Cell-in-Cell Phenomenon: A New Paradigm in Life Sciences

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Abstract: Cell-in-cell, a phenomenon characterized by one or more viable cells entering actively into another cell, was observed more than a century and has only attracted more attention in recent years and is becoming a new hot topic in the biological field, owing its biological significance in evolutionary as well as physiological and pathological relevance in development, homeostasis and diseases. In this paper we focus on the diversity, evolutionary conservatism and clinical implication of cell-in-cell as well as latest opinions on the research strategies. Based on the findings from our laboratory and other research groups three working models of cell-in-cell are also proposed.

Keywords: Cell-in-cell, cell-in-cell death, cell model, cell diversity, cell evolution.

INTRODUCTION

Thirty years ago, we observed that immune cells could enter tumor cells to form what we now know as cell-in-cell structure (CICs) [1]. Although the percentage of entry was very low (less than 5%), through electron microscope observation, we found out that over 70% of these immune cells underwent self-degradation inside the target tumor cells [1]. Back then, due to the Cultural Revolution, the access to scientific literature in China was very limited, nevertheless, we had already learnt that killer cells, such as cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells, could induce cytolysis of their target cells in a programmed way [2], which reminded us that the death of immune cell inside tumor cells might actually be a regulated biological process rather than an accidental event. This was verified after more than 30 years when Overholtzer and Brugge unveiled the long history of CICs in their thorough review [3]. In fact, due to the lack of proper research models and undefined biological significance, the phenomenon has long been ignored until 2000 when Brugge's group introduced a novel cell death pathway mediated by CICs and its relevance to cell transformation. Similar to other cell biological processes such as apoptosis, autophagy, or spontaneous cell fusion that were initially observed in the middle of the 20th century and drew greater attention later, studies on CICs have progressed greatly recently [4-14] and we owe it all to the biologists and clinicians and their dedication to reveal CICs' biological significance as well as physiological and pathological relevance. CICs thus is becoming a new hot topic in the biological field [3, 5-18].

CICs formation refers to the process of one or more cells actively entering another cell, forming a unique structure with distinct biological outcomes. CICs can be classified into homotypic and heterotypic, depending on the type of participating cells. The former occurs between autologous tumor cells, while the latter occurs between different cell lines, with immune cells entering tumor cells being the most common. These structures are not only observed in cell cultures, but also in tissues of low species such as *C. elegans* [19, 20] to mammals like humans, from physiological tissues, like the thymus [21-23] or liver [24-26] to pathologically inflammatory tissues [27-30]. Cells involved in CICs have various destinies. The entering cells (also referred to as the effector cells) can undergo mitosis inside the outer cells (also referred to as the target cells), or be released, or even fuse with the target cells. But most of internalized effector cells undergo cell death inside the target cell, termed cell-in-cell death [6-8]. Within the last decade, several forms of cell-in-cell death have been identified, with three of them representative, namely, cannibalism, entosis, and emperitosis [6, 7, 13]. Entosis has been even accepted by the Nomenclature Committee on Cell Death as a new form of cell death [31], which aroused great interests for scientists worldwide to work on this phenomenon. To further propel the researches on CICs, *Current Molecular Medicine* provides a valuable platform for distinguished scientists around the world to interpret its nature, scientific and medical values, and the perspective as well. In this review I intend to elucidate the latest opinions on CICs and the research strategies based on the findings from our laboratory and other research groups.

CELL-IN-CELL PHENOMENON IS DIVERSE AND EVOLUTIONARILY CONSERVED

Diverse Cell-in-Cell Formation in Higher Organisms

Since its first report a century ago, diverse forms of CICs have been described, including entosis, heterotypic cell cannibalism, emperitosis, homotypic cell cannibalism and phagocytosis and the like emperipolesis [4, 17, 13, 32, 33]. The pathway of the

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first two cell-in-cell deaths is lysosome-dependent cell death [6, 7], and the third one, which is elucidated and termed by us, is a typical caspase-dependent cell death [1, 8, 13]. Interestingly, we found that different immune cells may choose their ways to die inside tumor cells. For lymphocytes with killing activities including NK, and LAK and CIK, the cell-in-cell death is caspase-dependent and the process is very fast, which we coined as emperitosis (the combination of the word emperioplesis, meaning the entering of living lymphocytes into other cells, and the word apoptosis). For immune cells without killing activities like B cells, cell-in-cell formation resulted in a slow lysosome-dependent death, resembling what happens in entosis. Our further research revealed that the tumor target cells responded differently to different invaded immune cells, characterized by different rates of expansion of vacuoles wrapping the internalized immune cells. In case of internalized killer cells, the vacuoles of the target tumor cells expand more quickly than those invaded by non killer immune cells [13]. As a result, the killer immune cells were killed within the target tumor cells by re-uptaking granzyme B they released. These may represent a mechanism for tumor cells to go against immune attack, causing immune escape [16]. In contrast, by slowly killing the entrant nonlethal immune cells, the target cell may obtain benefits like "feed on" as found in cannibalism or entosis [6, 14].

