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ARTICLE

Multiple mechanisms determine the sensitivity of human-induced pluripotent stem cells to the inducible caspase-9 safety switch

Shigeki Yagyu[1,2], Valentina Hoyos[2], Francesca Del Bufalo[2,3] and Malcolm K. Brenner[2]

Expression of the *inducible caspase-9* (iC9) suicide gene is one of the most appealing safety strategies for cell therapy and has been applied for human-induced pluripotent stem cells (hiPSC) to control the cell fate of hiPSC. *iC9* can induce cell death of over 99% of iC9-transduced hiPSC (iC9-hiPSC) in less than 24 hours after exposure to chemical inducer of dimerization (CID). There is, however, a small number of resistant cells that subsequently outgrows. To ensure greater uniformity of the hiPSC response to iC9 activation, we purified a resistant population by culturing iC9-hiPSC with CID and analyzing the mechanisms by which the cells evade killing. We found that iC9-resistant hiPSC have significant heterogeneity in terms of their escape mechanisms from caspase-dependent apoptosis including reduced expression of *iC9* by promoter silencing and overexpression of BCL2. As a consequence, modifying a single element alone will be insufficient to ensure sustained susceptibility of *iC9* in all cells and prevent the eventual outgrowth of a resistant population. To solve this issue, we propose to isolate an iC9-sensitive population and show that this hiPSC line has sustained a uniform responsiveness to iC9-mediated growth control.

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INTRODUCTION

The *inducible caspase-9* (*iC9*) suicide gene[1,2] is an appealing safety switch for cell therapies. When used to control adoptively transferred T-cell therapy in patients, *iC9* induces swift and almost complete killing of both dividing and nondividing cells after exposure to an otherwise biologically inert dimerizing agent (chemical inducer of dimerization; CID, AP20187); has low spontaneous activation; and encodes a nonimmunogenic transgene product.[3–7] We recently reported a modification to the iC9-based system that could be used to control human-induced pluripotent stem cell (hiPSC) survival and thereby reduce the risks of oncogenic transformation or other adverse effects associated with undifferentiated hiPSC and their progeny.[8] In this system, *iC9* was transduced with a lentiviral vector and 95–99% of iC9-transduced hiPSC (iC9-hiPSC) were eliminated within 24 hours after exposure to the activating drug *in vitro*, and most teratomas derived from iC9-transduced hiPSC were eradicated by systemic administration of CID *in vivo*.[8] Of note, iC9 expression and sensitivity to cytotoxicity by the activating drug persisted unchanged during culture over time and after differentiation into the mesenchymal lineage.[8] Hence, the iC9 safety could be of value to ensure a safe application of hiPSC-based therapy.

Despite the ability of the iC9 system to control the fate of hiPSC, after exposure to the activating drug the small number of surviving hiPSC could regrow[8] and, given the potentially unlimited self-renewal capacity of undifferentiated hiPSC, lead to tumor growth or to other adverse events. An understanding of the mechanisms of resistance to CID is therefore required to ensure even greater efficacy of iC9-mediated apoptosis.

Since the residual iC9-hiPSC subpopulation may exploit apoptosis-resistance mechanisms that are attributable to both genetic and epigenetic heterogeneity within the original population,[9–12] we developed an iC9-mediated apoptosis-resistant hiPSC subpopulation and further studied the components that determine resistance to iC9-mediated apoptosis to develop approaches that deliver a more uniform response.

RESULTS

iC9 transgene expression was downregulated in iC9-resistant hiPSC

We previously established hiPSC lines that express the *iC9* transgene and *GFP* driven by *EF1α* core promoter from two hiPSC lines (iC9-TZ16 and iC9-TKCBSeV9). The iC9 could be activated in the presence of CID and induced rapid cell death in iC9-hiPSC, but ~1–5% of iC9-hiPSC remained viable.[8] To purify an iC9-hiPSC subpopulation that was resistant to iC9-mediated apoptosis (iC9-resistant TZ16, and iC9-resistant TKCBSeV9), iC9-hiPSC were cultured with 10 nmol/l of CID for 24 hours, then the medium was replaced with fresh mTeSR1 every day for 7 days, and the GFP-positive cells were enriched by fluorescence-activated cell sorting (Figure 1a). These resistant cells maintained high expression of pluripotent markers including OCT4, SOX2, SSEA-1, TRA-1–60, TRA-1–81, and alkaline phosphatase, and the ability to form teratoma in immunodeficient mice (Supplementary Figure S1), demonstrating their pluripotency. To test the sensitivity to CID of these iC9-resistant hiPSC, we cultured them in the presence of CID for 24 hours, and calculated the percentage of residual live cells (Annexin V negative, 7-AAD negative, and GFP positive). iC9-resistant hiPSC were not killed by CID,

