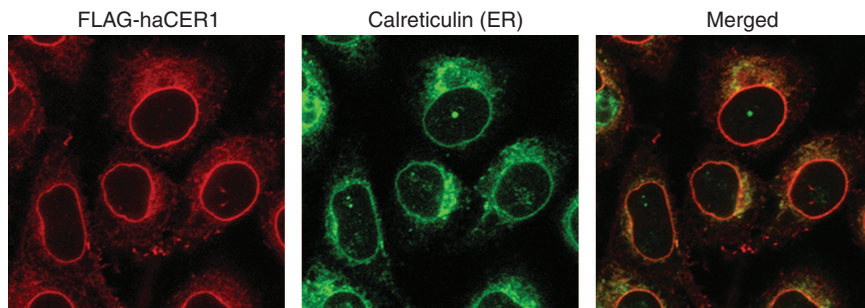
the haCER1 expression construct pcDNA3-Flag-haCER1 or the empty vector pcDNA3.1. Western blot analysis confirmed the expression of the recombinant haCER1 in HEKs transfected with pcDNA3-Flag-haCER1, but not with pcDNA3.1 (Figure 4a). *In vitro* activity assays showed that haCER1 overexpression caused a 4-fold increase in alkaline ceramidase activity towards D-e-C<sub>24:1</sub>-ceramide (Figure 4b). Electrospray ionization mass spectrometric (ESI)/MS/MS analysis revealed that haCER1 overexpression caused a 30.2 and 71.9% decrease in the levels of D-e-C<sub>24:0</sub>-ceramide and D-e-C<sub>24:1</sub>-ceramide, respectively, in HEKs. Interestingly, haCER1 expression caused 11.0 and 88.6% increases in the levels of D-e-C<sub>16</sub>-ceramide and D-e-C<sub>14</sub>-ceramide, respectively (Figure 4c). As ceramides can be converted to SPH, which in turn is phosphorylated to generate S1P, we determined the effect of haCER1 overexpression on the levels of both SPH and S1P. ESI/MS/MS showed that haCER1 expression caused 431.7 and 441.7% increases in the levels of SPH and S1P, respectively (Figure 4c). These results suggest that haCER1 upregulation increases the levels of SPH, S1P, and ceramides with a medium- or long-chain fatty acid but decreases the levels of ceramides with a very long-chain fatty acid, especially those with a very long-chain unsaturated fatty acid.



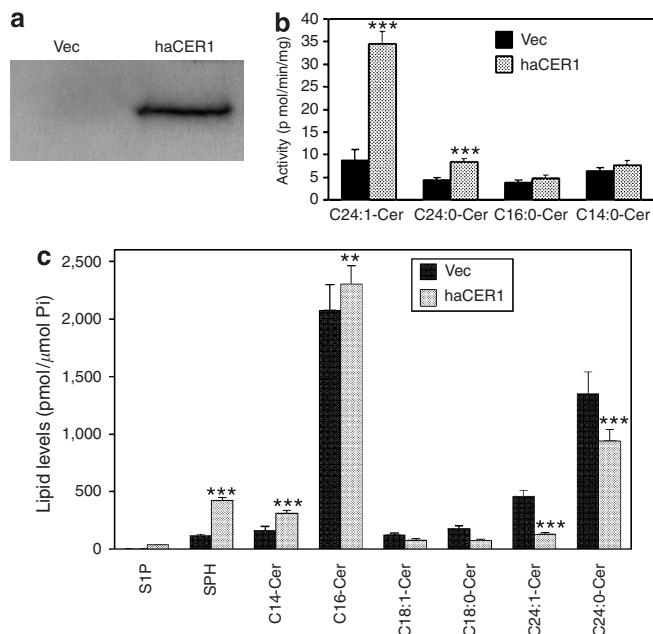
**Figure 2. haCER1 mRNA is expressed in epidermal keratinocytes and its expression is highly regulated by calcium and EGF.** (a) Multiple tissue mRNA blots (Origene, Rockville, MD and BD Biosciences) were hybridized with a <sup>32</sup>P-radiolabeled haCER1 DNA probe. The probed blots were exposed to X-ray films at -80°C for 3 days before the films were developed. After being stripped of the haCER1 probe, the mRNA blots were hybridized with the β-actin probe as a normalization control. (b) RT-PCR analysis was performed with RNA isolated from human neonatal foreskin keratinocytes (HEKs), HaCaT keratinocytes, A431 tumor cells, and human neonatal foreskin dermal fibroblast cells (DFB) using the primer sets specific for haCER1 or G<sub>3</sub>PDH as a normalization control. (c) HEKs grown in D-KFSM were treated or not treated with 1.8 mM CaCl<sub>2</sub> for 48 hours in the presence or absence of HKGS, EGF (20 ng/ml) before total RNA was isolated from the cells. The isolated RNA was quantified by qRT-PCR for the levels of haCER1 mRNA. High Ca, 1.8 mM CaCl<sub>2</sub>; and Low Ca, 0.06 mM. The data are the means ± SD of three independent experiments.

