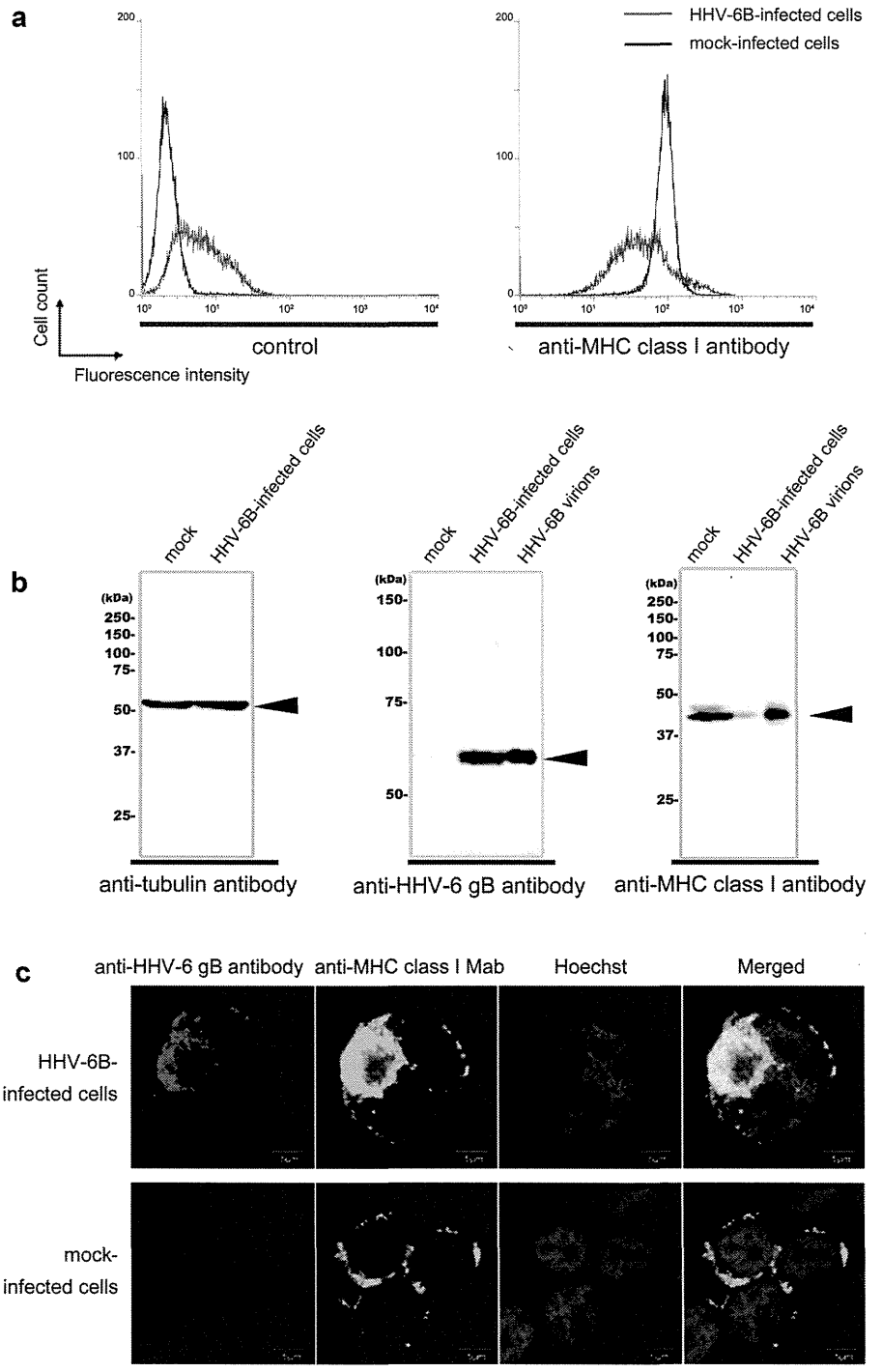


**Fig. 2. Expression of MHC class I in HHV-6B-infected cells.** (a) Expression of MHC class I on the surface of HHV-6B-infected cells is downregulated. HHV-6B-infected or mock-infected cells were harvested at 72 hr post-infection and fixed with 4% (w/v) paraformaldehyde. Fixed cells were stained with an anti-MHC class I antibody followed by staining with a secondary antibody prior to flow cytometric analysis. Control samples were incubated with the secondary antibody alone. Black histogram, mock-infected cells; blue histogram, HHV-6B- infected cells. (b) The total expression of MHC class I in HHV-6-infected cells was reduced. HHV-6B-infected or mock-infected cells were harvested at 72 hr post-infection and cell lysates prepared for western blotting. Purified HHV-6B virions were also used for western blotting. (c) MHC class I colocalizes with HHV-6B gB in intracellular compartments. HHV-6B-infected or mock-infected cells were harvested at 72 hr post-infection and fixed in cold acetone–methanol. Fixed cells were stained with antibodies against HHV-6 gB or MHC class I and with Hoechst33342. The stained cells were observed under a confocal microscope. The merged panels show the colocalized HHV-6 gB and MHC class I molecules. Single sections are shown. Scale bars: 5 micro meter.

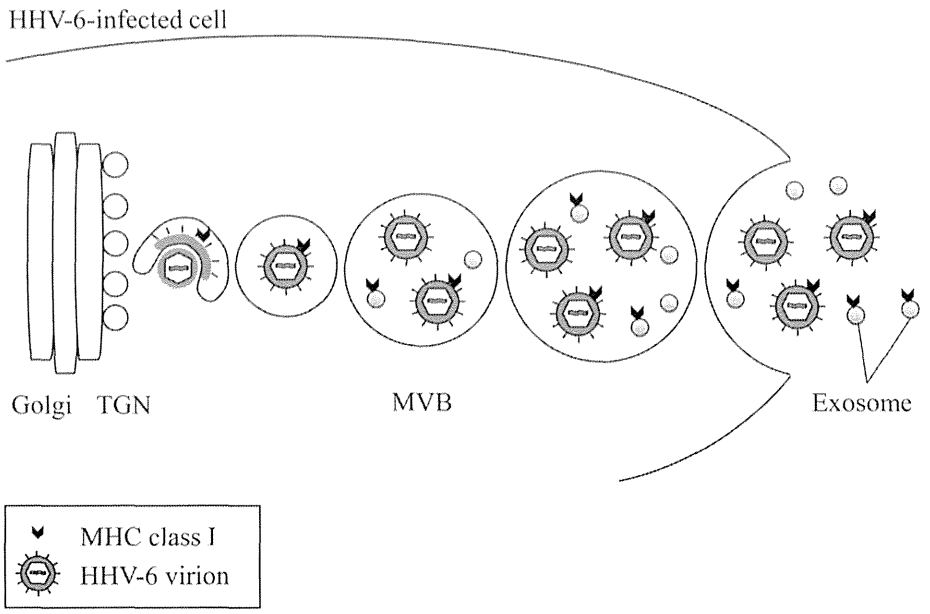


**Fig. 3.** Scematic representation of the fate of MHC class I molecules in HHV-6-infected cells. MHC class I molecules are transported to TGN- or post-TGN-derived vacuoles in HHV-6-infected cells and then incorporated into virions and intracellular small vesicles, which later become exosomes. Finally, MHC class I molecules are released from HHV-6-infected cells along with virions and exosomes.

within HHV-6-infected cells may show the combined characteristics of early and late endosomes. Recycling to early endosomes in HHV-6-infected cells may be modified or defective; therefore, several cellular proteins that use the same recycling system may be incorporated into virions and exosomes.

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## DISCLOSURE

The authors declare that they have no competing interests.

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Review

# Multimodal immunogenic cancer cell death as a consequence of anticancer cytotoxic treatments

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**Apoptotic cell death generally characterized by a morphologically homogenous entity has been considered to be essentially non-immunogenic. However, apoptotic cancer cell death, also known as type 1 programmed cell death (PCD), was recently found to be immunogenic after treatment with several chemotherapeutic agents and oncolytic viruses through the emission of various danger-associated molecular patterns (DAMPs). Extensive studies have revealed that two different types of immunogenic cell death (ICD) inducers, recently classified by their distinct actions in endoplasmic reticulum (ER) stress, can reinitiate immune responses suppressed by the tumor microenvironment. Indeed, recent clinical studies have shown that several immunotherapeutic modalities including therapeutic cancer vaccines and oncolytic viruses, but not conventional chemotherapies, culminate in beneficial outcomes, probably because of their different mechanisms of ICD induction. Furthermore, interests in PCD of cancer cells have shifted from its classical form to novel forms involving autophagic cell death (ACD), programmed necrotic cell death (necroptosis), and pyroptosis, some of which entail immunogenicity after anticancer treatments. In this review, we provide a brief outline of the well-characterized DAMPs such as calreticulin (CRT) exposure, high-mobility group protein B1 (HMGB1), and adenosine triphosphate (ATP) release, which are induced by the morphologically distinct types of cell death. In the latter part, our review focuses on how emerging oncolytic viruses induce different forms of cell death and the combinations of oncolytic virotherapies with further immunomodulation by cyclophosphamide and other immunotherapeutic modalities foster dendritic cell (DC)-mediated induction of antitumor immunity. Accordingly, it is increasingly important to fully understand how and which ICD inducers cause multimodal ICD, which should aid the design of reasonably multifaceted anticancer modalities to maximize ICD-triggered antitumor immunity and eliminate residual or metastasized tumors while sparing autoimmune diseases.**
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## Facts

- Accelerated progresses and discoveries in the field of oncology, immunology, and virology have made it possible to translate several emerging immunostimulatory strategies to treat malignant cancers towards promising clinical benefits.
- Profound understanding of the process of immunogenic cell death (ICD) induction by different ICD inducers such as certain chemotherapeutic agents and oncolytic viruses has highlighted the importance of immunological antitumor effects and proposed novel anticancer therapies.
- The execution of different types of programmed cell death (PCD), including apoptosis, autophagy, necroptosis, and pyroptosis, which are driven by a plethora of stimuli, was recently found to be regulated by orchestrated interactions among them, and importantly, some of these types of PCD exhibit an ICD property.
- Tumors and cancer cells treated with certain chemotherapeutic agents and oncolytic viruses can undergo ICD and release tumor-associated antigens (TAAs) accompanied by diverse danger-associated molecular patterns (DAMPs) and inflammatory cytokines to restore the tumor microenvironment and incite TAA-specific antitumor immunity.

## Open Questions

- What are the recent advances in the development of anticancer immunotherapeutic modalities in clinical settings?