[1]Department of Pediatrics, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan; [2]Center for Cell and Gene Therapy, Baylor College of Medicine, Texas Children's Hospital, and Houston Methodist Hospital, Houston, Texas, USA; [3]Hematology/Oncology Department, Bambino Gesù Children's Hospital, Rome, Italy.
Correspondence: S Yagyu (shigeky@koto.kpu-m.ac.jp)
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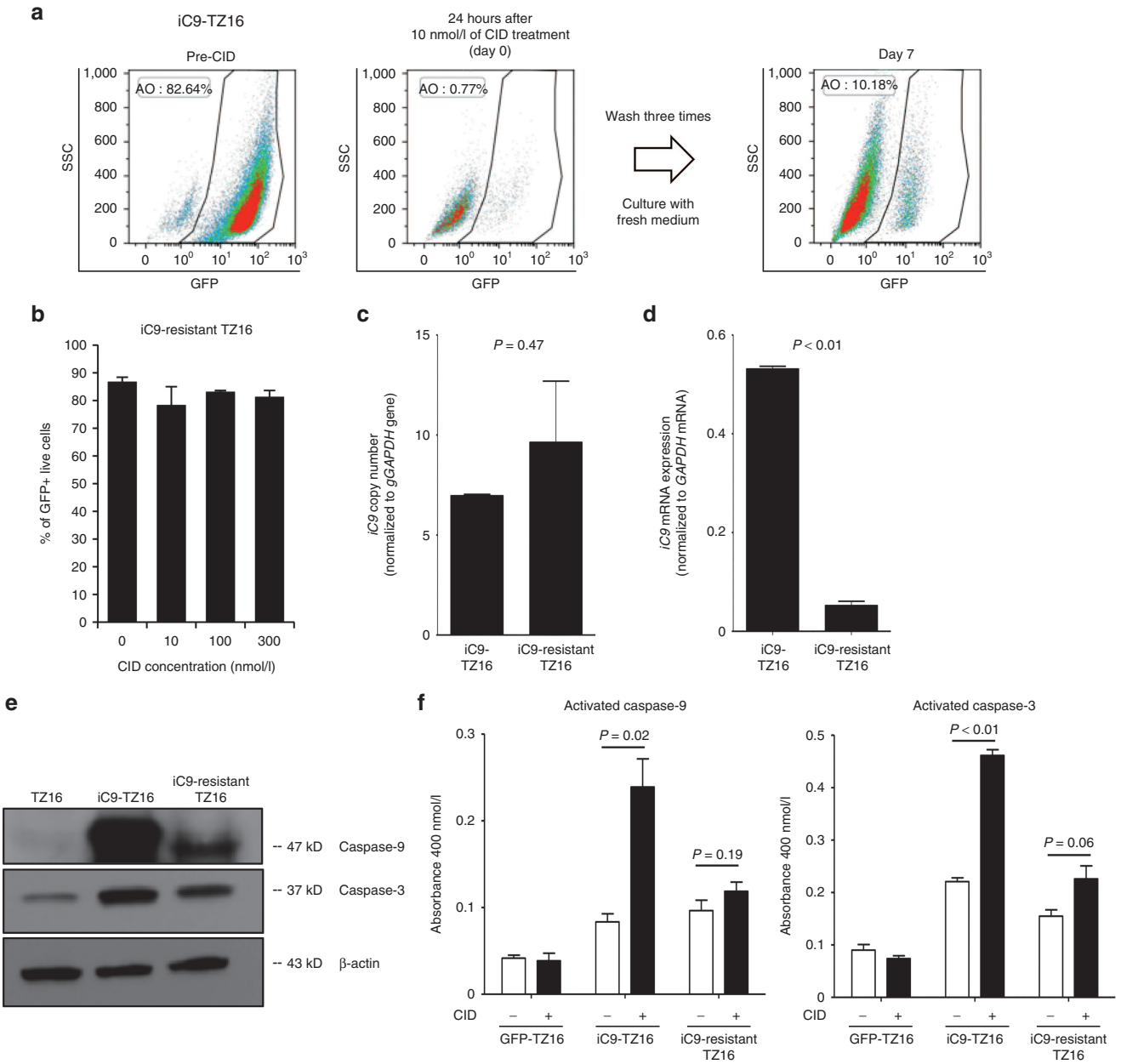

Figure 1 Isolation and characterization of iC9-resistant hiPSC. (**a**) Fluorescence-activated cell sorting for isolation of iC9-resistant hiPSC. (**b**) iC9-resistant hiPSC were treated with different concentration of CID, and the percentage of GFP-positive live cells was measured by Annexin V/7-AAD staining by flow cytometry. (**c**) The *iC9* transgene copy number in iC9-hiPSC and iC9-resistant hiPSC. The dosage of *iC9* transgene was normalized to that of *GAPDH* gene. (**d**) The mRNA expression of *iC9* transgene measured by real-time qPCR. The *iC9* expression was normalized by the expression of *GAPDH* gene. (**e**) The expression of caspase-9, cleaved caspase-9, caspase-3, and cleaved caspase-9 in hiPSC, iC9-hiPSC, and iC9-resistant hiPSC by Western blot. (**f**) Activation of caspase-9 and -3 was measured by colorimetric assay. iC9, inducible caspase-9; hiPSC, human-induced pluripotent stem cells

even at concentrations >30-fold higher than the concentrations that produced >95% killing of the parental line (% of live cells after 300 nmol/l of CID exposure; 81.33 ± 2.18% in iC9-resistant TZ16 and 71.57 ± 0.53% in iC9-resistant TKCBSeV9, respectively, Figure 1b, Supplementary Figure S2).

We determined *iC9* transgene transduction and expression in iC9-resistant hiPSC by measuring the copy number and mRNA expression of the transgene by quantitative polymerase chain reaction amplification and compared these values to parental iC9-hiPSC. *iC9* copy number was calculated from the ratio of *iC9* signal/*GAPDH* signal. iC9-sensitive and iC9-resistant hiPSC had similar transgene copy numbers (6.96 ± 0.07 in parental iC9-TZ16 versus 9.63 ± 3.06 in iC9-resistant TZ16, P = 0.46, and 1.448 ± 0.096 in parental iC9-TKCBSeV9 versus 1.158 ± 0.061 in iC9-resistant TKCBSeV9, P = 0.02, respectively, Figure 1c, Supplementary Figure S2). However, iC9-resistant hiPSC had significantly lower *iC9* mRNA (*iC9* signal/*GAPDH* signal; 0.52 ± 0.005 in parental iC9-TZ16 versus 0.04 ± 0.008 in iC9-resistant TZ16, P < 0.01, and 0.6491 ± 0.041 in parental iC9-TKCBSeV9 versus 0.005 ± 0.002 in iC9-resistant TKCBSeV9, respectively, P < 0.001, respectively, Figure 1d, Supplementary Figure S2), and protein expression (Figure 1e). Moreover, activation of caspase-9 and subsequently of caspase-3 in iC9-resistant hiPSC was not observed after CID exposure (Figure 1f). Of note, the sequence of the integrated *iC9* transgene was identical in iC9-resistant hiPSC