**haCER1 knockdown inhibits the calcium-induced generation of SPH and S1P but not ceramides in HEKs**

haCER1 overexpression increases the levels of SPH and S1P and decreases the levels of very long-chain ceramides. We investigated whether high calcium has a similar effect on the levels of ceramide, SPH, and S1P in HEKs; and if so, whether haCER1 knockdown blocks this effect. haCER1 expression in HEKs was downregulated by RNA interference. qRT-PCR analysis demonstrated that haCER1 expression was markedly downregulated by a haCER1-specific small interfering RNA (siRNA) (siCER1) compared with a control siRNA (siCON) (Figure 5a). HEKs transfected with siCON or siCER1 were grown in the presence of low calcium or high calcium. ESI/MS/MS showed that high calcium increased the levels of SPH, S1P, and all ceramide species; and haCER1 knockdown significantly inhibited the high-calcium-induced generation of SPH and S1P but not ceramides (Figure 5b). These results



**Figure 3. haCER1 is localized to the ER.** haCER1 coding sequence was tagged with an epitope tag (Flag) sequence and cloned into pcDNA3.1 to form the expression construct, pcDNA3-Flag-haCER1. This construct was transiently transfected into HaCaT cells and transfected cells were immunostained with antibodies (1:200) against the Flag peptide and calreticulin (an ER marker). Following incubation with anti-rabbit IgG and mouse IgG antibodies (1:300) conjugated with FITC and rhodamine, respectively, the immunostained cells were examined under a confocal laser-scanning microscope (LSM510, Zeiss).



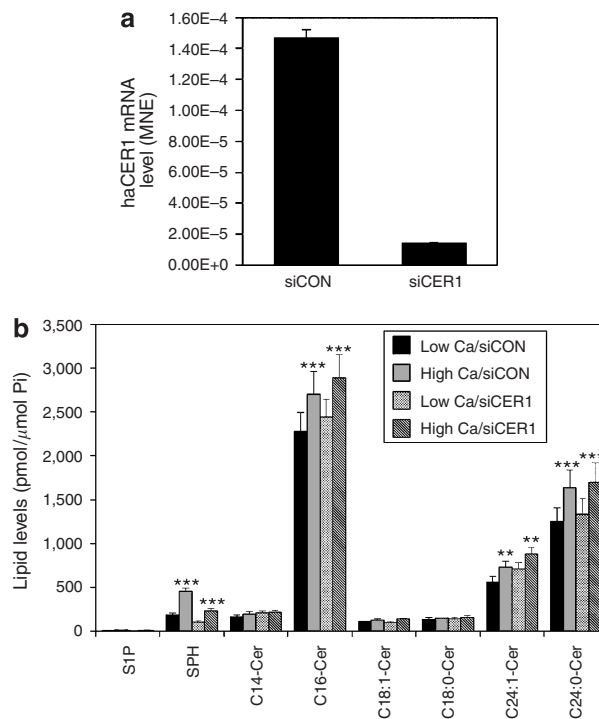
**Figure 4. haCER1 overexpression increases SPH and S1P but decreases very long-chain ceramides.** (a) HEKs grown in D-KSFM were transfected with pcDNA3-Flag-haCER1 or pcDNA3.1. Microsomes prepared from the above cells were subjected to Western blot analysis for expression of the Flag-tagged haCER1 using an anti-Flag antibody. Vec, the empty vector; and haCER1, pcDNA3-Flag-haCER1. (b) The microsomal preparations were measured for ceramidase activity towards various ceramides. (c) Lipids were extracted from the transfected HEKs grown to 95% confluence and were subjected to ESI/MS/MS analysis for the levels of ceramides, SPH, and S1P. The data are the means  $\pm$  SD of three independent experiments.

