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2 **Kaposi's Sarcoma-associated Herpesvirus vFLIP Promotes MEndT**

3 **to Generate Hybrid M/E State for Tumorigenesis**

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5 (Running title: KSHV induces MEndT for Tumorigenesis)

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21 **Key Words:** Kaposi's sarcoma (KS), Kaposi's sarcoma-associated herpesvirus

22 (KSHV), Mesenchymal stem cell (MSC), Mesenchymal-to-endothelial Transition

23 (MEndT), vFLIP.

24

1 **Abstract**

2 Kaposi's sarcoma (KS) is an angioproliferative and invasive tumor caused by Kaposi's
3 sarcoma-associated herpesvirus (KSHV). The cellular origin of KS tumor cells
4 remains contentious. Recently, evidence has accrued indicating that KS may arise
5 from KSHV-infected mesenchymal stem cells (MSCs) through
6 mesenchymal-to-endothelial transition (MEndT), but the transformation process has
7 been largely unknown. In this study, we investigated the KSHV-mediated MEndT
8 process and found that KSHV infection rendered MSCs incomplete endothelial
9 lineage differentiation and formed hybrid mesenchymal/endothelial (M/E) state cells
10 characterized by simultaneous expression of mesenchymal markers PDGFRA/Nestin
11 and endothelial markers PDPN/CD31. The hybrid M/E cells have acquired high
12 tumorigenic properties *in vitro* and the potential to form KS-like tumors after
13 transplanted in mice under renal capsules. These results faithfully recapitulate
14 Kaposi's sarcoma where proliferating KS spindle-shaped cells and the cells that line
15 KS-specific aberrant vessels were also found to exhibit a hybrid M/E state.
16 Furthermore, the genetic analysis identified KSHV-encoded FLICE inhibitory protein
17 (vFLIP) as a crucial regulator controlling KSHV-induced MEndT and generating
18 hybrid M/E state cells for tumorigenesis. Overall, KSHV-mediated MEndT that
19 transforms MSCs to tumorigenic hybrid M/E state cells driven by vFLIP is an
20 essential event in Kaposi's sarcomagenesis.

21

1 **Author Summary**

2 Kaposi's sarcoma manifests as multifocal lesions with spindle cell proliferation,
3 intense angiogenesis, and erythrocyte extravasation. Although the origin and true
4 malignant nature of KS remains contentious, it is established that KSHV infection
5 with concomitant viral oncogene expression in normal cell progenitors causes KS.
6 The mechanism of KSHV oncogenesis could be revealed through reproduction of KS
7 by infection of normal cells. This study reports that the KSHV infection of
8 mesenchymal stem cells initiates mesenchymal-to-endothelial transition (MEndT) that
9 generates mesenchymal/endothelial (M/E) hybrid state cells. The hybrid M/E cells
10 acquired high tumorigenic properties, including tumor initiation, angiogenesis,
11 migration, and the potential to form KS-like tumors after transplanted in mice. This
12 finding faithfully recapitulates Kaposi's sarcoma where proliferating KS spindle cells
13 and the cells that line KS-specific aberrant vessels are also found to exhibit the hybrid
14 M/E state. We also found that KSHV-encoded viral FLICE inhibitory protein (vFLIP)
15 plays a crucial role in promoting MEndT and the generation of M/E state cells. These
16 results provide a new layer of evidence for MSCs being the cell source of KS spindle
17 cells and reveal novel insight into KS pathogenesis and viral tumorigenesis.

18

1 Introduction

2 Kaposi's sarcoma (KS) is the most common neoplasm in AIDS patients. Kaposi's
3 sarcoma-associated herpesvirus (KSHV) is the causative agent of this malignancy [1].
4 KSHV is also associated with other malignancies, including primary effusion
5 lymphoma (PEL), multicentric Castleman's disease (MCD), and osteosarcoma[2-4].
6 Kaposi's sarcoma is a multicentric, oligoclonal neoplasm presenting clinically as red-
7 purplish spots localize mainly in the oral cavity or skin [5]. The histological features
8 of KS lesions are extremely complex. They consist of proliferating spindle tumor cells,
9 immature and leaky vessels, and prominent inflammatory infiltrate. The cellular
10 origin of KS spindle cells remains elusive. The current leading hypothesis is that KS
11 spindle cells may derive from endothelial lineage, as they bear pan-endothelial
12 markers (CD31, CD34, and CD36 and Factor VIII) and lymphatic endothelial markers
13 (VEGFR3, LYVE-1 and PDPN). However, KS spindle cells also express other
14 markers including smooth muscle cell (α SAM), macrophage (CD68), dendritic cell
15 (Factor XIII) and mesenchymal stem cell (Nestin and CD29) markers, suggesting that
16 KS cells do not faithfully represent an endothelial cell lineage [6]. Besides, KS
17 spindle cells display intriguing characteristics of progenitor or immature endothelial
18 cells – the expression of endothelial progenitor cell markers and lack of
19 Weibel-Palade bodies (WPB) regarded as a marker for mature vascular endothelium
20 [7, 8]. The remarkable heterogeneity of KS raised a hypothesis that KS spindle cells
21 may originate from mesenchymal stem cells (MSCs) or precursors of vascular cells [9,
22 10]. Recently, we found a series of evidence supporting the hypothesis. (i) An

1 immuno-histochemistry analysis showed that AIDS-KS spindle cells express
2 Neuroectodermal stem cell marker (Nestin) and oral MSC marker CD29, suggesting
3 an oral/craniofacial MSC lineage of AIDS-associated KS. (ii) KSHV infection of oral
4 MSCs effectively promotes multiple lineage differentiation, especially endothelial
5 differentiation *in vitro* and *in vivo*. (iii) Gene expression profiling analysis showed
6 that KSHV infection reprograms MSCs, resulting in mesenchymal-to-endothelial
7 transition (MEndT) and rendering KSHV-infected MSC the closest distance to
8 Kaposi's sarcoma in gene expression profile. (iv) When implanted in mice,
9 KSHV-infected MSCs were transformed into KS-like spindle-shaped cells with other
10 KS-like phenotypes [11]. Moreover, KSHV-infected primary rat embryonic
11 metanephric mesenchymal precursor cells (KMM) and mouse bone marrow-derived
12 MSCs (KP α (+)S) growth in KS-like conditions efficiently form KS-like tumor in
13 nude mice [10, 12]. Taken together, increasing evidence supports the notion that
14 Kaposi's sarcoma may arise from KSHV-infected MSCs through MEndT. However,
15 the underlying mechanism remains unclear.

16 MSCs have been identified as a population of hierarchical postnatal stem cells with
17 the potential to self-renew and differentiate into osteoblasts, chondrocytes, adipocytes,
18 cardiomyocytes, myoblasts and neural cells [13, 14]. MSCs can be induced to
19 endothelial-like cells with angiogenic cytokines, including VEGF, bFGF, and
20 angiopoietin [15]. The switch from mesenchymal to endothelial phenotype is referred
21 to as Mesenchymal-to-Endothelial Transition (MEndT), which is critical phases of
22 embryonic organic development and also contributes to diseases. In adult, MEndT

1 contributes to neovascularization by generating endothelial cell after cardiac injury
2 [16], and to cancer progression by enhancing angiogenesis [17]. Moreover, MEndT,
3 like its reverse process EndMT, is not a binary switch but a dynamic transition, which
4 generate many intermediate phenotypic states arrayed along the mesenchymal
5 (M)-to-endothelial (E) spectrum including mesenchymal-like (M), endothelial-like (E),
6 and hybrid M/E states. Studies suggested that tumor cells staying in different stages
7 have different roles in tumor progression [18-21].

8 KSHV can be found in all KS tumors and is present in all stages of KS (patch, plaque,
9 and nodular). In the early (patch) stage, KSHV is found in spindle-like cells
10 surrounding ectatic vessels, and in nodular KS, the virus is present in the vast majority
11 of spindle cells surrounding slit-like vessels [22]. The majority of KSHV in KS
12 lesions is in latent phase where only a limited number of latent genes are expressed. In
13 a small percentage of tumor cells, KSHV undergoes spontaneous lytic replication and
14 expresses a large number of viral genes. KSHV latency genes, including LANA,
15 vCyclin and vFLIP, are known to play roles in regulating cell proliferation and
16 apoptosis evasion, and endowing pro-angiogenic and inflammatory signals [6, 23].
17 Some lytic viral proteins, such as vGPCR and vIL-6, exhibit tumorigenic activities
18 and induce angiogenesis and inflammation [23-25]. vIL6 sufficiently induces BECs
19 differentiate to LECs via upregulating the expression of PROX1 [26], KSHV-encoded
20 miRNAs induce LEC-to-BEC reprogramming by downregulating MAF [27],
21 KSHV-initiated endothelial-to-mesenchymal transformation is mediated by vFLIP
22 and vGPCR through MT1-MMP in 3D LECs culture system [28]. Thus, many viral

1 genes are known to participate in KSHV-induced cell reprogramming and KS
2 oncogenesis.