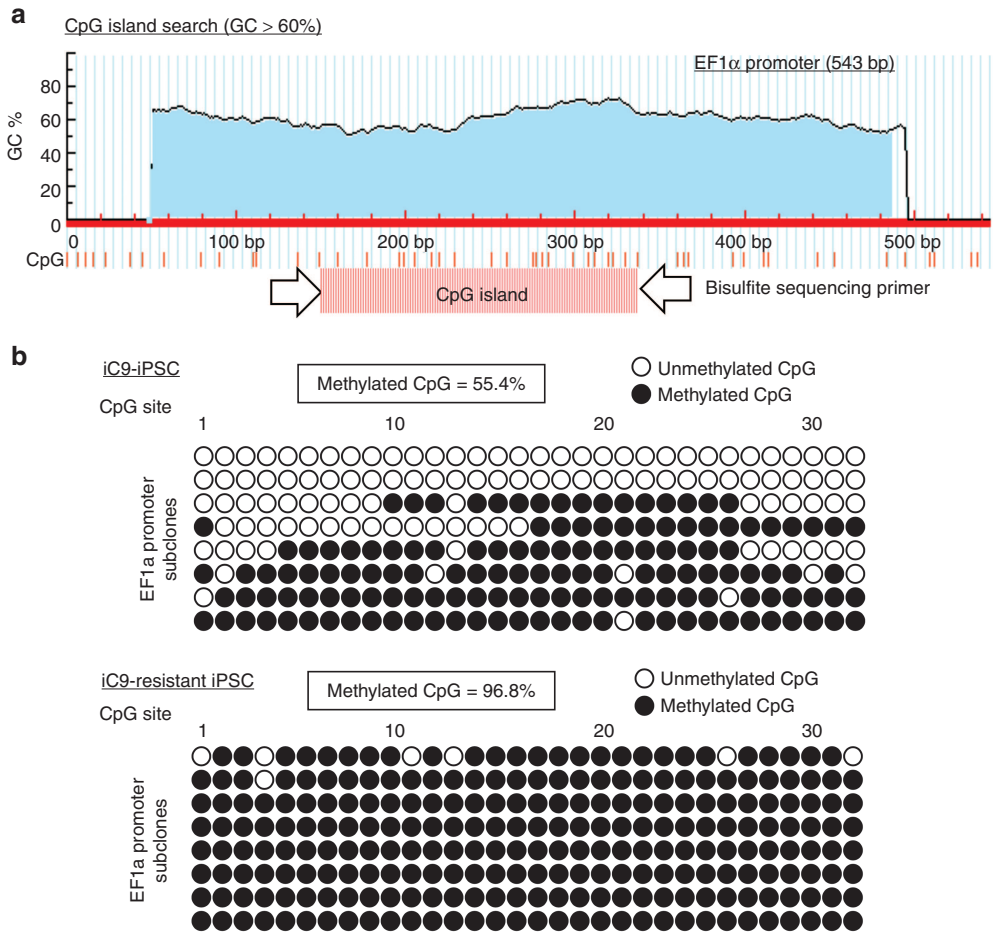

Figure 2 iC9-resistant hiPSC have *iC9* transgene silencing by promoter hypermethylaiton. (**a**) CpG island prediction for the *EF1α* core promoter. The *EF1α* core promoter contained approximately a 230-bp region wit.h more than 60% of GC content. (**b**) Methylation status of CpG sites in the *EF1α* core promoter of iC9-hiPSC and iC9-resistant hiPSC. The open and black circles represent unmethylated and methylated CpG sites, respectively. iC9, inducible caspase-9; hiPSC, human-induced pluripotent stem cells.

and in parental iC9-hiPSC, as confirmed by Sanger sequencing (data not shown). These data show that the failure of the *iC9* transgene to induce apoptosis in the resistant population after exposure to dimerizing drug is associated with downregulation of expression rather than lower transgene copy number or gene mutation.

EF1α promoter of *iC9* transgene was highly methylated in iC9-resistant hiPSC

Since iC9-resistant hiPSC had reduced transgene expression despite an *iC9* copy number similar to the parental line, we hypothesized that iC9 transgene expression was epigenetically silenced. Although we used the *EF1α* core promoter, which contains fewer methylatable CpG sites than the full-length human *EF1α* promoter sequence, there is nonetheless a 230-bp region with >60% GC content (Figure 2a). To investigate the methylation status of the *EF1α* core promoter in iC9 sensitive and resistant hiPSC, we used bisulfite sequencing of the CpG islands in the *EF1α* core promoter, and calculated the methylation status as the ratio of the number of methylated cytosines/the total number of cytosines in the CpG islands. As Figure 2b shows, the *EF1α* core promoter had higher levels of methylation in the dimerizer-resistant than the sensitive hiPSC (Figure 2b). Hence, epigenetic silencing of the *EF1α* core promoter could contribute to the lower *iC9* transgene expression observed in iC9-resistant hiPSC.

Reversibility of iC9 transgene silencing in iC9-resistant hiPSC

To determine whether an epigenetic modifier restored the expression and function of *iC9* transgene, we cultured iC9-resistant hiPSC with a demethylating agent, 5-aza deoxycytidine (5-aza-dC). Unfortunately, this agent was highly toxic to hiPSC even at concentrations as low as 0.1 µmol/l. Consequently, treatment with this and related agents served only to inhibit the growth of iC9-resistant hiPSC and did not augment iC9 expression in the dimerizer-resistant hiPSC (data not shown). As an alternative means of assessing the contribution of epigenetic modification to low transgene expression, we cultured iC9-resistant hiPSC with a member of a second class of epigenetic modifiers, the histone deacetyl transferase inhibitor (HDACi) LBH589. We measured changes in *iC9* mRNA expression by reverse transcription polymerase chain reaction over 48 hours, and found that *iC9* expression was restored in iC9-resistant hiPSC treated with 100 nmol/l of LBH589 (Figure 3a, iC9 signal/GAPDH signal; 0.026 ± 0.006 in iC9-resistant hiPSC, 0.075 ± 0.022 in iC9-resistant hiPSC with 100 nmol/l of LBH589, and 0.35 ± 0.01 in iC9-hiPSC, respectively). Upregulation of *iC9* enhanced CID-induced apoptosis in iC9-resistant hiPSC, but the extent of killing remained lower than the parental line, even in the absence of HDACi (Figure 3b, % of GFP-positive live cells; 83.72 ± 2.135 in CID (–)/LBH589 (–)versus 79.94 ± 1.72 in CID (+)/LBH589 (–), 60.96 ± 5.47 in CID (–)/LBH589 (+) versus 32.71 ± 3.37 in CID (+)/LBH589 (+), respectively).