suggest that haCER1 upregulation is accountable for the calcium-induced generation of SPH and S1P in HEKs.

#### haCER1 knockdown attenuates the calcium-induced growth arrest and differentiation of HEKs

As haCER1 expression in HEKs is markedly upregulated by high calcium, which potently induces growth arrest of HEKs, we tested whether haCER1 upregulation mediates the calcium-induced growth arrest of HEKs. HEKs grown in D-KSFM were transfected with siCON or siCER1. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays demonstrated that haCER1 knockdown slightly increased the growth rate of HEKs and significantly attenuated the high-calcium-induced decrease in the growth rate of HEKs (Figure 6a). [<sup>3</sup>H]thymidine incorporation assays showed that haCER1 knockdown slightly increased DNA synthesis in HEKs and significantly attenuated the high-calcium-induced inhibition of DNA synthesis (Figure 6b). These results suggest that haCER1 upregulation indeed play a role in the calcium-induced growth arrest of HEKs.

As high calcium also induces the differentiation of HEKs, we tested whether haCER1 upregulation also mediates the calcium-induced differentiation of HEKs. HEKs transfected with siCON or siCER1 were treated with high-calcium. Western blot analysis demonstrated that haCER1 knockdown

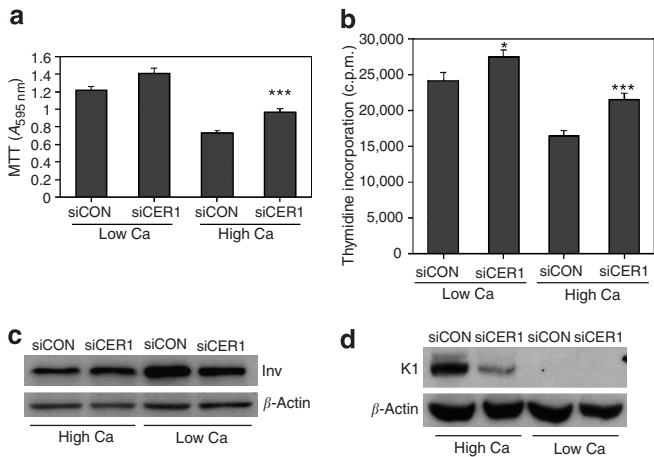


**Figure 5. haCER1 downregulation inhibits the calcium-induced generation of SPH and S1P but not ceramides.** (a) HEKs grown in D-KSFM were transfected with a control siRNA (Con siRNA) or haCER1-specific siRNA (haCER1 siRNA) at 10 nM. Seventy-two hours after the siRNA transfection, qRT-PCR analysis was performed to determine the expression of haCER2 in HEKs. (b) Twenty-four hours after siRNA transfection, HEKs were grown in the presence of low Ca<sup>2+</sup> or high Ca<sup>2+</sup> for 48 hours before lipid extraction and ESI/MS/MS analysis. The data are the means  $\pm$  SD of three independent experiments.

