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Perspective

Molecular mechanisms underlying cell-in-cell formation: core machineries and beyond

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Although cell-in-cell structures (CICs), with one or more cells present inside another cell, had been identified for a century, it was not until recent years that scientists started to uncover their pivotal roles in multiple biological processes, primarily via mediating the death of internalized cells. Meanwhile, considerable progresses were made on deciphering the mechanisms underlying their formation based on different models. Entosis was one of the best investigated CIC models, where cell internalization was coordinately driven by adherens junction and contractile actomyosin, the two spatially polarized and complementary core elements that were coupled by mechanical ring, a recently identified core element. Meanwhile, an expanding group of factors were found capable of regulating CIC formation by targeting these core elements. The elucidation of the molecular machinery controlling CIC formation enables synthetic engineering of cells used for clinical and research purposes. With the growing academic and translational interests in CICs, this perspective essay attempts to make an in-time updating of the latest progress on the molecular controls of CIC formation in the hope of moving the field forward.

CICs are important biological players

Despite sporadic reports in lower organisms and inflammatory tissues, tumors are the most representative tissues where CICs were documented. Almost every type of tumor tissue examined had been reported to have CICs (Huang et al., 2015b; Fais and Overholtzer, 2018). Recent advances in the technique of multiplex staining enabled the identification of a set of CIC subtypes that could be roughly divided into two major classes, i.e. the homotypic CICs (hoCICs), referring to structures formed among cells of the same types such as tumor cells, and the heterotypic CICs (heCICs), with cells internalized by another type of cells, such as leukocytes inside tumor cell (Huang et al., 2015a). Remarkably, CICs and their subtypes were found to be valuable as prognostic markers for early breast carcinoma (Zhang et al., 2019), pancreatic ductal adenocarcinoma (Huang et al., 2020), and head and neck squamous carcinoma (Fan et al., 2020). In addition to tumors, CICs were also present in other tissues under certain circumstances. For example, activated T cells could penetrate into hepatocytes to form heCICs (Benseler et al., 2011) and trophoblasts could internalize uterine luminal epithelial cells to facilitate implantation (Li et al., 2015). Actually, CICs are important players in a number of biological processes, including tumor evolution (Sun et al., 2014b), genome instability (Mackay et al., 2018; Liang et al., 2021; Rizzotto and Villunger, 2021), immune homeostasis (Benseler et al., 2011), virus transmission (Ni et al., 2015a, b), and embryonic development (Li et al., 2015).

CICs mediate inner cell death

Multiple mechanisms have been reported that account for CIC formation, such as entosis, emperitosis, and cannibalism (Fais and Overholtzer, 2018). Entosis was probably the best studied program that boosted the academic interest in CICs by the concept that CICs mediate a novel type of programmed cell death, which was clearly demonstrated by time-lapsed microscopy years ago. In brief, the internalized cells initially stayed alive and eventually ended up with three distinct outcomes: (i) whereas a majority of the inner cells (~50%–75%) underwent death evidenced by stop-moving, nuclear degradation, and the eventual disappearance within the enveloping cell, (ii) a portion of inner cells (10%–25%) stayed alive as evidenced by wandering around or occasionally dividing within the huge engulfing vacuoles, and (iii) some internalized cells (10%–25%) could even escape, seemly with no signs of damage, from the engulfing host. Interestingly, the death of the inner cells by entosis were largely not apoptotic

manifested by DNA break (TUNEL-positive) in the absence of the cleavage of caspase-3, a marker of apoptotic cell death (Overholtzer et al., 2007; Sun et al., 2014a). Moreover, during the death process, the microtubule-associated protein 1 light chain 3 alpha (LC3) was transiently recruited to the entotic vacuole before the inner cell death, which turned out to be dependent on the upstream activation of partial autophagic signaling involving in Atg5, Atg7, Vps34, and the vacuolar H^+/ATPase. Following LC3 recruitment, the entotic vacuoles matured into functional lysosomes as evidenced by the recruitment of lysosome-associated membrane glycoproteins (LAMPs), cathepsin import, and vacuolar acidification (Florey et al., 2011; Su et al., 2021). As such, the entosis was regarded as a novel mechanism of noncanonical cell death that was executed nonautonomously by the outer cells. It was even proposed as the type IV cell death apart from apoptosis (type I), necrotic cell death such as necroptosis, pyroptosis, and ferroptosis (type II), and autophagy (type III) (Martins et al., 2017; Galluzzi et al., 2018).

