Cell Research (2015) 25:1265-1268.

www.nature.com/cr

LETTER TO THE EDITOR

Implication of cell-in-cell structures in the transmission of HIV to epithelial cells

Cell Research (2015) **25**:1265-1268. doi:10.1038/cr.2015.119; published online 2 October 2015

Dear Editor,

Chronic virus infection, such as infection by Epstein-Barr virus (EBV), hepatitis B/C virus (HBV/HCV) and human immunodeficiency virus (HIV), constitutes major public health concerns. Although efforts in deciphering the mechanisms underlying their virological consequences have greatly improved clinical prevention and therapy, various challenges, such as virus tropism drifting, remain to be addressed to develop effective clinical interventions. While cell-free viruses usually infect target cells via binding to specific receptors, the presence of virions in non-susceptible cells has been reported with the mechanisms poorly understood [1]. Recently, we reported that EBV could infect non-susceptible nasopharyngeal epithelial cells (ECs) through the formation of cell-in-cell structures between ECs and internalized B lymphocytes that have been infected with EBV. This novel mechanism was termed in-cell infection [2]. Given the formation of similar heterotypic cell-in-cell structures between other EC-lymphocyte pairs, such as epithelial tumor cells and T lymphocytes [3], we wonder whether in-cell infection also plays a role in mediating transmission of other viruses.

HIV is well known to specifically infect $CD4^+$ immune cells including $CD4^+$ T lymphocytes, monocytes, macrophages and dendritic cells. The cell-surface CD4 serves as receptor for cell-free virus binding and such binding initiates effective infection [4]. Cell-to-cell infection via a specialized cell-cell contact region called virological synapse [5] has been argued as another way to transmit HIV between $CD4^+$ susceptible cells [1]. However, CD4-negative cells, such as colon ECs and vaginal ECs, were also found to contain HIV and are believed to serve as another productive reservoir [6], suggesting the existence of CD4-independent virus spreading mechanism. Exosomes released from HIV-infected host cells have been reported to facilitate productive HIV infection in non-susceptible cells by delivering cargos such as virus proteins and RNA species [7]. Based on our previous discovery of EBV in-cell infection [2], we hypothesized that formation of heterotypic cell-in-cell structures could also lead to transmission of HIV from internalized $CD4^+$ T cells to the non-susceptible ECs.

To test this hypothesis, we first examined the existence of heterotypic cell-in-cell structures in the co-culture of H9/IIIB cells, a T cell line chronically infected with HIV-1 IIIB (formerly called HTLV III), and cancer cell lines of epithelial origin including Caco2 (colon cancer cell line), MCF7 (breast cancer cell line) and PLC/PRF/5 (hepatoma cell line). We found that H9/IIIB cells (stained with CellTracker Orange) could efficiently penetrate into all epithelial cancer cell lines tested (stained with CellTracker Green), forming typical cell-in-cell structures. The frequencies of cell-in-cell structures increased gradually and reached the maximum by the end of the co-culture (24 h) (Figure 1A and 1B). Interestingly, compared with uninfected H9 cells, H9/IIIB cells showed enhanced ability to penetrate into Caco2 and MCF7 cells, but not PLC/PRF/5 cells, suggesting that HIV infection selectively promotes penetration of T cells into certain types of ECs (Figure 1B). We then stained these co-cultured cells with an antibody against the HIV p24 gag protein. As shown in Figure 1C and 1D, a portion of ECs co-cultured with H9/IIIB cells were positive for p24 gag. The frequency of p24 gag^+ ECs positively correlated with that of cell-in-cell structures observed in the co-culture, and more than 8% ECs were positive for p24 gag after 24 h co-culture (Figure 1C and 1D), suggesting the transmission of HIV to ECs. In agreement with this, transmission electron microscopy (TEM) revealed the presence of viral particles within the cytoplasm of ECs containing H9/IIIB cells, which had been degraded into corpse as indicated by a dashed circle (Figure 1E). In addition, we detected a high level of p24 gag protein in the supernatants of epithelial cancer cells even 4 days after H9/IIIB cells were removed from the co-culture (Figure 1F). Taken together, these data support the idea that formation of heterotypic cell-in-cell structures could mediate HIV transmission from $CD4^+$ T cells to otherwise non-susceptible ECs. Intriguingly, apoptotic H9/IIIB cells (as evidenced by DNA fragmentation) were often observed in p24 gag^+