The hypothesis that the process of CICs is an autonomous choice is also consistent with the latest finding from entosis [34, 35]. Sun *et al.* defined the entering cell as the "loser" and the host cell as the "winner", indicating different fates in cellular competition [34]. The CICs in multicellular organism is related to the cell's adaptivity to their new environments. In fact, be it homotypic or heterotypic, CICs reflects the competitive relationship between two cells. On the surface it is a life-and-death struggle, indeed it is a self-protective strategy of the cell to adapt to the new environment. The homotypic CICs helps tumor cells adapt to the rapid growth by maximizing and optimizing the resources in the niche [34]. The active selection role of cell-in-cell occurs not only at cellular level, but also at overall level. Benseler *et al.* had confirmed *in vivo* that CICs formation can be used as a tool for homeostasis maintenance [9]. They injected naïve auto-reactive T cells into B6 background mice that widely express H-2Kb, but the mice did not display any autoimmune pathological responses, such as hepatitis, enteritis, and inflammatory reaction of the skin, suggesting that there must be a mechanism de-activating the Des CD8+ T induced autoimmune pathological response. In order to explore the mechanism, they analyzed the dynamic distribution of cells injecting *in vivo*, and found that most of the Des CD8+ T cells were homed to lymph nodes 5 hours after the injection to B10.BR mice that did not express specific antigens; in those B6 mice that expressed specific antigens, Des CD8+ T cell were not homed to the lymph nodes, but remained in the liver and formed CICs with hepatocytes, and underwent self-degradation in an entosis-like way. It seems that Des CD8+ T cells entered the liver cells actively, rather than being engulfed by the liver cells. When impeding the entry with wortmannin to keep T cells in animals' peripheral blood, animals would suffer autoimmune diseases. Hence the authors named this process "suicidal emperipolesis", and believed they have discovered a new mechanism of T cell self-maintenance [9].

Cell-in-Cell Formation in *C. elegans*

The strides in the research of apoptosis and autophage-associated cell death in the last two to three decades owe not only to their participation in diseases, but also to their conservation in evolution. The CICs phenomenon's evolutionary track was also identified in lower organisms [19, 20]. One impressive research on the gonads of *C. elegans* came from Shaham's group [20]. As one of the most widely used models of research, *C. elegans* was extensively studied so that the generation and fate of each cell in development are well-pictured. In the development of the genital gland, the death of linker cells involves CICs formation. Linker cells are generated at 2nd larva stage (L2) in the middle of the body, then they migrate towards head and then go back and finally reach cloaca and die. Studies showed that, similar to entosis, the death of linker cells inside host cells is nonapoptotic. Linker cell must be first engulfed by the adjacent cells (U.lp or U.rp) with the same characteristics as normal cells, assuming it is the entrance of a live cell, making it a typical CICs [19]. Interestingly, Li *et al*. reported recently that trophoblast cells of blastocyst can "eat" the uterine luminal epithelium through an entotic process to achieve embryo implantation [36], which, together with the studies in *C. elegans*, indicates an important role of cell-in-cell in reproductive development.

Cell-in-Cell Formation in *Dictyostelium discoidum*

Looking at the early links along the biological evolution chain, Amoeba plays an important role as a unicellular organism. *Dictyostelium discoidum*, a branch of the amoeba family, has become an important model organism in basic cell biology and developmental biology, thanks to its unique biological features [37]. *Dictyostelium discoidum* has two types of reproduction: asexual and sexual. Interestingly, both involve CICs. During the suspension culture of amoeba cells, David *et al*. found that some cells failed to form tight cell aggregates, and could be easily separated. These cells were much larger than savage cells, contained numerous phagosomes, and some even contained intact amoeba cells. This phenomenon could be effectively induced by starvation. Interestingly, starved amoeba cells remained unicellular, thus failing to enter into the multicellular developmental stage [38]. This suggested that the phagocytic action might be providing cells with extra nutrition that prevented them from moving on to the next stage. Interestingly, phagocytosis seemed to be indispensable for *Dictyostelium discoidum*'s sexual reproduction. Its sexual reproduction begins with the fusion of two complementary haploidic gametes, resulting in a diploid

cell capable of phagocytizing adjacent cells. This cell is estimated to phagocytize hundreds of adjacent cells and eventually form a so-called marcrocyst. A marcrocyst is the product of sexual reproduction, which is large in size, has a cell wall with 3 layers. They also have the ability to digest adjacent amoeba cells. The innermost layer is composed of cellulose, and contains a macronucleus and several small nuclei. Such phagocytic phenomenon among amoebas is similar to cannibalism and entosis in mammal cells and may not be classified into phagocytosis [39].