3 Increasing evidence supports that KS derives from KSHV-infected MSC through
4 MEndT. However how KSHV infection drives mesenchymal stem cell for MEndT
5 process that leads to Kaposi's sarcoma was largely unknown. In this study, we
6 investigated the process of KSHV-mediated MEndT and the underlying mechanism.
7 We found that KSHV infection initiates an endothelial lineage, but incomplete
8 differentiation that generates tumor cells with hybrid mesenchymal and endothelial
9 phenotypes. Such hybrid M/E cells exhibit oncogenic properties and form KS-like
10 lesions in Kidney capsule transplantation. Finally, KSHV vFLIP was found to play
11 critical roles in KSHV-induced MEndT and oncogenesis. These findings further
12 support the hypothesis that KS tumor cells arise from KSHV-infected MSCs through
13 MEndT.

14 **Results**

15 **KSHV-positive spindle cells in Kaposi's sarcoma lesions display an intriguing** 16 **hybrid mesenchymal/ endothelial (M/E) phenotype**

17 KS tumors express heterogeneous markers characteristic of many cell lineages,
18 including endothelial markers and mesenchymal stem cell markers [11, 28-30]. We
19 wondered if the unique feature of KS truly reflects the simultaneous presence of
20 different lineage markers on the same tumor cell rather than the presence of distinct
21 subpopulations in different differentiation statuses. Toward this question, we

performed triple immunofluorescence assay on KS clinic samples for a mesenchymal marker (Nestin or PDGFRA), an endothelial marker (PDPN or CD31), and a KSHV marker (LANA). Samples from twelve AIDS patients, including early (macules/papules, n=7) and late KS lesions (nodules, n=5), were analyzed. We observed that LANA-positive spindle-shaped cells mostly expressed both mesenchymal stem cell markers (PDGFRA and Nestin) and endothelial markers (PDPN and CD31) simultaneously. In contrast, LANA-negative cells did not express endothelial markers PDPN and CD31 but only mesenchymal stem cell markers PDGFRA and Nestin (Figure 1A). Furthermore, the proportions of LANA+ PDPN+ CD31+ cells were significantly higher in the late KS lesions than in the early lesions (Figure 1B and C). The percentage of PDPN-positive cells correlates with the percentage of LANA-positive cells (Figure 1D). Almost all LANA-positive spindle cells expressed Nestin and CD31 regardless of early or late KS lesions; the proportions of LANA+ PDGFRA+ PDPN+ cells increase with KS progression (Figure 1E). These results indicate that (i) KS lesions contained a large number of tumor cells with hybrid mesenchymal/endothelial markers (M/E state) that may result from an incomplete mesenchymal-to-endothelial transition of MSCs; (ii) the M/E state is strongly associated with the presence of KSHV in the tumor cells; (iii) the proportion of KSHV-positive M/E hybrid cells increases with KS progression.

One of the most notable characteristics in KS lesions is abundant neovascularity, which results in a proliferation of irregular, jagged vascular channels accompanying with erythrocytes diapedesis. KS abnormal vessels differ from their normal

1 counterpart, displaying unique features characterized by slit-like and sieve-like
2 morphology. The tumor vascular channels surround and protrude into native vessels
3 resulting in characteristic promontory signs (Figure 1G). To characterize the KS
4 specialized vessels, we sought to determine the cellular origin of KS vessels by
5 analyzing the above triple immunohistochemistry images for specific marker profiles
6 of the vessels. In the KS adjacent normal blood and lymphatic vessels, vascular
7 endothelial cells are CD31+, PDPN-, PDGFRA-, and Nestin-; lymphatic endothelial
8 cells are CD31+, PDPN+, PDGFRA-, and Nestin-, whereas vascular smooth muscle
9 cells and pericytes are PDGFRA+, Nestin+, CD31-, PDPN- in both vessels (Fig. 1H).
10 This observation is consistent with the nature of normal blood vessels that are
11 composed of blood endothelial cells (BECs) and vascular smooth muscle cells
12 (VSMCs), and lymphatic vessels that are lined by lymphatic endothelial cells (LECs)
13 and a thin layer of smooth muscle cells (Figure 1F). In contrast, KS abnormal vessels
14 were made of lined LANA-positive cells that were either PDGFRA+, PDPN+ or
15 PDGFRA+, PDPN- when stained with PDGFRA and PDPN antibodies, and Nestin+,
16 CD31+ when staining with Nestin and CD31, suggesting that KS aberrant vessels are
17 created by KS tumor cells with hybrid M/E and xM phenotypes (Figure 1I). Thus, the
18 most noticeable difference in morphology and structure between KS abnormal vessels
19 and normal blood or lymphatic vessels is that KS specialized irregular jagged vessels
20 were lined by LANA+ spindle tumor cells rather than normalized endothelial cells
21 and these vessels are prone to leakage of fluid and extravasation of RBCs. Together,

1 these results indicate that KS spindle cells display a hybrid M/E state through MEndT
2 and KS abnormal vessels derive from KSHV-infected MSCs.

3 **KSHV infection induces MSCs to differentiate into mesenchymal/endothelial**
4 **hybrid state cells through MEndT *in vitro***

5 The triple immunofluorescence assay of KS lesions revealed the hybrid M/E
6 phenotype of KSHV-positive spindle-shaped tumor cells. This observation compelled
7 us to hypothesize that KS spindle cells arose from mesenchymal stem cells and
8 KSHV initiates an MEndT process converting cells from mesenchymal phenotype to
9 hybrid M/E phenotype. To prove this hypothesis, we attempted to reproduce this
10 process in cultured mesenchymal stem cells to investigate whether KSHV infection
11 could induce reprogramming of MSCs, leading to endothelial-like or M/E hybrid cells
12 and abnormal angiogenesis as observed in KS lesions. First, PDLSCs were grown in
13 2-D culture and infected with KSHV. The changes of the cells in mesenchymal and
14 endothelial cell markers were examined at different time points using Western blot,
15 RT-qPCR, and immunofluorescence assay (IFA). The result showed that some
16 mesenchymal markers, such as COL1A1, α SAM, and TAGLN, faded and endothelial
17 markers PROX1 and PDPN increased starting at the fourth day post-infection (Figure
18 2A). In consistent with KS lesions, mesenchymal stem cell markers PDGFRA and
19 Nestin remained unchanged after KSHV infection (Figure 2B). However, KSHV
20 infection did not result in the substantial expression of endothelial markers CD31 and

1 vWF, which are expressed in KS (Figure 2C), suggesting that the 2-D cell culture
2 system may not faithfully represent the MEndT in tumors.

3 Three-dimensional (3-D) organotypic cultures allow the mimic function of living
4 tissue and probably provide information encoded in tissue architecture. The 3-D
5 culture was used in mesenchymal stem cells in that MSC spheroids display enhanced
6 differentiation capability compared to 2-D culture [31, 32]. We established a 3-D
7 spheroid model by seeding mock- and KSHV-infected PDLSCs in a low attachment
8 condition. As time went by, PDLSCs formed a decentralized network, and then
9 numerous small aggregates progressively assembled into a single central spheroid
10 (Figure 2D). Once aggregated, the spheroid did not change in size but was generally
11 compacted. The expression spectrum of mesenchymal and endothelial cell markers in
12 mock- and KSHV-infected PDLSC spheroids was examined by IFA. As shown in
13 Figure 2E, the endothelial markers PDPN and CD31 were strongly induced, and
14 mesenchymal marker TAGLN was decreased in KSHV-PDLSC spheroids compared
15 with control spheroids. But PDGFRA expression remained unchanged between mock-
16 and KSHV-infected PDLSC spheroids. The transcription of mesenchymal and
17 endothelial markers in 2-D culture and 3D spheroids of KSHV-PDLSCs was
18 compared using RT-qPCR, and results showed that *PDPN*, *ICAM*, *PROX1*, *CD31*, and
19 *VEGFA* were dramatically up-regulated in 3-D spheroids, confirming the occurrence
20 of MEndT in KSHV-PDLSC spheroids (Figure 2F). Overall, the 3-D organotypic
21 culture provides the mimic function of living tissue allowing the development of
22 KSHV-infected MSCs into hybrid M/E cells closely resembling the spindle cells in

1 KS lesions. The result demonstrated that KSHV infection of MSCs sufficiently
2 induces MEndT (or incomplete MEndT), generating hybrid M/E KS-like cells in the
3 3-D environment.