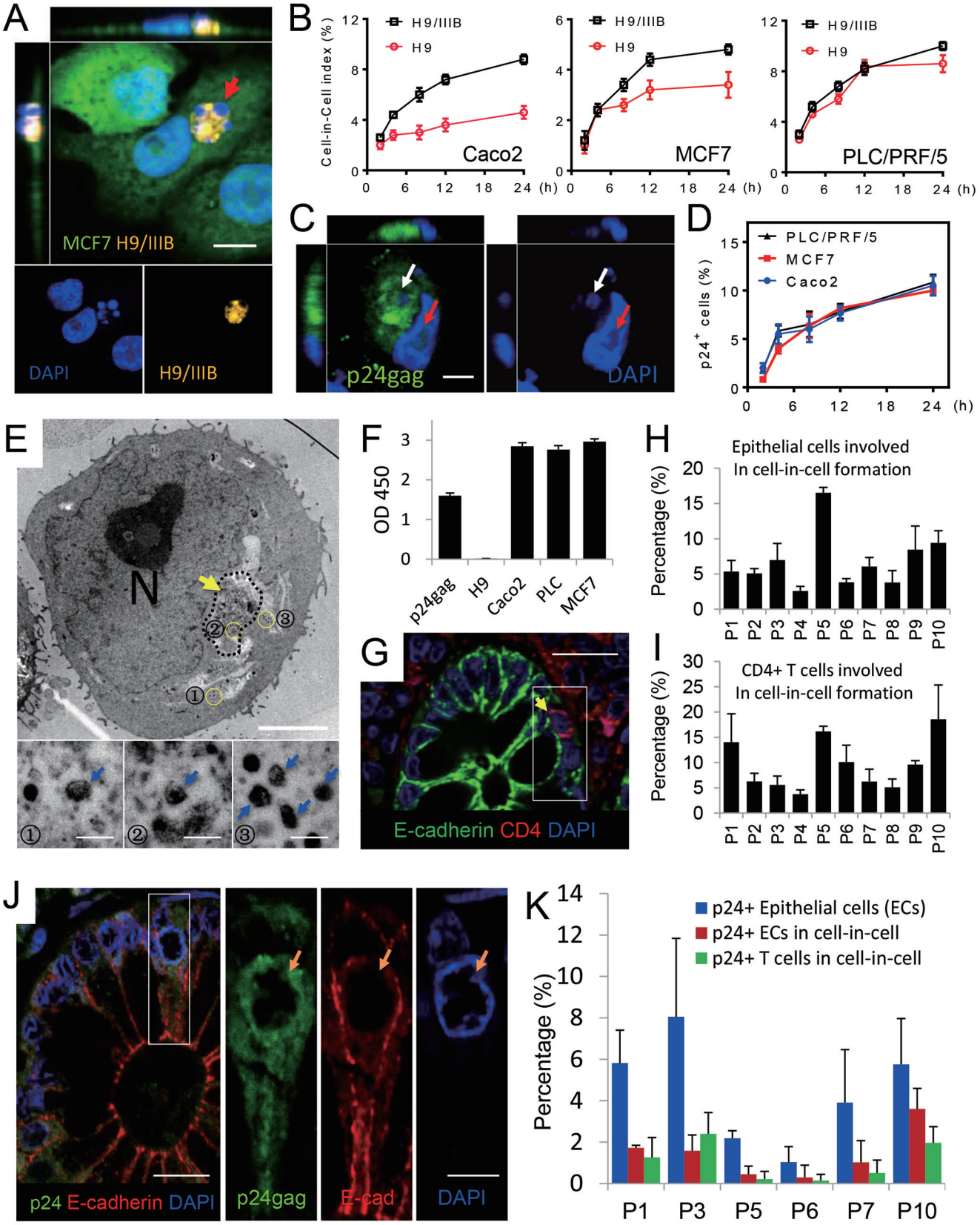
A
MCF7 H9/IIIB
DAPI
H9/IIIB
B
Cell-in-Cell index (%)
H9/IIIB
H9
Caco2
MCF7
PLC/PRF/5
(h)
C
p24gag
DAPI
D
PLC/PRF/5
MCF7
Caco2
p24+ cells (%)
(h)
E
N
①
②
③
F
OD 450
p24gag
H9
Caco2
PLC
MCF7
G
E-cadherin CD4 DAPI
H
Epithelial cells involved
In cell-in-cell formation
Percentage (%)
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10
I
CD4+ T cells involved
In cell-in-cell formation
Percentage (%)
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10
J
p24 E-cadherin DAPI
p24gag
E-cad
DAPI
K
p24+ Epithelial cells (ECs)
p24+ ECs in cell-in-cell
p24+ T cells in cell-in-cell
Percentage (%)
P1 P3 P5 P6 P7 P10