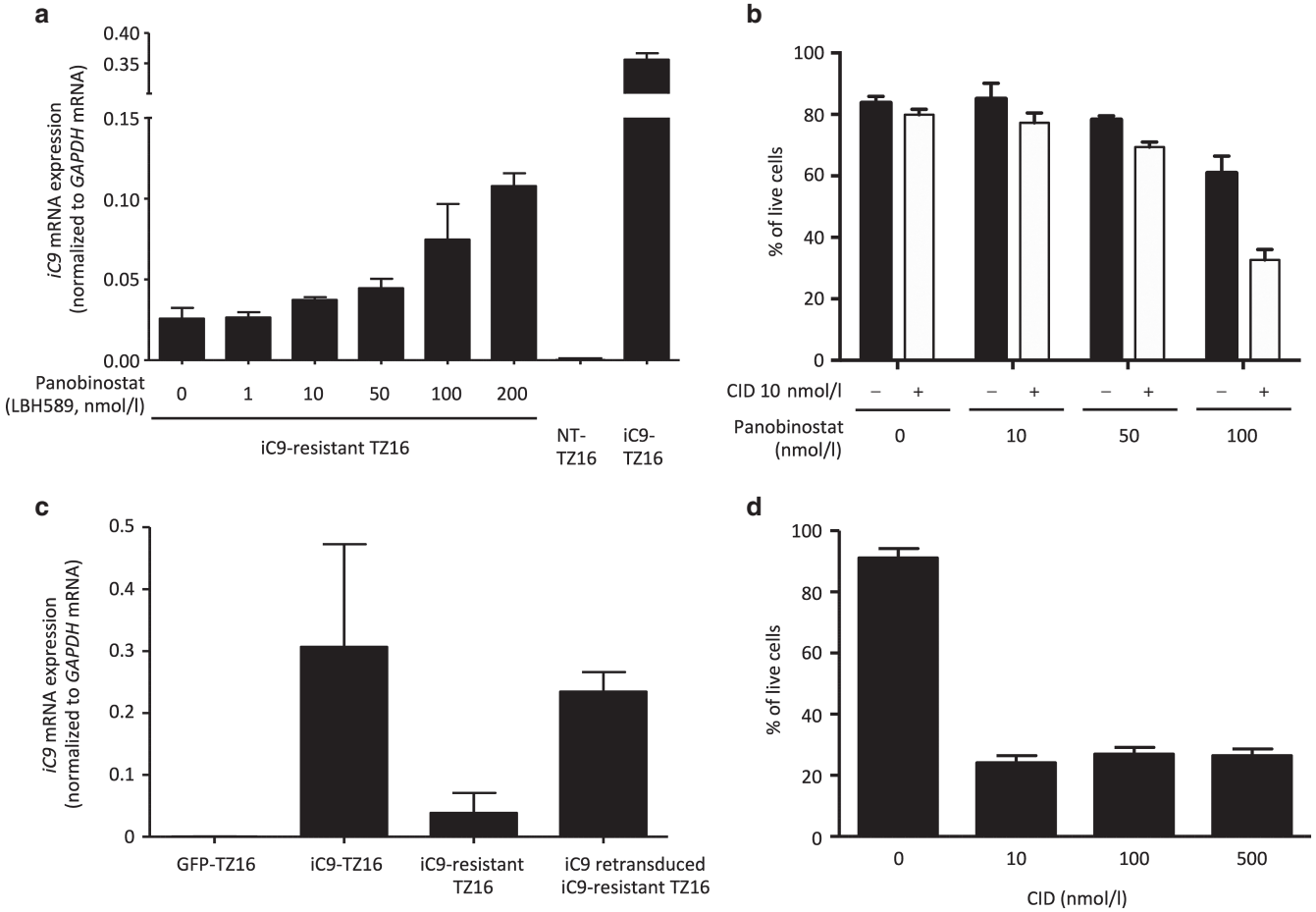

Figure 3 Restoration of iC9 transgene partially enhances iC9-mediated apoptosis in iC9-resistant hiPSC. (**a**) The expression levels of iC9 transgene in iC9-resistant hiPSC treated by HDAC inhibitor, LBH589. (**b**) The sensitivity of cytotoxicity by CID in combination with LBH589 was measured by flow cytometry. (**c**) The expression levels of iC9 transgene in iC9-resistant hiPSC retransduced with iC9 transgene. (**d**) The sensitivity of cytotoxicity by CID in iC9 retransduced iC9-resistant hiPSC was measured by flow cytometry. iC9, inducible caspase-9; hiPSC, human-induced pluripotent stem cells; HDAC inhibitor, histone deacetyl transferase inhibitor.

We next determined whether alternative, nonepigenetic manipulations to the resistant cells would be able to augment sensitivity to killing by iC9 activation. We first determined whether retransduction of the resistant cells to increase transgene copy number could restore sensitivity. We retransduced iC9-resistant hiPSC with a lentiviral vector encoding *iC9* and a selectable marker (*puromycin)*. The retransduced cells (iC9-retransduced hiPSC) were enriched for GFP positivity and by puromycin selection. The levels of *iC9* expression of iC9-retransduced hiPSC were comparable to the parental line (Figure 3c), but restoration of sensitivity to killing by CID was only incomplete (Figure 3d). Hence, augmentation of *iC9* expression alone does not fully restore sensitivity to iC9 activation, and while low expression of the *iC9* transgene due to epigenetic silencing certainly contributes to CID resistance, additional mechanisms that enhance resistance are also present.

Elevated levels of the antiapoptotic BCL2 protein impairs killing by iC9-mediated apoptosis

We next assessed the expression of antiapoptotic proteins including BCL2 and X-linked inhibitor of apoptotic protein (XIAP) in iC9-sensitive and resistant hiPSC and in nontransduced hiPSC cells as well. The iC9-resistant hiPSC had high expression of BCL2, while NT- or iC9-sensitive hiPSC had much lower levels. By contrast, the level of XIAP was similar between sensitive and resistant lines (Figure 4a). To determine whether overexpression of BCL2 contributes to the lower level of CID-induced apoptosis in iC9-resistant hiPSC, we cultured iC9-resistant hiPSC in combination with the BCL2 inhibitor, ABT737, and 10 nmol/l of CID for 24 hours, then measured the percentage of GFP-positive live cells by flow cytometry. The BCL2 inhibitor ABT737 augmented the activation of caspase-9 and subsequently of caspase-3 (Figure 4b), enhancing the sensitivity to cytotoxicity produced by CID (Figure 4c, % of GFP-positive live cells 24 hours after the culture with 10 nmol/l of CID and/or 1 µmol/l of ABT737; 91.79±1.38 in CID (–)/ABT737 (–) versus 87.13±4.73 in CID (+)/ABT737 (–), 44.56±18.37 in CID (–)/ABT737 (+) versus 15.08±7.30 in CID (+)/ABT737 (+), respectively).