Stepwise formation of entotic CICs

As part of the death process, the formation of entotic CICs was investigated extensively and could be empirically divided into three major steps including triggering, internalization, and closing. Although little insights were provided for the closing step, substantial progress was made in the triggering and internalization steps. It is now well accepted that entotic CIC formation could be initiated by three triggers, including matrix detachment, aberrant mitosis, and glucose starvation. Matrix detachment, simply by suspension culture, is more likely an artificial method to initiate CIC formation in a saturated manner and applicable to cells of different states. The underlying mechanism may involve the loss of counterbalance to cell–cell adhesion and downregulation of FAK signaling (Overholtzer et al., 2007; Ishikawa et al., 2015). In relatively lower frequency, the aberrant mitosis-induced CICs may occur in a more physiological context. Mitotic rounding (Durgan et al., 2017) and prolonged metaphase-induced activation of p53 signaling were proposed as the key factors that drove the internalization of aneuploid mitotic progenies (Liang et al., 2021). Glucose starvation induced entotic CIC formation under a harsh condition in a relative high frequency, particularly for tumor cells, which was involved in the activation of AMPK, a metabolic sensor of glucose availability (Hamann et al., 2017). Following the priming by the triggering factors, the process of CIC formation was then taken over by a set of molecular machineries that could be roughly classified as three core elements and a group of regulatory factors as described below.

Contractile actomyosin

A unique property for CIC formation by entosis is that the internalization process is believed to be driven by the cells that are being internalized (Overholtzer et al., 2007). Therefore, this active invasion process is different from the well-known phagocytosis, where the dead cell corpses, by exposing phosphoserine on cell surface, are passively engulfed by the outer phagocytes. At the molecular level, the active CIC invasion is dependent on the contractile actomyosin, one of the core elements that drive CIC formation, which is controlled by upstream RhoA–ROCKs signaling that regulates the phosphorylation of myosin light chain 2 (MLC2) (Overholtzer et al., 2007), and local activation of diaphanous-related formin 1 (mDia1) (Purvanov et al., 2014). Interestingly, subsequent studies indicated that the relative stiffness between internalized cells and the outer host cell determined whether and how CIC formation took place. Only when the intercellular differences in stiffness were strong enough to surpass the energy barrier could the CIC formation proceed, and the cells with higher RhoA activity and, therefore, stiffer would invade into their neighboring cells. Otherwise, the cell internalization process would either halt or occur in an opposite way (Sun et al., 2014b; Xia et al., 2014; Ning et al., 2015). This result actually suggested that both the inner and outer host cells would actively participate in the process of CIC formation, which was also supported by evidence from heCIC study (He et al., 2015). Remarkably, the contractile actomyosin that controls cell stiffness was asymmetrically localized at the rear cortical region away from the cell–cell contact (Figure 1), contraction of which generated inward forces that conceivably drove the internalization of cells with higher RhoA activity (Sun et al., 2014b).

Adherens junction

Similar to contractile actomyosin, adherens junction is another core element that displays asymmetric distribution within cells engaged in CIC formation. Moreover, adherens junction seems to be the switch of entotic CIC formation, because tumor cells that lack expression of adherens junction components, such as E-cadherin, P-cadherin, or α-catenin, failed to form CICs. Restoring the expression of these molecules in the corresponding cells, remarkably, could efficiently induce the formation of entotic CICs (Sun et al., 2014a; Wang et al., 2015). Intriguingly, increased cell–cell adhesion seemed to be only one of the contributors of CIC formation by restoring adherens junction, because some cells that could efficiently form cluster, indicating cell–cell adhesion, were incompetent of giving high frequency of CICs (Sun et al., 2014a). Under such circumstance, actomyosin tended to display an unpolarized distribution across the cell cortex, which resembled the pattern of actomyosin in cells with disrupted adherens junction, while adherens junction could still effectively form between adjacent cells that were treated with ROCK inhibitors such as Y27632, which suppressed actomyosin contraction (Overholtzer et al., 2007; Sun et al., 2014a). Further investigation indicated that adherens junction displayed a subcellular localization perfectly complementary to that of actomyosin (Figure 1), which actually

