Downregulation of Carbonic Anhydrase IX Promotes Col10a1 Expression in Chondrocytes

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Abstract
Carbonic anhydrase (CA) IX is a transmembrane isozyme of CAs that catalyzes reversible hydration of CO2. While it is known that CA IX is distributed in human embryonic chondrocytes, its role in chondrocyte differentiation has not been reported. In the present study, we found that Car9 mRNA and CA IX were expressed in proliferating but not hypertrophic chondrocytes. Next, we examined the role of CA IX in the expression of marker genes of chondrocyte differentiation in vitro. Introduction of Car9 siRNA to mouse primary chondrocytes obtained from costal cartilage induced the mRNA expressions of Col10a1, the gene for type X collagen α-1 chain, and Epas1, the gene for hypoxia-responsible factor-2α (HIF-2α), both of which are known to be characteristically expressed in hypertrophic chondrocytes. On the other hand, forced expression of CA IX had no effect of the proliferation of chondrocytes or the transcription of Col10a1 and Epas1, while the transcription of Col2a1 and Acan were up-regulated. Although HIF-2α has been reported to be a potent activator of Col10a1 transcription, Epas1 siRNA did not suppress Car9 siRNA-induced increment in Col10a1 expression, indicating that down-regulation of CA IX induces the expression of Col10a1 in chondrocytes in a HIF-2α-independent manner. On the other hand, cellular cAMP content was lowered by Car9 siRNA. Furthermore, the expression of Col10a1 mRNA after Car9 silencing was augmented by an inhibitor of protein kinase A, and suppressed by an inhibitor for phosphodiesterase as well as a brominated analog of cAMP. While these results suggest a possible involvement of cAMP-dependent pathway, at least in part, in induction of Col10a1 expression by down-regulation of Car9, more detailed study is required to clarify the role of CA IX in regulation of Col10a1 expression in chondrocytes.

Introduction
Carbonic anhydrases (CAs, EC 4.2.1.1) are zinc metalloenzymes that catalyze reversible hydration-dehydration of carbon dioxide and bicarbonate (CO2 + H2O ↔ HCO3− + H+). In higher vertebrates, at least 13 active CA isozymes have been identified, namely CAs I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, XIV, and XV. They are grouped by their subcellular localization, as CAs I, II, III, VII, and XIII reside in cytosol, VA and VB are found in mitochondria, VI is secreted in saliva and milk, and IV, IX, XII, XIV, and XV are anchored in the plasma membrane. In addition, 3 catalytic CA-related proteins (CARPs) have been reported, namely cytosolic CARP VIII, and extracellular CARPs X and XI [1]. Among these CA isoforms, CA IX has been shown to be overexpressed in solid tumors, and has roles in tumor growth promotion, metastasis, and poor responsiveness to radio- and chemotherapy [2], whereas its expression in normal tissues is generally quite low, except for some locations including gastric mucosa [3]. Inhibition of CA IX in tumor cells by chemicals or specific antibodies is regarded as a promising strategy for cancer treatment [4]. CA IX, with a molecular weight of 54/58 kDa, consists of an N-terminal proteoglycan-like domain, CA catalytic domain, transmembrane domain, and short cytoplasmic tail at the C-terminus [5], and forms a homodimer by a disulfide bridge between the catalytic domains [6]. It is known that C9AR, the gene coding for CA IX, is one of the most hypoxia-sensitive genes with a hypoxia-responsive element in its promoter region [7], while hypoxia inducible factor (HIF)-1α but not HIF-2α activates C9AR transcription [8]. The hypoxic environment of solid tumors is believed to be an important factor to induce CA IX overexpression.

In endochondral bone formation, mesenchymal cells migrate to sites of future skeletonogenesis and differentiate into chondrocytes. Chondrocytes proliferate and sequentially differentiate from resting chondrocytes into proliferating chondrocytes, and then into hypertrophic chondrocytes, which finally induce vasculature, then mineralize and undergo apoptosis. Cartilage is one of the most avascular tissues and its development is promoted under hypoxic conditions [9], while HIF-1α is distributed in the interior region of growth plates where highly hypoxic chondrocytes are
located [10]. It was also reported that HIF-1α plays important roles in the survival of chondrocytes as well as matrix production by chondrocytes [11]. HIF-1α directly promotes the expression of Sox-9, a master gene of chondrogenesis, by binding to its promoter region [12], thus it is required for normal development of cartilage [13]. In addition, it was recently reported that hypertrophic chondrocytes express mRNA for Epas1, the gene coding a transcription factor HIF-2α. HIF-2α is known to promote the expression of type X collagen α1 chain (Col10a1), a marker gene for hypertrophic chondrocytes, and those for terminal differentiated chondrocytes, such as matrix metalloproteinase-13 (Mmp13), and vascular endothelial growth factor A (Vegfa) [14].

We recently found that CA II, an isozyme of CA IX distributed in cytosol, regulates the differentiation of ameloblasts by modulating cytosolic pH [15]. Although immunohistochemical analysis revealed that CA IX is distributed in chondrocytes in human embryos [16], to the best of our knowledge, there is no report of the function of CA IX in chondrocytes. Considering that CA IX expression is induced by HIF-1α and CA IX is involved in tumor growth, it is considered important to investigate the possible involvement of CA IX in the growth, differentiation, and function of chondrocytes. In this study, we explored the expression of CA IX in epiphyseal cartilage in mice and its role in the expression of differentiation-related genes in chondrocytes in vitro.

Results

CA IX Was Distributed in Stationary and Proliferating Chondrocytes, but not in Hypertrophic Chondrocytes in Mouse Epiphyseal Cartilage

It has been reported that CA IX is distributed in chondrocytes in human embryos [16]. However, the role of this enzyme in differentiation and metabolism of chondrocytes has not been investigated. To gain insight into the function of CA IX in differentiation of chondrocytes in the growth plate, we first examined its immunohistochemical localization in epiphyseal cartilage from neonatal mouse tibia, and compared it with that of type II and type X collagens, marker proteins of chondrocytes and hypertrophic chondrocytes, respectively (Figure 1). Type II collagen was distributed throughout the cartilage (Figures 1A, D, G, and J), while type X collagen was localized in hypertrophic regions (Figures 1B, E, H, and K). On the other hand, CA IX immunoreactivity was detected in round and columnar proliferating chondrocytes, especially those in the central portion of the growth plate (Figure 1C). The protein expression of CA IX gradually decreased along with the progression of chondrocyte differentiation (Figures 1C, F, and I) and was scarcely detected in hypertrophic chondrocytes (Figure 1L), which was in clear contrast to type X collagen immunoreactivity (Figures 1B, E, H, and K).