DISCUSSION

The iC9 safety system can be used to reduce the consequences of oncogenic transformation in hiPSC. iC9-mediated apoptosis is produced by direct activation of the pro-apoptotic molecule caspase-9 in the late phase of the intrinsic apoptotic pathway.[2,3] iC9-mediated apoptosis can therefore circumvent many of the known anti-apoptotic mechanisms that act early in the apoptosis pathway. However, hiPSC are heterogeneous in terms of their sensitivity to *iC9*-mediated apoptosis, and we have previously shown that a subpopulation of

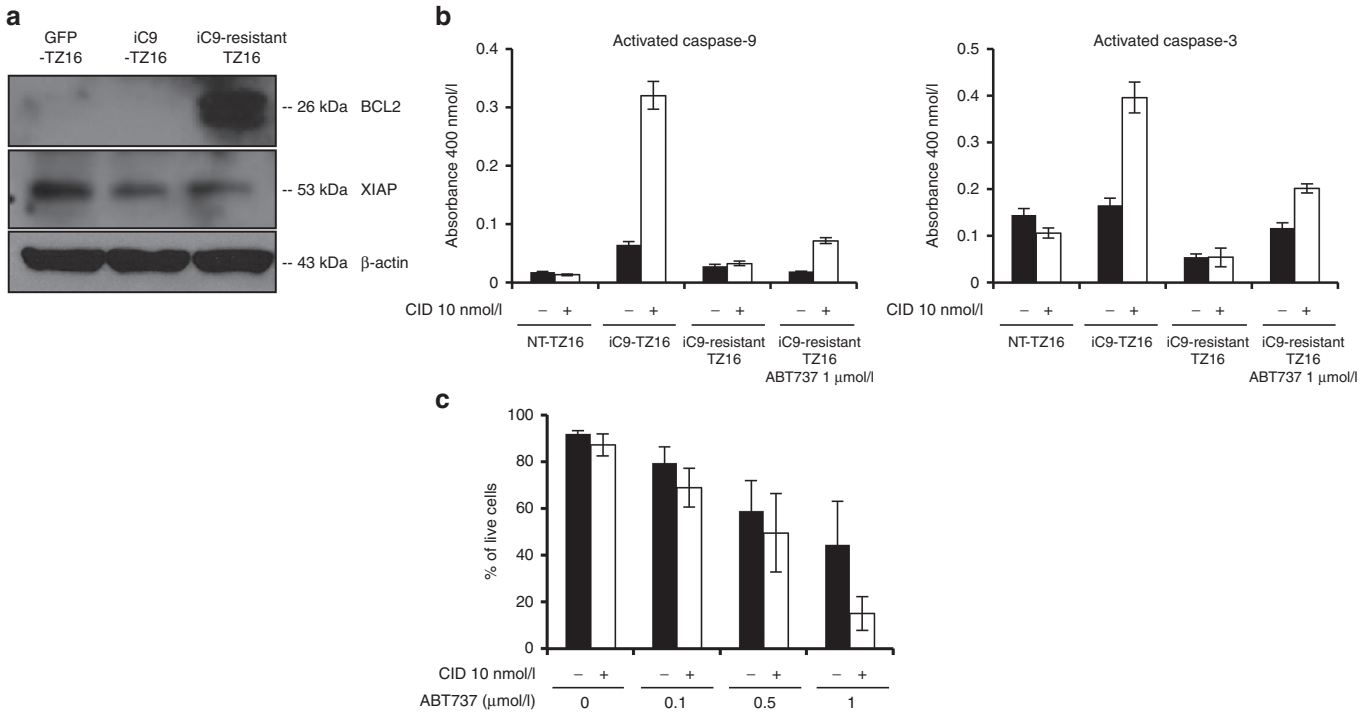

Figure 4 Anti-apoptotic BCL2 protein impairs killing effect by iC9-mediated apoptosis. (**a**) The expression of anti-apoptotic protein, BCL2 and XIAP in GFP-hiPSC, iC9-hiPSC, and iC9-resistant hiPSC by Western blot. (**b**) Activation of caspase-9 and -3 in iC9-resistant iPSC treated with CID in combination with BCL2 inhibitor, ABT-737, was measured by colorimetric assay. (**c**) The cytotoxicity of CID in combination with ABT737 in iC9-resistant hiPSC. iC9, inducible caspase-9; hiPSC, human-induced pluripotent stem cells; XIAP, X-linked inhibitor of apoptotic protein.

iC9-hiPSC remain viable even after exposure to the dimerizing drug that activates the iC9.[8] In this study, we explored whether a single and potentially reversible mechanism accounts for the resistance we observe, or whether a multiplicity of factors limits our ability to completely eradicate hiPSC. Here, we demonstrate that the mechanisms of iC9 resistance in hiPSC may indeed be multifactorial, including transgene silencing and overexpression of anti-apoptotic proteins, suggesting that introduction of the *iC9* transgene followed by subsequent (clonal) selection of stably CID-sensitive hiPSC to eliminate the multifactorial iC9-resistant subclones may be required for the maximum safety of hiPSC for human use.