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**Keywords:** ICD; DAMPs; apoptosis; necroptosis; immunotherapeutic anticancer agents; oncolytic virotherapy
**Abbreviations:** ICD, immunogenic cell death; PCD, programmed cell death; PAMPs, pathogen-associated molecular patterns; DAMPs, danger-associated molecular patterns; ecto-CRT, calreticulin exposure; HMGB1, high-mobility group protein B1; ATP, adenosine triphosphate; PS, phosphatidylserine; Hsp, heat-shock protein; ACD, autophagic cell death; ER, endoplasmic reticulum; ROS, reactive oxygen species; APCs, antigen-presenting cells; DCs, dendritic cells; TAAs, tumor-associated antigens; GM-CSF, granulocyte–macrophage colony-stimulating factor; PDT, photodynamic therapy; CVB3, coxsackievirus B3; CSCs, cancer stem cells
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- In response to diversified ICD inducers, how are DAMPs such as CRT, high-mobility group protein B1 (HMGB1), and ATP expressed by or released from the dying cancer cells?
- How do the diverse types of PCD differentially induce ICD to mount an efficient antitumor immunity?
- What are the prerequisites for an ideal ICD inducer to obtain an optimum level of ICD for long-lasting antitumor effects?
- It is vital to understand the molecular mechanisms of how ICD inducers, for example, infection with oncolytic viruses and resultant DAMPs, affect the host immune system. Can manipulation of ICD induction and/or combined strategies synergize with current or emerging oncolytic virotherapies?

The concept of immunogenic cell death (ICD) has recently been introduced to describe dying cancer cells that release endogenous danger molecules, the so-called damage-associated molecular patterns (DAMPs), after the exposure to certain cytotoxic agents to be recognized by antigen-presenting cells (APCs) such as dendritic cells (DCs) followed by formation of T-cell-mediated adaptive immunity.[1] Although it has long been considered that apoptotic cell death is tolerogenic, DAMPs have also been found to be released from cells undergoing apoptosis, providing a promising anticancer efficacy.[2–4] Therefore, comprehension of ICD induction gradually increases its significance, particularly in the field of cancer immunotherapy.

Overall prognosis of advanced cancer patients still remains dismal, thus making it imminent for oncologists to develop novel anticancer strategies. Recently, sipuleucel-T (Provenge, Dendreon, Seattle, WA, USA), indicated for patients with metastatic castration-resistant prostate cancer, received FDA's approval as the first therapeutic cancer vaccine.[5] In addition, extensive Phase II clinical trials have demonstrated that the oncolytic herpes simplex virus talimogene laherparepvec (T-Vec, Amgen Inc., Thousand Oaks, CA, USA)[6] and vaccinia virus JX-547 (Pexa-Vec, Jennerex Biotherapeutics, Inc., San Francisco, CA, USA),[7] both of which carry the gene encoding the immunostimulatory cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF), hold great promise for the treatment of advanced cancer patients. Furthermore, cytotoxic T-cell responses directed against oncolytic virus-infected cancer cells have been identified as an essential factor in the process of destruction of cancer.[8] Moreover, proinflammatory cytokines generated in the virus-infected cancer cells can restore the immunosuppressive tumor microenvironment.[9–11] Thus, oncolytic viruses are recently viewed as anticancer immunotherapeutic agents. These backgrounds make it imperative to update the molecular pathways and/or cellular constituents that regulate ICD.

Here, we review the progress of research on ICD, emphasizing how apoptotic, autophagic, and necroptotic cell death, called type 1, 2, and 3 PCD, respectively, are induced by various ICD inducers to achieve successful antitumor immunity. These multiple modes can be categorized by describing initiating events, intermediated changes, terminal cellular events, and their immunological responses, which are summarized in Table 1. In the later section, we outline the characteristics of anticancer agents and oncolytic viruses and how they induce diversified forms of cell death and interact with host's immune system.

## Apoptotic Cell Death as ICD

From ten million to billions of cells die per day as a consequence of normal tissue turnover,[12] which are vital for organisms to retain homeostasis.[13,14] Therefore, the existence of multiple modes of cell death in nature is not surprising. Apoptosis, type 1 PCD, is a specialized form of cell death, characterized by typical morphological changes, including chromatin condensation, nuclear fragmentation, and membrane blebbing (Table 1).[15] Apoptosis occurs ubiquitously in normal tissues and causes 'quiet' cell death that uses phosphatidylserine (PS) as an 'eat-me' signal to be quickly recognized by peripheral APCs. Although apoptotic cell death has been historically considered to be non-immunogenic,[16] recent studies unraveled that several anti-neoplastic agents, including doxorubicin,[1,17] oxaliplatin,[18,19] cisplatin,[20] and irradiation,[21,22,23] can trigger immunogenic apoptosis.[2] Mechanistically, the immunogenic apoptotic bodies induced by exposure to doxorubicin are sensed by APCs through their TLR-2/TLR-9-MyD88 signaling pathways.[17]

## DAMPs: as Effectors in ICD

The primary conceptual theory of the pattern recognition of pathogen-associated molecular patterns (PAMPs), such as viral or bacterial components, has failed to fully explain the consequence of immunogenicity. Thus, the secondary concept of DAMPs has been proposed, which could provoke an immune response.[24] Released DAMPs as hallmarks of ICD consisted of adenosine triphosphate (ATP), high-mobility group protein B1 (HMBG1), and exposed molecules on the outer membrane of dying cells such as CRT (ecto-CRT), heat-shock proteins (Hsp90 and Hsp70), and endoplasmic reticulum (ER) sessile proteins.[25,26,27] The excretion of DAMPs was considered to occur during necrosis under inflammatory and/or pathological conditions. However, DAMPs have recently been reported to be produced from apoptotic cancer cells treated with chemotherapy[1,18] or radiotherapy.[21]

## ICD Inducers

ICD inducers include multiple anticancer therapeutic modalities. It has been recently proposed that they can be classified into two categories, type I or II ICD inducers, based on their distinct actions to induce ER stress leading to apoptotic cell death (Tables 2 and 3).[27,28] The majority of ICD inducers such as chemotherapeutic agents (mitoxantrone,[29] anthracyclines,[2,30,31] oxaliplatin,[18,19] and cyclophosphamide[32]), shikonin,[33,34] the proteasome inhibitor bortezomib,[35] and 7A7 (an epidermal growth factor receptor-specific antibody),[36] cardiac glycosides,[37] and the histone deacetylase inhibitor (vorinostat)[38] are categorized as type I ICD inducers that primarily target cytosolic proteins, plasma membranes, or nucleic proteins. They also induce ER stress via collateral effects. Bortezomib, cardiac glycosides,

**Table 1** Comparison of multiple forms of programmed cell death and necrosis

| | Apoptosis (type 1 PCD) | Autophagic cell death (type 2 PCD) | Necroptosis (type 3 PCD) | Pyroptosis | Necrosis |
|---|---|---|---|---|---|
| Mode of cell death | Programmed | Programmed | Programmed | Programmed | Accidental |
| Initiators | TNF-α, FasL, or TRAIL, infectious pathogens | Nutrient deprivation, HDAC inhibitors, hypoxia, infectious pathogens | TNF-α, FasL, or TRAIL, microbial infections Ischemic injury | DAMPs, microbial infections | Toxins, infections, inflammation, trauma |
| Intermediate signalings | Mitochondrial pathway Caspase-3, -6, -7-dependent | Caspase-independent autophagosome formation Lysosomal protease | TNF receptor signaling JNK activation Caspase-independent RIP1/RIP3 necrosome | Nod-like receptors Caspase 1-dependent pyroptosome Inflammasome | - |
| Terminal cellular events | Non-lytic cell shrinkage DNA fragmentation apoptotic bodies | Non-lytic autophagic bodies | Non-lytic, loss of plasma membrane, swollen cellular organelles | Lytic, rapid loss of plasma membrane, cell swelling, pore formation | Lytic, plasma membrane rupture, leak of content |
| Inflammation Immunogenicity | Non-inflammatory + | Non-inflammatory + | Proinflammatory + + | Proinflammatory + + | Proinflammatory + + + |
| DAMPs released | Ecto-CRT HMGB1 and ATP release | HMGB1 and ATP release | Long genomic DNA IL-6 | HMGB1 and ATP release IL-1α, IL-1β, IL-6, IL-18, and TNF-α chemokines | HMGB1 and ATP release IL-1α, IL-33 mRNA, and genomic DNA |
| Eat-me signals | Ecto-CRT | LPC secretion PS exposure | LPC secretion PS exposure | PS exposure | PS exposure |

Abbreviations: TNF-α, tumor necrosis factor- α; FasL, Fas ligand; TRAIL, TNF-related apoptosis-inducing ligand; HDAC, histone deacetylase; IL-1α, interleukin-1α ; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-18, interleukin-18; IL-33, interleukin-33; ICD, immunogenic cell death; LPC, lysophosphatidylcholine; PS, phosphatidylserine; JNK, c-Jun N-terminal kinase.
The table gives a schematic overview of the multiple forms of cell death incuding apoptotic cell death (type 1 PCD), autophagic cell death (type 2 PCD), cell death induced by necroptosis (type 3 PCD), pyroptosis and necrosis. The extent of immunogenicity in each cell death subsection is scored as +, + +, and + + +, according to the expression levels of 'eat-me' signals and DAMPs emission. ICD in cancer can display different 'eat-me' signals, including ecto-CRT and LPC, on the cell membrane, as well as emission of DAMPs, ATP, and HMGB1. This peculiar ecto-CRT, which facilitates engulfment of TAAs from cancer cells by DCs, can only be found on cells that succumb to immunogenic apoptosis, whereas it is not present on cells dying in an immunologically silent manner. LPC secretion, PS exposure, and ATP release require autophagy induction. Numerous exquisite expression patterns shaped by the constituents of DAMPs and the interactive status of the immune system will predominantly determine the fate of subsequent immune responses, namely, immune tolerance or antitumor immunity

and shikonin effectively impede protumorigenic cytokine signaling.[27] Shikonin has been found to induce type 1 or 3 PCD, which is determined by caspase-8 activation as the 'decision-making switch'.[39] On the other hand, type II ICD inducers, which preferentially target the ER, include hypericin-based photodynamic therapy (PDT)[40,41,42] and oncolytic coxsackievirus B3 (CVB3).[9] Hypericin-based PDT is an anticancer therapy that utilizes hypericin to induce reactive oxygen species (ROS) in the vicinity of the ER.[43] Cancer cell infection with oncolytic viruses produce large amounts of viral proteins, which inevitably cause ER stress and ROS production to promote viral replication.[44,45] The quality and/or quantity of ER stress linking ROS triggered by ICD inducers may determine the ICD properties. Indeed, the previous finding that rigorous ROS-mediated ER stress augmented the release of DAMPs revealed an unrecognized role of RNA-dependent protein kinase (PKR)-like ER kinase (PERK) as a constituent of mitochondria-associated ER membranes to exert ROS-mediated mitochondrial apoptosis.[40,41,46] These observations indicate the superiority of type II ICD inducers with respect to immunological antitumor efficacies. However, further investigations to elucidate the precise interconnection between the ER stress and ROS production will be required to optimize antitumor immune responses.