Figure 1 Formation of cell-in-cell structure mediates HIV transmission from infected T cells to ECs. **(A)** Representative image for cell-in-cell structure formed between HIV-infected cells stained with CellTracker Orange and MCF7 cells stained with CellTracker Green 24 h after co-culture. Arrow indicates an internalized H9/IIIB cell. Scale bar, 10 μm. **(B)** Formation of cell-in-cell structures between H9 or H9/IIIB cells and ECs (Caco2, PLC/PRF/5 and MCF7). H9/IIIB: T cell line chronically infected with HTLV III; H9: uninfected control T cell line. Cell-in-cell structures were quantified at 6, 12, 18 and 24 h after incubation. About 200 ECs were counted at each time point. Data shown represent results from 3 independent experiments. **(C)** H9/IIIB cells were incubated with PLC/PRF/5 cells for 24 h and then stained with p24 gag antibody (green). White arrows indicate dying internalized H9/IIIB cell with apoptotic morphology; red arrows indicate p24 gag-positive outer PLC/PRF/5 cell. Scale bar, 10 μm. **(D)** Quantification of p24 gag-positive ECs after co-culture with H9/IIIB cells for the indicated time periods. **(E)** Electron micrographs of cell-in-cell structure formed between Caco2 and H9/IIIB (yellow arrow) cells. Cells were fixed, dehydrated and embedded in Epon resin followed by standard electron microscopic specimen processing as described in Supplementary information, Data S1. Bottom images show magnified regions circled in yellow in the upper image. Blue arrows indicate HIV virions. Scale bars, upper image, 5 μm; bottom images, 0.2 μm. **(F)** Presence of p24 gag in the supernatants of tumor cells (Caco2, PLC, MCF7) co-cultured with H9/IIIB cells. H9/IIIB cells were washed out 24 h after co-culture, and supernatants were collected 5 days after co-culture. p24 gag: standard sample protein (100 pg/ml). H9: supernatants from H9 cells as negative control. **(G)** Representative immunostaining image of cell-in-cell structure formed between $CD4^+$ T cell (CD4 in red) and colon EC (E-cadherin in green) in colon tissue from HIV-infected patient. Nuclei were stained with DAPI. Arrow indicates an internalized $CD4^+$ T cell. Scale bar, 20 μm. **(H-I)** Quantification of ECs (H) or $CD4^+$ T cells **(I)** involved in cell-in-cell structure formation in colon samples from 10 patients with HIV infection. **(J)** An EC (E-cadherin in red) in cell-in-cell structure detected in a colon sample from an HIV-infected patient is positive for p24 gag (green). Nuclei were stained with DAPI. Arrow indicates an internalized p24 gag-positive cell. Scale bars, left, 15 μm; right, 5 μm. **(K)** Quantification of p24 gag-positive cells in colon samples from HIV-infected patients. p24-positive ECs: the percentage of p24 gag-positive ECs in all ECs counted; p24-positive ECs in cell-in-cell: the percentage of p24 gag-positive ECs involved in the formation of cell-in-cell structures in all ECs counted; p24-positive T cells in cell-in-cell: the percentage of p24 gag-positive T lymphocytes involved in the formation of cell-in-cell structures in all T cells counted.

ECs (Figure 1C), suggesting that death of penetrated T cells may be involved in virus spread as seen in EBV in-cell infection [2].