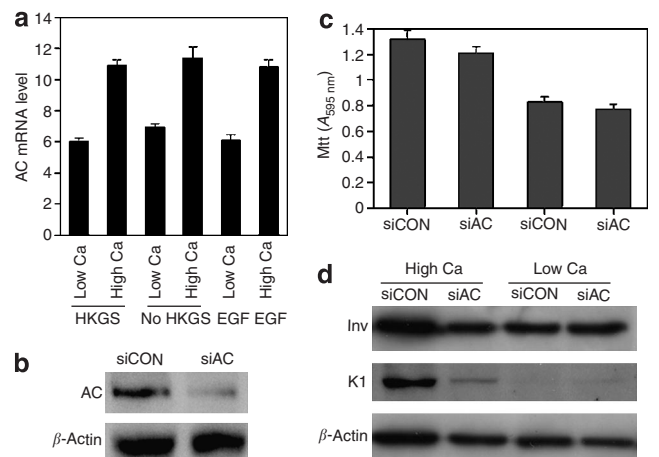
had no effect on the levels of keratin 1 or involucrin in HEKs grown in the presence of low calcium (Figure 6c). However, haCER1 knockdown inhibited the high-calcium-induced expression of both keratin 1 and involucrin in HEKs (Figure 6c). This suggests that haCER1 upregulation also a play role in the calcium-induced differentiation of HEKs.

#### Knockdown of acid ceramidase inhibits the calcium-induced differentiation of HEKs

We previously demonstrated that the expression of the acid ceramidase (AC) is also upregulated during the calcium-induced differentiation of HEKs (Houben *et al.*, 2006). As similar to haCER1, AC catalyzes the hydrolysis of ceramides, we investigated whether AC also plays a role in the calcium-induced growth arrest and differentiation of HEKs. qRT-PCR analysis demonstrated that treatment of HEKs with high calcium caused an 80% increase in the levels of AC mRNA and neither HKGS nor EGF had any effect on the basal levels of AC mRNA or the calcium upregulation of AC mRNA (Figure 7a). RNA interference was performed to knock down AC expression in HEKs. Western blot analysis showed that transfection of HEKs with an AC-specific siRNA markedly downregulated AC expression compared with transfection with siCON (Figure 7b). MTT assays revealed that AC



**Figure 6. haCER1 knockdown inhibits calcium-induced growth arrest of HEKs and calcium-induced expression of involucrin and keratin 1 in HEKs.** (a, b) HEKs were grown overnight in six-well plates containing D-KSFM before they were transfected with siCON or siCER1. At 24 hours after the siRNA transfection, the cells were treated with 1.8 mM CaCl<sub>2</sub> or water. Seventy-two hours after CaCl<sub>2</sub> treatment, the cells were subjected to (a) MTT assays or (b) [<sup>3</sup>H]thymidine incorporation assays. The data are the means ± SD of three independent experiments performed in duplicate. (c, d) HEKs were transfected with siCON or siCER1 and were treated with high-calcium as decreased in (a). At 48 hours after the addition of CaCl<sub>2</sub>, the cells were subjected to Western blot analysis for the levels of involucrin (Inv) and keratin 1 (K1). The data are the means ± SD of three independent experiments.



**Figure 7. AC knockdown inhibits the calcium-induced expression of involucrin and keratin 1.** (a) HEKs were grown in low- or high-calcium D-KSFM in the presence or absence of HKGS or EGF (20 ng/ml) before AC mRNA levels were determined by qRT-PCR as described in Figure 2. The data are the means ± SD of three independent experiments. (b) HEKs were transfected with siCON or AC-specific siRNA as described in Figure 5a. AC protein levels were determined by Western blot analysis using an anti-AC antibody. (c, d) HEKs transfected with siCON or AC-specific siRNA were grown in D-KSFM in the presence of low or high Ca<sup>2+</sup> before (c) MTT assays or (d) Western blot analysis for involucrin and keratin 1 as described in Figure 6.

knockdown slightly decreased the growth rate of HEKs grown in the presence of low or high calcium (Figure 7c). Western blot analysis found that AC knockdown slightly inhibited the calcium-induced expression of involucrin and keratin 1 (Figure 7d). These results suggest that AC upregulation plays a role in mediating the calcium-induced differentiation of HEKs without affecting the calcium-induced growth arrest.