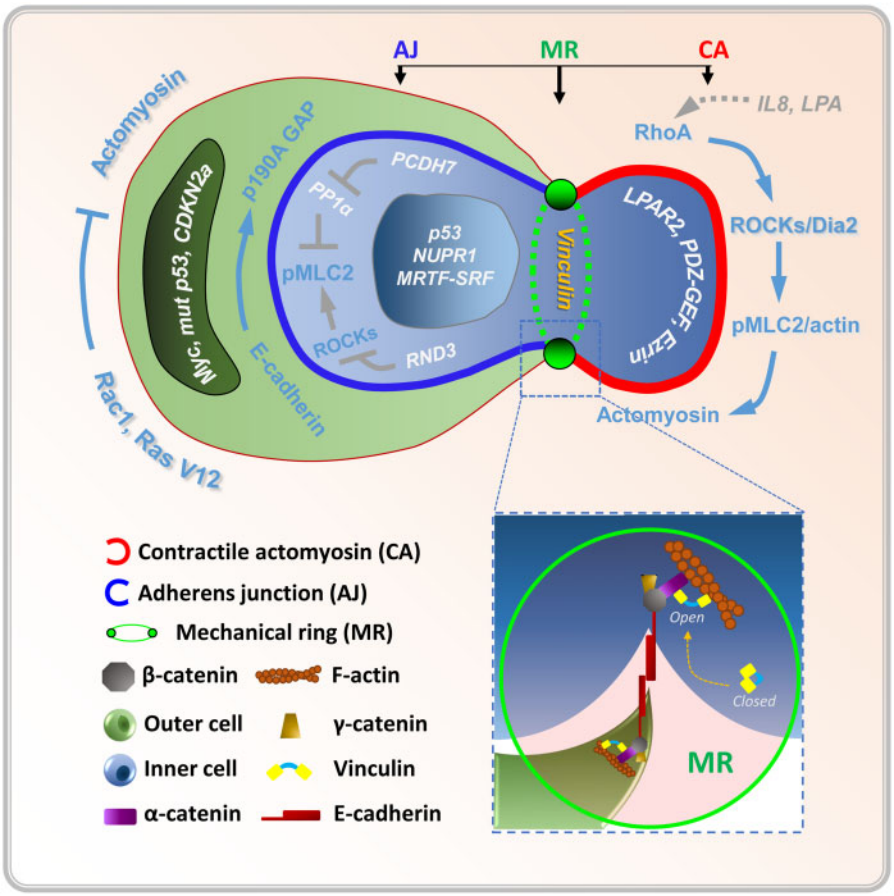

Figure 1 Molecular machineries regulating the formation of entotic cell-in-cell structures. The entotic cell-in-cell formation is regulated by three core elements (including contractile actomyosin, adherens junction, and mechanical ring) and a group of regulatory factors.

facilitated the establishment of a polarized distribution of actomyosin at the rear periphery by recruiting p190A RhoGAP. The p190A RhoGAP is a potent inhibitor of RhoA activity by converting RhoA-GTP to RhoA-GDP. Depletion of p190A RhoGAP can disrupt the asymmetric distribution of actomyosin leading to failed cell internalization (Sun et al., 2014a; Huang et al., 2015b). Therefore, a functional adherens junction, coupled to active contraction of polarized actomyosin, is critical for successful cell internalization to form CICs. This brought about a question on how adherens junction and contractile actomyosin, the two spatially compartmentalized and complementary core elements, are coupled.