Connecting dots of these findings, we may figure out a general picture of CICs in evolution and draw a conclusion that cell-in-cell formation may be a basic evolutionary model of cell interactions that can function as a tool in the development, homeostasis, and diseases of an organism. The conceptual "cell-in-cell evolutionary tool" helps scrutinize the evolution of life and individuals, and discover new mechanisms, new theories, and practical techniques.

CLINICAL IMPLICATION OF CELL-IN-CELL RESEARCHES

The earliest reports on CICs in the last century were based mainly on histopathological observations [7]. CICs may widely occur in tumors, virus infections, autoimmune diseases, and blood diseases, suggesting a close link between CICs and diseases [40-42]. Our inspection on various inflammatory tissues showed that almost all inflammation tissues we inspected have CICs with different quantities and forms [11]. Even in the same type of inflammation, such as hepatitis tissue, the properties of effector cells in CICs varied [43]. Nowadays, when you discuss CICs with pathologists, most of them would say: "I have noticed a lot of such structures, but I did not pay much attention to them". This reflects a state that CICs are common in pathological tissues.

At present, some researchers are focusing on the relationship between CICs and the clinical outcome and prognosis of diseases, especially in tumor patients [6, 44]. CICs exists in numerous types of tumors, including epithelium tumors such as breast cancer, lung cancer, liver cancer, pancreatic cancer, melanoma, prostate cancer, bladder cancer; non-epithelium tumors such as the rare mesotheliomas; hematopoietic tumors such as leukemia [3]. Pathological examinations show CICs are easily detected in tumoral fluids (hydrothorax fluid, ascites, urine, and pericardial effusion) [27, 45-47]. Some reports showed that the percentage of CICs was positively correlated with malignancy and tumor grades, suggesting that CICs formation may engage in tumor development and progression [27, 46, 48].

The real role of CICs in inflammation remains unknown. Scientists have found that in the CICs between homogeneous tumor cells, the invading cell could induce multiple nuclei in the target cell and aneuploidy spawn by interfering with its cytokinesis [10]. Aneuploidy is considered one checkpoint in tumorigenesis. We also found out that when lymphocytes entered tumor cells or histocytes, the frequency of multiple-nucleus cells induced is similar to that in homotypic cell-in-cell interaction [11]. Thus, we formulated a tentative hypothesis: through the mechanism mentioned above, the immune cells in an inflammatory tissue could rapidly induce chromosomal instability (CIN) of tissue cells through CICs. Some of these tissue cells with CIN may die or undergo immune clearance due to chromosomal aberrations or immunogen changes and some of them may further develop into cancer cells. Taking in mind the prevailing theory that inflammation is an accelerator of tumorigenesis, partially due to induced CIN, we speculate that CICs formation might be a 'fast track' from inflammation toward transformation [16, 49]. In fact, one of our recent experiments confirmed that given certain carcinogenic factors *in vitro*, the epithelial cells, after being penetrated by immune cells, display Epithelial-Mesenchymal Transition (EMT) features faster than those without CICs (unpublished data).

The pathological significance of CICs may go beyond inflammation-tumor transformation. Our study also found typical CICs in nasopharyngeal epithelial cells of nasopharyngeal carcinoma, along with intestinal epithelial cells of AIDS patients. The effector cells (B or T lymphocytes) in such CICs have consistency with tropism of the pathogenic viruses, that is, in CICs of nasopharyngeal carcinoma, the invading cells were CD20 positive B lymphocytes, and in AIDS, the invading cells were CD4 positive T lymphocytes, which are susceptible to EBV and HIV respectively. Actually, the virus antigen can also be detected in both outer epithelial cells and inner lymphocytes. This suggests that lymphocytes carrying viruses can transmit these viruses easily into non-susceptible cells like nasopharyngeal and intestinal epithelial cells *via* CICs, thereby changing the tropism of the viruses, which is considered as a tough clinical problem nowadays, especially in AIDS patients. Based on these findings, we are proposing a novel model of virus infection of non-susceptible cells, termed "in-cell infection", that is different from the most prevailing model of "the cell to cell infection" [50-52]. Although it is important to study CICs by analyzing clinical samples, there are still some difficulties. One of the prominent problems is that the CICs in tissues are less discernible than those *in vitro* cell lines, and the counting is highly subjective and tedious. It is necessary to develop new histochemical techniques for easy and accurate detection of CICs [53]. An automatic computerized counting system will greatly facilitate the study.

WORKING MODELS FOR CICs

The renewed interests on CICs in recent years have attracted more scientists like Xiaodong Wang to discuss its basic biological functions (the paper in this special issue). Only the National Science Foundation of China has funded five projects on cell-in-cell researches in 2014. Researches on CICs not only open a new window for life science, but also touch various

aspects of biology with its diversified forms and functions, like a kaleidoscope. As stated above, CICs, just like the basic life processes of apoptosis and autophagy, may be closely linked to development, homeostasis, and diseases. Here, we propose three models for this phenomenon based on current researches.

