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# CIIA Is a Novel Regulator of Detachment-Induced Cell Death

Kwang Je Kim<sup>1</sup>, Je-Wook Yu<sup>2</sup>, Hyun Sub Hwang<sup>1</sup>, and Eui-Ju Choi<sup>1</sup>

## Abstract

Detachment-induced cell death, or anoikis, is a type of apoptosis that occurs when epithelial cells lose their attachment to the extracellular matrix. Anoikis serves as a physiologic barrier to metastasis. Deviation from the tightly regulated mechanism of detachment-induced cell death might result in progression to metastatic cancer. Here, we investigated the function of CIIA in the regulation of anoikis. CIIA protein was upregulated in colon cancer tissue samples. Knockdown of CIIA in metastatic colorectal carcinoma SW620 and KM12SM cells promoted detachment-induced cell death through the regulation of caspase activation. Knockdown of CIIA also inhibited anchorage-independent growth in soft agar and colony formation after suspension stress. These observations suggest that CIIA is a novel negative regulator of anoikis. *Cancer Res*; 70(15); 6352–8. ©2010 AACR.

## Introduction

Cancer is one of the most dangerous human diseases. Cell immortality, abnormal cell growth regulation, self-sufficient cell growth, cellular evasion of apoptosis, sustained angiogenesis, cell invasion, and metastasis are well-defined hallmarks of tumors (1). Due primarily to these characteristics of tumor cells, successful cancer treatments remain elusive. Metastasis, in particular, poses one of the major difficulties in cancer treatment. Normal cells die when they lose attachment to the extracellular matrix. However, many malignant cancer cells avoid detachment-induced cell death and acquire metastatic potential. Anoikis serves as a physiologic barrier to metastasis (2–4). Identification of the critical regulators of anoikis might provide important information leading to the development of more effective treatments for malignant metastatic cancer.

There are many signaling pathways involved in anoikis (5). Many cancer cells evade detachment-induced cell death through the amplification of survival signals or inhibition of cell death signals. One of the major apoptosis pathways, the caspase cascade, is also involved in anoikis (3, 6, 7). Caspase activation cascades are associated with two pathways in cell death, the death receptor-mediated extrinsic pathway and the mitochondria-mediated intrinsic pathway. In the extrinsic pathway, a death-inducing signaling complex is formed to activate initiator procaspase-8 (8). In the mitochondria-mediated intrinsic pathway, a signaling complex called the apoptosome is formed to activate initiator procaspase-9 (8, 9). In response to a number of diverse apoptotic stimuli, cyto-

chrome *c* is released from the mitochondria into the cytoplasm. Upon binding of released cytochrome *c* to Apaf1, Apaf1 forms heptameric oligomers through interactions of its CARD domain. Initiator procaspase-9 is recruited to the Apaf1 oligomer through the CARD domain and then activated (10, 11). Activated initiator caspases subsequently induce the activation of effector caspases that leads to apoptosis. Under detachment conditions, caspase activation occurs; however, in metastatic cancer cells, caspase activation is circumvented for survival (7, 12, 13).

Previously, we reported that CIIA functions as an anti-apoptotic protein that blocks tumor necrosis factor- $\alpha$ - and H<sub>2</sub>O<sub>2</sub>-induced cell death (14). Here, we showed that CIIA inhibits detachment-induced apoptosis through the regulation of caspase activation. CIIA protein was upregulated in colon cancer tissue samples, and knockdown of CIIA induced anoikis in metastatic colorectal carcinoma cells. These results show that CIIA is a novel negative regulator of detachment-induced cell death.

## Materials and Methods

### Cell culture, transfection, and induction of anoikis

Colon cancer SW480, SW620, KM12C, and KM12SM cells were kindly provided by Dr. S.G. Chi (Korea University, Seoul, Korea). SW480 and SW620 cells were purchased from American Type Culture Collection. KM12C and KM12SM cells were from the Korean Cell Line Bank in 2008. SW620 cells were derived from a lymph node metastasis of SW480 colon cancer cells (15). KM12SM cells are metastatic variants from the KM12C cells (16). Madin-Darby canine kidney (MDCK) cells were purchased from the Korean Cell Line Bank in 2002. 293T cells were provided by Dr. D.S. Lim (Korea Advanced Institute of Science and Technology, Daejeon, Korea; cells were purchased from American Type Culture Collection) in 2007. SW620 and SW480 cells were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS). KM12SM, MDCK, and 293T cells were in DMEM supplemented with 10% FBS. KM12C cells were grown in MEM supplemented with 10% FBS. SW480, SW620, KM12C, and KM12SM cells were transfected by electroporation using a Microporator (Invitrogen). MDCK cells were transfected