## Calreticulin Exposure

In response to specific chemotherapeutic agents, oncolytic viruses, and vorinostat, ecto-CRT has been found only on cells succumbing to immunogenic apoptosis.[2,9,38] This 'eat-me' signal promotes phagocytosis by DCs, thereby facilitating their tumor antigen presentation and incitement of TAA-specific cytotoxic T cells.[2,47] It has been shown that blockade of CRT inhibits phagocytosis of anthracycline-treated tumor cells by DCs and impairs their immunogenicity in mice.[2,47] In general, CRT exposure during ICD is an earlier process occurring within a few hours than PS externalization.[48] The ecto-CRT induction capacity of ICD inducers has been shown to depend on the properties of ER stress and ROS production.[2,37,49] Cancer cells can induce ecto-CRT followed by disturbance of the ER structure with GADD34 activation and PERK phosphorylation. It has been shown that depletion of PERK abolishes anthracycline-driven ecto-CRT and immunogenicity of cellular death (ER stress module),[19] and that caspase-8 acts upstream of apoptotic proteins Bax and Bak, and subsequent cleavage of its substrate Bap31 (apoptotic module) is indispensable for ecto-CRT induction.[19] Furthermore, a direct interaction between ecto-CRT and ERp57 was shown to be required for their cotranslocation to the cell surface (Figure 1).[29] Unlike the release of HMGB1 and ATP,

**Table 2** Classification of type I ICD inducers determined by their major targets to provoke antitumor responses

| Anticancer agents | Type of cell death induced | DAMPs | Major targets by ICD inducers | Preclinical observations for inciting antitumor immunity |
|---|---|---|---|---|
| Cytotoxic agents (mitoxantrone, oxaliplatin, anthracyclines) | Apoptosis, autophagic cell death, necroptosis | Ecto-CRT, ERp57, HMGB1, and ATP release | Nucleus (DNA or DNA-related proteins for cell mitosis) | *In vivo* antitumor effect is mitigated by depletion of $CD8^+$ T cells. Immunogenicity requires ecto-CRT in prophylactic tumor vaccination mouse models. |
| Cyclophosphamide (CTX) | Apoptosis | Ecto-CRT, HMGB1 release | Nucleus (DNA) | Metronomic doses of CTX deplete Treg from bed and tumors, CTX modulates DCs to produce IL-12 |
| Shikonin | Apoptosis, necroptosis | Ecto-CRT, ecto-Hsp70 | Cytosol (pyruvate kinase-M2 protein) | DCs incubated with shikonin increase Th1 cells but decrease Treg cells |
| Bortezomib | Apoptosis, autophagic cell death | Ecto-Hsp90 | Cytosol (26S proteasome) | Cytotoxicity of NK cells against bortezomib-treated cells increased |
| 7A7 (EGFR-specific antibody) | Apoptosis | Ecto-CRT, ERp57, ecto-Hsp70, ectp-Hsp90 | Cell surface receptor (EGFR) | Contribution of $CD4^+$ T and $CD8^+$ T to 7A7-triggered suppression of metastasis in mice model |
| Cardiac glycosides | Apoptosis | Ecto-CRT HMGB1 and ATP release | Cell surface ($Na^+/K^+$-ATPase, enzyme) | Prophylactic antitumor immunity is partially dependent on $CD8^+$ T cells accompanied with Th17 cells |
| UVC irradiation | Apoptosis, necroptosis, necrosis | Ecto-CRT and ERp57, HMGB1 and ATP release | Nucleus (DNA) | UVC-treated cells increase susceptibility to attack by NK cells and total splenocytes |
| Vorinostat (HDAC inhibitor) | Apoptosis Autophagic cell death | Ecto-CRT | Nucleus (chromatin structure) | Promote the differentiation of $CD8^+$ T cells to memory cells |

Abbreviations: Ecto-CRT, calreticulin exposure; DAMPs, damage-associated molecular patterns; ICD, immunogenic cell death, HMGB1; high-mobility group protein B1; Hsp, heat-shock protein; Treg, regulatory T cells; DCs, dendritic cells; IL-12, interleukin-12; NK, natural killer; EGFR, epidermal growth factor receptor; ATP, adenosine triphosphate; UVC, ultraviolet C

**Table 3** Classification of type II ICD inducers determined by their major targets to provoke antitumor responses

| Anticancer agents | Type of cell death induced | DAMPs | Major targets by ICD inducers | Preclinical observations for inciting antitumor immunity |
|---|---|---|---|---|
| PDT with hypericin | Apoptosis, autophagic cell death dependent on Bax/Bak, necroptosis | Ecto-CRT, ecto-Hsp70, ectp-Hsp90, HMGB1, and ATP release | ER (ROS generation) | PDT -hypericin therapy provokes antitumor immunity in both prophylactic and therapeutic murine tumor models. Same therapy-treated tumor cells result in phenotypic maturation of DCs and robust $CD4^+$T and $CD8^+$T cell expansion |
| CVB3 | Apoptosis | Ecto-CRT, HMGB1 translocation, ATP release | ER (ROS generation) | Intratumoral CVB3 administration markedly recruited NK cells and granulocytes, both of which contribute to the antitumor effects as shown by depletion assays, macrophages, and mature DCs into tumor tissues |
| Ad5/3-hTERT-E1A-hCD40L: chimeric Ad5/3 capsid, an hTERT promoter and human CD40L | Apoptosis | Ecto-CRT, HMGB1 release, ATP release | ER (ROS generation) | In two syngeneic mouse models, murine CD40L induced activation of APCs, leading to increased IL-12 production in splenocytes, associated with induction of the Th1 cytokines IFN-$\gamma$, RANTES, and TNF-$\alpha$. Tumors treated with Ad5/3-CMV-mCD40L displayed an enhanced presence of macrophages and cytotoxic $CD8^+$ T cells |
| Edmonston strain MV | Apoptosis | IL-6 production, HMGB1 release | ER (ROS generation) | Coculture of MV-infected melanoma cells with human DCs led to both CD80 and CD86 upregulation on them. $CD8^+$ T cells cocultured with tumor cell-loaded and MV-infected DCs degranulated CD107a to target tumor cells with functional killing activity |

Abbreviations: PDT, photodynamic therapy; CVB3, coxasackievirus B3; MV, measles virus; ROS, reactive oxygen species; ER, endoplasmic reticulum; hTERT, telomerase reverse transcriptase; hCD40L, human CD40 ligand; Th1, T helper type 1; RANTES, regulated and normal T cell expressed and secreted; TNF-$\alpha$, tumor necrosis factor-$\alpha$; IL-6, interleukin-6

ecto-CRT could be one of the determinants that distinguishes between immunogenic and non-immunogenic cell death.[47]

### HMGB1

HMGB1, one of the DAMPs, is a DNA-binding protein originally known as a nuclear non-histone chromatin-binding protein.[50] Although extracellular HMGB1 had been deemed to be released mainly from the nucleus during necrosis,[42] it was found to be excreted from cells undergoing late stage of apoptosis and autophagy.[30,51] HMGB1 inhibition in cancers undergoing immunogenic apoptosis impaired their ability to incite antitumor immunity in a prophylactic vaccination model.[30] HMGB1 initiates potent inflammation by stimulating the production of proinflammatory cytokines[52] from APCs via its binding to different surface receptors including receptor for advanced glycation end-products (RAGE), TLR2, TLR4, TLR9, and TIM3 (Figure 1).[53,54] Importantly, the binding of HMGB1 to TLR4 on APCs was required to suppress tumor development, which is consistent with clinical study showing that breast cancer patients harboring a single-nucleotide polymorphism (Asp299Gly) in the *TLR4* gene undergo an early relapse after anthracycline treatment.[30,55,56] In contrast, secreted HMGB1 could induce a protumor inflammation to facilitate tumor progression.[57] In addition, HMGB1 expression is significantly associated with overall survival of patients with bladder cancer.[58] As HMGB1 is an intrinsic sensor of oxidative stress,[59] the immunomodulatory properties of HMGB1 might be determined by its redox status.[60,61] Indeed, reduced HMGB1 production from dying cells was shown to trigger the immunogenic DCs, whereas oxidized HMGB1 during apoptosis fails.[51] As the extracellular space is usually oxidative under physiological conditions but is unpredictably variable under pathogenic conditions,[62] the unstable redox status of the tumor microenvironment might account for these inconsistent findings. However, the observation that the tumor microenvironment tends to be pro-oxidative[63] implies that a therapeutic approach using antioxidants to decrease ROS production would be favorable to stimulate antitumor immunity. Importantly, many anticancer agents, including chemotherapy,[30] radiation,[22] or oncolytic viruses,[9,64,65] have been shown to induce HMGB1 release from cancer cells, highlighting the significance of further addressing the mechanism of how these modalities affect the redox status of HMGB1.

### Adenosine Triphosphate

Extracellular ATP released from apoptotic cells is another important factor in ICD induction. ATP signaling recognized by P2Y2 receptors on phagocytes as a 'find-me' signal enables