4 **KSHV-induced MEndT leads to incomplete endothelial differentiation**

5 Then, we asked if KSHV-induced hybrid M/E state cells have acquired functional
6 characteristics of endothelial cells in addition to expressing endothelial markers. To
7 this end, we evaluated KSHV-infected PDLSCs for their endothelial characteristics.
8 Matrigel tubule formation assay was carried out for the acquisition of endothelial and
9 angiogenesis property and showed that KSHV-infected PDLSCs exhibited increased
10 ability to form capillary-like structures in comparison to mock-infected PDLSCs
11 (Figure 3A). Uptake of acetylated low-density lipoprotein (Ac-LDL) is a hallmark of
12 endothelial cells and macrophages [33]. KSHV-infected PDLSCs were found to
13 possess an Ac-LDL uptake capacity similar to HUVECs (Figure 3B). KSHV induced
14 a notable outgrowth in KSHV-infected PDLSC spheroids whereas rare sprouting was
15 observed in mock-infected PDLSCs (Figure 3C). Interestingly, KSHV-infected
16 PDLSCs spontaneously formed many vessel-like structures in the 3D spheroids, but
17 such structures were not seen in mock-infected PDLSC spheroids (Figure 3D).
18 Immunofluorescence staining of these lumens showed the expression of
19 pan-endothelial marker CD31 in KSHV-infected PDLSC spheroids but not in control
20 spheroids (Figure 3E).

1 To investigate the endothelial differentiation grade of KSHV-infected PDLSCs *in vivo*,
2 KSHV-infected PDLSCs mixed with Matrigel were injected into C57BL/6 mice.
3 Seven days later, the matrigel plugs were striped. Immunohistochemistry analysis
4 using antibodies against CD34 (mature and immature blood vessel marker), hCD105
5 (immature blood vessel marker), and hCD31 (mature blood vessel marker),
6 respectively, showed enhanced staining for CD34, hCD105, and hCD31 in the
7 Matrigel plug from the mice transplanted with KSHV-infected PDLSCs (Figure 3F).
8 Meanwhile, an enhanced number of leaky blood vessels and neo-vascular cavity were
9 observed in KSHV-infected PDLSC plugs, suggesting that a fraction of
10 KSHV-infected PDLSCs can differentiate into immature and mature vessels, and may
11 also induce mouse endothelial cells to participate in neovascularization to form leaky
12 vessels. Weibel–Palade bodies (WPBs) are endothelial cell specialized organelles. We
13 checked whether KSHV-infected PDLSCs displayed WPBs using a transmission
14 electron microscope. Weibel–Palade bodies were not observed in both
15 KSHV-infected PDLSCs and uninfected control PDLSCs (Figure 3G). Taken together,
16 our findings indicate that KSHV infection can reprogram MSCs to acquire endothelial
17 markers and functions through MEndT. However, KSHV-induced endothelial lineage
18 differentiation is incomplete as cells retain specific mesenchymal markers and lack
19 endothelial cell specialized organelles Weibel–Palade bodies (WPBs), exhibiting
20 striking resemblance to KS spindle cells [8].

21 **Characterization of KSHV-infected MSCs residing in intermediate hybrid M/E** 22 **state**

1 To characterize KSHV-infected MSCs residing in different phenotypic states along
2 the mesenchymal–endothelial spectrum, we used mesenchymal (PDGFRA) and
3 lymphatic endothelial (PDPN) double markers to track the KSHV-mediated MEndT
4 program. The PDGFRA/PDPN profiles of mock- and KSHV-infected PDLSCs were
5 analyzed by flow cytometry (Figure 4A). LECs were included as a reference. PDLSCs
6 were positive for PDGFRA but very few expressed PDPN, whereas LECs were
7 PDGFRA-negative and PDPN-positive. KSHV infection of MSCs generated a small
8 percentage (5%) of PDGFRA- PDPN+ (xE) cells, 60% of PDGFRA+ PDPN+ (M/E
9 hybrid) cells and 35% of PDGFRA+ PDPN- (xM) cells. Considering the KSHV
10 infection rate was between 70-80%, most KSHV-infected MSCs were believed to be
11 converted to M/E and xE cells. Similarly, the KSHV-infected PDLSCs in 3-D culture
12 spheroids generated a large number of M/E hybrid state cells and rare xE state cells
13 (Figure 4B).

14 To characterize these subpopulations, we isolated xM, hybrid M/E, and xE cells from
15 KSHV-infected PDLSCs using PDGFRA/PDPN antigen marker combination and
16 tracked their M and E status during propagation *in vitro*. Highly pure xM (PDGFRA+
17 PDPN-) and hybrid M/E (PDGFRA+ PDPN+) were collected, and the status of xM
18 and hybrid M/E cells were found sustainable after ten days culture (Figure 4C),
19 indicating both xM and M/E subpopulation were low-plastic and resided stably in an
20 intermediate phenotypic state *in vitro*. These xM and M/E subpopulations were
21 characterized for their mesenchymal and endothelial status by Western blot,
22 RT-qPCR, and immunofluorescence (IFA) analyses (Figure 4D-F). Hybrid M/E state

1 cells, as expected, displayed a mixture of mesenchymal and endothelial traits,
2 including the simultaneous expression of PDGFRA and PDPN together with other M
3 and E markers. Some mesenchymal cytoskeletal markers (COL1A1, TAGLN, and
4 α SAM) were down-regulated in M/E cells compared to xM cells in mRNA and
5 protein levels, revealed by RT-qPCR, Western blot, and IFA. On the other hand,
6 levels of several endothelial markers (PROX1, VCAM, EDNRA, and ICAM) were
7 significantly elevated in M/E cells relative to xM cells (Figure 4D–F).

8 **Hybrid M/E state cells exhibit highest oncogenic properties and the potential to** 9 **form KS-like tumors after ectopic transplantation**

10 The striking resemblance between KS spindle cells and KSHV-mediated M/E state of
11 MSCs suggests that KS originates from KSHV-infected MSCs, and the M/E state
12 cells have acquired KS tumorigenic properties during the mesenchymal-to-endothelial
13 transition driven by KSHV. To confirm this speculation, we examined the M/E and
14 xM state cells for their tumorigenic potentials including malignant transformation,
15 migration/invasion, and angiogenesis. Soft agar colony formation assay, the standard
16 tumorigenicity test, was used to evaluate cellular anchorage-independent growth
17 under a low-nutrient and -oxygen microenvironment [34, 35]. Mock- and
18 KSHV-infected PDLSCs, as well as xM and hybrid M/E cells isolated from
19 KSHV-infected PDLSCs, were subjected to colony forming assay. KSHV-infected
20 PDLSCs produced seven colonies, but mock-infected PDLSCs failed to develop any

1 visible colonies. Hybrid M/E state cells formed more colonies than xM state cells
2 (Figure 5A).

3 It was reported that KSHV infection of MSC increases its migration and invasion
4 capability, which was proposed to be responsible for the tendency of KS occurring in
5 injured or inflamed sites of the body [36]. To assess the migration ability of distinct
6 phenotypic state cells, mock- and KSHV-infected PDLSC spheroids were seeded on
7 adherent culture plates, and observed for spindle-shaped cells migrating from the
8 spheroids. We found that more KSHV-infected PDLSCs moved away from their
9 spheroids than mock-infected spheroids. When they were plated on nonadherent
10 surfaces, no migration was observed with both mock- and KSHV-infected spheroids
11 (Figure 5B). Then the migrating cells were analyzed for PDPN/PDGFRα expression
12 profile to determine their MEndT status. Results showed that the migrating cells were
13 mainly hybrid M/E state cells (Figure 5C). The invasion abilities of KSHV-PDLSC
14 and M/E cells were assayed using a transwell apparatus as illustrated in Figure 5D.
15 Cells that permeated the Matrigel and reached the other side of the membrane were
16 analyzed by flow cytometry. The number of migrated cells from KSHV-infected
17 PDLSCs spheroids was significantly greater than mock-infected spheroids (Figure
18 5E). The invaded cells were almost entirely hybrid M/E state cells (Figure 5F). In
19 addition, we compared migration and invasion properties of M/E hybrid and xM state
20 cells using transwell assays. The result showed that KSHV infection enhanced
21 PDLSC's migration and invasion ability, and hybrid M/E state cells have a higher
22 capacity to migrate and invade than xM state cells (Figure 5G). Furthermore,

1 angiogenic capabilities of hybrid M/E and xM were also analyzed using a Matrigel
2 tubulogenesis assay, and hybrid M/E cells exhibited a higher ability to form
3 capillary-like structures in Matrigel stroma than xM cells (Figure 5H).