To ascertain differentiation-dependent changes in CA IX distribution, we quantitatively analyzed the expressions of Col2a1, Col10a1, and Car9 mRNA in regions that contained round proliferating chondrocytes, columnar proliferating chondrocytes, and hypertrophic chondrocytes in slices of epiphyseal cartilage prepared by laser microdissection (Figure 2A). While the expression of Col2a1 mRNA showed a tendency to decrease with progression of differentiation (Figure 2B), the expression of Col10a1 mRNA was prominent in hypertrophic region (Figure 2C). On the other hand, the highest level of Car9 mRNA expression was detected in regions containing round proliferating chondrocytes, while that was decreased in a differentiation-dependent manner and became very low in hypertrophic regions, where Col10a1 mRNA was highly expressed (Figure 2D). These results clearly indicate that the expression of Car9 mRNA is dependent on the differentiation stage of chondrocytes and down-regulated in hypertrophic chondrocytes. It was previously reported that transcription of the Car9 gene is promoted by HIF-1α [8], a transcription factor known to reside in chondrocytes in the growth plate proliferating zone at early differentiation stages [10,14]. Therefore, it is conceivable that the expression of CA IX protein in epiphyseal cartilage is regulated by HIF-1α at the transcriptional level.

Car9 siRNA Induced Increased Expression of Col10a1 mRNA in Primary Chondrocytes

Our finding of differentiation stage-dependent expression of CA IX in growth plate specimens (Figures 1 and 2) raised the possibility that this enzyme is involved in chondrocyte differentiation. To explore that possibility, we examined the effects of Car9 siRNA on the expression of several genes in primary chondrocytes, including marker genes of chondrocyte differentiation. As shown in Figure 3A, the Car9 siRNA used in this study significantly suppressed the expression of Car9 mRNA (p = 0.05). In addition, fluorescent immunostaining for CA IX showed a lowered expression of CA IX protein after introduction of Car9 siRNA (Figure 3B). Proliferation of chondrocytes was not suppressed significantly by introduction of Car9 siRNA (Figure 3C, p = 0.01). Next, we examined the expressions of marker genes related to differentiation of chondrocytes, including Col2a1, Col10a1, Vegfa, and Mmp13, using reverse transcription (RT)-polymerase chain

![Figure 1. Localization of CA IX in epiphyseal cartilage.](Image 130x175)
CA IX Activity Was Not Involved in Regulation of Col10a1 and Epas1 Expressions

We previously reported that CA II, the most abundant CA in cytosol, plays a pivotal role in differentiation of ameloblasts via intracellular pH-dependent regulation of c-Jun N-terminal kinase (JNK) activity [15]. To clarify that the decreased enzymatic activity of CA IX is involved in up-regulated expressions of mRNAs for Col10a1 and Epas1 in Car9-silenced chondrocytes, cells were cultured in the presence or absence of an inhibitor for CA IX, 4-(2-aminoethyl)-benzenesulfonyl fluoride, after introduction of Car9 or control siRNA. As shown in Figure 4E, the CA IX inhibitor did not affect the mRNA expression of either Col10a1 or Epas1. In addition, we determined intracellular and extracellular pH values in primary chondrocytes after introduction of Car9 or control siRNA. Despite the fact that CA IX is one of the enzymes that regulates cellular pH, Car9 siRNA showed little effect on intracellular or extracellular pH in chondrocytes (Figures 4G and H). These results indicate that the catalytic activity of CA IX is dispensable for regulation of expression of these genes.

Col10a1 Expression Was Up-regulated by Car9 siRNA in Primary Chondrocytes Cultured in a Hypoxic Condition

In a separate experiment, we examined the effect of Car9 silencing on mRNA expressions of Car9, Col10a1, Sox5, Sox6, Sox9, and Epas1 in chondrocytes cultured under a hypoxic condition (Figure 5). There was a scant difference between the increments in Col10a1 expression in chondrocytes under hypoxia as compared to chondrocytes under normoxia. As with Col10a1 expression, oxygen tension did not affect the level of Epas1 expression. In contrast, Car9 siRNA lowered Epas1 mRNA levels in chondrocytes cultured under hypoxia (Figure 5).

Forced Expression of CA IX did not Affect Expression of Col10a1 or Epas1 in chondrocytes

To examine whether overexpression of CA IX suppresses the expression of Col10a1 and Epas1 mRNAs in chondrocytes contrary to the reactions after introduction of Car9 siRNA (Figures 3 and 4), we introduced an expression plasmid of CA IX into chondrocytes. However, forced expression of CA IX did not change the mRNA levels of either Col10a1 or Epas1 in Chondrocyte Differentiation

Figure 2. Expression of Car9 mRNA in epiphyseal cartilage.

Frozen sections of lower limbs excised from 1-day-old postnatal ddY mice were microdissected and analyzed for the mRNA expressions of Col2a1, Col10a1, and Car9.

A. Regions consisting of round-shaped proliferating chondrocytes (1), columnar proliferating and prehypertrophic chondrocytes (2), and hypertrophic chondrocytes (3) were isolated from epiphyseal cartilage sections sliced by laser microdissection. B. Expression of Col2a1 mRNA in the microdissected specimens (1, 2, 3). C. Expression of Col10a1 mRNA in the microdissected specimens (1, 2, 3). D. Expression of Car9 mRNA in the microdissected specimens (1, 2, 3). The expression level of each gene was normalized to that of Gapdh and expressed as a value relative to that obtained in region 1. Data are expressed by boxplots (the sample maximum, the upper quartile, the median, the lower quartile, and the minimum observation). 

reaction (PCR) (Figure 3D). Expression of Col2a1, expressed by chondrocytes irrespective of their differentiation stage, was not changed by Car9 silencing. The Col10a1 gene is known to be expressed in hypertrophic chondrocytes, and we found that its mRNA expression was up-regulated by introduction of Car9 siRNA. On the other hand, the expressions of Vegfa and Mmp13, both of which are specifically observed in terminally differentiated hypertrophic chondrocytes in growth plates, were not affected by Car9 siRNA. To ascertain the augmented expression of Col10a1 in Car9-silenced chondrocytes, the expression level of Col10a1 mRNA was quantitatively analyzed using real-time RT-PCR along with those of Col2a1 and aggrecan (Acan) (Figures 3E–G). Car9 siRNA caused an 8-fold increase in Col10a1 expression (Figure 3F). Acan as well as Col2a1 is known to be expressed by both proliferating and hypertrophic chondrocytes. While the mRNA level of Col2a1 was not affected (Figure 3E), that of Acan was suppressed to one-half by introduction of Car9 siRNA (Figure 3G). In parallel with the lowered expression of Acan mRNA, introduction of Car9 siRNA induced a 29.6% decrease in the amount of Alcian blue bound to the chondrocyte culture at 4 days after the siRNA introduction (Figure 3H), indicating that the amounts of proteoglycans including aggrecan were decreased by Car9 silencing.