To examine the physiological relevance of our findings, sigmoid specimens from 10 HIV-infected patients were stained with antibodies against CD4 (for $CD4^+$ T cells) and E-cadherin (for ECs). Surprisingly, substantial infiltration of $CD4^+$ lymphocytes into sigmoid tissue was observed. Typical cell-in-cell structures could be readily identified in all sigmoid specimens examined (Figure 1G). Quantification analysis showed that about 7% ECs on average (2.6%-16.5%) were penetrated in specimens from different donors (Figure 1H). Among all infiltrating $CD4^+$ T lymphocytes, averagely 9.5% (3.8%-18.5%) were found inside ECs (Figure 1I), indicating that $CD4^+$ T cells actively penetrated into colon ECs in AIDS patients. Next, we stained these samples with antibodies against HIV p24 gag and E-cadherin. Samples from 6 patients showed detectable p24 gag staining. In these samples, a small number of lymphocytes (about 1%, ranging from 0.2% to 2.4% of total lymphocytes counted) penetrated into ECs and were positive for p24 gag (green columns in Figure 1K). Interestingly, we also found comparable numbers of ECs (about 1.5%, ranging from 0.3% to 3.6% of total ECs) were penetrated by lymphocytes and were positive for p24 gag (red columns in Figure 1K). In some cell-in-cell structures, both lymphocytes and ECs were p24 gag-positve (Figure 1J). These data are consistent with our in vitro data that HIV-infected T lymphocytes spread viruses directly to ECs within the cell-in-cell structure. It is worth to note that about 4.5% (1%-8%) of total ECs in these specimens were positive for p24 gag, and a portion of them lacked the cell-in-cell structures (compare the blue with the red columns in Figure 1K). One possible explanation for this observation is that the inner cells (lymphocytes) eventually underwent cell death and were cleared by the outer cells (ECs). Taken together, we conclude that formation of cell-in-cell structures may function as a novel pathway for direct transmission of HIV from infected T lymphocytes to non-susceptible $CD4^-$ ECs.

Unlike cell-free virus infection, CD4 receptor is not required for this type of virus spread as the internalized $CD4^+$ T cells would be cleared within the ECs. Thus, together with our work on EBV [2], we propose that the formation of cell-in-cell structures may have broader implications in spreading of different types of viruses. Future work based on this model would shed new light on how viruses evolve to adapt to new hosts and transmit between susceptible and non-susceptible cells. It should be noted that both cell-to-cell and cell-in-cell mechanisms could potentially contribute to EC infection in our cell co-culture system; actually cell-in-cell infection is more like an updated version of cell-to-cell infection as target and effector cells could make contact from head to toes within a limited space, thus leading to more efficient virus transmission [2]. While polarity has been implicated in HIV transmission via cell-to-cell [8] or exosome-mediated mechanisms [9], its role in in-cell infection of HIV or EBV remains to be investigated. Nev-

ertheless, polarity proteins, such as Par3 and Lgl, were found to be important regulators of cell-in-cell structure formation [10]. Therefore, it is conceivable that cellular polarity might influence in-cell infection through certain mechanisms.

Over the past century, the detection of cell-in-cell structures, characterized by the presence of one or more viable cells within another cell, had been documented in various human diseases with tumor being the most studied one [11]. Recently, a similar phenomenon was discovered in the development of lower organisms, such as *C. elegans* [12], suggesting that cell-in-cell structure formation is evolutionarily conserved. Moreover, the formation of homotypic cell-in-cell structure by entosis may mediate competition among tumor cells and thus promote clonal selection and tumor evolution [13-15]. Our work on HIV and EBV identifies a novel function of cell-in-cell structure formation. It is conceivable that future investigation would reveal more critical roles of cell-in-cell structures in complex biological processes.

Acknowledgments

We thank Drs Yu-quan Wei, Yong-tang Zheng, Shi-bo Jiang, Xin-wen Chen, Ying Wang, Jianning Deng and Chun-gen Pan for their constructive comments on this work. We also thank Dr Songlin Wang for technical assistance in immunostaining and Dr Daoming Zhuang for help in culturing H9/IIIB cells. This study was supported by the National Natural Science Foundation of China (81273197 and 81471578 to XNW, and 81472588 to QS), the National Basic Research Program of China (2015CB553704 to QS), the Pioneer Project for Young Scientist of Military Biomedicine (14QNP119 to QS), and the National Major Scientific and Technological Special Project for "Significant New Drugs Development" (2015ZX09501-009 to QS).

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)