4 Then we evaluated the capability of KSHV-infected PDLSCs in forming KS-like
5 tumors *in vivo* using a 3D spheroids ectopic transplantation model (Figure 6A). Mock-
6 or KSHV-infected PDLSC spheroids embedded within the scaffold were transplanted
7 under the kidney capsule of immunocompromised mice. Kidneys were harvested after
8 four weeks for immunohistochemical analysis (Figure 6B). Hematoxylin and eosin
9 staining showed spindle-shaped cells, abundant new vessel networks, slit-like
10 vascular spaces with leaky erythrocytes, haemosiderin accumulation, and
11 mononuclear cell infiltrates in the region of inoculation of KSHV-infected MSC
12 spheroids, reminiscent of typical characteristics in KS lesions. In contrast, these tumor
13 properties were not seen in mock-infected MSC spheroids transplant (Figure 6C). The
14 sections were stained with antibody against KSHV antigen LANA and proliferation
15 marker Ki-67 and results showed that the KSHV-infected graft spindle-shaped cells
16 expressed LANA and Ki-67 (Figure 6D).

17 The mock- and KSHV-infected PDLSC implants were subjected to triple IFA for
18 mesenchymal and endothelial markers with antibodies against LANA, PDGFRA,
19 PDPN or LANA, Nestin, CD31. The result showed that cells in KSHV-infected
20 PDLSC implants co-expressed PDGFRA and PDPN, as well as Nestin and CD31,
21 while mock-infected implants expressed very low levels of PDPN and CD31 (Figure

6E). The triple IFA results closely resemble those observed in KS lesions (Figure 1A) and strongly suggest that KSHV-infected MSCs can be transformed into KS-like cells through MEndT.

vFLIP plays a crucial role in the acquisition of hybrid M/E state and tumorigenesis

To understand how KSHV promotes MEndT and what viral gene products involves in the process to generate M/E state cells, a class of viral genes, including K1, vIL-6, RTA, K8, PAN, K12, vFLIP, v-Cyclin, LANA, and vGPCR, were ectopically expressed in PDLSCs by transducing their lentiviral vectors. Among these viral genes, vFLIP was found to be able to induce the expression of endothelial markers PDPN, ICAM, and VEGFA (Figure 7A). The expression of vFLIP in PDLSCs also notably increased the proportion of hybrid M/E state (PDGFRA⁺ /PDPN⁺) cells (Figure 7B). In addition, immunofluorescence staining revealed the upregulation of endothelial marker PDPN in vFLIP expressing cells compared to control PDLSCs (Figure 7C). Furthermore, vFLIP expression enhanced the angiogenesis of PDLSCs (Figure 7D). In 3D organotypic cultures, vFLIP-expressing PDLSCs spheroids generated a large number of hybrid M/E state cells (Figure 7E). vFLIP- expressing PDLSCs and control spheroids were transplanted into nude mice under the kidney capsule. Hematoxylin and eosin staining showed that spindle-shaped cells, microvessels containing red blood cells, and infiltration of the inflammatory cells were frequently observed in the grafting region of vFLIP-spheroids rather than control spheroids (Figure 7F).

1 Meanwhile, vFLIP- expressing PDLSC and control implants were stained for
2 PDGFRA and PDPN, and results showed vFLIP- expressing PDLSC implants
3 co-expressed PDGFRA and PDPN, while control implants did not express PDPN
4 (Figure 7G).

5 A "loss-of-function" approach was used to verify the contribution of vFLIP to
6 initiating MEndT and generating hybrid M/E status. The expression of vFLIP was
7 silenced using a CRISPR/Cas9-mediated gene knockout with a double-gRNA
8 lentivirus in KSHV-infected PDLSCs [37]. A series of paired gRNAs were designed
9 targeting the flanking regions of the ORF71 gene to create ORF71 knockout (KO)
10 (Figure 8A). Satisfied knockout efficiency was achieved with gRNAs 2 and 3, which
11 removed ORF71 from the KSHV genome in KSHV-infected PDLSCs (Figure 8B and
12 C). The ORF71 KO mutant KSHV was analyzed for its MEndT capability in PDLSCs.
13 We found that PDGFRA+PDPN+ cells were notably reduced in vFLIP-knockout cells,
14 suggesting that vFLIP KO prevented KSHV from inducing the MEndT process
15 (Figure 8D). In 3D organotypic cultures, vFLIP-KO KSHV failed to induce PDPN
16 expression in KSHV-PDLSCs spheroids (Figure 8E). Then we examined if
17 vFLIP-KO affects the oncogenic properties of KSHV-infected PDLSCs, including
18 malignant transformation, migration/invasion, and angiogenesis properties. Colony
19 forming assays were performed and results showed a significant reduction in the
20 number of colonies from vFLIP-knockout KSHV-infected PDLSCs compared with
21 control KSHV-infected PDLSCs (Figure 8F). 3D migration assay were carried out,
22 and results showed that vFLIP knockout resulted in fewer cells moved away from

1 spheroid in KSHV-PDLSCs. The number of migrated cells from KSHV-infected
2 PDLSCs spheroids was significantly less than wild-type virus-infected spheroids in a
3 3D spheroid transwell assay (Figure 8G). A tubule formation assay showed decreased
4 angiogenesis with vFLIP-knockout KSHV-infected PDLSCs (Figure 8H).

5 The contribution of vFLIP in promoting MEndT and tumorigenesis was evaluated *in*
6 *vivo* using mice kidney capsule implant model. vFLIP- knockout and control
7 KSHV-infected PDLSC spheroids were transplanted into the kidney capsule. The
8 transplants were harvested at four weeks post-transplantation and analyzed by
9 Hematoxylin and eosin staining. The result showed spindle-shaped cells, slit-like
10 vessels containing red blood cells, and erythrocytes diapedesis in the implants of
11 wild-type KSHV-infected PDLSC spheroids, while these tumor properties were not
12 seen in vFLIP- knockout KSHV-infected PDLSC spheroids transplants (Figure 8I).

13 The sections were subjected to triple IFA with antibodies against LANA, PDGFRA,
14 and PDPN. Results showed that deletion of vFLIP completely abolished
15 KSHV-induced MEndT and the generation of hybrid M/E state cells *in vivo* (Figure
16 8J). Taken together, these results indicated that KSHV-encoded vFLIP is crucial in
17 the generation of M/E hybrid state cells and tumorigenesis via MEndT in MSCs.

18 **Discussion**

19 Prior studies have suggested that KS may arise from KSHV-infected
20 mesenchymal stem cells (MSCs) through an MEndT process [10-12]. In this study,
21 we investigated the MEndT process in KSHV-infected MSCs and characterized

1 distinct differentiation subpopulations along the mesenchymal-to-endothelial
2 spectrum. We found that KSHV promotes incomplete endothelial differentiation of
3 MSCs to generate mesenchymal/endothelial (M/E) hybrid state cells, and the hybrid
4 M/E cells acquired highest tumorigenic properties *in vitro* and *in vivo*. This finding
5 faithfully recapitulates Kaposi's sarcoma where proliferating KS spindle cells and the
6 cells that line KS-specific aberrant vessels are also found to exhibit the hybrid M/E
7 state. Furthermore, we revealed that KSHV-encoded viral FLICE inhibitory protein
8 (vFLIP) plays a crucial role in promoting MEndT and the generation of M/E state
9 cells. These findings provide a new layer of evidence for MSCs being the cell source
10 of KS spindle cells and reveal novel insight into KS pathogenesis and viral
11 tumorigenesis.