**DISCUSSION**

haCER1 is the ortholog of the maCER1 that we identified previously (Mao et al., 2003). haCER1 and maCER1 share an 88% sequence identity. Similar to maCER1, haCER1 is highly expressed in skin. With RT-PCR, we further revealed that haCER1 is expressed in HEKs but not in dermal fibroblast cells, suggesting that haCER1 is an epidermis-specific enzyme. We experimentally proved that haCER1 is a bona fide ceramidase that prefers ceramides with a very long-chain unsaturated fatty acid as substrates both *in vitro* and in cells.

The data present in this study strongly suggest that haCER1 plays a role in mediating the calcium-induced growth arrest and differentiation of epidermal keratinocytes. First, we demonstrated that haCER1 was activated and upregulated by high-calcium, a potent inducer of growth arrest, and differentiation of epidermal keratinocytes, but was down-regulated by EGF, which promotes the proliferation of HEKs. Second, the RNA interference-mediated downregulation of haCER1 inhibited the calcium-induced growth arrest of HEKs as well as the calcium-induced expression of keratin 1 and involucrin in HEKs. The role of haCER1 in mediating the

differentiation of keratinocytes is further supported by our previous studies showing that haCER1 mRNA is much more abundant in the suprabasal layers with differentiated keratinocytes than in the basal layer with proliferating keratinocytes; and the levels of haCER1 mRNA are increased more than 100-fold in HEKs in the advanced stage of differentiation (Houben et al., 2006).

haCER1 upregulation mediates the calcium-induced growth arrest and differentiation of HEKs probably by generating SPH and S1P. This is supported by several lines of evidence. First, similar to high-calcium treatment, the ectopic expression of haCER1 increased the levels of SPH and S1P in HEKs. Second, haCER1 knockdown inhibited the calcium-induced generation of SPH and S1P and the calcium-induced growth arrest and differentiation of HEKs. Third, SPH (Wakita et al., 1994), its analogs dihydro-sphingosine (Tolleson et al., 1999) and PHS (Kim et al., 2006), and S1P (Kim et al., 2004) have been shown to induce growth arrest or differentiation of keratinocytes.

High-calcium treatment also increased the levels of D-e-C<sub>14</sub>-ceramide, D-e-C<sub>16</sub>-ceramide, D-e-C<sub>24:0</sub>-ceramide, and D-e-C<sub>24:1</sub>-ceramide in HEKs, but haCER1 knockdown failed to inhibit the calcium-induced generation of these ceramides, suggesting that haCER1 upregulation is not accountable for the calcium-induced generation of ceramides. Ectopic expression of haCER1 caused a decrease in the levels of very long-chain ceramides (D-e-C<sub>24:0</sub>-ceramide and D-e-C<sub>24:1</sub>-ceramide) in HEKs grown in the presence of low calcium, whereas the high-calcium-induced upregulation of haCER1

failed to do so, suggesting that high calcium not only upregulates haCER1 but also increases substrates of haCER1. Ceramides, as discussed earlier, have been implicated in the growth arrest of keratinocytes in response to vitamin D. Currently, it remains unclear whether the calcium-induced generation of ceramides plays a role in the growth arrest and differentiation of HEKs. However, it is certain that the calcium-induced generation of ceramides provides haCER1 with unlimited substrates. Therefore, the generation of very long-chain ceramides at least plays an indirect role in the haCER1-mediated cellular responses in HEKs by serving as the precursor of SPH and S1P. This is supported by our recent studies showing that overexpression of haCER2, the homolog of haCER1, enhances the generation of SPH from the hydrolysis of very long-chain ceramides, leading to the growth arrest of HeLa cells (Xu *et al.*, 2006).

Our previous studies demonstrated that in addition to haCER1, HEKs express four other ceramidases including the AC (Houben *et al.*, 2006). We showed that high calcium also moderately upregulates the mRNA levels of AC, although to a lesser extent (Houben *et al.*, 2006). This finding is confirmed in this study. The calcium co-upregulation of AC and haCER1 indicates that AC and haCER1 may have a similar role in the calcium-mediated growth arrest or differentiation of HEKs. Indeed, AC knockdown also inhibited the calcium-induced expression of keratin 1 or involucrin in HEKs, although with a lesser potency than haCER1 knockdown. It has been shown that AC upregulation increases the levels of SPH but not S1P (Monick *et al.*, 2004). We demonstrated that AC overexpression caused an increase in the levels of both SPH and S1P in HeLa cells (unpublished data). These results suggest that AC upregulation mediates the calcium-induced differentiation of HEKs probably by generating SPH and S1P as well. This further supports that haCER1 mediates the calcium-induced differentiation of HEKs through generating SPH and/or S1P.