Epas1 Expression Was Up-regulated by Car9 siRNA in Primary Chondrocytes

Although Sox5, Sox6, and Sox9 are genes for well-known transcription factors that control chondrocyte differentiation, their expressions were not shown to be influenced by Car9 siRNA (Figures 4A–C). It was recently reported that HIF-2α, a transcription factor encoded by Epas1, is expressed in terminally differentiated growth plate chondrocytes [17] and activates Col10a1 gene [14]. We examined the effects of introduction of Car9 siRNA on the mRNA expressions of Epas1 and other hypoxia-related transcription factors, namely Hif1α and Hif3α, using RT-PCR (Figure 4D). While the expression of Hif1α mRNA in primary chondrocytes was not affected, that of Epas1 was up-regulated by introduction of Car9 siRNA. Hif3α mRNA was not detected in chondrocytes regardless of the level of Car9 expression. Real-time RT-PCR analysis revealed that Car9 siRNA had little influence on the mRNA expressions of Sox5, Sox6, and Sox9 (Figures 4A–C), whereas it induced a 3-fold increase in the mRNA level of Epas1 (Figure 4E).
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No significant difference between the values was indicated at any time point (p = 0.05). D. At 48 hours after introduction of siRNAs, expressions of mRNAs for Col2a1, Col10a1, Vegfa, and Mmp13 were analyzed by RT-PCR. E-G. The expressions of Col2a1 (E), Acan (F), and Col10a1 (G) were quantitatively analyzed by real-time RT-PCR. The expression level of each gene was normalized to that of Gapdh. Data are expressed by boxplots (n = 4) in the fold change by introduction of Car9 siRNA (the sample maximum, the upper quartile, the median, the lower quartile, and the minimum observation). P-values determined by two-tailed Mann-Whitney U-test are indicated. H. At 4 days after introduction of Car9 or control siRNA, chondrocyte cultures were stained with Alcian blue. Alcian blue bound to the cell matrix was extracted and determined spectrophotometrically (n = 4). A P-value determined by two-tailed Mann-Whitney U-test is indicated. Typical photographs are shown above the columns. doi:10.1371/journal.pone.0056984.g003

Figure 3. Effects of Car9 siRNA on chondrocyte proliferation and expressions of marker genes of chondrocyte differentiation. Car9 siRNA or control siRNA was introduced into primary chondrocytes isolated from the rib cages of 1-day-old postnatal ddY mice. A. The expression of Car9 mRNA was analyzed by real-time RT-PCR at 48 hours after introduction of Car9 siRNA or control siRNA, with the level normalized to that of Gapdh. Data are expressed as the mean ± SD (n = 4) for fold changes caused by introduction of Car9 siRNA. P-value obtained by two-tailed Mann-Whitney U-test (n = 4) indicated that the Car9 siRNA used in this study significantly lowered the expression level of Car9 mRNA (p = 0.05). B. Expression of CA IX protein in chondrocytes was detected by immunocytochemical staining at 48 hours after introduction of control siRNA (left 2 panels) or Car9 siRNA (right 2 panels). Lower 2 panels show the results obtained without primary antibody against CA IX. Bar, 50 μm. C. Car9 siRNA (unfilled square) or control siRNA (filled square) was introduced to chondrocytes on day 0. Proliferation of chondrocytes was assessed spectrophotometrically using CellTiter 96® Aqueous One Solution. Data are expressed as the mean ± SD (n = 4). At each time point, Mann-Whitney U-test with Bonferroni correction was performed to evaluate the difference between control siRNA- and Car9 siRNA-introduced cells.
effect on the expression of Col10a1 was small. H89, an inhibitor of protein kinase A (PKA), up-regulated the expression of Col10a1 mRNA in both Car9 siRNA- and control siRNA-introduced cells, while its effect on the expression of Epas1 mRNA was only marginal. On the other hand, 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of phosphodiesterase (PDE), suppressed augmentation of Col10a1 expression by Car9 siRNA. LY294002, a phosphatidylinositol 3-kinase (PI3K) inhibitor, increased the basal expression level of Epas1 in control siRNA-introduced cells. Also, a Src Kinase Inhibitor 1 [4-(4'-Phenoxyanilino)-6,7-dimethoxyquinazoline] suppressed the expression of Col10a1 and Epas1 in Car9 siRNA-introduced cells. Jervine, a hedgehog signaling pathway inhibitor, did not show a marked effect on the expression of Col10a1 or Epas1 in either Car9 siRNA- or control siRNA-introduced cells. These results imply that various intracellular signaling systems are involved in expression of Col10a1 and Epas1 mRNAs in chondrocytes. Specifically, there is a possibility that p38 MAPK and PI3K are involved in the expression of Epas1, and that the cAMP-dependent pathways negatively regulate Col10a1 expression. In addition, it is also indicated that Src-dependent

Figure 4. Effects of Car9 siRNA on expressions of transcription factors related to chondrocyte differentiation. Car9 siRNA (+) or control siRNA (-) was introduced into mouse primary chondrocytes. A-C. The expressions of Sox5 (A), Sox6 (B), and Sox9 (C) were quantitatively analyzed by real-time RT-PCR at 48 hours after introduction of the siRNAs. The expression level of each gene was normalized to that of Gapdh. Data are expressed by boxplots (n = 4) for fold changes caused by introduction of Car9 siRNA (the sample maximum, the upper quartile, the median, the lower quartile, and the minimum observation). D. The expressions of mRNAs for Hif1a, Epas1, Hif3a, and Gapdh were analyzed by RT-PCR at 48 hours after introduction of Car9 siRNA or control siRNA. E. The expression of Epas1 was quantitatively analyzed by real-time RT-PCR at 48 hours after introduction of the siRNAs. Data are expressed by boxplots. F. Twenty-four hours after introduction of Car9 siRNA or control siRNA, primary chondrocytes were additionally cultured for 24 hours in the presence or absence of 50 μM 4-(2-aminoethyl)-benzenesulfonamide, an inhibitor of extracellular CAs. Then, the expressions of mRNAs for Car9, Col2a1, Col10a1, Epas1, and Gapdh were analyzed by RT-PCR. G and H. Intracellular (G) and extracellular (H) pH values were determined in primary chondrocytes at 48 hours after introduction of Car9 siRNA and control siRNA. Data are expressed as the mean ± SD of 4 experiments. P-values determined by two-tailed Mann-Whitney U-test are indicated.