Selection Model

Based on the biological effects, the selection model is proposed [16] (Fig. **1**). In this model, cells enter target cells to be cleared or released with new biological features and functions. Typical example is that the positive-negative selection of Naïve T cells in thymic nurse cells and its clearance in adult liver cells [54]. Besides TNC, effector cells releasing from CICs are also common in most cell systems, although the frequency is much low than cell-in-cell death. The issues on the effector cell-escaping from the target cell and its biological characteristics and functional changes may also need further study. Developing new methods to efficiently capture these escaping cells would be challenging but promising. Recently, we found that chimeric antigen receptor T cells (CAR T) raised in culture with different CD markers (CD4 or CD8) and cytotoxic activities die in their tumor target cells by choice (i.e. entosis or emperitosis) and shown an effector fate-determined anti-tumor activities.

Stress Model

The stress model emphasizes the changes of the target cells and their biological impact on the microenvironment where CICs forms (Fig. **2**). In this model, the effector cell enters the target cell and changes its biological characteristics through cell-in-cell interaction, ultimately leading to phenotypic changes in the target cell, thereby triggering a biological response to create a new niche that may generate a series of physiological or pathological reactions, which further extend throughout the whole body. The biological changes in target cells have gained lesser attentions than those in effector cells, but some examples are available to illustrate its significance. One example would be the entrance of the immune cell into a histocyte or a tumor cell and the consequential aneuploid changes. Recently, Powell *et al*. found that macrophages could fuse with tumor cells through the CICs [55]. The resulting hybrid cells display marked changes in their surface molecules, hence producing new tumor cells with stronger metastatic ability. According to our data, about 3% of the cells in CICs turned into fused cells, not only changing the membrane characteristic of the target cell, but also their genetic materials, which could possibly be a "natural" mechanism of gene variation and thereby produce new biological effects [56]. Hepatitis, especially hepatitis B has a very high morbidity rate in China and has two extreme clinic types – acute necrotic hepatitis and symptomless carriers of hepatitis B virus, the underlying etiopathogenesis still very puzzling. Because we found out that leukocyte-hepatocyte CICs could be detected in most inflammatory tissues in different stages, we would hypothesize that under certain conditions, the leukocytes of different types or in different activated states penetrate into the liver epithelial cells. After intracellular interaction, the epithelial cells as target cells may up-regulate or down-regulate their co-stimulatory or inhibitory immune molecules on their cell surfaces, creating a niche that is either inflammatory or immune-tolerant, thereby inducing a holistic response favoring either acute inflammation (in case of acute necrotizing hepatitis) or immune-tolerance (in case of symptomless carriers of the hepatitis B virus). Exemplification of hepatitis B for the 'stress model' reminds us to focus more on the changes in target cells after cell-in-cell interaction and its subsequent biological outcomes. Hu *et al*. screened cell-in-cell structures in different pathological tissues from patients with different types of hepatitis and the results support this corollary [43].

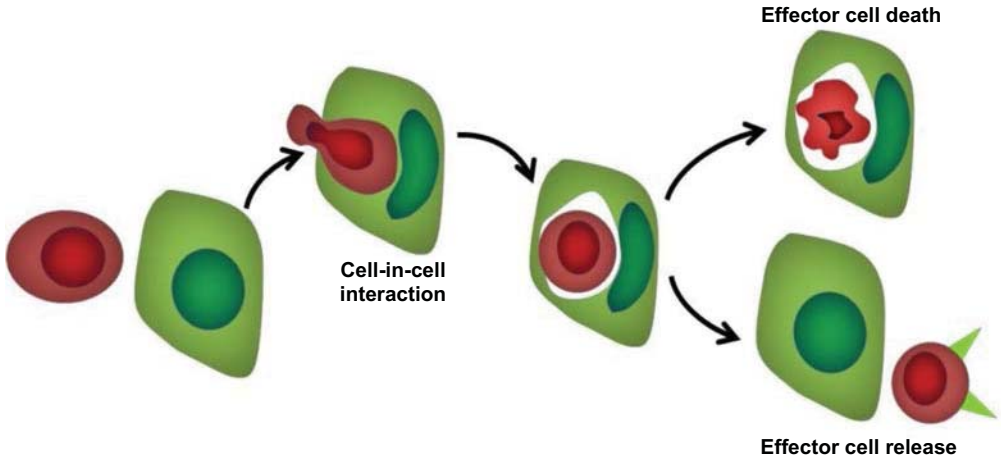

Fig. (1). A cell-in-cell selection model. Diagram depicts effector cell (red cell) penetrates into the cytoplasm of host cell (green cell) to form cell-in-cell structure. After cell-in-cell interaction between effector cell and host cell, some effector cells display self-degradation in different pathways, entosis, cannibalism or emperitosis (upper panel), while others release from the host cell and acquire new properties (low panel), resulting different biological activities.