12 Kaposi's sarcoma is a multifocal neoplasm characterized by proliferating
13 KSHV-infected spindle-shaped tumor cells, aberrant capillaries, and infiltration of
14 inflammatory cells. KS spindle cells exhibit extraordinarily heterogenic populations
15 revealed by a variety of cell markers, including vascular and lymphatic endothelial,
16 mesenchymal, smooth muscle, and hematopoietic precursor markers [38]. The origin
17 of the spindle-shaped KS cell lineage remains contentious. Currently the most widely
18 accepted theory is that KS cells may derive from the endothelial cell lineage [39]. The
19 other models for KS cell origin include that KSHV infects circulating endothelial
20 progenitor cells [40, 41] or multi-potential mesenchymal stem cells [11, 42] and
21 drives differentiation of these cells towards KS phenotypes. Recently, we and others
22 found a series of evidence that favors the model of KS originating from mesenchymal

1 stem cells [11]. Both endothelial and mesenchymal models agree on that KS spindle
2 cells reside in an intermediate phenotypic state along the mesenchymal-endothelial
3 spectrum, which is associated with malignant phenotypes such as tumor initiation,
4 migration and evasion. The endothelial origin model suggests that terminally
5 differentiated lymphatic endothelial cells can convert into KS precursors through an
6 endothelial-to-mesenchymal transition (EndMT) [28], while mesenchymal origin
7 model proposes that KSHV-infected MSCs undergo a mesenchymal-to-endothelial
8 transition (MEndT) process to acquire KS malignant phenotypes [11]. In this study,
9 we demonstrated that KSHV infection of MSCs initiates an incomplete MEndT
10 process and generates highly oncogenic hybrid M/E state cells, which recapitulates
11 KS that comprises hybrid M/E state spindle-shaped tumor cells. The robust
12 recapitulation of KS in our *in vitro* and *in vivo* mouse model strongly supports the
13 theory that KSHV-infected mesenchymal stem cells are the cellular origin of KS.
14 Although there exists a possibility that hybrid M/E KS can arise from both
15 undifferentiated MSCs through MEndT or terminally differentiated lymphatic
16 endothelial cells (LEC) through EndMT, of which both lead to sarcomagenesis, we
17 failed to prove that KSHV-infection could render LEC the M/E hybrid state in cell
18 culture model. In particular, KSHV infection of LEC could not induce PDGFRA and
19 Nestin expression, failing to recapitulate the KS phenotype (data not shown).

20 One of the signature characteristics of KS is their abundant neovascularity, which
21 present clinically as red-purple lesions. Neovascularity in KS begins at very early
22 stage (patch stage) prior to establishment of a mass. The KS-specialized new vessels

1 are prone to leakage of fluid and extravasation of red blood cells [23]. KSHV-infected
2 MSCs, implanted in mice under kidney capsule, were observed to proliferate and form
3 slit-like vascular spaces that contain erythrocytes, resembling to KS abnormal vessels.
4 More interestingly, in both kidney capsule implant and KS lesions, the abnormal
5 vessels were found to be made of hybrid M/E state (PDGFRA+PDPN+) cells,
6 suggesting that KSHV-infected MSC implants in mice accurately recapitulate KS in
7 neovascularity property. Why does KS aberrant neoangiogenesis form leaky
8 capillaries? Our finding may provide an explanation that KS specialized irregular
9 jagged vessel are lined by LANA positive cells with hybrid M/E phenotype derived
10 from KSHV-infected MSCs, unlike normal blood or lymphatic vessels that are
11 composed of blood or lymphatic endothelial cells plus a thin layer of vascular smooth
12 muscles. Abnormal neoangiogenesis is also observed in other solid tumors. In a
13 process termed “vascular mimicry” (VM), which has been found in breast cancer,
14 melanoma and nasopharyngeal carcinoma, tumors create their own, tumor cell-lined
15 channels for fluid and blood transport [43]. In light of that both KS aberrant vessels
16 and VM are poorly formed by tumor cells and leaking red blood cells and fluid, KS
17 tumor cell-lined vessels can be considered as a new type of vascular mimicry and may
18 have similar pathological roles as VM in tumor development and metastasis.

19 MEndT, like its reverse process EndMT, is a process of multiple and dynamic
20 transition and generate distinct phenotypes including mesenchymal-like (xM),
21 endothelial-like (xE), and hybrid M/E states. KSHV-induced MEndT appears to be an
22 incomplete differentiation process that accumulates hybrid M/E state cells as the

majority of population and allows a small percentage of cells progress to endothelial-like (xE) state. These heterogeneities, to some degree, reflect the complex phenotypes of KS tumor cells that is not encountered in normal tissues [44]. Moreover, the hybrid M/E state cells display greatest tumorigenic ability including malignant transformation, angiogenesis, migration, and invasiveness compared to other subpopulations, and form KS-like lesions in kidney capsule transplantation. Interestingly, as epithelial or carcinoma cells progress toward high-grade malignancy, they often activate epithelial-to-mesenchymal transition (EMT). It has been reported recently that in breast cancer and nasopharyngeal carcinoma, the cells residing in intermediate hybrid E/M (bearing both epithelial and mesenchymal cell markers) state along the epithelial-to-mesenchymal spectrum exhibit the highest tumorigenicity [20]. Therefore, it is likely that acquisition of a hybrid E/M state, regardless of MEndT or EMT process, is essential for tumorigenicity of both carcinoma and sarcoma.

KSHV possesses two life cycles, latent and lytic. Although both latent and lytic cycles contribute to Kaposi's sarcomagenesis [45, 46], the KSHV mode of infection in KS lesions is predominantly latent and the expression of latent genes (i.e., LANA, vCyclin and vFLIP) is sufficient in viral genomic persistence and cell transformation [47]. In this study, we found that vFLIP plays a crucial role in initiating MEndT and the generation of hybrid M/E cells. FLIP family proteins are known to be inhibitors of death receptor (DR)–induced apoptosis [48, 49]. Previous studies revealed a role for vFLIP in binding to I κ B kinase γ (IKK γ), thereby inducing NF- κ B activation [50-52]. Furthermore, the pathogenic role of vFLIP has been explored in mice by expressing it

1 in B cells and endothelial cells. When expressed in B cells, vFLIP was found to
2 induce B cell transdifferentiation resulting in expansion of the macrophage/DC
3 compartment [53]. When expressed in endothelial cells, mice developed pathological
4 abnormalities with appearance of elongated spindle-like cells, profound
5 proinflammatory phenotype, and expansion of myeloid cells. Although no skin
6 KS-like lesion was observed on these mice, this study has shown that some
7 characteristics of KS can be induced by vFLIP [54]. Our current study showed that
8 the expression of vFLIP in mesenchymal stem cells resulted in acquisition of KS
9 characteristics including spindle-shaped cells and aberrant neovascularity through
10 initiating MEndT and generation of M/E state cells. This study revealed the
11 mechanism of KSHV transforming MSCs to KS tumors through MEndT and
12 demonstrated the central role of hybrid M/E state cells in KS tumorigenesis.

13 **Materials and Methods**

14 **Ethics statement and study approval**

15 The collection of human samples and the use of periodontal ligament stem cells
16 (PDLSCs) in our research were approved by the Medical Ethics Review Board of Sun
17 Yat-sen University (approval no. 2015-028). Written informed consent was provided
18 by study participants. The animal experiments in this study were approved by Animal
19 Ethics Review Board of Sun Yat-sen University (approval no. SYSU-IACUC-
20 2020-000317) and carried out strictly following the Guidance suggestion of caring

laboratory animals, published by the Ministry of Science and Technology of the People's Republic of China.

Cell culture

Periodontal ligament stem cells (PDLSCs) were isolated from the periodontal ligament tissues (cells from 5 individuals were pooled to offset individual differences) and maintained in alpha minimal essential medium (α -MEM, GIBCO Life Technologies) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 200 mM L-glutamine (Sigma) and antibiotics (HyClone). Human dermal lymphatic endothelial cells (LECs, ScienCell) were cultured in endothelial cell medium (ECM, ScienCell) plus supplements (ECGS, ScienCell). Human umbilical vein endothelial cells (HUVEC) were purchased from the China Center for Type Culture Collection (CCTCC, China) and cultured in endothelial cell growth medium 2 BulletKit (ScienCell). Human embryonic kidney (HEK) 293T cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were cultured in a humidified 5% CO₂ atmosphere at 37°C.