Although haCER1 and AC have a similar role in mediating the calcium-induced differentiation of HEKs, they differ in regulating the growth of HEKs. We demonstrated that haCER1 knockdown promoted the proliferation of HEKs and attenuated the calcium-induced growth arrest of HEKs, whereas AC knockdown failed to do so. Consistently, we found that the calcium-induced upregulation of haCER1 but not AC was inhibited by the growth factor EGF. The different role of haCER1 and AC in regulating cell growth may be related to their cellular localizations, because haCER1 and AC are localized to the ER and lysosomes, respectively.

Our studies suggest that an inhibition or downregulation of haCER1 could lead to aberrant growth and differentiation of keratinocytes, which have been shown to be associated with many skin diseases, including psoriasis. Interestingly, the *haCER1* gene (19p13.3) is localized in a psoriasis susceptibility locus, *PSORS6* (19p13) (Hensen *et al.*, 2003). It is interesting to know whether haCER1 dysregulation is implicated in psoriasis and other skin diseases.

In conclusion, our studies demonstrated that the expression of haCER1 and AC is upregulated by  $\text{Ca}_o^{2+}$ .  $\text{Ca}_o^{2+}$  binds to its receptor calcium-sensing receptor and elevates the

levels of  $\text{Ca}_i^{2+}$ , which, in turn, activates the preexisting haCER1 and upregulates haCER1 and AC. The upregulation of haCER1 and AC enhances the hydrolysis of ceramides that are increased by calcium, augmenting the generation of SPH and S1P, which induce the growth arrest and differentiation of keratinocytes.

## MATERIALS AND METHODS

### Lipid preparation

D-erythro-SPH, D-erythro-dihydrosphingosine, and D-ribo-PHS were purchased from Avanti Polar-Lipids Inc. (Alabaster, AL). Ceramides used in this study were synthesized in the Lipidomics Core at the Medical University of South Carolina (MUSC) as described (Usta *et al.*, 2001).

### Cell culture

HEKs (Cascade Biologics Inc.) were cultured in D-KSFM with HKGS (Cascade Biologics Inc., Portland, OR). HaCaT keratinocytes provided by Dr Norbert E Fusenig (German Cancer Research Center, Heidelberg, Germany) and A431 cells (American Type Culture Collection, ATCC) were cultured in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum. Dermal fibroblast cells in human neonatal foreskin were provided by Dr Maria Trojanowska (MUSC) and were cultured in DMEM containing 10% fetal bovine serum. Fetal bovine serum and all media were from Invitrogen Inc. (Carlsbad, CA).

### Cloning of haCER1

A Blast search of the NCBI GenBank with the maCER1 protein sequence as a query revealed a human expressed sequence tag with an accession number of BG698821 that encodes a polypeptide highly homologous to maCER1. Compared with the maCER1 sequence, this polypeptide lacks the N-terminal sequence. Thus, rapid amplification of cDNA ends (5'-RACE) was performed to obtain the N-terminal-coding sequence as described (Mao *et al.*, 2003). The first round PCR was conducted with human neonatal skin keratinocyte cDNA as a template using the adaptor primer AP1 (BD Biosciences Inc., San Jose, CA) and a gene specific primer 5'-CAGTAGCGGACTTTGAGGGTTTC-3'. The second round PCR was performed with the first round PCR products as a template using the adaptor primer AP2 and a nested gene-specific primer 5'-TGAG GGTTTCACCTGGCATCTC-3'. The single resulting 5'-RACE product was cloned into a vector pCR2.1 (Invitrogen Inc.). DNA sequencing revealed the translation initiation site (ATG) in the extended expressed sequence tag. The full-length open reading frame was amplified from the skin keratinocyte cDNA using the primers 5'-GG ATCCATGCCTAGCATCTTCGCCTATCAGAG-3'/5'-GAATTCTCAG CAGTCCTGTGCATCACCC-3' and cloned into a PCR cloning vector pCR2.1 (Invitrogen Inc.). The open reading frame was subsequently cloned to a yeast expression vector pYES2-Flag to generate the yeast expression construct pYES2-Flag-haCER1. The coding sequence for the Flag-tagged haCER1 was subcloned from pYES2-Flag-haCER1 to a mammalian expression vector pcDNA3.1-Zeo (Invitrogen Inc.) to generate a mammalian expression construct pcDNA3-Flag-haCER1. Both expression constructs were verified by DNA sequencing.