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pathways may be involved in the expression of both Col10a1 and Epas1.

While the effects of H89 and IBMX on Col10a1 expression suggested an involvement of the cAMP-dependent pathways in induction of Col10a1 in Car9-silenced chondrocytes, cAMP levels in chondrocytes introduced with Car9 siRNA and its control siRNAs were determined at 36 hours after introduction of the siRNAs. The amount of cAMP in the Car9-silenced chondrocytes was significantly lower than that in control chondrocytes (Figure 8B, \( a = 0.05 \)). While a significant difference was not observed, there was a lowering trend in the PKA activity in Car9-silenced chondrocytes compared with the control siRNA-introduced cells (Fig. 8C). To further investigate the involvement of the cAMP-dependent pathway in induction of Col10a1 and Epas1 expressions after Car9 silencing, we examined the effects of 8-bromoadenosine-3',5'-cyclic monophosphate sodium salt (Br-cAMP), a membrane-permeable analog of cAMP, on the expressions of Col10a1, Car9, and Epas1 in chondrocytes with or without introduction of Car9 siRNA. Br-cAMP did not affect the expression of Car9 in the control or Car9 siRNA-introduced chondrocytes (Figure 8D). On the other hand, Br-cAMP significantly suppressed the increased expression of Col10a1 mRNA induced by Car9 silencing (Figure 8E, \( a = 0.05 \)), while it up-regulated the expression of Epas1 mRNA in chondrocytes irrespective of Car9 expression level (Figure 8F, \( a = 0.05 \)). These results suggest that the cAMP/PKA pathway is one of the possible mechanisms involved in regulation of Col10a1 expression by CA IX in chondrocytes.

Discussion

In the present study, we found that CA IX was distributed among proliferating chondrocytes in mouse epiphyseal cartilage, while its expression was down-regulated in hypertrophic chondrocytes. In addition, introduction of Car9 siRNA up-regulated the expression of mRNA for Col10a1 in mouse primary chondrocytes in vitro. Taken together, it is reasonable to conclude that CA IX suppresses the expression of Col10a1, a typical gene expressed in hypertrophic chondrocytes, both in resting and proliferating chondrocytes. Since the forced expression of CA IX had no effect on proliferation of chondrocytes and expression of Col10a1 in chondrocytes, there is a possibility that the amount of endogenous CA IX in chondrocytes is sufficient for supporting their proliferation and suppression of Col10a1 expression. In addition to the change in Col10a1 expression, lowered expression of Acan mRNA (Figure 3G) and reduced Alcian blue binding (Figure 3H) by introduction of Car9 siRNA, indicating a possibility that CA IX plays a role in regulation of the expression of not only Col10a1 but also Acan gene.

Resting and proliferating chondrocytes in growth plates express HIF-1\( \alpha \) [13], whereas hypertrophic chondrocytes express HIF-2\( \alpha \) [17]. Replacement of HIF-1\( \alpha \) with HIF-2\( \alpha \) in growth plates is important for chondrocyte differentiation and skeletal development. HIF-1\( \alpha \) binds to the Sox9 promoter to activate its expression [12]. Since Sox9 is a master gene of chondrocyte differentiation [18], HIF-1\( \alpha \) is required for chondrogenic differentiation of mesenchymal stem cells and maintenance of chondrocyte phenotypes [12,19]. On the other hand, it is also known that SOX-9 suppresses hypertrophic differentiation of chondrocytes [20,21], while HIF-2\( \alpha \) reportedly plays important roles in hypertrophic differentiation of chondrocytes.
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That is, HIF-1α is regarded to be more vulnerable to oxygen than HIF-2α, which causes preferential distribution of the latter in hypertrophic regions which are less hypoxic as compared to resting and proliferating regions of growth plates [10,22]. Since Car9 is known to be transactivated by HIF-1α, but not HIF-2α [7,8], it was not unexpected that the expressions of Car9 mRNA and CA IX protein were observed in proliferating chondrocytes and then down-regulated in hypertrophic chondrocytes (Figures 1 and 2).

HIF-2α is regarded as a regulator of the expression of marker genes of hypertrophic chondrocytes, including Col10a1, Vegfa, and Mmp13 [14]. In this study, the expressions of Col10a1 and Vegfa were up-regulated by Car9 siRNA (Figures 3F and 4E), whereas those of Vegfa and Mmp13 were not affected (Figure 3D). In addition, Epas1 siRNA did not suppress Car9 siRNA-induced increment in Col10a1 expression (Figure 7C). These results indicate that Car9 siRNA induced Col10a1 transcription through a mechanism independent from the increased expression of Epas1 mRNA. Lowered expression of HIF-2α protein was confirmed in the chondrocytes introduced with Epas1 siRNA and those with both Car9 and Epas1 siRNAs (Figure 7D), which indicates that the increased expression of Col10a1 mRNA in Car9-silenced chon-

Figure 7. Independent regulation of expressions of Epas1 and Col10a1 by CA IX. Car9 siRNA (siCar9) and Epas1 siRNA (siEpas1) were simultaneously introduced into primary chondrocytes. The expression levels of mRNAs for Car9 (A), Epas1 (B), and Col10a1 (C) were quantitatively analyzed by real-time RT-PCR. The expression level of each gene was normalized to that of Gapdh and is shown as the relative value to that obtained with cells introduced with only control siRNA (leftmost column in each graph). Data are expressed as the mean ± SD (n = 4). At each time point, Mann-Whitney U-test with Bonferroni correction was performed to evaluate the difference between control plasmid- and CA IX-expression plasmid-transfected cells. No significant difference between the values was indicated at any time point (p > 0.01). C-F. The expressions of Col2a1 (C), Acan (D), Col10a1 (E), and Epas1 (F) were quantitatively analyzed by real-time RT-PCR, with the expression level of each normalized to that of Gapdh. Data are expressed by boxplots (n = 6) for fold changes caused by introduction of Car9 siRNA (the sample maximum, the upper quartile, the median, the lower quartile, and the minimum observation). P-values determined by two-tailed Mann-Whitney U-test are indicated. doi:10.1371/journal.pone.0056984.g007

differentiation of chondrocytes and endochondral ossification [14,17]. Transition from HIF-1α to HIF-2α in growth plates has been explained by the differential stability of these HIF proteins.