KSHV production and infection

iSLK.219 cells (kindly provided by Dr. Ke Lan of Wuhan University) were induced for KSHV reactivation by treating with 1 mg/mL doxycycline and 1 mmol/L sodium butyrate for 5 days. The culture supernatants were filtered through a 0.45- μ m filter and centrifuged at 100,000 g for 1 h. The rKSHV.219 pellet was resuspended in 1x

1 PBS in 1/100 volume and stored at -80°C until use. Virus infection was carried out as
2 previous procedure [11].

3 **Spheroid generation**

4 Mock- and KSHV-infected PDLSCs were seeded in non-adherent 96-well plates
5 pre-coated with 0.5% agarose at 15,000 to 20,000 cells per well. The spheroids were
6 grown at 37°C up to 4 days in a humidified atmosphere with 5% CO₂. Spheroid
7 generation in non-adherent plates was captured using ZEISS microscope. To collect
8 spheroids, the media containing the spheroids was transferred to 15 ml conical tube,
9 then washed twice with PBS, and centrifuged at 1000 rpm for 5 min. For histology
10 analysis, the spheroids were collected following by agarose pre-embedding.

11 **Tubule formation assay**

12 96-well plates were coated with Matrigel (BD Biosciences, 354234) and incubated at
13 37°C for 30 min for solidification. Cells were plated onto Matrigel-coated wells at a
14 density of 8×10^4 cells/ well with 100 µl αMEM without FBS, and incubated at 37°C
15 with 5% CO₂ for 8 h. The images of tube formation were captured using a ZEISS
16 fluorescence microscope and analyzed with NIH Image J software.

17 **AcLDL uptake assay**

18 To assess the ability of cells to take up acLDL, PDLSCs were starved overnight, and
19 then 4 µg/ml Dil-acLDL (Yeasen Biotechnology, Shanghai, 20606ES76) was added

1 to the medium. After 4h culture, cells were washed three times with PBS, fixed, and
2 analyzed under a ZEISS fluorescence microscope.

3 **Spheroid sprouting assay**

4 Mock- and KSHV-infected PDLSCs were seeded in non-adherent 96-well plates
5 pre-coated with 0.5% agarose at 2,000 cells per well. After 12-18 hours incubation,
6 the spheroids were harvested and mixed with Matrigel. The mixture was placed in a
7 48-well plate and incubated at 37°C for 1h for solidification, followed by adding
8 medium. Four days later, the sprouting of the spheroids was observed under a ZEISS
9 microscope and recorded.

10 **Flow cytometry analysis and cell sorting**

11 Cells were detached from plates with 0.25% trypsin and washed once with 1xPBS.
12 Then the cells were fixed with 4% paraformaldehyde for 20 min and incubated with
13 APC-anti-human PDPN (eBioscience, 17-9381-42), and PE-anti-human PDGFRA
14 (Sino Biological, 10556-MM02-P) for 30 min at 4°C. After washing with PBS, cells
15 were subjected to flow cytometry analysis. Data were analyzed using CytExpert or
16 FlowJo softwares.

17 To sort xM, M/E and xE state cells, KSHV-infected PDLSC were trypsinized and
18 resuspended in ice-cold 1xPBS containing 2% FBS at 1×10^7 cells per 100 μ l.
19 PDGFRA-PE and PDPN-APC antibodies in 1:50 dilution were added into
20 suspensions. GFP-positive cells were sorted for the first round, and

1 PDGFRA+/PDPN- or PDGFRA+ /PDPN+ cells were isolated by 20% cutoffs for the
2 second rounds of sorting. Isolated cells were allowed to culture for no more than two
3 weeks.

4 **Cell migration/invasion assay**

5 Cell migration and invasion assays were performed using 24-well transwell chambers
6 with filter membranes 12 µm pore size (Millipore Corporation, PIXP01250). Cells
7 were detached with trypsin-EDTA, washed once with 1xPBS, and then resuspended
8 in serum-free medium. 3×10^4 cells were placed in transwell insert, and the medium
9 containing 20% FBS was added to the lower chamber. After 24h incubation,
10 non-migration cells were removed with a cotton swab. The migrated cells were
11 stained and counted under a ZEISS microscope. The cell invasion assay was
12 performed by following the same procedures as cell migration assay except that
13 transwell inserts were precoated with cooled Matrigel (BD Biosciences).

14 For 3D spheroids migration assay, the spheroids were collected, washed with 1xPBS,
15 and resuspended in serum-free medium. 5-10 spheroids were seeded in adherent
16 24-well plates or nonadherent 24-well plates precoated with 0.5% agarose. After 48h
17 incubation, images were captured under a ZEISS microscope. To detect the status of
18 migrated cell away from spheroid, the spheroids were detached by Ophthalmic
19 forceps, and the remaining migrated cell were collected for flow cytometric analysis
20 after staining with PDGFRA-APC and PDPN-PE antibodies.

1 3D spheroids invasion assay was performed using 24-well transwell chambers with
2 filter membranes 12 μ m pore size. 5-10 Spheroids resuspended in serum-free medium
3 were seeded in an insert that has been coated with Matrigel (BD Biosciences). Lower
4 compartment was added with medium containing 20% FBS. Plates were then
5 incubated at 37°C for 48h to allow cells to migrate. Non-migration cells were
6 removed with a cotton swab. The migrated cells were stained and counted under a
7 ZEISS microscope. To detect the status of invaded cell away from spheroids, cells on
8 the lower side of the membrane were collected for flow cytometric analysis after
9 staining with PDGFRA-APC and PDPN-PE antibodies.

10 **Soft agar colony formation**

11 To determine cellular tumor transformation activity, 24-well plates coated with 0.5%
12 agarose medium were prepared. After the agar is solidified, a total of 2,000 cells,
13 suspended in α MEM medium supplemented with 0.3% agarose and 20% FBS, were
14 seeded onto the soft agar coated 24-wells. Cells in soft agar were incubated at 37°C in
15 a 5% CO₂ incubator for 3-4 weeks, and fresh culture medium was added to each well
16 every 3 to 4 days. Colonies larger than the average size of control colonies were
17 counted.

18 **Western blotting**

19 Cell lysates were prepared as previously described [11]. Whole cell extracts of 30 μ g
20 protein were resolved in SDS-PAGE and transferred onto nitrocellulose membranes.
21 The membranes were blocked with 5% non-fat milk/PBS for 30 min and incubated

1 with primary antibodies as follows: anti-PDGFR α (Cell Signaling, 3174T),
 2 anti-COL1A1 (Proteintech, 67288-1-Ig), anti-ACTA2 (α -SMA, ABclonal, A7248),
 3 anti-SM22 (TAGLN, Proteintech, 10493-1-AP), anti-PROX1 (Boster, BA2390),
 4 anti-PDPN (Proteintech, 11629-1-AP), anti-VEGF-A (Immunoway, YT5108),
 5 anti-VCAM (Proteintech, 11444-1-AP), anti-ETAR (EDNRA, Santa Cruz,
 6 sc-135902), anti-ICAM (Proteintech, 10831-1-AP), anti- β -actin (Sigma, A5441).
 7 Anti-IR Dye 800 or Dye 680 anti-rabbit or anti-mouse IgG antibodies (LI-COR
 8 Biosciences) were used as the secondary antibodies. An Odyssey system (LI-COR
 9 Biosciences) was used for detection of proteins of interest.

10 **RT-qPCR**

11 Total RNA was extracted by Ultrapure RNA Kit (CW BIO, CW0581) in accordance
 12 with manufacture's instructions. cDNA was synthesized by reverse transcription.
 13 cDNA was diluted 5 times and subjected to real-time PCR using LightCycler 480
 14 SYBR Green I Master (Roche) with specific primers for the genes of interest.
 15 GAPDH gene was used for calibration. The primer sequences used for RT-qPCR are
 16 listed in Table 1. All real-time PCR was done in triplicate.

17 **Matrigel plug assay**

18 To evaluate the contribution of KSHV-infected PDLSC in angiogenesis ex vivo,
 19 female C57BL/6J mice (4–6 weeks old, n = 5) were purchased from the Animal
 20 Center of Sun Yat-sen University. 5-10 $\times 10^6$ cells in 100-200 μ l medium were
 21 thoroughly mixed with 500 μ l Matrigel, and subsequently engrafted to animals by

1 inguinal injection. At day 7 post-engraftment, mice were sacrificed and Matrigel
2 plugs were removed. The plugs were fixed in 4% paraformaldehyde and embedded in
3 paraffin. The tissue sections were stained with CD34, hCD105 and hCD31 to
4 visualize vascularization.