The coding sequence and protein sequence of haCER1 have been submitted to the GenBank under the accession number of AF347024.

### Semi-quantitative RT-PCR analysis

Total RNA was isolated from various cell types using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Five microgram total RNA from each cell sample was reversely transcribed into cDNA as described (Mao *et al.*, 2003). One-tenth of the transcribed cDNA was subjected to PCR analysis for the levels of haCER1 mRNA under the condition of one cycle of 94°C for 30 seconds, 30 cycles of 94°C for 20 seconds, 60°C for 30 seconds, 72°C for 50 seconds, and one cycle of 72°C for 5 minutes. PCR analysis for the levels of glyceraldehyde 3 phosphate dehydrogenase (G<sub>3</sub>PDH) mRNA was performed under the same conditions but with one-thirtieth of the transcribed cDNA. The PCR primer sets used were 5'-TGGACTGGTGTGAGAGCAACTTC-3'/5'-TCAGCAGTCC TTG TCATCACCC-3' (haCER1) and 5'-TGAAGTCCGGAGTACACG GATTTGGT-3'/5'-CATGTGGGCCATGAGGTCCACCAC-3' (G<sub>3</sub>PDH). PCR products were verified by DNA sequencing.

### qRT-PCR analysis

Real-time PCR was performed on an iCycler system (BioRad Inc., Hercules, CA) using primers 5'-GCCTAGACTCTTCGCCTATCAG-3'/5'-GGAAGTTGCTCTCACACCACT-3', haCER1; 5'-TGATGCTTGAC AAGGCACCA-3'/5'-GGCAATTTTCATCCACCACC-3', AC; and 5'-CAATGTTCGGTGCAATTCAGAG-3'/5'-GGATCCCATTCTACCCT GTG-3',  $\beta$ -actin. Standard reaction volume was 25  $\mu$ l, including 12.5  $\mu$ l of iQ™ SYBR Green Supermix (Bio-Rad), 10  $\mu$ l of cDNA template, and 2.5  $\mu$ l of a primer mixture. The initial PCR step was 3 minutes at 95°C, followed by 40 cycles of a 10 seconds melting at 95°C and a 45 seconds annealing/extension at 60°C. The final step was 1 minute incubation at 60°C. All reactions were performed in triplicate. Real time RT-PCR results were analyzed using Q-Gene software, which expresses data as mean normalized expression (Muller *et al.*, 2002). Mean normalized expression is directly proportional to the amount of mRNA of the target gene (haCER1) relative to the amount of mRNA of the reference gene ( $\beta$ -actin).

### Northern blot analysis

Northern blot analysis was performed as described (Mao *et al.*, 2001). Briefly, the haCER1 coding sequence amplified by PCR was radiolabeled by [<sup>32</sup>P]-dCTP using a random priming kit (Amersham, Piscataway, NJ). The radiolabeled DNA probe was hybridized to a multiple human tissue mRNA blot (BD Biosciences Inc.). After being stripped of the haCER1 probe, the mRNA blot was hybridized with a radioactive  $\beta$ -actin probe (BD Bioscience).