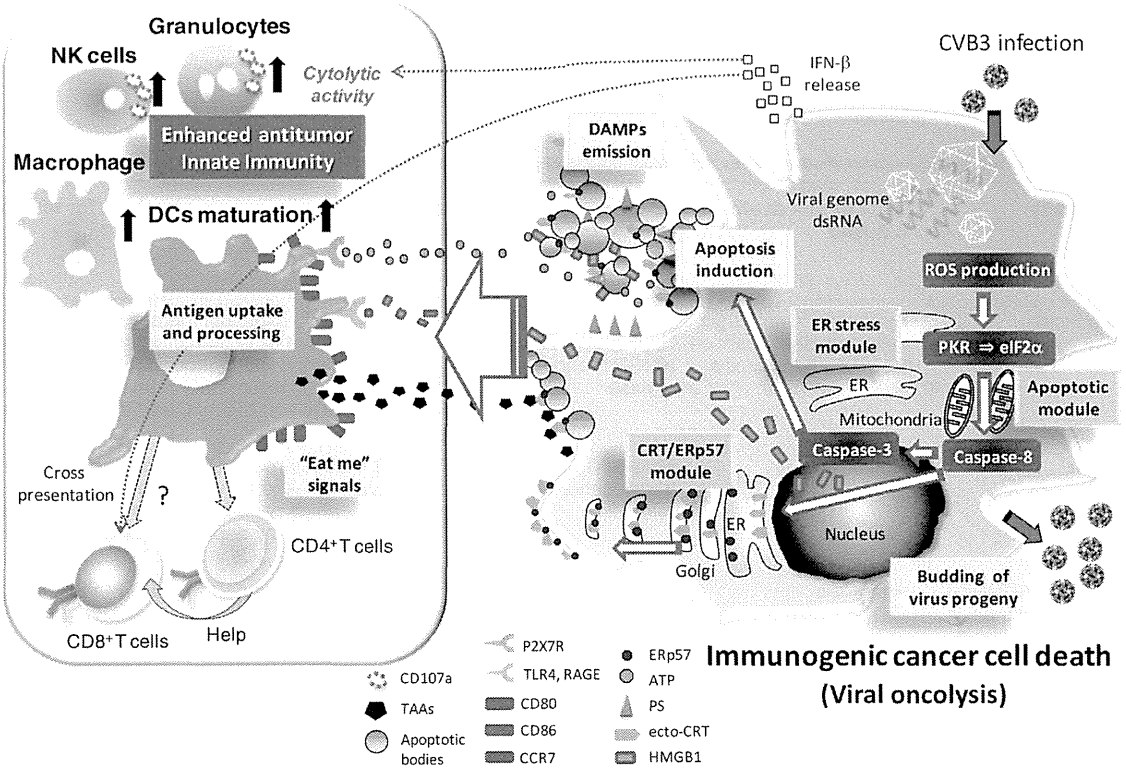


**Figure 1** Oncolytic virus (CVB3) infection-triggered cancer cell death induces innate immune cell-mediated antitumor immunity. Intratumoral CVB3 infection-activated natural killer (NK) cells and granulocytes with enhanced expression of CD107a, a cytolytic degranulation marker, have been found to contribute to substantial antitumor effects as evidenced by NK cell and granulocyte depletion assays. Upon CVB3 infection, tumor cells can partially induce ecto-CRT on human tumor cells during early apoptosis, whereas majority of other viruses subvert ICD by circumventing ecto-CRT induction, and followed by robust release of DAMPs, including ATP and HMGB1, during later stages of cell death, which facilitates maturation of DCs via binding to Toll-like receptor 4 (TLR4)/RAGE and $P_2 \times _7R$, respectively. Viral genomes and/or viral progenies also stimulate DCs for their activation. Mature DCs may then efficiently phagocytose TAAs simultaneously released from dying cells and ultimately cross-present them to $CD8^+$ T cells with the support with $CD4^+$ T cells to elicit substantial antitumor immunity. Although ATP secretion relies on autophagic machinery, the other forms of cancer cell death, such as autophagic cell death and necroptosis, triggered by CVB3 infection have not yet been fully investigated

them to migrate into inflamed sites.[66] Indeed, ATP released from cancer cells treated with chemotherapeutic agents is essential for effective antitumor immune responses.[67] In addition, small interfering RNA-mediated inhibition of autophagic machinery abolishes ATP release from chemotherapy-treated tumor cells and mitigates the antitumor response.[68] Radiotherapy triggers ATP release from dying tumor cells through its interaction with the P2 $\times$ 7 purinergic receptor,[69] possibly resulting in the activation of the NLRP3–ASC–inflammasome axis and subsequent secretion of IL-1$\beta$.[70]

We and others recently showed that oncolytic viruses induce secretion of extracellular ATP from human cancer cells (Figure 1).[9,65] Unlike ecto-CRT induction, the release of ATP and HMGB1 is triggered by a range of death-inducing stimuli, and is not restricted to induction in apoptotic cell death.[47] Although ATP production is required for efficient vaccinia virus production[71] and facilitates HIV infection through its interaction with P2Y2 receptors,[72] there is little knowledge of how oncolytic viruses provoke ATP release.

### Autophagic Cell Death

Autophagy physiologically has catabolic roles, particularly in cell survival.[73] However, persistent autophagy causes a caspase-independent form of cell death that is, morphologically defined as autophagic cell death (ACD), termed as type 2 PCD, through lysosomal proteinase-regulated elimination of cellular organelles.[74,75] Autophagy sometimes directs itself to cellular death, either in cooperation with apoptosis or as a back-up system, and thus is deemed as a cellular program with a 'double-faced' role.

Interestingly, the key molecules of autophagy and apoptosis pathways are intricately intertwined with shared several molecules including regulatory genes such as *p53* and *p19ARF*.[76] This crosstalk therefore can be viewed as a significant clue to understand the fate of dying cancer cells from therapeutic view points. Although ACD occurs without chromatin condensation but with massive autophagic vacuolization,[77] autophagy, often disabled in cancer, has been shown to be required for induction of immunogenicity.[68] First, dying cells in embryoid bodies that lack autophagy-related gene are unable to express the 'eat-me' signals and secrete lower levels of the 'come-get-me' signal, lysophosphatidylcholine.[78] Second, autophagy deficiency hinders ATP secretion from dying cancer cells, resulting in the impairment of DC recruitment and formation of adaptive immunity responses (Table 1).[68] Third, the inability of autophagy-deficient cancer cells to provoke antitumor immunity after chemotherapy can be reverted by suppression of extracellular ATP-degrading enzymes.[68,79] Therefore, immunogenicity of ACD could be mediated by subtle spatiotemporal alterations in the treated cancer cells.

Novel strategy of autophagy inhibition is therapeutically effective for eliminating apoptosis-resistant cancer cells based on the rationale that growing tumors may harness autophagy as an adaptation to resist therapeutic stresses.[80,81,82] Hence, more efforts should be made to elucidate the intricate interaction between autophagy inhibition and resulting effects on the immunogenicity.

### Necrotic Cell Death and Necroptosis

Necrotic cell death is induced by external factors such as toxins, cancer, infections, and trauma, and is morphologically characterized by cellular swelling, rupture of the plasma membrane, and loss of cytoplasmic contents.[83] Understanding the immunogenicity of necrotic cell death is becoming important because it frequently induces robust inflammatory reactions to mount protective immune responses (Table 1).[84,85,86] Although necrosis has long been viewed as non-PCD, its execution was shown to be controlled by specific signal-transduction pathways and catabolic mechanism.[87,88,89] This alternative form of necrotic PCD, aptly termed necroptosis (type 3 PCD), is induced by tumor necrosis factor (TNF) receptor signaling that involves activation of the receptor-interacting protein (RIP) family. Upon inhibition of apoptotic pathway by the caspase inhibitor, activation of RIP1 and RIP3 kinase leads to mitochondrial instability and cell death.[90,91] Phosphorylated RIP1 and RIP3 generate a molecular complex called the necrosome, which initiates necroptosis. ROS production under necroptosis has been shown to facilitate TNF-$\alpha$-induced cell death by sustaining c-Jun N-terminal kinase activation.[92] Intriguingly, necroptosis can also be executed via stimulation by apoptosis-inducible ligands such as TNF-$\alpha$, FasL, or TRAIL (Table 1). Notably, cytotoxic agents are shown to induce necrotic cell death in apoptosis-defective cancer cells,[93] probably because necroptosis is principally induced when a cell cannot die via apoptotic pathways.[94] On the other hand, conventional therapy-resistant cancer stem cells (CSCs) have a higher antiapoptotic activity than that of their counterparts.[95,96] Therefore, it would be vital to clarify the key machinery of not only the necroptosis induction in cancer cells for CSC-directed therapeutic application but also the resultant immunogenicity to modulate antitumor immunity.

### Pyroptosis

Pyroptosis is a recently indentified form of PCD stimulated by microbial infections and non-infectious stimuli such as myocardial infarction and cancer. In contrast to apoptosis, pyroptosis is uniquely mediated by caspase-1 activity triggered by the formation of a cytosolic complex termed the 'inflammasome', resulting in highly inflammatory outcomes (Table 1). Pyroptotic cells represent morphological characteristics, some of which are shared with apoptosis and necrosis.[97] The function of activated caspase-1 is to cleave proteolytically the proforms of the proinflammatory cytokines, IL-1$\beta$ and IL-18, to their active forms.[97] Although pyroptosis has been intensively studied in the context of bacteria-infected macrophages,[98] it can also be triggered in human cancer cells infected with recombinant herpes simplex virus 2 (HSV-2) (Table 4).[99] Pyroptotic cancer cells induced by microbial infection have been recently shown to facilitate phagocytosis by macrophages, presumably through their PS exposure and ATP release.[100] Accordingly, the caspase-1-dependent generation of proinflammmatory cytokines and other DAMPs could be essential factors to provide a suitable inflammation for ICD induction.

**Table 4** DNA oncolytic viruses and their differential properties to induce either multiple forms of cell death or antitumor immunity

| Oncolytic viruses | Type of cancer cells | Type of cell death induced | DAMPs | Possible mechanism of antitumor immunity |
|---|---|---|---|---|
| hTERT-Ad: CRAds regulated by human hTERT promoter | Human glioma, cervical and prostate cancer | Autophagy | NA | NA |
| hTERT-Ad | Human lung cancer | Autophagy via E2F1-miR-7-EGFR | NA | NA |
| OBP-702: p53-armed hTERT-Ad | Human osteosarcoma | Apoptosis Autophagy | NA | NA |
| CRAd-S-RGD: Ad5 carrying the RGD motif and survivin promoter | Human glioma cells | Autophagy | NA | NA |
| Ad5/3-hTERT-E1A-hCD40L: chimeric Ad5/3 capsid with hTERT promoter | Murine urothelial carcinoma, melanoma | Apoptosis | Ecto-CRT, ATP and HMGB1 | Enhanced recruitment of macrophages and $CD8^+$ T cells |
| ZD55-IFN-$\beta$: Oncolytic adenovirus carrying IFN-$\beta$ | Human hepatoma, breast cancer | Apoptosis Necroptosis | NA | NA |
| Vaccinia virus | Human colon, breast, ovarian cancer | Not apoptosis Possibly necrosis | HMGB1 release | NA |
| vSP: antiapoptosis genes, SPI-1- and SPI-2-deleted vaccinia virus | Murine colon adenocarcinoma | Apoptosis Necrosis | HMGB1 release | NA |
| HSV2: Human simplex virus 2 | Human endometrial cancer | Apoptosis Necrosis | HMGB1 release | NA |
| HSV-1716: a replication-restricted mutant herpes simplex virus | Murine ovarian cancer | NA | NA | Intratumoral injection induced IFN-$\gamma$, CXCL9 and CXCL10 with increase in NK and $CD8^+$ T cells |
| HSV-2 mutant $\Delta$PK: ICP10PK-deleted HSV-2 virus | Human melanoma cells | Apoptosis Pyroptosis | NA | Dominant induction of $CD4^+$ Th1 cells |

Abbreviations: hTERT, telomerase reverse transcriptase; CRAds, conditionally replicative adenoviruses; miR-7, microRNA-7, EGFR, epidermal growth factor receptor, ecto-CRT, calreticulin exposure; DAMPs, damage-associated molecular patterns; ICD, immunogenic cell death, HMGB1; high-mobility group protein B1;ATP, adenosine triphosphate; IFN-$\beta$, interferon-$\beta$; HSV, herpes simplex virus; IFN-$\gamma$, interferon-$\gamma$; CXCL9, chemokine (C–X–C motif) ligand 9; CXCL10, chemokine (C–X–C motif) ligand 10; NK, natural killer cells; NA, not assessed; Th1, T helper type 1