Figure 6. Effects of forced expression of CA IX on chondrocyte proliferation and mRNA expression of Col2a1, Acan, Col10a1, and Epas1. Primary mouse chondrocytes were transfected with a CA IX-expression plasmid (+) or its control plasmid (-). A. At 48 hours after transfection, the expression of CA IX (58kDa) was assessed by western blot analysis using anti-mouse CA IX antibody. CA IX band is indicated by an arrowhead. B. Proliferation of chondrocytes introduced with CA IX-expression plasmid (filled square) and control (unfilled circle) plasmids was assessed spectrophotometrically using CellTiter 96 Aqueous One Solution. Data are expressed as the mean ± SD (n = 4). At each time point, Mann-Whitney U-test with Bonferroni correction was performed to evaluate the difference between control plasmid- and CA IX-expression plasmid-transfected cells. No significant difference between the values was indicated at any time point (p = 0.01). C-F. The expressions of Col2a1 (C), Acan (D), Col10a1 (E), and Epas1 (F) were quantitatively analyzed by real-time RT-PCR, with the expression level of each normalized to that of Gapdh. Data are expressed by boxplots (n = 6) for fold changes caused by introduction of Car9 siRNA (the sample maximum, the upper quartile, the median, the lower quartile, and the minimum observation). P-values determined by two-tailed Mann-Whitney U-test are indicated. doi:10.1371/journal.pone.0056984.g006

differentiation of chondrocytes and endochondral ossification [14,17]. Transition from HIF-1α to HIF-2α in growth plates has been explained by the differential stability of these HIF proteins.
Cytotoxic (Figure 7C) was induced by some mechanisms other than HIF-2α. Further studies are required to clarify the mechanisms of Col10a1 induction in Epas1-silenced chondrocytes. Treatment with Car9 siRNA alone also lowered the expression level of HIF-2α (Figure 7D), even though Car9 silencing increased the expression level of Epas1 mRNA (Figure 7B). While there is a possibility that CA IX facilitates translation of the Epas1 gene or suppresses degradation of HIF-2α protein through an unknown mechanism, further studies are also required to explain the discrepancy between the increased Epas1 mRNA level and decreased HIF-2α protein level in Car9-silenced chondrocytes. At least it can be mentioned that HIF-2α does not play an important role in the induced expression of Col10a1 mRNA in the Car9-silenced chondrocytes.

It has been reported that CA II, one of the isozymes of CA IX, plays a critical role in ameloblast differentiation via intracellular pH-dependent regulation of JNK activity [15]. However, Car9 siRNA did not induce a significant change in intracellular or extracellular pH in primary chondrocytes in the present experimental settings (Figures 4G and H, α = 0.05). In addition, SP600125, a JNK inhibitor, did not affect the mRNA expression of Col10a1 (Figure 8A), indicating that CA IX modulates Col10a1 expression by a mechanism different from that shown in ameloblasts [15].

Among the inhibitors tested in this study, IBMX, an inhibitor of PDE, suppressed the increment in Col10a1 expression induced by Car9 siRNA (Figure 8A), suggesting a possible involvement of cyclic nucleotides such as cAMP and/or cGMP in that suppressed expression. Several reports have indicated the role of cGMP-dependent kinase II in promotion of hypertrophic differentiation of chondrocytes [23–25]. Therefore, it seems unlikely that cGMP participates in suppression of increased expression of Col10a1 in
Epas1 cAMP enhanced Car9 dependent pathways plays a role in induction of 8E), which reinforces the idea that down-regulation of cAMP-regulation of 8A). Although the requirement of MEK/ERK pathways for 8A). In the present study, inhibition of PKA augmented the 8A). In addition, our finding of a decrease in cellular level of cAMP in 8A) and Car9-siRNA-introduced chondrocytes indicated that CA IX is involved in maintenance of cAMP level in chondrocytes (Figure 8B). Furthermore, Br-cAMP suppressed the augmented expression of Col10a1 mRNA in chondrocytes harboring Car9 siRNA (Figure 8E), which reinforces the idea that down-regulation of cAMP-dependent pathways plays a role in induction of Col10a1 expression in Car9-silenced chondrocytes. On the contrary, Br-cAMP enhanced Epas1 expression in both control and Car9-silenced chondrocytes (Figure 8F), indicating that CA IX suppresses Epas1 expression via a mechanism other than cAMP-dependent pathways.

Several reports have noted biological functions of CA IX aside from its enzymatic activity. For example, it was reported that the proteoglycan-related sequence within the CA IX molecule mediates cell adhesion [30,31] and that CA IX forms a complex with β-catenin [31]. It is known that the Wnt/β-catenin cascade plays a crucial role in chondrocyte differentiation [25,32]. In addition, CA IX reportedly functions as a molecular chaperone [33]. Thus, it is suggested that CA IX suppresses the expression of Col10a1 at least in part via maintenance of the cAMP-dependent pathway in a manner independent of its enzymatic activity resides in the extracellular domain. Further detailed studies are required to clarify how CA IX regulates intracellular cAMP level.

Although more detailed studies on translation, post-translational modifications including phosphorylation, degradation, and molecular interaction of signaling molecules are needed to confirm that CAIX regulates expression of Col10a1 expression, our present experiments using inhibitors of signaling molecules hint that several intracellular signaling pathways other than cAMP-dependent ones possibly play a part in the expressions of Col10a1 and Epas1 mRNAs in chondrocytes. For instance, treatment with a Src kinase inhibitor [Src Kinase Inhibitor I] lowered the expression of Col10a1 as well as that of Epas1 in Car9-silenced chondrocytes (Figure 8A). It was shown that treatment with PP2, another Src kinase inhibitor, increased the expression of both early and late differentiation markers including Col10a1 in a chondrogenic cell line ATDC5 [34]. Since it is reported that CA IX interacts with β-catenin [31], one of the substrates of Src kinase [35], there may be an interaction between CA IX and Src in regard to β-catenin. As for Epas1, it was reported that Src kinases mediate the hypoxia-induced expression of Epas1 mRNA in human lung adenocarcinoma cells [36]. On the other hand, inhibition of p38 MAPK and PI3K increased the expression of Epas1 in the control chondrocytes without Car9 silencing in our experiments (Figure 8A). Although the requirement of MEK/ERK pathways for Epas1 expression under hypoxia has been reported [37], to the best of our knowledge, the role of p38 MAPK or PI3K in regulation of Epas1 expression have not been studied.