While incorporation of a suicide gene is necessary to increase the safety of hiPSC, previous reports suggested that hiPSC are somewhat resistant to transgene introduction by retroviral or lentiviral transduction[13] and that expression of a suicide gene in iPSC would be rapidly silenced by epigenetic modulation.[14] For example, a previous suicide gene study for nonhuman iPSC showed that *iC9*-transduced murine/rhesus iPSC lost transgene expression during differentiation due to human *EF1α*-derived promoter hypermethylation.[15] In our studies, we used a core human *EF1α* promoter with few CpG sites that should be less prone to silencing by methylation and, therefore, able to produce more stable long-term transgene expression than other constitutive promoters derived from *cytomegalovirus* (*CMV*) promoter or *spleen-focus foaming virus* (*SFFV*).[14,16] We indeed demonstrated that the expression of *iC9* transgene driven by human *EF1α* core promoter was sustained at high levels in >99% of transduced undifferentiated hiPSC, as well as in their differentiated progeny.[8] However, since hiPSC may contain multiple mutations that occur prior to reprogramming or evolve with passaging,[9–12] heterogeneity within the hiPSC population produced diversity in terms of transgene silencing, making possible the selection of a CID-resistant subpopulation. This population is highly methylated and expresses low levels of the suicide gene that are insufficient for the cells to be killed by even 30-fold higher concentrations of dimerizer drug (Figure 1b). As has been reported previously, undifferentiated pluripotent cells are highly sensitive to demethylating agents such as 5-aza-dC,[17,18] and so we were prevented by toxicity from assessing the effects of reversing methylation. Although this limitation prevents us from concluding that the hypermethylation of human EF1α core promoter contributed to transgene silencing and thus to iC9-resistance, we used high concentrations (200 nmol/l) of panobinostat, a potent deacetylator of histone proteins, as an alternative means of studying the impact of epigenetic modifiers. A concentration of panobinostat that can reverse histone acetylation[19] only modestly increase iC9 gene expression. It is possible that additional deacetylation with still higher drug concentrations would have further enhanced expression, but such concentrations are themselves toxic.[20] Recent studies have shown that the incorporation of a ubiquitous chromatin opening element adjacent to the human *EF1α* promoter[14] or the use of a completely CpG-free *EF1α* promoter[21] may further reduce the risk of transgene silencing and produce stable transgene expression. Although these results are encouraging, further detailed examination of the resistant hiPSC suggested that sustaining transgene expression at high levels may be necessary for effective killing, but it alone is not sufficient. Sensitivity to CID-induced cytotoxicity was not restored in the resistant hiPSC even when expression levels were increased to those observed in iC9-sensitive hiPSC by retransduction (Figure 3c,d). In malignant cells, sensitivity to iC9-mediated apoptosis may correlate with the expression of XIAP, a direct caspase-9 inhibitor.[22] We did not find evidence for this association in the resistant population studied, and second mitochondria-derived activator of caspases (SMAC) mimetics, which antagonize XIAP, did not enhance CID-induced apoptosis in iC9-resistant hiPSC (Figure 4a and Supplementary Figure S3). We found instead that the

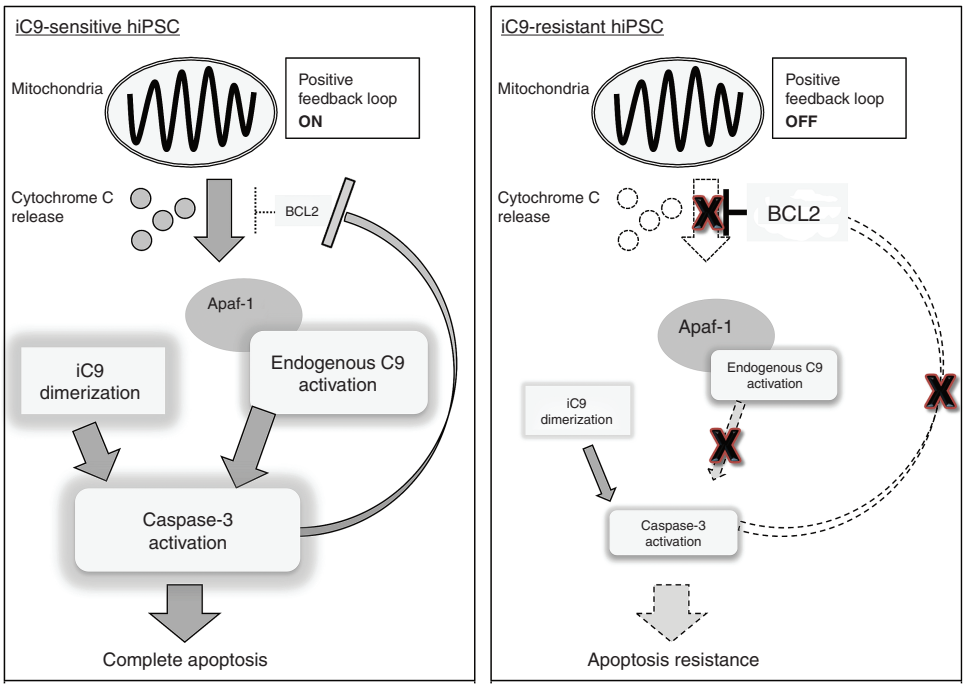

Figure 5 Schematic images of resistant mechanisms of iC9-mediated apoptosis in iC9-hiPSC. The iC9-resistant hiPSC overexpressed BCL2. Decreased activation of caspase-9 in the resistant cells cannot fully inhibit overexpressed BCL2, which fails to turn on the positive feed back pathway and hence fails to induce complete apoptosis. iC9, inducible caspase-9; hiPSC, human-induced pluripotent stem cells.

antiapoptotic protein BCL2 was upregulated. The family of BCL2 proteins regulates the intrinsic apoptotic pathway by blocking the mitochondrial disruption that triggers the activation of caspase-9, a component of the intrinsic or mitochondrial apoptosis signaling cascade.[23,24] Activation of iC9 acts in the terminal part of the intrinsic apoptosis pathway, downstream of the activity of BCL2, but recent studies have shown that the caspase-9 signaling cascade induces also mitochondrial disruption through the cleavage of BCL2 family protein, thereby amplifying the intrinsic apoptotic pathway through the activation of endogenous caspase-9 protein.[25,26] Blockade of this positive feedback loop with high levels of BCL2 may raise the threshold for apoptosis.[27] Given that iC9-resistant hiPSC also had lower iC9 expression, the BCL2 inhibitor may be insufficient to overcome upregulated BCL2, and the positive feedback loop may fail to be established (Figure 5). If this explanation is correct, BCl2 knockdown may be an alternative means of increasing sensitivity to iC9.

iC9-resistant hiPSC are likely derived from a subpopulation of hiPSC that have multiple escape mechanisms from caspase-dependent apoptosis including reduced expression of *iC9* and overexpression of BCL2.[28] As a consequence, modifying a single, or even multiple elements may be insufficient to ensure sustained susceptibility of iC9 in all cells and to prevent the eventual outgrowth of a resistant population; indeed, we failed to eliminate iC9-retransduced iC9-resistant hiPSC by both CID and BCL2 inhibition (Supplementary Figure S4). Instead, selection of iC9-hiPSC populations that are stably sensitive to the dimerizing drug may be preferable to ensure that the infused hiPSC line has the desired properties.[8] Single-cell–derived iC9-hiPSC clones that have sustained sensitivity can indeed be generated[8] if independent studies of additional lines and clones show that the mechanisms of resistance we identify here are indeed generalizable. Then clinical implementation of hiPSC therapy may be made safer by assessing *in vivo* elimination of cloned hiPSCs with apparently stable sensitivity to the dimerizing drug.