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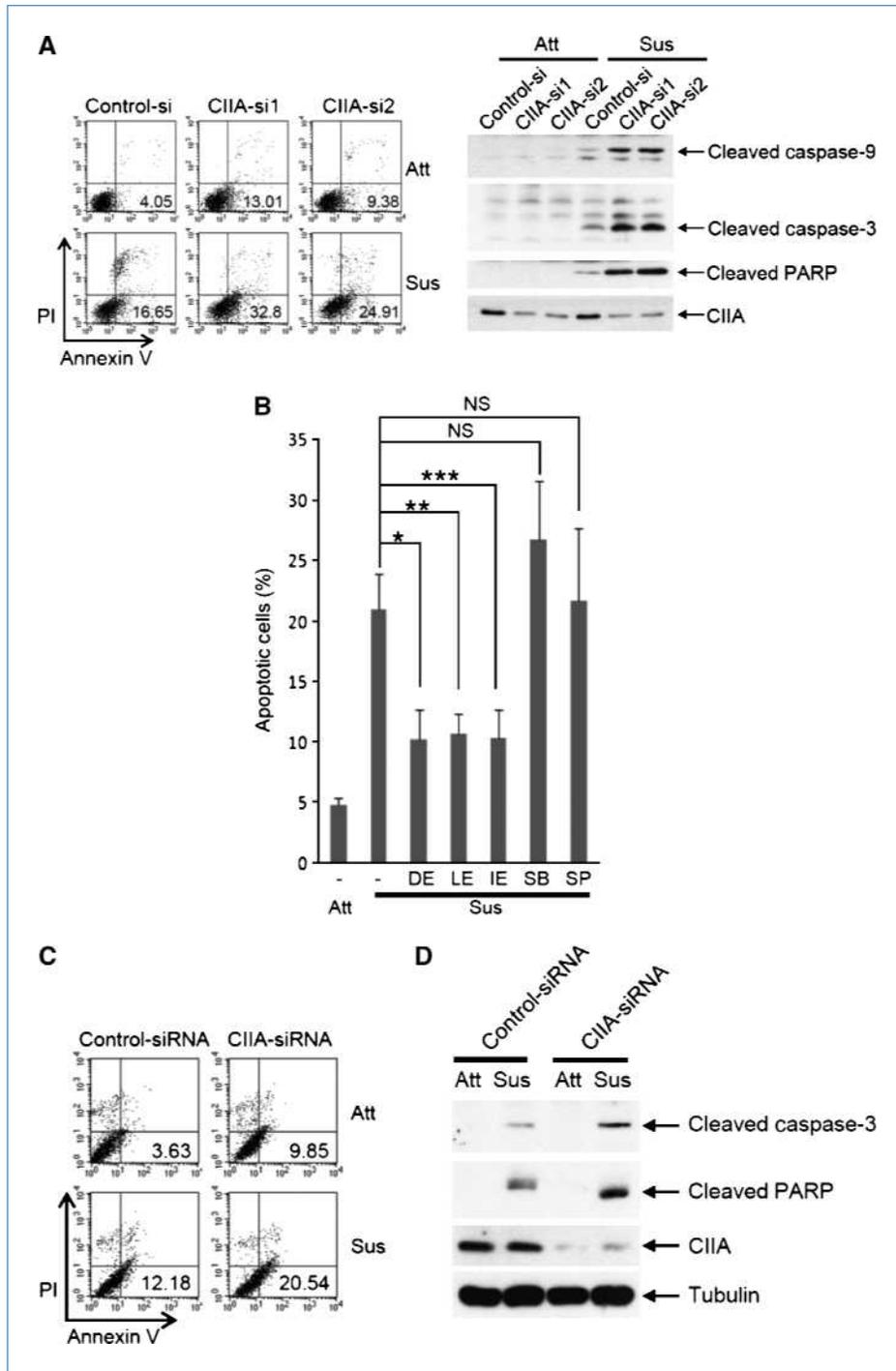
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with the use of LipofectAMINE (Invitrogen). 293T cells were transfected with polyethylenimine (Sigma-Aldrich). For the induction of anoikis, cells were cultured in suspension on dishes coated with poly-2(hydroxyethyl methacrylate) (10 mg/mL in ethanol).