From the results obtained in this study, we propose a novel role for CA IX in cartilage development that is down-regulating Col10a1 mRNA expression in stationary and proliferating chondrocytes (Figure 9). Hence, down-regulation of CA IX in hypertrophic regions of growth plates triggers Col10a1 expression. On the other hand, the forced expression of CA IX had no effect on the expression of Col10a1 in chondrocytes, suggesting a possibility that the amount of endogenous CA IX in chondrocytes is sufficient for suppression of Col10a1 expression.

Materials and Methods

Ethics Statement

All animal experiments were approved by the Ethical Board for Animal Experiments of Showa University (Approval No. 11027), Tokyo, Japan.

Mice

We used ddY mice provided by Japan SLC Inc. (Hamamatsu, Japan). They were housed in a specific pathogen-free environment.

Preparation of Frozen Fixed Samples

Lower limbs were dissected from 1-day-old postnatal ddY mice, embedded in OCT compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan).

Figure 9. Schematic representation of possible roles of CA IX in regulation of Epas1 expression in chondrocytes. Blue and red lines represent pathways already known and those indicated in this study, respectively. Arrows with equilateral-triangular heads and T-shaped bars show facilitation and suppression of the pathways, respectively. HIF-1α induces the expression of Car9 mRNA and hence that of CA IX protein [8]. It is known that cAMP-dependent pathways inhibit hypertrophic differentiation of chondrocytes including the expression of Col10a1 [26-29]. On the other hand, it is reported that HIF-2α, encoded by Epas1 gene, transactivates the Col10a1 gene [14]. We propose that CA IX suppresses the expression of Col10a1 mRNA partly via a cAMP-dependent manner based on the following observations. Col10a1 expression was induced by introduction of Car9 siRNA (Figure 3G). The cAMP level was lowered by Car9 siRNA (Figure 8B). Inhibition of PKA by H89 augmented the expression of Col10a1 induced by Car9 silencing (Figure 8A). In addition, inhibition of PDE by IBMX and activation of PKA by Br-cAMP suppressed the expression of Col10a1 expression induced by Car9 silencing (Figures 8A and 8D). While Car9 siRNA also enhanced the expression of Epas1 mRNA (Figure 4F), HIF-2α does not mediate the Col10a1 induction by Car9 silencing (Figure 7C). It is partly because Car9 siRNA lowers HIF-2α protein via an unknown mechanism (Figure 7D). Epas1 siRNA rather enhanced the expression of Col10a1 mRNA by an unknown mechanism (Figure 7C). It is reported that Src kinase mediates the expression of Epas1 and Col10a1 [34,36]. In this study, Src kinase inhibitor I lowered the induction of Epas1 and Col10a1 expressions after Car9 silencing (Figure 8A), which may indicate a possibility that CA IX suppresses the Src kinase-mediated pathways. Augmentation of Epas1 expression in the presence of the inhibitors of p38 MAPK (SB202190) and PI3K (LY294002) suggests a possible inhibition of Epas1 expression by these kinases (Figure 8A).

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Tokyo, Japan), and immediately snap frozen in isopentane cooled in liquid nitrogen. The specimens were then made into frozen blocks and stored at -80°C. Frozen samples prepared for laser microdissection were sliced using a cryomicrotome (MICROM International GmbH, Walldorf, Germany) at a thickness of 7 μm, then each tissue section was affixed to a slide to which an original thin film (provided by Meiwa Shoji Co., Ltd., Tokyo, Japan) had been attached using silicone adhesive (GE Toshiba Silicone, Tokyo, Japan). Frozen tissues for immunohistochemistry were sliced at a thickness of 4 μm. Sliced samples were stored at -40°C until use.

Laser Microdissection and cDNA Preparation from RNA Isolated from Micro-dissected Samples
The sliced samples were air-dried at room temperature for 2 to 3 minutes and fixed in 100% methanol for 3 minutes. The sections were then washed with 50% ethanol, stained with LCM Staining Kit (Ambion, Carlsbad, CA, USA), and washed with 100% ethanol, then air-dried, after which they were subjected to microdissection using a PALM Micro Beam (P.A.L.M. Microlaser Technologies, Bernried, Germany) with a 337-nm nitrogen laser. The targets were regions that mainly consisted of round proliferating chondrocytes, columnar proliferating chondrocytes with pre-hypertrophic chondrocytes, and hypertrophic chondrocytes (Figure 2A). Each region was collected from 4 serial sections. There are estimated to be approximately 500–1000 microdissected cells per region. Total RNA was extracted from the laser micro-dissected tissues using an RNAeasy Micro Kit (Qiagen, Valencia, CA, USA). At the final step of extraction, RNA was eluted in 17 μl of DNase/RNase-free water (Invitrogen Co., Carlsbad, CA, USA). RT reaction was performed using High Capacity RNA-to cDNA MasterMix (Applied Biosystems, Carlsbad, CA, USA) with 16 μl of eluted RNA solution.

Immunohistochemistry of Type II Collagen
Frozen sections at 4 μm thick were air-dried for 10 minutes, fixed by incubation for 5 minutes in ice-cold acetone, and washed 3 times in phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked by treatment for 30 minutes in methanol containing 1% hydrogen peroxide. The samples were then treated for 15 minutes with Protein Block Serum-Free Ready-to-Use (DAKO, Tokyo, Japan) before incubation for 1 hour at 37°C with anti-chicken type II collagen monoclonal IgG (10R-C135A, Fitzgerald Industries International, Acton, MA, USA) at a dilution ratio of 1:100. After washing 3 times with PBS, the primary antibody bound to the samples was visualized by treatment for 30 minutes at room temperature with DAKO EnVision + System-HRP Labeled Polymer Anti-Mouse (K4000, DAKO) and subsequent incubation with a DAB substrate kit (K3468, DAKO). After washing with PBS, the slides were observed under a light microscope.