## DAMPs Induced by Infection with Oncolytic Viruses

Because oncolytic viral infection can produce abundant PAMPs, including viral proteins and nucleic acids, followed by the release of DAMPs and the entire repertoire of TAAs from treated tumors,[101] oncolytic virus-triggered ICD may be more effective for induction of antitumor immunity. As viruses have developed sophisticated machineries to evade apoptotic cell death and interfere with ER stress and autophagy responses for their survival,[102,103] ICD may have played an essential role in the everlasting war between viruses and their hosts. We and other groups have found that many oncolytic viruses can induce apoptotic cell death and/or necrosis in cancer cells,[9,104,105,106] supporting their immunostimulatory potential to augment antitumor efficacy (Tables 4 and 5).[107,108] CVB3 infection induces multiple DAMPs including ecto-CRT, HMGB1 translocation from nuclei, and ATP release from human lung cancer cells. Importantly, intratumoral CVB3 administration can prominently recruit cytolytic degranulation marker CD107a-mobilized NK cells and granulocytes, and mature DCs into the tumor bed (Figure 1).[9,27] As pathogenic viruses have developed their strategies to subvert ecto-CRT and circumvent ICD induction,[109] it is noteworthy that CVB3 infection can induce ecto-CRT accompanied by other DAMPs.[9] Furthermore, we demonstrated that both NK cells and granulocytes substantially contributed to the CVB3-mediated antitumor efficacy as evidenced by *in vivo* depletion assays.[9]

Upon intratumoral replication of oncolytic viruses, resultant alterations in tumor microenvironment may restore the compromised antitumor immunity, presumably through induction of IFNs and/or cytokines that activate NK cells and APCs.[110,111] Although tumor-infiltrating DCs were impaired at maturation by immunosuppressive IL-10, $PGE_2$, and transforming growth factor $\beta$ produced from tumor cells,[112] unidentified components in the culture media from reovirus-infected cancer cells facilitated maturation of DCs.[113]

Recent studies delineated that oncolytic viruses such as vaccinia, measles, HSV-2, and adenovirus cause the release of HMGB1.[64,65,114,115,116,117] Although HMGB1 interacts with viral components and may modulate viral replication,[117] the molecular mechanisms of how each oncolytic virus differentially produces these DAMPs remain largely elusive.

## Multimodal PCD Induced by Oncolytic Viruses

We showed that approximately 20% of CVB3-mediated cytotoxicity of A549 cells resulted from apoptotic cell death.[9]

**Table 5** RNA oncolytic viruses and their differential properties to induce either multiple forms of cell death or antitumor immunity

| Oncolytic viruses | Type of cancer cells | Type of cell death induced | DAMPs | Possible mechanism of antitumor immunity |
|---|---|---|---|---|
| Edmonston vaccine strain of MV | Human melanoma | NA | IL-6 HMGB1 release | Human DC maturation Priming an adaptive T-cell response |
| MV-NPL: genetically engineered MV | Human renal cell carcinoma | Apoptosis | NA | NA |
| MV-CEA:Edmonston vaccine MV genetically engineered to produce CEA antigen | Human breast cancer | Apoptosis | NA | NA |
| CVB3 | Human non-small-cell lung cancer | Apoptosis | Preapoptotic ecto-CRT, HMGB1 translocation, ATP release | Phenotypic activation of immature DCs and lytic NK cells in tumors. Deletion of NK and granulocytes abrogated the CVB3-induced *in vivo* antitumor immunity |
| NDV | Human glioma | Autophagy | NA | NA |
| Reovirus | Human multiple myeloma | Apoptosis Autophagy | NA | NA |
| Live-attenuated poliovirus | Human neuroblastoma | Apoptosis | NA | NA |
| M51R: M protein mutant VSV | Human glioblastoma multiforme | Apoptosis | NA | NA |
| Interferon-sensitive VSV (AV3 strain) | Human prostate cancer | Apoptosis | NA | NA |

Abbreviations: MV, measles virus; CVB3, coxasackievirus B3; NDV, New castle disease virus; CEA, carcinoembryonic antigen; VSV, vesicular stomatitis virus

This induction was presumably due to the capacity of CVB3 infection to induce PKR-mediated phosphorylation of eIF2 and caspase-8-mediated activation of proapoptotic mediator, caspase-3 (Figure 1).[118,119] Other DNA and RNA oncolytic viruses have been reported to induce apoptotic cancer cell death (Tables 4 and 5). However, there are only two reports showing that virus-induced ecto-CRT was correlated with enhanced intratumoral infiltrations of immune subpopulations, which accounted for the '*in vivo*' remarkable antitumor immunity.[9,65]

Several studies showed that recombinant oncolytic adenoviruses induced ACD in human malignant glioma cells,[120] brain tumor stem cells,[121] osteosarcoma cells,[105] and lung cancer cells.[122] Newcastle disease virus also triggered autophagy in glioma cells to promote its viral replication.[123] Reovirus-mediated oncolysis of multiple myeloma was reported to be orchestrated via upregulation of autophagy.[124] Because cancer cells are largely refractory to apoptotic inducers but vulnerable to necroptosis,[39] overcoming anticancer drug resistance may be achieved by activation of necroptotic rather than apoptotic pathways, where the former might be the intrinsic 'Achilles heel' of cancers.[125] So far only recombinant adenovirus has been shown to facilitate both necroptotic and apoptotic cell death with a synergistic effect on cancer cells when combined with doxorubicin (Tables 4 and 5).[126] In addition, most oncolytic viruses may induce pyroptotic cancer cell death accompanied by abundant proinflammatory cytokines and DAMPs. Accordingly, some oncolytic viruses may induce multimodal ICD, allowing them to be a plausible modality as promising agents of immunotherapy.

## Strategies to Enhance the Potentials of ICD Induced by Oncolytic Viruses

Besides DAMPs, massive production of type I IFNs (IFN-$\alpha/\beta$) upon oncolytic viral infection can be a potent immunomodulator through their indirect immunostimulatory effects on neutrophils and T cells,[127,128] as well as through their direct antiproliferative effects.[129] Despite a creation of multimodal ICD by oncolytic viruses to facilitate antitumor immunity, much attention should be paid to the preferential antiviral immunity that might impede direct viral oncolysis-mediated tumor destruction. To avoid this, cyclophosphamide is shown to retard immune removal of oncolytic viruses, enhancing the persistence of viral infection.[130] Another promising strategy to overcome antiviral immunity could be potentiating immune responses by gene modification of oncolytic viruses to arm them with immunostimulatory cytokines, such as GM-CSF, IL-2, IL-12, and IL-15. Indeed, the results of clinical trials of the GM-CSF gene-harboring oncolytic vaccinia virus JX-594 and the GM-CSF gene-harboring oncolytic herpes virus talimogene laherparepvec demonstrated that a clinical benefit can be accomplished by combined respective oncolytic activity with the recruitment of immune cells.[6,7,131] The combination of adoptive T-cell therapy with oncolytic viruses is shown to elicit an increased antitumor effect.[131,132] Collectively, the design of combinatorial therapies of oncolytic viruses with immunotherapeutic modalities may

hold the key to mount maximally a multifaceted attack against cancers.

## Conclusions

Although mechanism of ICD induction is a very complicated process, we need to elucidate how dying cells become much more stimulatory in shaping antitumor immune responses than was ever expected. Notably, intermediate death processes, including caspase activation, mitochondrial degradation by autophagy, ROS production, and oxidative modification of DAMPs, have been found to fine-tune the balance between antitumor tolerance and immunity, providing implications in manipulation of ICD.

Four forms of PCD, apoptosis, autophagy, necroptosis, and pyroptosis, may jointly decide the fate of cells of malignant cells. However, in terms of immunogenicity, investigations of only apoptotic cell death in cancer cells have just begun. Therefore, further elucidation of determinants of respective PCD-inducing pathway and characterization of resultant ICD should aid to develop novel anticancer strategies. A recent review advocates a list of characteristics for an ideal ICD inducer,[27] as follows: (1) efficiently activates apoptosis or necrosis leading to emission of multiple DAMPs and TLR agonists;[133,134] (2) irrelevant in drug-efflux pathways;[135] (3) can induce ER stress;[134] (4) has negligible suppressive or inhibitory effects on immune cells;[136] (5) counteracts immunosuppressive responses;[136,137] and (6) directly targets not only the primary tumor but also metastases.[138] No ideal ICD inducer exists, but it is important to seek for ideal combinatorial therapies that could achieve these properties. Of the currently known relevant ICD inducers, those that meet most of these properties include mitoxantrone, hypericin-PDT, and shikonin. However, diverse oncolytic viruses could be the promising ICD inducer as we gain more knowledge about the properties yet to be investigated. Evidently, they can destroy conventional therapy-resistant CSCs,[139] possibly through their ability to induce distinctive PCD and/or modification to express genes that target CSC-specific signaling pathways underpinning their cell survival.[140]

Gaining more detailed insights into the mechanisms of ICD induction, to be perceived by the immune system, will not only ameliorate the development of promising anticancer agents or combinatorial therapies but also offer useful knowledge in various life science fields including virology, immunology, and clinical medicine.

## Conflict of Interest

The authors declare no conflict of interest.

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# Characterization of common marmoset dysgerminoma-like tumor induced by the lentiviral expression of reprogramming factors

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**Key words**

Common marmoset, FGFR, regenerating medicine, reprogramming factor, tumorigenesis

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**Recent generation of induced pluripotent stem (iPSCs) has made a significant impact on the field of human regenerative medicine. Prior to the clinical application of iPSCs, testing of their safety and usefulness must be carried out using reliable animal models of various diseases. In order to generate iPSCs from common marmoset (CM; *Callithrix jacchus*), one of the most useful experimental animals, we have lentivirally transduced reprogramming factors, including POU5F1 (also known as OCT3/4), SOX2, KLF4, and c-MYC into CM fibroblasts. The cells formed round colonies expressing embryonic stem cell markers, however, they showed an abnormal karyotype denoted as 46, X, del(4q), +mar, and formed human dysgerminoma-like tumors in SCID mice, indicating that the transduction of reprogramming factors caused unexpected tumorigenesis of CM cells. Moreover, CM dysgerminoma-like tumors were highly sensitive to DNA-damaging agents, irradiation, and fibroblast growth factor receptor inhibitor, and their growth was dependent on c-MYC expression. These results indicate that DNA-damaging agents, irradiation, fibroblast growth factor receptor inhibitor, and c-MYC-targeted therapies might represent effective treatment strategies for unexpected tumors in patients receiving iPSC-based therapy.**

The development of a technology to generate iPSCs from differentiated somatic cells by the transduction of a set of transcription factors, OSKM, made a significant impact in the field of basic research for regenerative medicine, in light of their potential use as a cell source for transplantation therapy for various kinds of incurable diseases.[1,2]

However, the low efficiency of iPSC generation, the need to induce their efficient differentiation into specific cell types, and the risk of tumor formation in recipients transplanted with iPSC-derived functional cells have hindered the clinical application of iPSCs.[3] The transduction of transcription factors, including the oncogene *c-MYC*, insertional mutation of the genome caused by virus vectors, and genomic instability due to the stress of long-term culture for reprogramming, might contribute to tumor development when iPSC-derived functional cells are applied to clinical practice.[4] Although the technology used to generate iPSCs has improved with the use of OSK without M,[5] non-viral vectors,[6] other molecules such as mRNA or miRNA,[7] and chemicals,[8] tumor formation in recipients remains a major concern.[9] Despite these issues, reprogramming factor-related tumor cells have not been well-characterized to date.