Mechanical ring

Our recent work identified the third core element that interfaced between the adherens junction and contractile actomyosin, where the element formed a ring-like structure in three-dimension serving like an entry for the internalizing cells (Figure 1). The ring-like structure was clearly a multimolecular complex, as evidenced by transmission electron microcopy imaging and colocalization analysis by structured illumination microscopy, that contained a group of adhesion and cytoskeleton molecules. These molecules included E-cadherin, α-catenin, γ-catenin, Ezrin, and F-actin, but seemed to avoid MLC2, the critical component of actomyosin (Wang et al., 2020b). Interestingly, vinculin, an adhesion protein that could sense and transmit force (Dumbauld et al., 2013), was highly enriched in the ring-like structure, where vinculin turned out to be in an open form that was functional in coordinating force transmission between cells by FRET analysis. Therefore, the vinculin-enriched structure was named as mechanical ring, which was supported by the fact that the FRET values changed upon mechanical manipulation by the treatments of compounds that either enhanced (LPA) or inhibited (Y27632) actomyosin contraction. Importantly, vinculin depletion dramatically suppressed the mechanical ring formation and CIC formation, suggesting an essential role for mechanical ring in CIC formation, in which the C-terminal tail domain of vinculin, responsible for binding to F-actin, seemed to be the critical mediator, as ectopic expression of the truncated tail domain also significantly inhibited CIC formation. Moreover, vinculin depletion resulted in disrupted polarization of actomyosin that penetrated into the cell–cell contact, suggesting a key role for mechanical ring in coordinating adherens junction and contractile actomyosin to promote CIC formation. In agreement, both disrupting cell–cell adhesion by EGTA, a calcium chelator, and inhibiting actomyosin contraction by Y27632, a ROCK inhibitor, compromised the mechanical ring evidenced by the disappearance of the vinculin ring leading to aborted CIC formation (Wang et al., 2020b).

Regulatory factors

In addition to the above core elements, an ever-growing number of factors were identified as regulators of entotic CIC formation that generally targeted at least one of the above core elements. This perspective essay put forth a summarized framework with several representative factors, whereas for a more comprehensive list, the readers are recommended to read the other excellent reviews (Fais and Overholtzer, 2018; Mackay and Muller, 2019). Based on the subcellular localizations, the regulator factors could be roughly divided into three subcategories, including extracellular, membrane, and intracellular factors.

Extracellular factors are attractive candidates for therapeutic purpose as they could be directly applied to and taken

over by cells. By comparative expression profile analysis, IL8, an inflammatory cytokine that recruits leukocytes, such as neutrophil, was identified as a positive regulator of entotic CIC formation, which was believed to be a direct effect attributed to increased cell–cell adhesion via upregulating the expression of P-cadherin and γ-catenin (Ruan et al., 2018a). Serum was proposed to contain factors regulating cannibalistic CIC formation previously (Brouwer et al., 1984), which was supported by a recent study showing that LPA, a serum component, could effectively promote entotic CIC formation. LPA, primarily via the signaling axis of LPAR2–G12/13–PDZ RhoGEF, regulated the dynamics of polarized actomyosin, in which the formin mDia1 was involved (Purvanov et al., 2014). Meanwhile, LPA treatment also promoted the formation of the mechanical ring in terms of both integrity and strength, which was likely correlated with the speed of CIC formation (Wang et al., 2020b).

The membrane is the place where intercellular adhesion and mechanical transmission occur that mediates CIC formation. Different types of molecules were identified in recent years, including membrane lipids and proteins. Cholesterol is a structural component of the cell membrane, which was recently found to be a negative regulator of entotic CIC formation. The effect was correlated with a significant suppression of actomyosin contraction indicated by decreased phospho-MLC (pMLC) (Ruan et al., 2018b). PCDH7 is a transmembrane protein belonging to the cadherin superfamily that also contains E-cadherin and P-cadherin; however, in contrast to E-cadherin and P-cadherin, PCDH7 suppressed entotic CIC formation associated with compromised cell–cell adhesion, which was actually attributed to local activation of actomyosin contraction, via inactivating PP1α, at cell–cell contact region that counteracts cell internalization (Wang et al., 2020a).