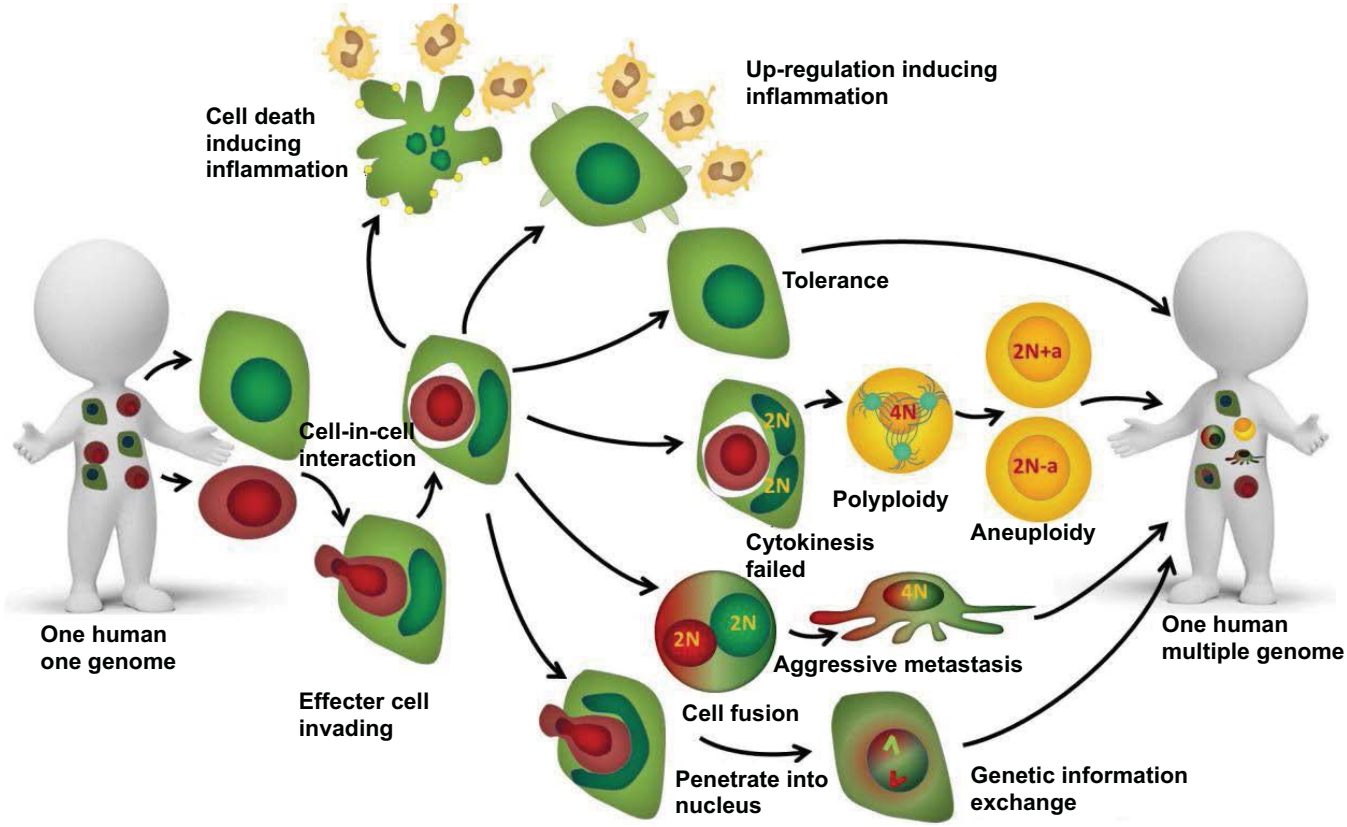

Fig. (2). A cell-in-cell stress model. In this model, effector cell/cells (red cell) inside host cell (green cell) may change the biological properties of the host cell through cell-in-cell interactions, which may result in sequentially the niche changes around the host cell and systemic immune shifting. For example, expressing up-regulating molecules on the host cell membrane may induce inflammation (up panel); otherwise, expressing co-inhibitory molecules on the host cell membrane may induce immune tolerance (middle panel). In some cases, entered effector cell /cells change genomes of the host cell, thereby providing a new way to form new cells with different biological features (low panel). There are different pathways leading to multigenomes in host cell: cytokinesis failure of host cell due to cell-in-cell structure results in polypolid cell formation and subsequent following bipopar division often leads to chromosome missegregation and produce aneuploidy; cell fusion between effector cell and host cell can lead to metastatic cell formation; in other case, effector cell can penetrates into the nuclei of host cell and may result in genetic information exchange; finally through all the above ways, somatic cell genomes may be changed from one genome into multiple genomes.