5 **Kidney capsule transplantation**

6 Spheroids were collected and washed once with 1xPBS. Approximate 100 spheroids
7 were placed on a single $3 \times 2 \times 2$ -mm sterile gelfoam scaffold to culture for 1 day in
8 medium. Recipient female nude mice (6–8 weeks old, n=3-5) were weighed and
9 anesthetized with isoflurane by strictly following the animal care Guidelines. Via
10 flank incisions, kidneys were exteriorized and a small incision was made in the renal
11 capsule. Spheroids/scaffold/pellets were placed under the renal capsule, and the
12 wound was stitched up. The kidney capsule grafts were harvested 28 days after
13 transplantation.

14 **Plasmids**

15 The KSHV genes K1, vIL6, RTA, K8, PAN, K12, LANA, vCyclin and vGPCR were
16 amplified by PCR using the cDNA prepared from iSLK.219 as templates and cloned
17 into the Bgl II and EcoR I restriction sites of the pMSCV-puro lentiviral vector
18 (Addgene plasmid # 68469). vFLIP were cloned by inserting its PCR fragment into
19 the modified pMSCV-puro-3HA vector at Bgl II and Xho I restriction sites. All these
20 constructs were confirmed by DNA sequencing. Lentivirus was produced by

1 co-transfection of 293T cells with expression vector pMSCV and PIK packaging
2 plasmid at a 1:1 ratio.

3 **CRISPR-Cas9-mediated knock out of KSHV vFLIP expression**

4 The gRNAs targeting the 5' and 3' regions of vFLIP were designed by an online
5 CRISPR design tool (<http://crispr.mit.edu>) using the KSHV genomic sequences
6 (NCBI Reference Sequence: NC_009333.1). The gRNA sequences were subcloned
7 into the BsmBI restriction sites of CRISPR/Cas9 vectors lentiCRISPR v2 (Addgene
8 plasmid # 52961). Lentivirus was produced by triple transfection of 293T cells with
9 the sgRNA expression LentiCRISPR-v2 vector and the packaging plasmids psPAX2
10 (Addgene plasmid # 12260) and pMD2.G (Addgene plasmid # 12259) at a ratio of
11 5:3:2. PDLSCs were transduced with two gRNA/Cas9-expressing lentivirus, one for 5'
12 regions and the other for 3' regions of vFLIP gene, followed by puromycin selection
13 for 1 week. The selected PDLSCs acquired the ability to knock out viral vFLIP when
14 KSHV infects the PDLSC.

15 To verify ORF71 KO efficiency, total DNA was isolated using a HiPure Tissue DNA
16 Mini kit (Magen, D3121) in accordance to manufacture's instruction, and analyzed by
17 PCR using PrimeSTAR (TaKaRa, R040A). The primers used for monitoring the
18 efficiency of vFLIP knockout are Pr1 (ACCCTGCGTAAACAACCG) and Pr2
19 (ACCCAAAGACTGGCTCAT). The relative mRNA level of ORF71 was also
20 determined by RT-qPCR using specific KSHV ORF71 primers 5'-

1 GGATGCCCTAATGTCAATGC -3' (forward) and 5'-

2 GGCGATAGTGTGGGAGTGT -3' (reverse).

3 **Immunofluorescence analysis**

4 Cells were grown on glass-coverslip overnight, fixed with 4% paraformaldehyde for

5 15 min, washed with 1xPBS and permeabilized in 0.1% Triton X-100 for 15 min.

6 After blocking with 1% BSA for 30 min, the samples were incubated with primary

7 antibodies: anti-LANA (Abcam, ab4103), anti-SM22 (TAGLN, Proteintech,

8 10493-1-AP), anti-PDPN (Proteintech, 11629-1-AP), anti-vWF (Proteintech,

9 11778-1-AP), anti-CD31 (Proteintech, 11265-1-AP), anti-VCAM (Proteintech,

10 11444-1-AP). After washing, samples were incubated with secondary antibody

11 Donkey anti-Rabbit IgG Alexa Fluor 555 (Life, A-31572) or Goat anti-Rat IgG Alexa

12 Fluor 555 (Life, A-21434) for 1 hour. Images were taken using a ZEISS microscope.

13 The same procedure was used for immunofluorescence analysis of paraffin-embedded

14 3D spheroids, except that 3D spheroids were first de-paraffinized, rehydrated and

15 antigen retrieval.

16 Triple immunostaining for KS tissues or 3D spheroids implant sections was

17 performed using a mouse anti-PDGFRα (Immunoway, 4G11, YM3688) or

18 anti-Nestin (Santa Cruz, sc-23927) antibody, a rabbit anti-PDPN (Proteintech,

19 11629-1-AP) or anti-CD31 (Proteintech, 11265-1-AP) antibody and a rat anti-LANA

20 antibody (Abcam, ab4103). Anti- rabbit IgG Alexa 647- (Invitrogen, A-21244), Anti-

21 mouse IgG Alexa 488- (Invitrogen, A-28175) and Anti- rat IgG Alexa 555 (Invitrogen,

1 A-21434)-conjugated antibodies were used for secondary antibodies. Nuclei were
2 counterstained with Hoechst 33342 (Sigma). For each KS specimen, the number of
3 cells was counted from DAPI+ nuclei from 4-8 pictures of representative areas (100 ×
4 120 μm) by using AxioScan Z1. A total of 246 to 724 cells were manually counted
5 from every study specimen. The percentage of Nestin/ CD31 or PDGFRA/PDPN
6 single positive, double positive or double negative cells in LANA-positive were
7 counted on 4-8 successive fields from per specimen manually.

8 **Immunohistochemistry analysis**

9 KS tissues, Matrigel plug or 3D spheroids implant sections were deparaffinated and
10 rehydrated. After antigen retrieval with 0.1M citrate buffer (pH 6.0), Sections were
11 block with 5% BSA for 30 min. The sections were incubated with primary antibodies:
12 anti-LANA (Abcam, ab4103), anti-CD34 (Proteintech, 14486-1-AP), anti-human
13 CD105 (Proteintech, 67075-1-Ig), anti- human CD31 (PECAM-1, Immunoway,
14 PT0035), anti-Ki-67 (Cell Signaling, 9129). DAB super sensitive reactivity system
15 (Maxim Biotechnology, Fujian, KIT-9720) was used for antigen detection.

16 **Statistical analysis**

17 Statistical analyses were performed by two-tailed Student's t-test using GraphPad
18 prism 8.0 (GraphPad Software, Inc.), or the Mann-Whitney U test in IBM SPSS
19 Statistics 18 software to determine the statistical differences between the experimental
20 and control groups. P < 0.05 was considered statistically significant. *P<0.05,

1 **P<0.01 and ***P<0.001; NS, not significant (P > 0.05). Data were graphed as mean
2 ± SEM.

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Figure 1. Co-expression of mesenchymal and endothelial markers in KS tissue.

(A) Representative immunofluorescence images of AIDS-KS lesion tissues (lower) and their adjacent normal skin tissues (upper) stained with PDGFRA or Nestin (green), PDPN or CD31 (red), and LANA (yellow) antibodies. The nuclei were counterstained with Hoechst 33342 (blue). Higher magnifications of the white boxed areas are also showed underneath. Scale bars in the original images represent 100 μ m, and in the enlarged images represent 20 μ m. **(B)** Co-localization of mesenchymal (Nestin and PDGFRA), endothelial (PDPN and CD31), and KSHV markers (LANA) in KS early lesions (patch and plaque) and late lesions (nodular). Boxed areas are enlarged. Scale bar, 50 μ m. **(C)** Percentage of LANA, PDGFRA, PDPN, Nestin and CD31 positive cells in 4–8 successive fields composed mostly of spindle tumor cells and vessels in KS early (n=7 samples) or late lesions (n=5 samples). **(D)** Spearman's test shows correlation between LANA expression and cells positive for PDPN in early and late KS tumors. **(E)** Percentage of Nestin-/CD31-, Nestin+/CD31-, Nestin+/CD31+, Nestin-/CD31+ and PDGFRA-/PDPN-, PDGFRA+/PDPN-, PDGFRA+/PDPN+, PDGFRA-/PDPN+ cells in LANA+ spindle tumor cells in early or late KS lesions. **(F)** and **(G)** The difference in morphology and structure between normal vessels **(F)** and KS specialized vessels **(G)**. Higher magnifications of the black boxed areas are shown underneath. Scale bar, 200 μ m (upper), 50 μ m (lower). **(H)** and **(I)** Expression patterns of mesenchymal and endothelial markers in normal **(H)** and KS abnormal vessels **(I)**. Samples were stained with antibodies against PDGFRA/Nestin (green), PDPN/CD31 (red), and LANA (yellow), and the nuclei were counterstained with Hoechst 33342