A number of intracellular molecules had been reported to be capable of regulating entotic CIC formation. Apart from the cytoskeletal molecules that are conceivable CIC executors, such as vinculin as part of the mechanical ring (Wang et al., 2020b) and microtubule (Xia et al., 2014), some oncogenic proteins were found important regulator. Small GTPase family members, including KRas, Rac1, CDC42, and RND3, could more or less directly regulate RhoA–ROCK signaling to control either the identity or frequency of internalization (Sun et al., 2014b; Durgan et al., 2017; Liang et al., 2021); cell cycle regulator primarily regulated CIC frequency, and the internalization as well, by targeting actomyosin contraction (CDNK2A, CDC20, CUEDC2), microtubule cytoskeleton (Aurora A, TIP150-MACK), or adherens junction (CDKN2A) (Xia et al., 2014; Liang et al., 2018, 2021). Nuclear or nuclear-shuffling proteins, such as p53, Myc, NUPR1, and MRTF-SRF, primarily through controlling the expression of downstream effectors then the core elements, participated into the regulation of entotic CIC formation (Mackay et al., 2018; Mackay and Muller, 2019; Liang et al., 2021). Additionally, aberrant expression of polarity proteins, including PAR3, Lgl1/2, and CDC42, functioned as an efficient inducer of entosis by altering the balance of RhoA–ROCK signaling between cells (Wan et al., 2012; Durgan et al., 2017).

Conclusion and remarks

Though considerable progress has been made over the past decade, the mechanistic studies on CIC formation and subsequent death are still in their infancy. This was largely attributed to technical challenges including the lack of a unique biochemical marker to specifically read out CICs-mediated death and the limited ways of CIC characterization (primarily by microscopic imaging at present). Hence, a high-throughput screening, based upon biochemical assays, has not been available yet. Recent progress in image recognition by machine learning or artificial intelligence (AI) may shed light on this technical bottle neck. We recently made attempts to identify CICs by the algorithms of convolutional neural network and obtained ideal results in recognizing CICs on cytospin slides (specificity and sensitivity: >97%, respectively) (Tang et al., 2021). Thus, it is expected that a high-content screening, combined with AI recognition of CICs, for biochemical or genetical regulators of CIC formation, would be a feasible way to speed up the mechanistic studies of CIC-mediated death.

Meanwhile, several important issues should be taken into account for future investigations. First, unlike other death mechanisms that can be completed in just one cell, the CIC-mediated death is involved in at least two cells, which adds another layer of complexity to the molecular regulation. In fact, most of the above elements and factors could function in a competition-specific manner, i.e. preferentially impacting either the inner or outer cells. Second, most of the regulatory factors were found generally targeting the first two or both core elements. Few were reported for the third core element of the mechanical ring, because it was just proposed recently. It will be interesting to identify regulatory factors that function via the mechanical ring, either alone or synergistically with other core elements. Third, though CIC formation could be altered by targeting the above identified elements/molecules, up to date, a robust gain-of-function phenotype was only reported for the case of E-cadherin or P-cadherin expression in cancer cells null in the expression of the corresponding genes. Identifying more molecular switches and potent enhancers are helpful for directed engineering of cells for diagnostic and therapeutic purposes. Fourth, previous studies had shed light on the steps of triggering and internalization but little was reported on closing step, which is the final step that concludes the CIC formation process. One hypothesis is that the closing step might resemble that occurs in endocytosis, just in a much larger scale. Consistent with this idea, either treatment of dynamin inhibitor or depletion of clathrin light chain, both of which led to inhibited endocytosis, could compromise entotic CIC formation (unpublished data), although it is unclear whether this is a direct effect from failed closing or secondary

from inhibited endocytosis. Another hypothesis is that the closing step might be similar to the step of abscission taking place in cell division, where the complex of endosomal sorting complex required for transport, a multifunctional machine for protein trafficking and membrane scission, was involved (Gatta and Carlton, 2019). In agreement with this idea, a recent study demonstrated that a small tail was frequently cut off from the internalized cell during the final closing step of entotic CIC formation (Lee et al., 2019); however, the underlying molecular controls remain to be elucidated. Moreover, despite many mechanistic insights that had been obtained, genetic engineering of CICs for technical and therapeutic purposes was relatively rare. Two Japanese groups had made interesting exploration in utilizing CICs as vectors to transfer therapeutic reagents such as oncolytic viruses (Onishi et al., 2016; Kojima and Fussenegger, 2018), which provided a proof-of-principle example, though preliminary and to be optimized, for the potential applications of engineered CICs. It is conceivable that more promising strategies aiming for technical and therapeutic purposes would be available with efforts endeavored in the future.

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