**RNA interference**

To generate cells that stably expressed CIIA short inhibitory



**Figure 2.** Knockdown of CIIA induces anoikis in SW620 and KM12SM cells. A, suspension-induced apoptosis was examined in SW620 cells expressing CIIA-si1 and CIIA-si2 by fluorescence-activated cell sorting analysis using Annexin V/PI staining after 48 h of suspension culture (left). Cleavage of caspase-9, caspase-3, and PARP was examined by Western blot analysis after 48 h of suspension culture (right). B, detachment-induced apoptosis of CIIA-si1 cells in the presence of the indicated inhibitors (DE, Z-DEVD-FMK; LE, Z-LEHD-FMK; IE, Z-IETD-FMK; SP, SP600125; SB, SB203580) after 24 h, the suspension culture was analyzed by fluorescence-activated cell sorting. Columns, mean of two analyses (\*, \*\*, and \*\*\*,  $P < 0.01$ ; NS, no significance). C, suspension-induced apoptosis was examined in KM12SM cells transfected with control or CIIA siRNA oligonucleotides as in A. D, cleavage of caspase-3 and PARP was examined by Western blotting after 48 h of suspension culture in control or CIIA siRNA-expressing KM12SM cells.

Noble agar). SW620 cells ( $2 \times 10^4$  cells) in 1.0 mL of top agar (RPMI medium containing 10% FBS and 0.4% Noble agar) were added to each well and incubated for 20 days. Colonies were stained with 0.005% crystal violet and counted.

**Colony-forming assay**

SW620 cells were cultured in poly-2(hydroxyethyl methacrylate)-coated dishes for 48 hours, and then transferred to

six-well culture plates (300 cells/well). After 15 days, colonies were stained with 0.05% crystal violet.