Immunohistochemistry of Type X Collagen
Frozen sections at 4 μm thick were air-dried for 10 minutes, fixed by incubation for 5 minutes in ice-cold ethanol, and washed 3 times in PBS. Endogenous peroxidase activity was blocked by treatment for 10 minutes in methanol containing 3% hydrogen peroxide. The samples were treated for 10 minutes at 43°C with 0.5% bovine serum albumin in PBS, washed 3 times with PBS, and incubated for 30 minutes at room temperature in PBS containing 2 mg/ml hyaluronidase from bovine testes (Sigma-Aldrich). After stopping the hyaluronidase activity with fetal bovine serum (FBS) (Invitrogen), the samples were incubated for 45 minutes at 37°C with anti-rat type X collagen rabbit polyclonal antibody (No. 234196, Calbiochem, Darmstadt, Germany) at a dilution ratio of 1:30 and washed 3 times with PBS. The primary antibody was visualized following treatment for 30 minutes at room temperature with DAKO EnVision + System-HRP Labeled Polymer Anti-Rabbit (K4003, DAKO) and subsequent incubation with a DAB substrate kit (DAKO). After washing with PBS, the slides were observed under a light microscope.

Immunohistochemistry of CA IX
Frozen sections at 4 μm thick were air-dried for 10 minutes, fixed by incubation for 10 minutes in 4% paraformaldehyde, pH 7.4, and washed 3 times in PBS. After blocking the endogenous peroxidase activity by 3% hydrogen peroxide, the samples were treated with Protein Block Serum-Free Ready-to-Use (DAKO). The samples were then incubated for 1 hour at room temperature with anti-mouse CA IX antibody (M-100 anti-mouse CA IX rabbit polyclonal antibody, sc-25600, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After washing with PBS, the primary antibody was visualized following treatment for 30 minutes at room temperature with DAKO EnVision + System-HRP Labeled Polymer Anti-Rabbit (DAKO) and subsequent incubation with a DAB substrate kit (DAKO). After washing with PBS, the slides were observed under a light microscope.

Cell Culture
Primary chondrocytes were isolated from costal cartilages of 1-day postnatal ddY mice (Japan SLC). Briefly, rib cages were excised from the mice and digested to remove soft tissues for 1 hour at 37°C with 1 mg/ml collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) in a mixture of Dulbecco’s modified Eagle’s medium and Ham F12 medium (DMEM/F12), containing antibiotics and 2.5% FBS. Cartilage specimens were washed with PBS and further digested for 15 hours at 37°C in the same medium containing 1 mg/ml collagenase A. The resultant cell suspension was filtered through a 70-μm cell strainer to remove debris. Primary chondrocytes were washed by centrifugation and used in the experiments without passage. Experimental conditions for introduction of the siRNAs and the plasmids into and cultivation of chondrocytes were described below. For cell proliferation assay, chondrocytes were plated in 96-well plates at a density of 1×10^4 cell/well. Cell proliferation was assessed spectrophotometrically using CellTiter 96® Aqueous One Solution (Promega, Madison, WI, USA), according to the manufacturer’s instructions. In some experiments, isolated chondrocytes were cultured under a hypoxic condition (5% CO2 and 95% N2) using an INVIVO2 hypoxia workstation (Ruskinn Technology Ltd., Bridgend, UK).

Introduction of siRNAs for Car9 and Epas1 into Chondrocytes
Stealth™ siRNAs for mouse Car9 and Epas1 and their control non-silencing siRNA were purchased from Invitrogen. The sense and antisense sequences of Stealth™ siRNAs for Car9 and Epas1, designed using BLOCK-iT™ RNAi Designer (Invitrogen), are shown in Table S1. Each siRNA (30 pmol) was introduced into 40–50% confluent chondrocytes cultured in antibiotics-free DMEM/ F12 containing 2.5% FBS in 6-well plates using Lipofectamine RNAiMAX (Invitrogen) by reverse transfection under 5% CO2–95% air (a normoxic condition) or 5% CO2–95% N2 (a hypoxic condition). After 24 hours of incubation, the culture medium was changed to fresh DMEM/F12 + 2.5% FBS containing antibiotics. In some experiments, Epas1 siRNA (30 pmol) and Car9 siRNA (30 pmol) was added to the culture medium.
pmol) were simultaneously introduced into chondrocytes in the same manner as described above. Expression of Car9 and Epas1 mRNAs was evaluated by RT-PCR and real-time RT-PCR, as described below.

**Immunological evaluation of CA IX knockdown**

CA IX protein level in Car9-silenced cells was visualized by immunocytochemistry. Briefly, primary chondrocytes introduced with Car9 siRNA or its control siRNA were fixed by treatment with 4% paraformaldehyde (pH 7.4) for 10 minutes at 4°C, then incubated for 1 hour with a 4% solution of Block Ace™ (Dainippon Pharma, Osaka, Japan). Next, the cells were incubated overnight at 4°C with 0.5 μg/ml M-100 anti-mouse CA IX rabbit polyclonal antibody (Santa Cruz) in 0.4% Block Ace solution. The cell-bound primary antibody was visualized under a fluorescence microscope after incubation with Alexa Fluor® 488 donkey anti-rabbit IgG (Invitrogen).

**Western Blot Analysis**

An expression plasmid for CA IX was prepared by insertion of a RIKEN Mouse FANTOM® clone of Car9 cDNA (clone ID 9130714F03, GenBank accession AK136579.1) purchased from DNASFORM (Yokohama, Japan) into pcDNA3.1/His C (Invitrogen). The CA IX expression plasmid and its control plasmid (pcDNA3.1/His C) [4 μg] were transfected into 40–50% confluent chondrocytes cultured in 6-well plates using Lipofectamine 2000 (Invitrogen). Forced expression of CA IX protein was confirmed by Western blot analysis.