### haCER1 expression in yeast cells

The Flag-tagged haCER1 was expressed in yeast cells as described (Mao *et al.*, 2001). Briefly, the expression construct pYES2-Flag-haCER1 or the empty vector pYES2-Flag was transformed into the yeast strain  $\Delta ypc1\Delta ydc1$ . Expression of the Flag-tagged haCER1 was induced by 2% galactose. Microsomes were prepared and were subjected to Western blot analysis and ceramidase activity assays.

### haCER1 expression in mammalian cells

The expression construct pcDNA3-Flag-haCER1 or the empty vector pcDNA3.1 was transfected into HEKs using Lipofectamine and PLUS reagents (Invitrogen) according to the instructions of the manufacturers. Expression of the Flag-haCER1 was confirmed by Western blot analysis using an anti-Flag tag antibody (Sigma).

### Protein concentration determination

Protein concentrations were determined with BSA as a standard using a BCA protein determination kit (Pierce Inc., Rockford, IL) according to the manufacturer's instructions.

### Western blot analysis

Western blot analysis was performed as described (Hu *et al.*, 2005).

### Ceramidase activity assay

Ceramidase activity was determined by the release of sphingoid bases (SPH, dihydro sphingosine, or PHS) from ceramides, dihydroceramide, or phytoceramides. Briefly, 20  $\mu$ l of microsomes (1  $\mu$ g/ $\mu$ l proteins) in an assay buffer (25 mM Tris-HCl, pH 8.0, containing 5 mM CaCl<sub>2</sub>) was mixed with 20  $\mu$ l of substrate in the assay buffer containing 0.3% Triton X-100, and the mixture was incubated at 37°C for 30 minutes. The reactions were stopped by extraction with chloroform and methanol. Sphingoid bases were determined by high performance liquid chromatography (HPLC) with D-e-C<sub>17</sub>-SPH as an internal standard as described (Usta *et al.*, 2001).

### Immunocytochemistry

Cells were co-stained with antibodies against the Flag peptide (1:200) and calreticulin (1:200), followed by anti-rabbit and mouse IgG antibodies (1:200) conjugated with rhodamine and FITC, respectively, as described (Mao *et al.*, 2001). The immunostained cells were examined under a confocal laser-scanning microscope (Carl Zeiss, Inc., Thornwood, NY).

### ESI/MS/MS lipid analysis

ESI/MS/MS analysis of sphingolipids was performed on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer, operating in a Multiple Reaction Monitoring (MRM)-positive ionization mode as described (Bielawski *et al.*, 2006). Levels of sphingolipids were normalized to total phosphate, which was determined as described (Van Veldhoven and Bell, 1988).

### siRNA transfection

An siCON (UAAGGUAUGAAGAGAUACUU/GUAUCUCUUCUAU GCCUUUUU); a siCER1, siCER1 (GGCCUGUUCUCCAUGUA UUUUU/AAUACAUGGAGAACAGGCCUU); and an AC-specific siRNA (AAAUAUCAACCUAUCCUCCUUCUU/GAAGGAGGAUAG GUUGAUUUU) were chemically synthesized in Dharmacon (Lafayette, CO). HEKs cells were transfected with each siRNA at a concentration of 10 nM using Lipofectamine and PLUS reagents (Invitrogen Inc.) according to the manufacturer's instructions.

### MTT assay

Viable cells were determined using an *in vitro* toxicology assay kit based on MTT (Sigma Inc.) according to the manufacturer's instructions.

### [<sup>3</sup>H]Thymidine incorporation assay

Thymidine incorporation assays were performed as described (Rao and Otto, 1992). After being labeled with [<sup>3</sup>H]thymidine (1  $\mu$ Ci/ml) for 6 hours, the cells were washed three times with the ice-cold phosphate-buffered saline, incubated in 5% trichloroacetic acid at 37°C for 20 minutes, and then washed twice with 100% ethanol. The ethanol insoluble DNA was dissolved in 1% SDS and 0.3 N NaOH,



and incorporation of [<sup>3</sup>H]thymidine into DNA was determined by scintillation counting.

### Statistical analysis

Student's *t*-test was applied for statistical analysis. Data were presented as mean ± SD. Statistically significant differences were reported as \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

### CONFLICT OF INTEREST

The authors state no conflict of interest.

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