MATERIAL AND METHODS

hiPSC and reagents

Human iPSC, TZ16, was provided by the Human Stem Cell Core at Baylor College of Medicine (BCM Houston, TX). Human iPSC, TKCBSeV9, was kindly provided from the Stem Cell Bank of the Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, the University of Tokyo (Tokyo, Japan). Chemical inducer of dimerization (CID; AP20187, ARIAD Pharmaceuticals, Cambridge, MA, chemically virtually identical compound to AP1903 which was used for the clinical trials[5,7]) was purchased from Clontech Laboratories (Mountain View, CA). 5-aza-deoxycytidine was purchased from ApexBio Technology (Houston, TX). HDAC inhibitor, LBH589 and BCL2 inhibitor, ABT-737, were purchased from Selleck Chemicals (Houston, TX).

Establishment of iC9-mediated apoptosis-resistant hiPSC

We cultured hiPSC on a BD Matrigel-coated plate (CORNING, Corning, NY) with mTeSR1™ medium (STEMCELL Technologies, Vancouver, Canada) as described previously.[8] We generated *iC9*-expressing hiPSC (iC9-hiPSC) by transduction of lentiviral *iC9* expression vector, *pCDH-EF1α-iC9.2A.GFP*, and enrichment of the transduced cells for GFP expression using fluorescence-activated cell sorting as described previously.[8] To establish iC9-hiPSC subpopulations that were resistant to iC9-mediated apoptosis (iC9-resistant hiPSC), we cultured iC9-hiPSC with 10 nmol/l of CID for 24 hours. We then replaced the medium with fresh mTeSR1, which was replaced every day for 7 days. We enriched the GFP-positive cells using the MoFlo cell sorter (Beckman Coulter, Brea, CA).

In vitro apoptosis study

Twenty-four hours after CID exposure, we harvested and stained the cells with Annexin V-PE and 7-amino actinomycin D (7-AAD)

according to the manufacturer's instruction (Annexin V: PE Apoptosis Detection Kit I, BD Pharmingen™). The percentage of Annexin V negative, 7-AAD negative, and GFP-positive cells were quantified as live cells by flow cytometry (Gallios™, Beckman Coulter) and analyzed with Kaluza® Flow Analysis Software (Beckman Coulter).

Copy number and mRNA expression of *iC9* transgene

We determined the copy number and expression of the *iC9* transgene by quantitative polymerase chain reaction amplification as previously described.[5,8] Briefly, we extracted DNA and total RNA from each sample by QIAamp DNA mini kit and RNeasy mini kit (Qiagen, Venlo, Netherlands), respectively, as per the manufacturer's protocol. mRNA was transcribed into cDNA by iScript cDNA synthesis kit (Bio-rad, Hercules, CA) as per the manufacturer's protocol. For amplification, we used the iQ5 Real-time PCR Detection System (Bio-rad), iTaq Universal SYBR Green Supermix (Bio-rad), and specific primer sets that amplify the *iC9* transgene but not endogenous human *CASP9* gene. The real-time PCR reaction used one cycle of 95 °C for 30 seconds, followed by PCR amplification with 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. We normalized the gene dosage of *iC9* to that of *GAPDH*, and the mRNA expression of *iC9* was also normalized to that of *GAPDH*. Primer sequences are shown in Supplementary Table S1.

Caspase colorimetric assay

We determined the activation of caspase-9 and caspase-3 by a Caspase-9/-3 Colorimetric Assay Kit (Biovision, Milpitas, CA) according to the manufacturer's instruction. Briefly, cells were incubated with or without 10 nmol/l of CID for 2 hours, then harvested and lysed to purify proteins. We incubated 100 µg of total protein with LEHD-pNA substrate for caspase-9 assay and DEVD-pNA substrate for caspase-3 assay, respectively, at 37 °C for 2 hours. Samples were analyzed by Infinite® 200 PRO (TECAN, Männedorf, Switzerland) at 400-nm wavelength.

Western blot

We lysed cells to obtain proteins, and 30 µg of total protein were separated by SDS-PAGE. After blocking with 5% nonfat dried milk, we incubated the membrane with primary antibodies against Caspase-9, Caspase-3, BCL2, and XIAP (all from Cell Signaling Technologies, Danvers, MA) according to the manufacturer's instructions, then washed and incubated the membrane with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA). We visualized Immunoreactive bands using Super Signal™ West Dura Extended Duration Substrate (Thermo Scientific, Waltham, MA).