**Treatment of Chondrocytes with Chemicals**

Br-cAMP (Cat. No. B5386), a membrane-permeable derivative of cAMP, 4-[2-aminoethyl]benzenesulfonylamide (Cat. No. 275247), an inhibitor of extracellular CaAs, IBMX (Cat. No. 15879), a PDE inhibitor, SP600125 (Cat. No. S5567), a JNK inhibitor, and LY294002 (Cat. No. L9908), a PI3K inhibitor, were purchased from Sigma-Aldrich. Src Kinase Inhibitor I (Cat. No. 567805), a PKA inhibitor, and Jervine (Cat. No. 420210), a hedgehog signaling pathway inhibitor, were obtained from Calbiochem (La Jolla, CA, USA). These inhibitors were added to cultures at 24 hours after introduction of the siRNAs and the cells were cultured for an additional 24 hours in their presence. The concentrations of the reagents in culture medium were as follows: Br-cAMP, 0.2 mM; 4-[2-aminoethyl]benzenesulfonylamide, 50 μM; IBMX, 0.1 mM; SP600125, 30 μM; LY294002, 20 μM; Src kinase Inhibitor I, 20 μM; SB202190, 20 μM; H89, 10 μM; and Jervine, 10 μM.

**Determination of cAMP Content in Chondrocytes**

cAMP was extracted from the cultured chondrocytes with 0.1 M HCl at 36 hours after introduction of Car9 and control siRNAs. cAMP content was quantified using a Cyclic AMP EIA Kit (Cayman Chemical Co., Ann Arbor, MI, USA), according to the manufacturer's instructions. The cAMP content was corrected for cellular protein determined using a BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

**Measurement of PKA Activity in Chondrocytes**

PKA activity in the lysates of chondrocytes was determined using a PKA Kinase Activity assay kit (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. The PKA activity was checked by agarose-gel electrophoresis. Concentration and quality of RNA were analyzed using NanoDrop® Spectrophotometer ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Fresh RNA samples having the ratio of absorptions at 260 nm vs. 280 nm (260/280) higher than 1.8 were applied to RT reactions. cDNA was synthesized in a 10 μl reaction mixture containing 2 μg of total RNA using Superscript® III reverse transcriptase (Invitrogen) and a random hexamer (Invitrogen) following the manufacturer's instructions. Since amplification was not detected in the PCR reactions using RT reaction products obtained in the absence of reverse transcriptase, we did not treat the RNA samples with DNase.

**PCR Analysis**

cDNA samples obtained as described above were diluted to 1:5 with DNase/RNase-free water (Invitrogen). Each diluted cDNA sample (2 μl) was added to a total of 20 μl reaction mixture containing GoTaq® Green Master Mix (Promega) and appropriate primers. The PCR primers, synthesized and supplied by Invitrogen, were designed using Primer3 software (http://primer3.sourceforge.net); based on the reported sequences of their mRNAs. The sequences of the primers, amplification sites, and accession numbers of Car9, Col2a1, Col10a1, Acan, Mmp13, Vegf, Hif1a, Epas1, Hif3a, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in Table S2. The PCR products were separated on 1.0% agarose gels and stained with ethidium bromide.

**Quantitative Real Time PCR Analysis**

Quantitative real time PCR was performed and reported according to the MIQE guidelines [39]. cDNA samples were diluted to 1:10 with DNase/RNase-free water. Quantitative real time PCR analyses were performed using TaqMan® Gene Fast Universal PCR Master Mix (Applied Biosystems) with a StepOne® Real-Time PCR System (Applied Biosystems). The program was StepOne® Software (ver. 2.0, Applied Biosystems). The total reaction volume was 10 μl including 2 μl of the diluted cDNA. The thermo-cycling parameters employed were as follows: holding for 20 seconds at 95°C, followed by 40 cycles of a denaturation at 95°C for 1 second and annealing and elongation at 60°C for 20 seconds. The probe and primer pairs for Car9, Col2a1, Acan, Col10a1, Sox5, Sox6, Sox9, Epas1, and GAPDH were supplied by Applied Biosystems. Their assay numbers were listed in Table S3. All real time PCR runs were accompanied by a dilution series to
calibrate PCRs for empirical efficiency. The expression level of each gene was normalized against that of Gapdh and expressed as the relative value for each experiment. Gapdh is one of the widely-used reference genes. In this study, Ct values of each gene were stable based on the amounts of mRNA among different total RNA samples from cartilage tissue. Plasmids constructed with Car9 siRNA and its control siRNA. Additional information about real time PCR performed in this study is summarized in Table S4.

Measurements of Intracellular and Extracellular pH

The pH value of the culture medium samples was determined as extracellular pH using a pH electrode (Horiba Ltd., Kyoto, Japan). Intracellular pH was determined as described previously [39]. Briefly, primary chondrocytes were detached by collagenase digestion, washed with PBS, resuspended in HEPES-buffered saline containing 5 mM glucose (HBSG) and, and treated for 30 minutes with 10 μM BCECF-AM (Dojindo Laboratories, Kumamoto, Japan). After washing with HBSG, cells were resuspended in HBSG and the fluorescence intensity of BCECF in the cells was measured using a Hitachi F-4000 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan). The cells were alternately excited at 455 or 505 nm with a 150 W xenon lamp and fluorescence emission was monitored at 530 nm at intervals of 5 seconds. The 505/455 excitation ratio corresponded to a specific intracellular pH. Calibration of pH was performed using cells permeabilized in buffers containing 10 μM nigericin (N7143, Sigma-Aldrich), 130 mM KCl, 10 mM NaCl, 1 mM MgSO4, and 10 mM MOPS, with various pH values.

Statistical Analysis

Online software programs were used for statistical analyses [40,41]. Nonparametric Mann Whitney U-test was used for comparisons between the results from 2 groups. Steel-Dwass test, a non-parametric post-hoc test, was used for all the pairwise comparisons of the results from 3 or more groups. The P-values were shown in the figures. The values used for drawing figures and statistical analyses employed in this study are listed in Tables S5 and S6, respectively.

Supporting Information

Table S1 Sense and antisense sequences of Stealth™ siRNAs for Car9 and Eps1.

Table S2 Primers for RT-PCR.

Table S3 TaqMan® probes used in this study. See details at the Web site of TaqMan® Assays (http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/PCR/real-time-PCR/real-time-PCR-assays/taqman-gene-expression.html?kwcid=TC13009|taqman%20probe||S1|b|11989269653).

Table S4 Real time PCR quality control.

Table S5 List of the data analyzed statistically in this study.

Table S6 Statistical analysis performed for drawing the Figures.

Author Contributions

Conceived and designed the experiments: YM TM. Performed the experiments: TM GY AY KY TA MH TS MT FI NI. Analyzed the data: KY TT KM KB RK. Wrote the paper: YM TM GY RK.
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