Competition Model

The competition model is based on Sun's latest findings recently published in *Cell Research* [34]. In his research, the entering cell and target cell were named "winner" and "loser", indicating different fates in competition. CICs in multicellular tissues are often related with the cells' adaptation to a new environment. The phenomenon and theories on cell competition originated from early studies on fruit flies. Researchers found that in the embryonic development of fruit flies, less fit cells are eliminated through a short-distance interaction to ensure optimal cells for tissue formation. Only recently this phenomenon has been confirmed in mammals. In fact, be it homotypic or heterotypic, the CICs reflect a competitive relationship between two cells. It's a self-protective strategy of the cell to adapt to a new environment. The CICs between homotypic cells helps tumor cells adapt to the rapid growth by maximizing and optimizing the resources in the microenvironment. In the CICs between immune cells and histocytes/tumor cells at inflammatory or tumor parts, the immune cells die, demonstrating a new mechanism for tumor cells to resist the killing of immune cells, thereby gaining an edge in the competition. Compared to phagocytosis, a process in high organisms executed by specialized cells like macrophage or granulocytes, CICs keep more features of unicellular organisms and execute completely locally for their own survival by "eating" neighboring cells, as seen in microglial phagocytosis of live neuron during inflammation, ischaemia and neurodegeneration [57, 58].

FUTURE EFFORTS ON CELL-IN-CELL RESEARCH

Extensive Communication and Cooperation are Necessary

The lack of proper research systems is leading cause of the sporadic researches on CICs in history. The last decade witnessed some *in vitro* cell-in-cell research systems, but given the low percentage of CICs, the studies were hard to be conducted

systematically. At present, the cell lines used in major laboratories have a high CICs forming frequency, but the selections of the cell line are mostly occasional. For questions like, "Which cell lines are more suitable for cell-in-cell model?", "What determines the entering or being entered?", "Do different cell-in-cell systems share common mechanisms?", much more work is needed to get clearer answers. The three carefully studied types of cell-in-cell death are characterized very similarly through cell entrance and intracellular deaths of effector cells. However, after a closer look, the distinctions are clear [17]. We and Fais *et al*. all found out that in cannibalism and emperitosis between tumor cells and lymphocyte interactions, lymphocyte entry needs ezrin, but they vary in subsequent intracellular death by different molecular mechanisms [5, 8]. Overholtzer *et al*. found that in the entotic process of breast cancer cells MCF7, detached cells enter the adjacent cells depending on adherens junctions mediated by the cadherins (E- and/or P-cadherin) and the contraction of actomyosin regulated by RhoA-ROCK [7]; but Cano *et al.* found that Nupr1 played a greater role in the pancreatic cancer cell PDCA, indicating minutia exists in molecular mechanisms [59].

We screened over a hundred of available cell lines in an attempt to discover patterns of CICs formation, but the findings seemed to be more complicated [11]. Our screening shows that the penetration of one cell into another is commonly seen among different cell lines, and that some are less penetrable than others. In general, cells able to form homotypic CICs are more prone to be penetrated. But immune cells, fresh isolated or their leukemia cell lines, are rarely penetrated by either homotypic or heterotypic cells. In other words, immune cells including their leukemia cells are more prone to enter into solid tumors of various kinds, but are seldom penetrated. Interestingly, solid tumor cells from different origins are also able to form heterotypic CICs. In most cases, cell penetration is highly directional, *i.e.*, cell A can be penetrated by cell B, C, or both, but A could not penetrate B or C (Fig. **3**). In homotypic CICs, the entrant can also be sequentially penetrated by another cell from a different origin, forming a nested CICs (unpublished data) (Fig. **4**). Our findings also indicated that stem cells, including tumor stem cells are more prone to penetration. For example, a hepatocarcinoma stem cell Hep-12 isolated from metastasis showed high frequency of CICs with NK cells, compared to its parental cancer cells Hep-11 isolated from patient primary lesions (15% *vs* 3%). One phenomenon of CICs deserves special attention: CICs formation could occur cross species. Cells from human beings, mice, rats, monkeys, and even dogs are able to form heterotypic CICs with each other (Fig. **5**). In some cases, species differences in target cells cause minor effects on the entrance of effector cells. In one of our experiments, human NK92 cells entered into the mesenchymal stem cells of a human, mouse, and rat all at a high rate of over 10% (unpublished data). This species-cross phenomenon is rare in biology, once again suggesting that CICs formation is an evolutionarily conserved process and some of the basic mechanisms are conserved during evolution. Another possibility for this in-distinction in forming CICs is that the penetration of effector cells does not rely on the commonly-known membrane receptor-ligand recognition model, but on a more primitive and direct model. The recent finding of Yao *et al*. and Sun *et al*. offers an explanation [34, 60]. They found that cells' ability to form CICs depends on their rigidities. That is, stiff cells enter soft cells. The softer the cell, the higher the percentage of entrance is generated. Interestingly Huang *et al.* [61, 62] also found out that vesicles from tumor cells could enter other tumor cells selectively, implementing a treatment effect, and the selection depends on the rigidity of the tumor cells [61]. The softer the target cell, the easier it is penetrated. These findings provide useful information to analyze the differences in CICs formation. After compiling all of the data mentioned above, it seems that the mechanisms of CICs formation are diversified; much more about them remains to learn.