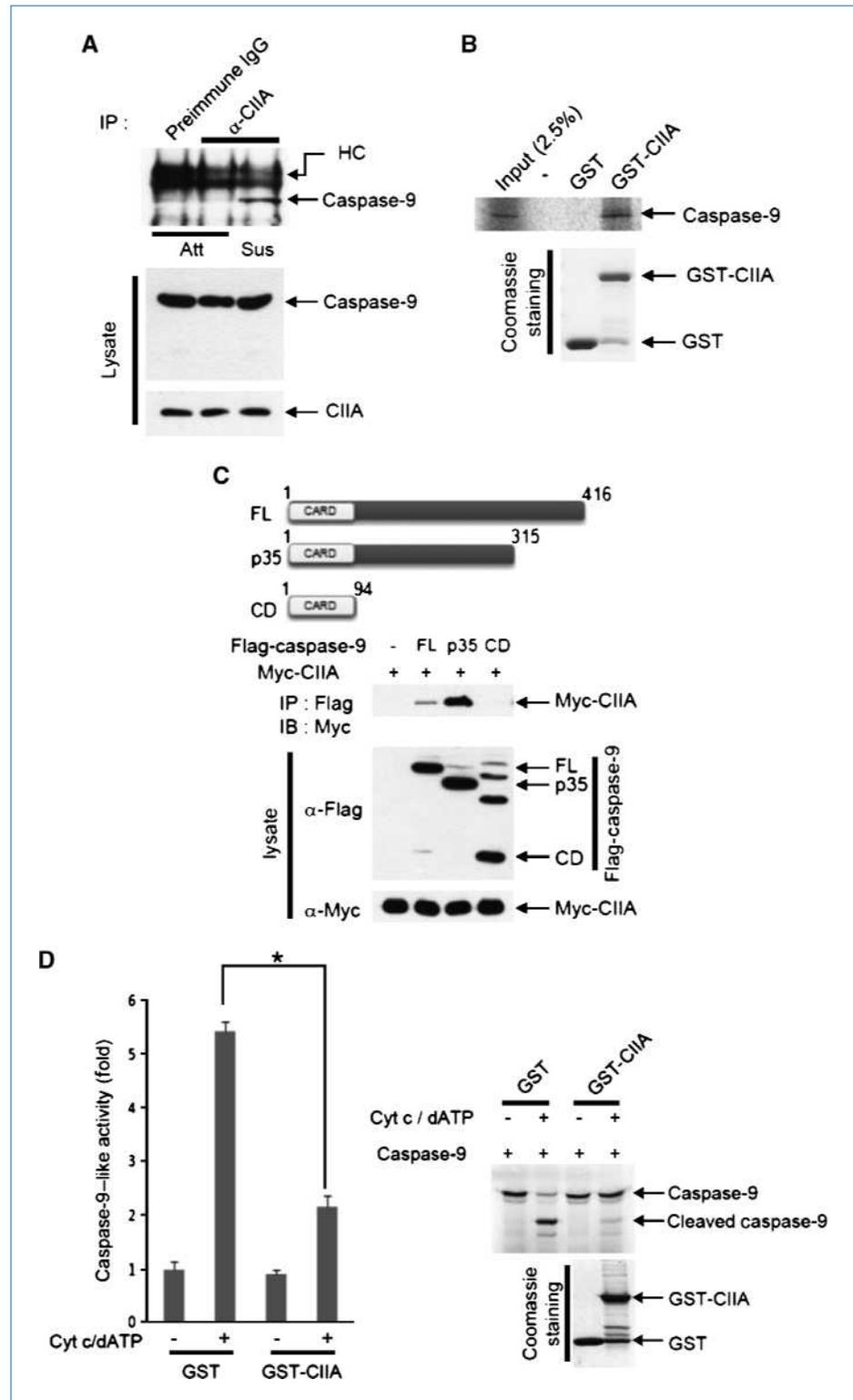
**Immunohistochemistry**

To determine the levels of endogenous CIIA in normal and colon cancer tissues, premade AccuMax Array colon cancer tissues with liver metastasis slides (ISU ABXIS CO. LTD., Seoul, Korea) were immunoreacted with anti-CIIA antibody

(Santa Cruz Biotechnology). Immunoreactive proteins were visualized with fast 3,3'-diaminobenzidine tetrahydrochloride dehydrate tablets (Sigma) after signal amplification using avidin-biotin-peroxidase complex (Vector Laboratories).

Images were acquired by Olympus DP71 microscopy using DP controller software (Olympus). Relative expression levels of CIIA were quantified by Adobe Photoshop CS-based image analysis using the equation  $(1 - \text{mean of the staining intensity})$

**Figure 3.** CIIA inhibits caspase-9 activation. A, the interaction of CIIA and caspase-9 in attached and 24-h suspension conditions was examined by coimmunoprecipitation. HC, heavy chain. B, *in vitro*-translated  $^{35}\text{S}$ -labeled caspase-9 was applied to GST-CIIA on glutathione-conjugated beads. Bead-bound  $^{35}\text{S}$ -labeled caspase-9 was collected and analyzed by autoradiography. C, 293T cells were transfected for 48 h with a vector encoding Myc-CIIA together with vectors for Flag-tagged caspase-9 variants [FL, full-length (amino acids 1–416); p35, p35 fragment (amino acids 1–315); CD, CARD domain (amino acids 1–94)]. Cell lysates were immunoprecipitated with anti-Flag antibody, and the resulting pellets were subjected to immunoblot analysis with anti-Myc antibody. D, left, 293T cell lysates were added with cytochrome c (0.5  $\mu\text{mol/L}$ ) and dATP (100  $\mu\text{mol/L}$ ) as indicated, and assayed for caspase-9 activity by colorimetric assay (\*,  $P < 0.01$ ). Right,  $^{35}\text{S}$ -labeled caspase-9 was incubated for 1 h at 37°C with cytosolic S-100 solution obtained from 293 cells in the presence of cytochrome c, dATP, and either GST or GST-CIIA as indicated. Cleavage of caspase-9 was analyzed by autoradiograph.



of the selected region / mean of the staining intensity of background)  $\times$  100.

## Results and Discussion

### CIIA is upregulated in colon cancer tissues

We previously reported that CIIA is an antiapoptotic protein (14). Many antiapoptotic proteins are frequently upregulated in cancer cells as compared with their normal counterparts, and contribute to the ability of cancer cells to evade cell death. We investigated the protein levels of CIIA in colon cancer tissues by immunohistochemistry using premade AccuMax Array slides. CIIA was upregulated in colon cancer tissues and corresponding liver metastasis colon cancer tissues as compared with normal tissues in each patient examined (P1–P9; Fig. 1). Furthermore, the protein levels of CIIA were higher in metastatic colon cancer tissues than in nonmetastatic colon cancer tissues (Fig. 1B). CIIA expression was also higher in metastatic SW620 or KM12SM cells than in their nonmetastatic counterpart SW480 or KM12C cells (Supplementary Fig. S1).