The CM (*Callithrix jacchus*) has several advantages as an experimental laboratory primate, including ease of handling, being inexpensive to house and feed, and a high reproductive rate.[10] Therefore, CM and CM-derived iPSCs represent useful experimental tools for testing the clinical utility of iPSC-based regenerative medicine *in vivo* and *in vitro*.

In this study, we attempted to generate iPSCs from CM fibroblasts, and inadvertently produced immature malignant tumor cells. We therefore analyzed the biological characteristics of these cells *in vitro* and *in vivo*. The results may provide useful information for the development of strategies to deal with tumors unexpectedly formed in patients treated with iPSC-based therapies.

## Materials and Methods

**Cell culture, induction of reprogramming, and proliferation assay.** Common marmoset ARCs, CM ESCs, and iPS A cells derived from fetal liver cells (provided by Erika Sasaki, KEIO-REKEN Research Center for Human Cognition, Keio University, Tokyo, Japan) were maintained in DMEM/F12 (Sigma-Aldrich, St. Louis, MO, USA) containing 20% Knockout Serum Replacement (Gibco, Carlsbad, CA, USA), 0.1 mM non-essential amino acid (Gibco), 1 mM L-glutamine (Nacalai Tesque, Kyoto, Japan), 1% antibiotic–antimycotics (Nacalai Tesque), 0.4 mM 2-mercaptoethanol (Sigma Aldrich), and 0.12% sodium hydroxide (Nacalai Tesque). The CM DGs were maintained in DMEM/F12 containing 10% FBS at 37°C in a 5% humidified $CO_2$ atmosphere. Detailed descriptions of the cell culture, reprogramming method, and proliferation assay are provided in Figures 1 and 5.

**Plasmids and lentiviral vector production.** Human OCT3/4, SOX2, KLF4, or c-MYC was inserted into CSIV-CMV-MCS-IRES2-Venus lentiviral vectors (kindly provided by Hiroyuki Miyoshi, Riken, Tsukuba, Japan). Short hairpin RNAs targeting OCT3/4, SOX2, and c-MYC were obtained from Addgene (Cambridge, MA, USA), and shRNA targeting KLF4 was obtained from Applied Biological Materials (Richmond, BC, Canada). Lentiviruses were produced as previously described.[11]

**Microarray analysis.** Total RNA from AGM fibroblasts, ARCs, and iPS A cells were isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA was reverse-transcribed, biotin-labeled, and hybridized for 16 h to a marmoset genome oligonucleotide custom array Marmo2 (in preparation),[12] which was subsequently washed and stained in a Fluidics Station 450 (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. Detailed protocols of microarray analysis are provided in Figures 2 and S5.

**DNA-damaging treatments.** The CM DGs were treated with 1 μg/mL MMC (Kyowa Hakko Kirin, Tokyo, Japan) or 10 μg /mL cisplatin (Sigma-Aldrich) for 1 h at 37°C. For irradiation, CM DGs were irradiated (20 Gy) using Gammacell 40 (Atomic Energy, Chalk River, Ontario, Canada). At 24 h after treatment, the cells were stained with propidium iodide (Nacalai Tesque), and the proportion of dead cells was analyzed as the sub-$G_1$ population by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA).

**Statistical analysis.** Statistical analyses were carried out with the GraphPad Prism 5.0d software package (GraphPad Software, La Jolla, CA, USA). Statistical analyses were carried out using a two-tailed unpaired Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant.

Additional information is provided in Supporting information.

## Results

**Characteristic of aorta-gonado-mesonephros fibroblast-derived colonies formed by transduction of reprogramming factors.** To generate CM-derived iPSCs, reprogramming factors (OSKM) were transduced into AGM fibroblasts using lentiviral vectors (Fig. 1a). Then OSKM-transduced cells were transferred to mouse embryonic fibroblast feeder cells on day 7 post-infection, and cultured in medium for CM ESCs. We found that the cells formed sphere-like structures on day 17 post-infection (Fig. 1b). Moreover, these colonies showed AP activity

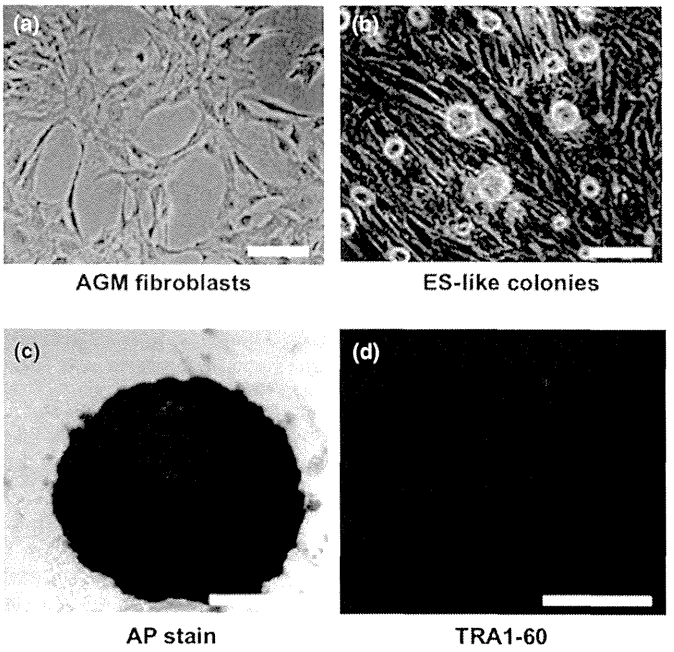


**Fig. 1.** Characterization of aorta-gonado-mesonephros (AGM) fibroblast-derived colonies formed by transduction of reprogramming factors. Representative phase-contrast images of (a) AGM fibroblasts and (b) abnormally reprogrammed cells (ARCs) forming round-shaped colonies. (c) Representative image showing expression of alkaline phosphatase (AP) activity in ARCs. (d) Immunocytochemical staining showing expression of TRA1-60 in ARCs. Bar = 100 μm.

(Fig. 1c), and expressed ESC markers such as TRA1-60, SALL1, LIN28, and DPPA4 (Figs 1d, S1). These results suggested that the reprogrammed AGM fibroblasts formed immature, round iPSC-like colonies.

**Chromosome abnormality and tumor-forming ability in abnormally reprogrammed cells.** Given that *KLF4* and *c-MYC* are well-known oncogenes,[13,14] transduction with OSKM transcription factors may cause cell transformation and chromosome instability.[15] We carried out karyotype analysis of the colony-forming cells to determine if OSKM-transduced AGM fibroblasts exhibited chromosome instability. The normal karyotype of CM cells is 44 autosomes and two sex chromosomes (46, XX or 46, XY).[16] However, the round colony-forming cells contained 44 autosomes, one X chromosome, and an abnormal marker chromosome (mar), with deletions of chromosome 4q, and were therefore denoted as 46, X, del (4q), +mar (Fig. 2a, right panel). The karyotype of the parental AGM fibroblasts was 46, X, +mar (Fig. 2a, left panel). These results suggested that the reprogramming stress induced by OSKM might have caused the deletion of 4q, although the possibility that the stress of long-term *in vitro* culture might have resulted in chromosome instability could not be excluded. These colony-forming cells were named ARCs.

To examine the ability of ARCs to differentiate into three germ layers like ESCs, we carried out an *in vitro* differentiation assay based on the protocol for human ESC differentiation.[17,18] Unlike human ESCs, ARCs did not differentiate into neural progenitors, cardiomyocytes, or hepatic cells (Fig. S2, Video S1).

We carried out *in vivo* differentiation assays by injecting ARCs into the testes of SCID mice. Approximately 6 weeks after injection, 11 of 18 mice injected with ARCs showed

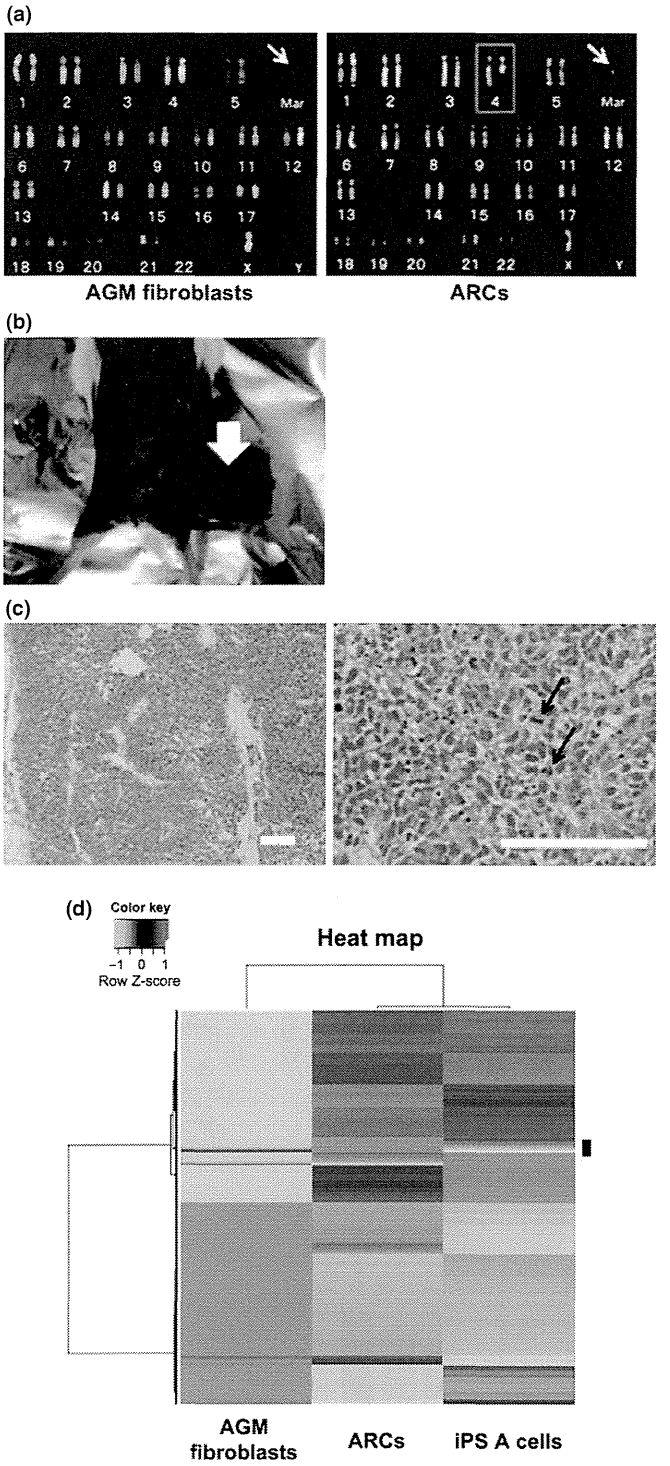


**Fig. 2.** Chromosome abnormality and tumor-forming ability in abnormally reprogrammed cells (ARCs). (a) Karyotype analyses of aorta-gonado-mesonephros (AGM) fibroblasts (left panel) and ARCs (right panel). Arrows indicate marker chromosome. Blue outline indicates the deletion of 4q. Mar, marker chromosome. (b) Representative photograph of dysgerminoma-like tumor (arrow) formed by transplantation of ARCs into SCID mice. (c) Hematoxylin-eosin staining of dysgerminoma-like tumor tissues. Arrows in right panel indicate mitotic figures in tumor cells. Bar = 100 μm. (d) Microarray analysis. Gene expressions in AGM fibroblasts, ARCs, and normal induced pluripotent stem (iPS) A cells were analyzed by unsupervised hierarchical clustering. A heat map using probes showing differential expression levels in each cell line is shown. Red indicates upregulation; green indicates downregulation. The black bar on the right side of the heat map shows candidate differentially expressed probes in ARCs.