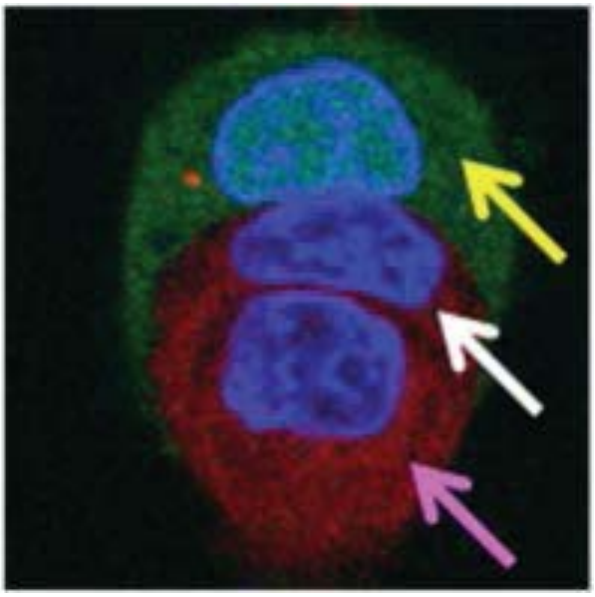

Fig. (3). Cell penetration in cell-in-cell structure may be highly directional. Three different types of cells form special cell-in-cell structure. In the mix-culture, cell A (green cell, white arrow) can be penetrated by both cell B (black cell, yellow arrow) and cell C (red cell, pink arrow). However, we hardly can observe cell A penetrates into cell B or cell C.

Therefore, it is necessary to establish a communication platform to exchange information on the features of CICs formation and variation of different cell lines, and specify cell lines as model cell lines to conduct more precise investigation on the molecular network. Systemic study may also need advanced CICs enrichment technique, actually He *et al*. now can enrich the CICs to 90% purity through flow cytometry [63]. Besides cell-in-cell death, other features and fates of the cells involved in CICs warrant further study.

In Vivo Animal Model Holds the Key

The study of a biological phenomenon is ultimately for the purpose of developing new diagnostic and therapeutic strategies for human diseases. Thus the *in*

vivo animal models are crucial. Most observations on CICs in early days were based on the pathological tissues of patients, but were sporadic and random.

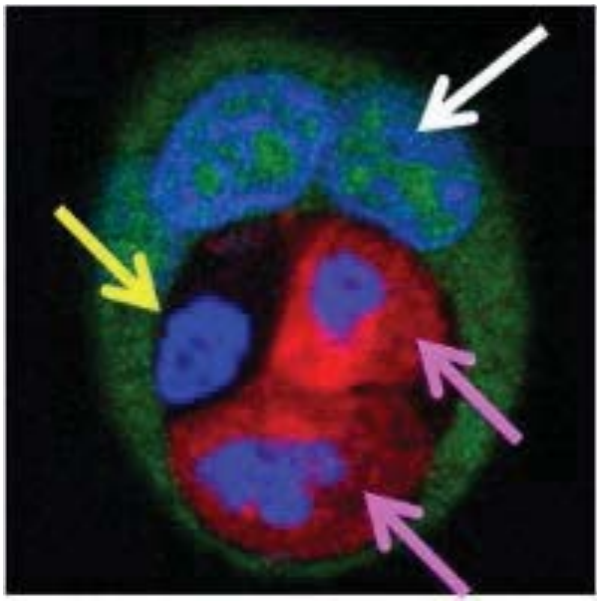

Fig. (4). A nested cell-in-cell structure. One cell (low red cell, pink arrow) penetrates into the second host cell (middle cell, white arrow), both of them penetrate into the third one (upper green cell, yellow arrow).