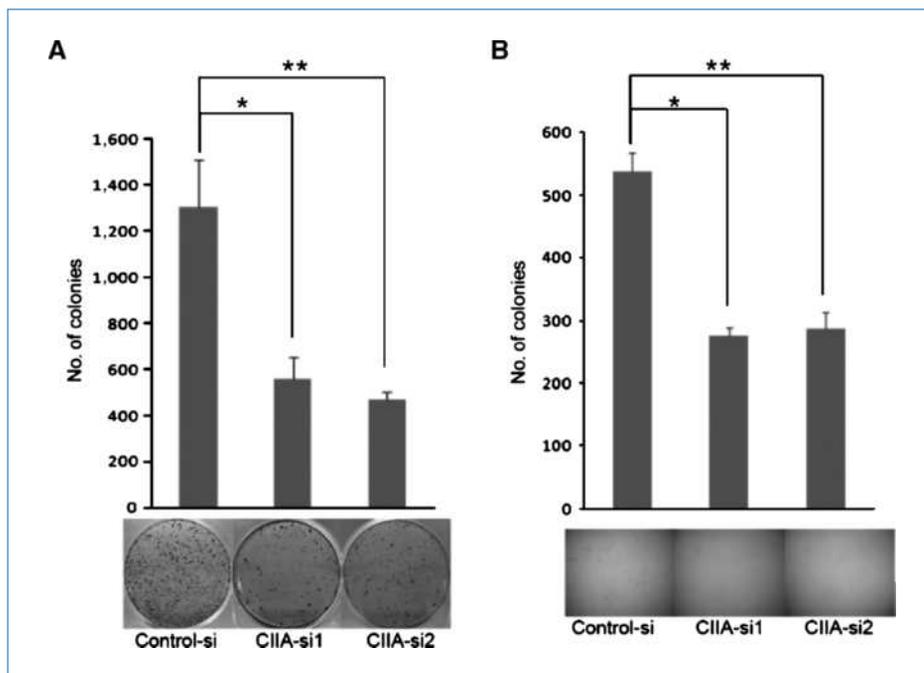
### Knockdown of CIIA induces anoikis in metastatic colon cancer cells

To better understand the possible role of CIIA in cancer development, we examined the effect of CIIA on detachment-induced apoptosis in metastatic cancer SW620 cells. We first examined the effect of siRNA-mediated CIIA depletion on anoikis resistance in SW620 cells. We generated two SW620 cell lines (CIIA-si1 and CIIA-si2) that stably expressed CIIA siRNA.

Depletion of CIIA expression by RNA interference (RNAi) promoted detachment-induced apoptosis and the cleavage of caspase-9, caspase-3, and PARP in SW620 cells (Fig. 2A). Next, we examined whether caspase activation might mediate anoikis in CIIA knockdown SW620 cells. Inhibitors of caspase-3, caspase-8, and caspase-9 (Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK, respectively) suppressed detachment-induced apoptosis in CIIA knockdown SW620 cells (CIIA-si1), whereas inhibitors of *c-Jun* NH<sub>2</sub>-terminal kinase and p38 (SP600125 and SB203580, respectively) had no effect (Fig. 2B). We confirmed the inhibitory effect of CIIA on detachment-induced apoptosis in SW620 cells transiently transfected with CIIA siRNA oligonucleotides (Supplementary Fig. S2A). Furthermore, siRNA-mediated CIIA depletion promoted the cleavage of caspase-9, caspase-3, and PARP in the cells (Supplementary Fig. S2A and B). These results suggested that CIIA depletion converts anoikis-resistant SW620 cells to an anoikis-susceptible phenotype through caspase activation. The promoting effects of CIIA siRNA on detachment-induced apoptosis (Fig. 2C) as well as cleavage of caspase-3 and PARP (Fig. 2D) were also observed in another metastatic colon cancer cell line, KM12SM cells. Furthermore, overexpression of CIIA inhibited detachment-induced caspase activation and apoptosis in MDCK cells (Supplementary Fig. S3).