Bisulfite conversion and sequencing

To investigate the methylation status of the human *EF1α* promoter region, we treated 500 ng of genomic DNA with sodium bisulfite using an EZDNA methylation kit (Zymo Research, Irvine, CA) following the manufacturer's protocol, and subjected bisulfite converted DNA to bisulfite sequencing PCR, which amplified the CpG islands in the human *EF1α* promoter sequence using the appropriate primer sets. We subcloned the PCR products into pCR blunt vector using Zero Blunt® PCR cloning kit (Life Technologies, Carlsbad, CA), and then sequenced purified (plasmid) DNA from individually picked transduced colonies using M13 primer sets. We analyzed the methylation status with the BiQ Analyser software[29] using the following formula: the number of methylated cytosine/the number of total cytosine in the CpG islands of the human *EF1α* promoter region. Primer sequences are shown in Supplementary Table S1.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1. Spencer, DM, Wandless, TJ, Schreiber, SL and Crabtree, GR (1993). Controlling signal transduction with synthetic ligands. *Science* **262**: 1019–1024.
2. Fan, L, Freeman, KW, Khan, T, Pham, E and Spencer, DM (1999). Improved artificial death switches based on caspases and FADD. *Hum Gene Ther* **10**: 2273–2285.
3. Straathof, KC, Pulè, MA, Yotnda, P, Dotti, G, Vanin, EF, Brenner, MK *et al*. (2005). An inducible caspase 9 safety switch for T-cell therapy. *Blood* **105**: 4247–4254.
4. Tey, SK, Dotti, G, Rooney, CM, Heslop, HE and Brenner, MK (2007). Inducible caspase 9 suicide gene to improve the safety of allodepleted T cells after haploidentical stem cell transplantation. *Biol Blood Marrow Transplant* **13**: 913–924.
5. Di Stasi, A, Tey, SK, Dotti, G, Fujita, Y, Kennedy-Nasser, A, Martinez, C *et al*. (2011). Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med* **365**: 1673–1683.
6. Zhou, X, Di Stasi, A, Tey, SK, Krance, RA, Martinez, C, Leung, KS *et al*. (2014). Long-term outcome after haploidentical stem cell transplant and infusion of T cells expressing the inducible caspase 9 safety transgene. *Blood* **123**: 3895–3905.
7. Zhou, X, Dotti, G, Krance, RA, Martinez, CA, Naik, S, Kamble, RT *et al*. (2015). Inducible caspase-9 suicide gene controls adverse effects from alloreplete T cells after haploidentical stem cell transplantation. *Blood* **125**: 4103–4113.
8. Yagyu, S, Hoyos, V, Del Bufalo, F and Brenner, MK (2015). An inducible caspase-9 suicide gene to improve the safety of therapy using human induced pluripotent stem cells. *Mol Ther* **23**: 1475–1485.
9. Gore, A, Li, Z, Fung, HL, Young, JE, Agarwal, S, Antosiewicz-Bourget, J *et al*. (2011). Somatic coding mutations in human induced pluripotent stem cells. *Nature* **471**: 63–67.
10. Young, MA, Larson, DE, Sun, CW, George, DR, Ding, L, Miller, CA *et al*. (2012). Background mutations in parental cells account for most of the genetic heterogeneity of induced pluripotent stem cells. *Cell Stem Cell* **10**: 570–582.
11. Abyzov, A, Mariani, J, Palejev, D, Zhang, Y, Haney, MS, Tomasini, L *et al*. (2012). Somatic copy number mosaicism in human skin revealed by induced pluripotent stem cells. *Nature* **492**: 438–442.
12. Mills, JA, Wang, K, Paluru, P, Ying, L, Lu, L, Galvão, AM *et al*. (2013). Clonal genetic and hematopoietic heterogeneity among human-induced pluripotent stem cell lines. *Blood* **122**: 2047–2051.
13. Santoni de Sio FR, Gritti A, Cascio P, Neri M, Sampaolesi M, Galli C *et al*. (2008). Lentiviral vector gene transfer is limited by the proteasome at postentry steps in various types of stem cells. *Stem Cells* **26**: 2142–2152.
14. Pfaff, N, Lachmann, N, Ackermann, M, Kohlscheen, S, Brendel, C, Maetzig, T *et al*. (2013). A ubiquitous chromatin opening element prevents transgene silencing in pluripotent stem cells and their differentiated progeny. *Stem Cells* **31**: 488–499.
15. Wu, C, Hong, SG, Winkler, T, Spencer, DM, Jares, A, Ichwan, B *et al*. (2014). Development of an inducible caspase-9 safety switch for pluripotent stem cell-based therapies. *Mol Ther Methods Clin Dev* **1**: 14053.
16. Hong, S, Hwang, DY, Yoon, S, Isacson, O, Ramezani, A, Hawley, RG *et al*. (2007). Functional analysis of various promoters in lentiviral vectors at different stages of *in vitro* differentiation of mouse embryonic stem cells. *Mol Ther* **15**: 1630–1639.
17. Biswal, BK, Beyrouthy, MJ, Hever-Jardine, MP, Armstrong, D, Tomlinson, CR, Christensen, BC *et al*. (2012). Acute hypersensitivity of pluripotent testicular cancer-derived embryonal carcinoma to low-dose 5-aza deoxycytidine is associated with global

DNA damage-associated p53 activation, anti-pluripotency and DNA demethylation *PLoS One* **7**: e53003.
18. Wongtrakoongate, P, Li, J and Andrews, PW (2014). Aza-deoxycytidine induces apoptosis or differentiation via DNMT3B and targets embryonal carcinoma cells but not their differentiated derivatives. *Br J Cancer* **110**: 2131–2138.
19. George, P, Bali, P, Annavarapu, S, Scuto, A, Fiskus, W, Guo, F *et al.* (2005). Combination of the histone deacetylase inhibitor LBH589 and the hsp90 inhibitor 17-AAG is highly active against human CML-BC cells and AML cells with activating mutation of FLT-3. *Blood* **105**: 1768–1776.
20. Scuto, A, Kirschbaum, M, Kowolik, C, Kretzner, L, Juhasz, A, Atadja, P *et al*. (2008). The novel histone deacetylase inhibitor, LBH589, induces expression of DNA damage response genes and apoptosis in Ph-acute lymphoblastic leukemia cells. *Blood* **111**: 5093–5100.
21. Klug, M and Rehli, M (2006). Functional analysis of promoter CpG methylation using a CpG-free luciferase reporter vector. *Epigenetics* **1**: 127–130.
22. Ando, M, Hoyos, V, Yagyu, S, Tao, W, Ramos, CA, Dotti, G *et al*. (2014). Bortezomib sensitizes non-small cell lung cancer to mesenchymal stromal cell-delivered inducible caspase-9-mediated cytotoxicity. *Cancer Genef Ther* **21**: 472–482.
23. Yang, J, Liu, X, Bhalla, K, Kim, CN, Ibrado, AM, Cai, J *et al.* (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* **275**: 1129–1132.
24. Kluck, RM, Bossy-Wetzel, E, Green, DR and Newmeyer, DD (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275**: 1132–1136.
25. Cheng, EH, Kirsch, DG, Clem, RJ, Ravi, R, Kastan, MB, Bedi, A *et al*. (1997). Conversion of Bcl-2 to a Bax-like death effector by caspases. *Science* **278**: 1966–1968.
26. Chen, M, Guerrero, AD, Huang, L, Shabier, Z, Pan, M, Tan, TH *et al*. (2007). Caspase-9-induced mitochondrial disruption through cleavage of anti-apoptotic BCL-2 family members. *J Biol Chem* **282**: 33888–33895.
27. Guerrero, AD, Schmitz, I, Chen, M and Wang, J (2012). Promotion of caspase activation by caspase-9-mediated feedback amplification of mitochondrial damage. *J Clin Cell Immunol* **3**: doi:10.4172/2155-9899.1000126.
28. Barese, CN, Felizardo, TC, Sellers, SE, Keyvanfar, K, Di Stasi, A, Metzger, ME *et al*. (2015). Regulated apoptosis of genetically modified hematopoietic stem and progenitor cells via an inducible caspase-9 suicide gene in rhesus macaques. *Stem Cells* **33**: 91–100.
29. Bock, C, Reither, S, Mikeska, T, Paulsen, M, Walter, J and Lengauer, T (2005). BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. *Bioinformatics* **21**: 4067–4068.

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