tumor formation (Fig. 2b), whereas no mice injected with AGM fibroblasts showed tumor formation (0/3, data not shown). Staining with H&E revealed that the tumors were relatively homogenous, with high cellular density, necrosis, and pleomorphism, indicating their malignant phenotype (Fig. 2c). In addition, tumors were composed of nests and sheets of uniform round or polygonal cells with abundant, clear to faintly eosinophilic cytoplasm with well-demarcated cytoplasmic borders and a delicate network of thin-walled blood vessels in the tumor nests (Fig. 2c, right panel). Furthermore, immunohistochemical analyses revealed that the tumor cells were focally and weakly immunopositive for vimentin, and immunonegative for the differentiation markers cytokeratin, S100, desmin, α-smooth muscle actin, and neuron-specific enolase (data not shown). Tumor tissues also expressed c-KIT, but not CD30 or CD45 (Fig. S3). These molecular expression profiles implied that the tumor was equivalent to human malignant dysgerminoma, rather than other types of immature tumors such as embryonal carcinoma, yolk sac tumor, or teratoma.[19,20] The tumor was named CM DG.

We next carried out soft agar assays to determine if ARCs were transformed and showed anchorage-independent growth as a result of ectopic expression of reprogramming factors. The ARCs were cultured in 0.5% agarose-containing medium for 20 days, and the number of colonies was counted. The ARCs formed many colonies, compared with parental AGM fibroblasts (Fig. S4a and data not shown). These results strongly suggested that ARCs were transformed during reprogramming, and acquired the capacity for anchorage-independent growth. To clarify the contribution of reprogramming factors that could transform AGM fibroblasts, we transduced various combinations of these factors into AGM fibroblasts, and examined if the transduced cells were transformed by the colony formation assay on mouse embryonic fibroblasts, AP staining assay, and soft agar assay. The iPSC-like colonies were found in OSKM- and OSM-transduced cells (OSKM, 30 ± 3/5000; OSM, 6 ± 0/5000), but they were not found at all when OSK, OS, OM, SM, O, S, K, or M were transduced (Fig. S4b). AP activity was found in both OSKM- and OSM-transduced cells, although OSM-transduced cells showed weaker AP activity than OSKM-transduced cells (Fig. S4c). In soft agar assay, the anchorage-independent growth was found in both OSKM- and OSM-transduced cells (Fig. S4d; OSKM, 160 ± 23/1000; OSM, 163 ± 10/1000). These results indicated that the simultaneous expression of OCT3/4, SOX2, and c-MYC was at least required for the transformation of AGM fibroblasts, while KLF4 did not play a major role in the transformation of AGM fibroblasts.

Tomioka *et al.* reported the establishment of CM iPSCs (iPS A cells) showing normal karyotype.[12] To characterize the gene expression in ARCs, we carried out microarray analyses using mRNA from ARCs, iPS A cells, and AGM fibroblasts. According to the clustering pattern and the heat map, 171 probes that showed higher expression levels in ARCs as compared to other cells were selected as candidate differentially expressed genes in ARCs (Fig. 2d). Moreover, we focused on the genes specifically highly expressed in ARCs compared to those in iPS A cells, and the top seven genes highly expressed in ARCs were selected (*ZFHX4*, *PCDH19*, *NFIX*, *HOXC8*, *STMN2*, *SERPINA3*, and *CXORF67*). Then we validated these data by semiquantitative RT-PCR analyses, and five genes (*ZFHX4*, *NFIX*, *HOXC8*, *STMN2*, and *CXORF67*) were confirmed to be more expressed in ARCs than those in controls (Fig. S5). The high expression of these five genes might be characteristics of ARCs.

**Characteristic of CM DGs.** We then surgically removed CM DGs and cultured them *in vitro* to examine their biological characteristics. The CM DGs could grow infinitely in a semi-floating state in the culture dish, and showed continuous expression of Venus fluorescent protein (Fig. 3a,b). We generated five CM DG cell lines (CMY401, CMY402a, CMY402b, CMY403a, and CMY403b) from five independent tumors formed by the injection of ARCs into SCID mice, and found that all four transduced reprogramming factors were integrated into their genomes (Fig. S6a). Both endogenous and exogenous reprogramming factors were expressed in these cell lines (Fig. 3c,d).

**Effects of DNA-damaging agents and irradiation on CM DGs.** Dysgerminomas are generally sensitive to cisplatin and irradiation.[21,22] We therefore examined the effects of DNA-damaging agents such as MMC and cisplatin on CM DGs. Three CM DG cell lines (CMY402a, CMY402b, and CMY403a) were treated with MMC for 1 h, and the proportion of dead cells was analyzed by flow cytometry at 24 h after treatment. The percentage of cells with a sub-$G_1$ DNA content was taken as a measure of dead cells in the population. The proportion of dead cells in MMC-treated CM DG cultures was significantly higher than that in controls (MMC-treated AGM fibroblasts) (Figs 4a, S7). Similar results were obtained when these three cell lines were treated with cisplatin or irradiation (Figs 4b,c, S8, S9). These results suggested that CM DGs were

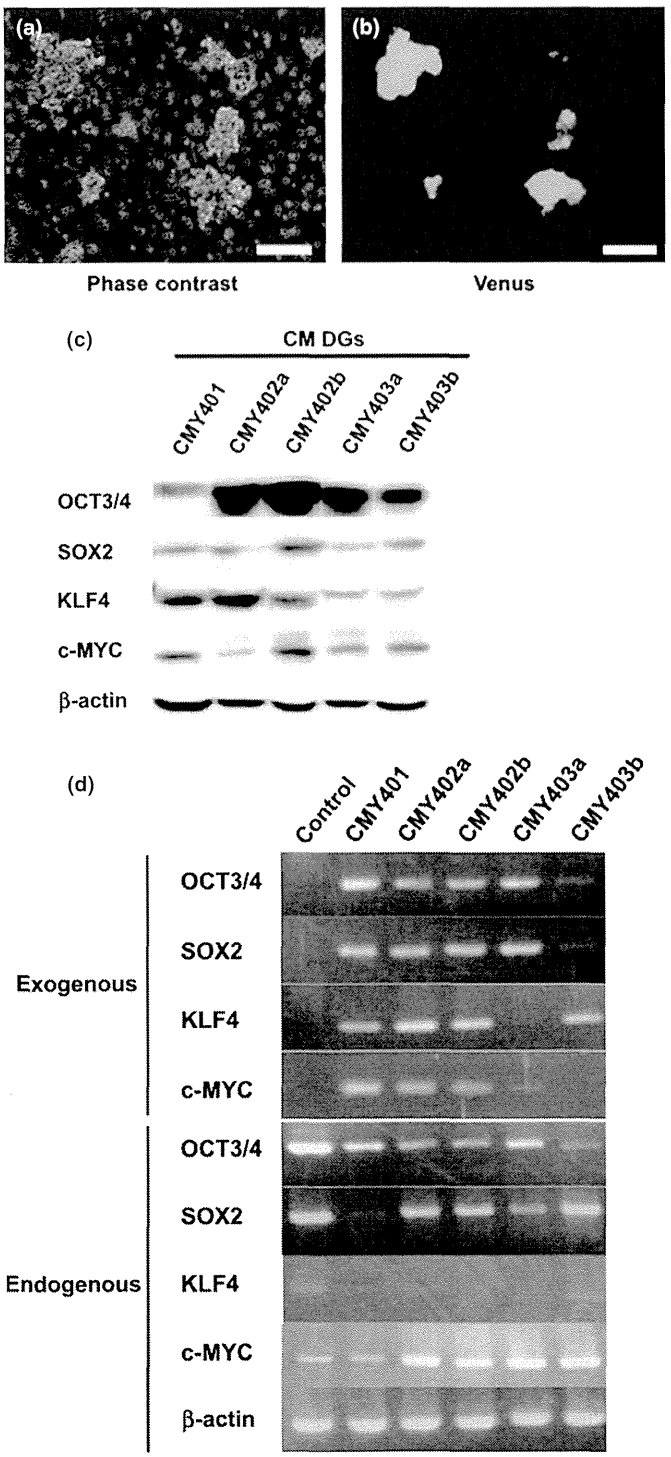


**Fig. 3.** Characterization of common marmoset dysgerminoma-like (CM DG) cells in culture. (a) Representative phase-contrast image of CM DGs. (b) Immunofluorescent image of Venus expression in CM DGs. Bar = 100 μm. (c) Western blot analysis showing expression of reprogramming factors in CM DG cell lines. (d) RT-PCR analysis showing the expression of endogenous or exogenous reprogramming factors in CM DGs. Cj11 (CM embryonic stem cell line) was used as control.