### CIIA inhibits the activation of caspase-9

Given that RNAi-mediated CIIA depletion promoted detachment-induced caspase activation and apoptosis, we next examined a possible action of CIIA on the activation of caspase-9. We first examined the interaction of endogenous CIIA and caspase-9 in SW620 cells. The interaction of



**Figure 4.** RNAi-mediated CIIA depletion inhibits anchorage-independent growth. A, after 48 h of suspension culture, the indicated cells (300 cells) were seeded on a six-well culture plates and maintained for 15 d. Colonies were stained with 0.05% crystal violet and counted. B, cells were seeded on a six-well plate ( $2 \times 10^4$  cells/well) and maintained for 20 d. The number of colonies in each well was calculated as the sum of four independent regions. Columns, mean of three determinants (\* and \*\*,  $P < 0.01$ ).

endogenous CIIA and caspase-9 was detected under suspension growth conditions (Fig. 3A). Furthermore, ectopically expressed CIIA bound to full-length and p35 fragment of caspase-9, but not to its CARD domain (Fig. 3C). *In vitro* binding data also showed that caspase-9 directly bound to CIIA, as a GST-fusion protein, but not to GST (Fig. 3B). Moreover, overexpressed CIIA inhibited oligomerization of recombinant caspase-9 in transfection studies (Supplementary Fig. S4). Together, these data suggest that CIIA physically interacts with caspase-9. We next examined whether CIIA affected the activation of caspase-9 triggered by cytochrome *c* and dATP. Cytochrome *c*/dATP-mediated caspase-9 activation was significantly reduced by GST-CIIA (Fig. 3D). These results suggested that CIIA interacts with caspase-9 in cells under suspension culture and inhibits the activation of caspase-9 and downstream caspases such as caspase-3.

Given that caspase-8 as well as caspase-9 might be associated with detachment-induced apoptosis in CIIA-depleted SW620 cells (Fig. 2B), we examined the cleavage of caspase-8 and caspase-9 at various times after suspension culture in control and CIIA-depleted SW620 cells (Supplementary Fig. S5A). The time course of the activation was similar between caspase-8 and caspase-9. Moreover, a caspase-8 inhibitor Z-IETD-FMK did not block the detachment-induced activation of caspase-9 in CIIA-depleted SW620 cells (Supplementary Fig. S5B). These results thus suggested that caspase-8 activation was not involved in caspase-9 activation induced by detachment in CIIA-depleted cells.

### CIIA is necessary for anchorage-independent growth

Next, we carried out colony-forming assays after suspension culture, and examined anchorage-independent growth in soft agar (Fig. 4A and B). The colony formation assay was carried out to quantify living cells after growth in suspension. Anchorage-independent growth is one of the characteristics of transformed cells, including metastatic cancer cells. After 48 hours of suspension culture, cells were plated in fresh dishes for 15 days. CIIA knockdown cells (CIIA-si1 and CIIA-si2) formed fewer colonies than control SW620 cells (Fig. 4A). Similarly, when we examined colony formation in soft agar, CIIA knockdown cells formed fewer colonies than control cells (Fig. 4B). Thus, CIIA facilitated anchorage-independent growth.

The caspases are the major signaling players in anoikis. Activation of the caspase cascade is also one of the main targets of cancer treatment (13). Many reports have shown that antiapoptotic proteins that negatively regulate the caspase cascade, such as XIAP, Bcl-2, Bcl-xL, and MCL-1 are frequently overexpressed in cancer cells, and loss or inhibition of these antiapoptotic proteins is involved in anoikis (12, 13, 18, 19). Our results showed that CIIA inhibits detachment-induced apoptosis through the negative regulation of caspase activation. CIIA was upregulated in colon cancer and corresponding liver metastasis colon cancer tissues as compared with normal tissues. Knockdown of CIIA promoted detachment-induced apoptosis in anoikis-resistant colon cancer cells. These results suggest that CIIA is a novel inhibitor of anoikis. CIIA induces epithelial-mesenchymal transition and cell invasion (20). Thus, it is conceivable that CIIA induces cancer cell differentiation to metastatic cancer cells through the inhibition of anoikis and induction of invasion. Because anoikis serves as a physiologic barrier that inhibits metastasis, the induction of anoikis has important implications in cancer treatment. On the basis of our findings in this study, we propose that CIIA functions as a novel regulator of anoikis, and may be a possible target for metastatic cancer treatment.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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