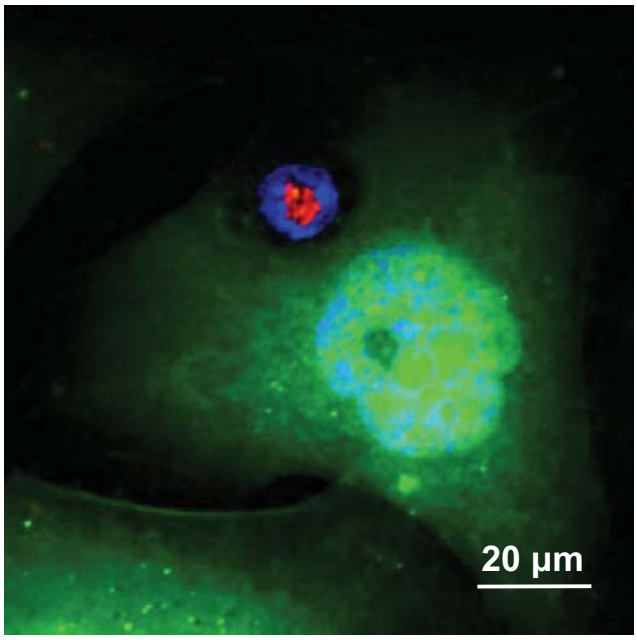

Fig. (5). Cell-in-cell structures formation could occur cross species. A granulocyte (red cell) with a typical doughnut nucleus (blue: DAPI) from C57BL/6 mice penetrates into a human CNE-2 cell (green cell).

The recent studies are somehow linked with diseases [9, 45, 64], but still lack an ideal, stable and reproducible *in vivo* model that can be applied to the comprehensive physiological and pathological studies. According to our studies on a variety of inflammatory issues, CICs are rare in normal tissues, but common in inflammatory ones, and are inducible [11]. For example, injecting mice with PolyI:C + D-GaIN to induce acute hepatitis. The NK (CD56 positive)-hepatocyte CICs were detected in the livers of these mice in just two days. Other inducers may induce other types of CICs. In human hepatitis specimens of different stages, cells forming CICs vary greatly. At the early stage, lymphocyte-hepatocyte cells are more common; in the late stage, granulocyte-hepatocyte cells are the most common. If we can build such a stable animal model, it would be conducive for cell-in-cell studies.

The study on a new biological phenomenon depends on model animals, among which nematodes and zebrafish are the most classical and common. Fortunately, as mentioned above, CICs that bears on important physiological functions has been observed in nematodes [19, 20]. At present, despite the unknown formation mechanism and divided opinions over the fates of entering cells, selecting this animal as an *in vivo* model is no doubt a progress in cell-in-cell studies. If the process of linker cell death is stable and reproducible [19], we could use this physiological process as *in vivo* indicators and discover its molecular networks through gene knockout, so as to improve the overall cell-in-cell researches.

Omics Screening will Fuel Cell-in-Cell Research

Although research on CICs strode forward in the past decade, the overall research system is limited to cell biology and molecular biology – short of the involvement of systemic biology. Omics technologies, which have been widely applied in life sciences, are rarely adopted. In retrospect of the history of apoptosis and autophage, apart from establishing qualified *in vivo* and *in vitro* research models, identifying inhibitors in every step of the process is a major driver to the research progresses. By screening ~200,000 chemical compounds, Xiaodong Wang *et al*. identified the RIP3 inhibitors [65, 66], which contribute greatly to the study on necroptosis. If the specific inhibitors in every step, including cell entrance, cell-in-cell death, mitosis and release, can be identified, they would not only improve our understanding of its mechanism, but also pave the way for identifying intervention targets. At present, there have been some inhibitors, such as wortmannin, which inhibits CICs formation by impeding the PI3K signaling [9]. Y27632, a ROCK kinase inhibitor, has also been proven to prevent cell entrance or cell-in-cell death [7]. However, these inhibitors are not specific. In most cases, the inhibitors also inhibit conjugate formation between two cells, a key step for a variety of cell functions. Thus it is hard to confirm the *in vivo* function of certain CICs formation. Benseler *et al*. first used wortmannin to prevent naïve T from entering hepatocytes *in vivo,* demonstrating that formation of CICs between these T cells and hepatocytes is one of the mechanisms to remove noxious immune cells and achieve homeostasis. Although wortmannin treatment led to increased naïve T cells in the peripheral blood of the tested animal, there was no proof of decreasing CICs within the liver cells [9]. Therefore, wortmannin did not count as a specific inhibitor. Plus it is highly

toxic, and may not be appropriate for other *in vivo* experiments. High throughput screening for chemicals may provide an efficient route to identify these inhibitors.

CONCLUSION

There is an old Chinese proverb, “I am born to be useful”, similar to a Western saying, “That is reasonable” by the German philosopher, Hegel. CICs, as a unique and old phenomenon in cell biology, must play some key roles in life, which will be unveiled further. Currently, we’ve seen the tip of the iceberg in the cell-in-cell phenomenon; it’s still long way from revealing its molecular structures, formation mechanism, fate choices and clinical implication etc. To fulfill this, more energetic researchers, new technologies, and possibly sharing strategies used in apoptosis and autophage researches are needed. Importantly, the research on the cell-in-cell phenomenon has unquestionably opened a new window showing a new field for life science.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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