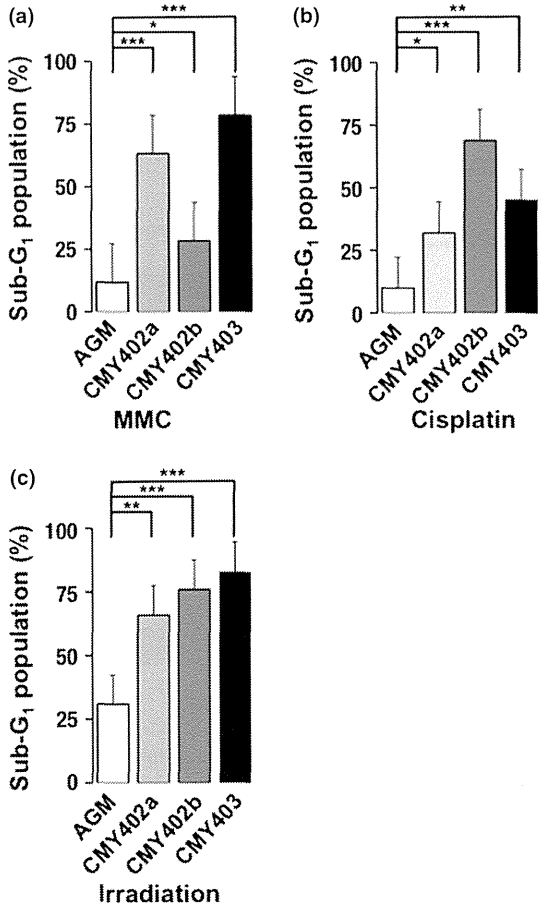


**Fig. 4.** Effects of DNA-damaging agents and irradiation on common marmoset dysgerminoma-like cells (CM DGs). The cells were treated with (a) mitomycin C (MMC), (b) cisplatin, or (c) irradiation, and the proportions of sub-$G_1$ populations in aorta-gonado-mesonephros (AGM) fibroblasts or CM DG cell lines were analyzed by FACS. Results are shown as means ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

more sensitive to DNA damage than their parental AGM fibroblasts.

We carried out inverse PCR analyses to identify the integration sites of the lentiviral vectors expressing reprogramming factors in CM DGs. OCT 3/4-, SOX2-, KLF4-, and c-MYC-expressing lentiviral vectors were integrated into 5, 12, 5, and 9 genomic sites, respectively (Fig. S6b, Table S1). The possibility that multiple integrations of lentiviral vectors into the genome caused chromosome instability, leading to the formation of CM DGs, could therefore not be excluded.

**Dependence of CM DGs growth on c-MYC and bFGF signalings.** To address the question of whether proliferation of CM DGs was dependent on reprogramming factors, we observed the proliferation rate after suppression of each reprogramming factor by shRNA (Fig. S10). Suppression of c-MYC or all four reprogramming factors greatly inhibited the proliferation of CM DGs, indicating that the growth of CM DGs was highly dependent on c-MYC (Fig. 5a).

The proliferation of human ESCs is known to be promoted by bFGF signaling.[23] We examined the possibility that the growth of CM DGs might also be enhanced by bFGF signaling by analyzing the proliferation of CM DGs cultured in medium with or without bFGF. The growth of CM DGs was highly dependent on bFGF (Fig. 5b). Consistent with these results, BGJ398, an inhibitor for FGFR 1 to 4, remarkably inhibited the growth of CM DGs in a dose-dependent manner (Fig. 5c). Moreover, FACS analyses revealed that the sub-$G_1$ population, representing dead cells, was increased in the presence of BGJ398 (Fig. S11). It should be emphasized that the $IC_{50}$ of BGJ398 (59 nM) was lower for CM DGs than for their parental AGM fibroblasts and control CM skin fibroblasts (Fig. 5d), indicating higher sensitivity of CM DGs. These results suggested that the growth of CM DGs was dependent on bFGF signaling, and therefore FGFR inhibitor could be used to control the growth of the reprogramming factor-induced tumor.

## Discussion

In this study we investigated the characteristics of ARCs and CM DGs generated in the reprogramming process of CM AGM fibroblasts by Yamanaka factors.

A normal iPSC line of iPS A cells, showed the expression of ES markers, pluripotency, and flattened morphology, like human iPSCs.[20] In contrast, ARCs showed sphere-like structures, like mouse iPSCs.[1] This morphological difference between iPS A cells and ARCs might be useful to select "true" iPSCs derived from CM, although the underlying molecular mechanisms responsible for this morphological difference remain unknown.

We found, by microarray analyses, that the gene expression pattern in ARCs was more similar to that in iPS A cells than that in AGM fibroblasts, suggesting that reprogramming processes have been done in ARCs by the transduction of reprogramming factors. We also found that genes such as *ZFHX4*, *NFIX*, *HOXC8*, *STMN2*, and *CXORF67* were highly expressed in ARCs. It should be noted that, among these, *HOXC8* is known to be a transcriptional factor related to tumorigenesis.[24] Therefore, these candidates of markers might be useful to predict the tumorigenic potential of iPSCs. Further evaluation is required to confirm our hypothesis.

The original AGM fibroblasts had an abnormal marker chromosome (mar; Fig. 2a, left panel). Although tumor formation was not evident caused in SCID mice (data not shown), this chromosome instability might also be one of the inducers of

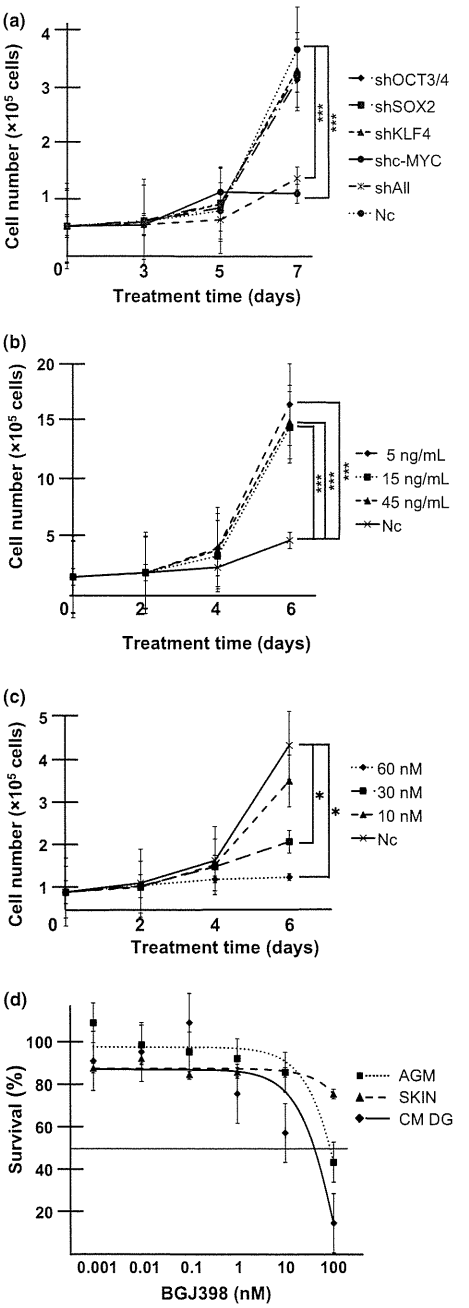


**Fig. 5.** Dependence of common marmoset dysgerminoma-like (CM DG) cell growth on c-MYC and basic fibroblast growth factor (bFGF) signaling. (a) Inhibition of CM DG growth by knockdown of c-MYC. Cells ($3 \times 10^4$) were seeded on 24-well plates and transduced with shRNA targeting OCT3/4, SOX2, KLF4, c-MYC, or all reprogramming factors (shAll). Cell growth curves were analyzed by cell counts at the indicated time points. Results are shown as means ± SD. ***$P < 0.001$. Nc, negative control (mock vector). (b) Growth rate of CM DGs was promoted by the addition of bFGF. Cells were cultured in the presence or absence (Nc) of bFGF. Cell numbers were counted at the indicated time points. Results are shown as means ± SD. ***$P < 0.001$. (c) FGFR inhibitor suppressed CM DG growth. Cells were cultured in the presence or absence (Nc) of the FGFR1-4 inhibitor BGJ398; bFGF was added at 5 ng/mL. Cell numbers were counted at the indicated time points. Results are shown as means ± SD. *$P < 0.05$. (d) CM DGs, aorta-gonado-mesonephros fibroblasts (AGM), and CM skin fibroblasts (SKIN) were treated with different concentrations of BGJ398 for 3 days, and the growth-inhibitory effects were analyzed by MTS assay. The $IC_{50}$ for CM DGs was lower than those for parental AGM fibroblasts and control CM skin fibroblasts. Results are shown as means ± SD.

carcinogenesis during the reprogramming process. Thus, needless to say, to generate "safe" iPSCs, validation of the karyotype of the original cells is needed. Moreover, ARCs lost chromosome 4q and X or Y, and possessed an abnormal marker chromosome (mar). Various tumor suppressors including human tumor suppressor gene 1, large tumor suppressor 1 and P36 transformed follicular lymphoma gene have been identified on chromosome 4q in CM cells (Table S2), suggesting that loss of these tumor suppressors might have induced the transformation of CM AGM fibroblasts during reprogramming, although the possibility that translocation of chromosome 4q occurred during the reprogramming process caused the transformation of cells could not be excluded.

It is also possible that the continuous activation of ectopically-transduced transcription factors, including the oncogene *c-MYC*, might have contributed to cell transformation, as described previously.[25,26] Indeed, CM DGs overexpressed c-MYC, and their growth was highly dependent on c-MYC expression, suggesting that downregulation of c-MYC might represent a possible strategy for inhibiting the growth of reprogramming factor-related tumors.

Insertional mutation caused by the integration of lentiviral vectors into the genome might also have promoted cell transformation. Lentiviral vectors expressing reprogramming factors were integrated into at least 31 different genomic sites in CM DGs, some of which were in the vicinity of protein-encoding genes. Moreover, the expression of reprogramming factors transduced by lentiviral vectors continued for over a year in ARCs (data not shown). A safer method, without genome integration, is therefore required for the delivery of reprogramming factors to somatic cells to generate iPSCs applicable for transplantation therapies. Although Sendai virus vectors or transfection of DNA or mRNA may be safer methods,[27] these are lengthy processes that can take more than 1 month to obtain iPSCs,[28] which could also cause stress and lead to genomic instability and subsequent tumor formation. More sophisticated, safer, and more rapid methods of reprogramming might be desirable.

Common marmoset DGs resembled human dysgerminomas in terms of both their pathology and sensitivity to irradiation and DNA-damaging agents.[21,22] In addition, the growth of CM DGs was significantly inhibited by an FGFR1-4 inhibitor. Therefore irradiation, chemotherapy, and FGFR1-4 inhibitors might be effective strategies for controlling human dysgerminomas, and also for tumors that develop in patients treated with iPSC-based therapies.

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## Disclosure Statement

The authors have no conflicts of interest.

## Abbreviations

| | |
|---|---|
| AGM | aorta-gonado-mesonephros |
| AP | alkaline phosphatase |
| ARC | abnormally reprogrammed cell |
| bFGF | basic fibroblast growth factor |
| CM | common marmoset |
| DG | human dysgerminoma-like cell |
| ESC | embryonic stem cell |
| FGFR | fibroblast growth factor receptor |
| iPSC | induced pluripotent stem cell |
| K | KLF4 |
| M | c-MYC |
| MMC | mitomycin C |
| O | OCT3/4 |
| S | SOX2 |

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