- 1 (blue). Scale bar, 50 μ m. Error bars represent mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$,
- 2 *** $p < 0.001$; the Mann-Whitney U test.
- 3

Figure 2. KSHV infection initiates a differentiation process converting MSCs

from mesenchymal phenotype to M/E hybrid phenotype. (A) Cell lysates from

mock- and KSHV-infected PDLSCs at indicated time points were immunoblotted for

PDGFRα, COL1A1, α-SMA, TAGLN, PROX1, PDPN, VEGFA and β-actin. **(B)**

Relative mRNA levels of mesenchymal and endothelial related genes in mock- and

KSHV-infected PDLSCs (K-PDLSCs) after 4 days infection. **(C)** Mock- and

KSHV-infected PDLSCs were immunostained for LANA, TAGLN, PDPN, vWF and

CD31 at 4 days post-infection. Scale bar, 50 μm. **(D)** A time course of K-PDLSC

aggregating to form spheroid in no-adherent plate. Scale bar, 500 μm. **(E)** Expression

1 of LANA, TAGLN, PDPN, vWF and CD31 in mock- and KSHV-infected PDLSC
 2 spheroid. Scale bar, 50 μ m. (F) The mRNA expression level of *TAGLN*, *α -SAM*,
 3 *Nestin*, *PDGFRA*, *PDPN*, *ICAM*, *PROX1*, *CD31*, and *VEGFA* was analyzed by RT-
 4 qPCR in K-PDLSC spheroids in comparison with their parallel 2D culture.
 5

Figure 3. Hybrid M/E state is enriched through KSHV-mediated MEndT and displays partial endothelial characteristics. (A) Tubule formation assays were performed with mock- and KSHV-infected PDLSCs (K-PDLSCs). Scale bar, 500 μ m. (B) Mock- and KSHV-infected PDLSCs were incubated with DiI-acLDL for 4 hours. DiI-acLDL uptake (red), as well as KSHV-GFP infection (green), were analyzed with a fluorescence microscope. Scale bar, 100 μ m. (C) Mock- and KSHV-infected PDLSC spheroids were embedded into Matrigel, and the sprouting length was analyzed by a Zeiss fluorescence microscope. Scale bar, 200 μ m. (D) H&E staining of

1 PDLSC and K-PDLSC spheroid sections. Scale bar, 50 μ m. **(E)** The expression of
2 CD31 in the spheroids of PDLSC and K-PDLSC was detected by IFA. Scale bar, 50
3 μ m. **(F)** Immunohistochemical analysis of Matrigel plug of mock- and
4 KSHV-infected PDLSCs from implanted mice for CD34, hCD105 and hCD31. Scale
5 bar, 50 μ m. **G**, Mock- and KSHV-infected PDLSCs, along with HUVECs, were
6 examined for Weibel–Palade bodies (WPBs) under transmission electron microscope.
7 Scale bar, 1 μ m. Original magnification, $\times 2$ (enlarged insets).

8

Figure 4. KSHV-induced hybrid M/E state cells were characterized as an intermediate state in mesenchymal-to-endothelial differentiation spectrum. (A)

Mock- and KSHV-infected PDLSCs, and LECs were examined for PDGFRA and PDPN expression profile by flow cytometry analysis. Three subpopulations (xM, hybrid M/E and xE) were quantification based on the PDGFRA and PDPN profiles ($n = 3$ independent experiments). Statistical analyses were performed using two-tailed Student's test, and P-values were calculated by GraphPad Prism. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (B) PDGFRA and PDPN expression in PDLSC and K-PDLSC spheroids were analyzed by IFA. Scale bar, 50 μ m. (C) K-PDLSCs were stained for PDGFRA and PDPN and sorted by flow cytometry. The purified xM and M/E populations were cultured for 10 days and their PDGFRA/PDPN profiles were examined for their phenotypic plasticity. (D) Western blot analysis of xM, M/E and

1 LECs for their mesenchymal and endothelial markers. (E) The expression profiles of
 2 mesenchymal and endothelial markers in hybrid M/E and xM state cells were
 3 analyzed at mRNA level by RT-qPCR. (F) IFA analysis of LANA, TAGLN and
 4 VCAM in xM and M/E state cells. Scale bar, 50 μ m.
 5

Figure 5. KSHV endows PDLSCs with tumorigenic Properties and M/E exhibits the highest tumorigenicity. (A) Soft agar colony formation assay to determine anchorage-independent cell growth in PDLSCs, K-PDLSCs, xM and M/E state cells. Scale bar, 200 μ m. **(B)** Images of PDLSC and K-PDLSC spheroids 48h after transferring onto nonadherent or adherent plates. Scale bar, 200 μ m. **(C)** FASC analysis of the migrated cells detached from K-PDLSC spheroids for PDGFRA/PDPN profiles. **(D)** Illustration of spheroids Transwell invasion assay. **(E)** Quantitation of the number of invaded cells from PDLSC and K-PDLSC spheroids. **(F)** PDPN/PDGFRA expression profiles of the invaded cells from K-PDLSC spheroids. **(G)** Transwell migration and invasion assays of PDLSC, K-PDLSC, xM and M/E state cells. Quantitation of cell migration and invasion was shown on the right. **(H)** Tubule formation assays were performed with xM and M/E cells. HUVECs were included as a positive control. Error bars represent mean $\pm$ SEM. n = 3 (unless otherwise indicated). Statistical analyses were performed using two-tailed Student's test. *p < 0.05, **p < 0.01, ***p < 0.001.

2 **Figure 6. Ectopic transplantation of PDLSC and K-PDLSC spheroids in mice**
3 **under kidney capsule. (A)** Schematic diagram illustrating the process of
4 transplanting PDLSC and K-PDLSC spheroids under renal capsule of a nude mouse
5 kidney. **(B)** The kidneys transplanted with PDLSC and K-PDLSC spheroids were
6 harvested after 28 days. ($n = 3-4$ mice for each group). **(C)** Representative images of

- 1 H&E staining of PDLSC and K-PDLSC spheroids transplants. Scale bar, 200 μ m. **(D)**
- 2 IHC staining of PDLSC and K-PDLSC spheroids transplants for LANA and Ki-67. **(E)**
- 3 Immunofluorescent overview of PDGFRA, PDPN, Nestin, CD31 and LANA
- 4 expression in PDLSC and K-PDLSC spheroids under the renal capsule. Scale bar, 50
- 5 μ m.
- 6

Figure 7. vFLIP promotes MEndT of MSCs and enhances formation of hybrid M/E state cells and tumorigenesis. (A) Expression of PDGFRA, PDPN, ICAM and VEGFA in PDLSCs transduced with their expression vector was analyzed by Western blotting. **(B)** vFLIP-, vCyclin- and LANA-expressing PDLSCs were examined for the proportion of hybrid M/E state cells using FASC. **(C)** Immunofluorescence staining of

1 vFLIP-expressing PDLSCs for PDPN expression. Scale bar, 20 μm . **(D)**

2 vFLIP-expressing PDLSCs were subjected to a tubule formation assays. Scale bar,

3 200 μm . **(E)** vFLIP-expressing PDLSC spheroids were examined by IFA for

4 PDGFRA/PDPN expression profiles. Scale bar, 50 μm . **(F)** Representative images of

5 H&E staining of PDLSC and vFLIP-PDLSC spheroids transplanted in mice under

6 kidney capsule. Scale bar, 100 μm (upper) and 20 μm (lower). ($n = 3-4$ mice for each

7 group). **(G)** Immunofluorescent overview of PDGFRA/PDPN expression in PDLSC

8 and vFLIP-PDLSC spheroids under renal capsule. Scale bar, 50 μm .

9

Figure 8. Silencing of vFLIP expression results in abolishment of KSHV-induced

MEndT and tumorigenesis. (A) Schematic diagram depicting the CRISPR/Cas9

editing sites in the KSHV genome. The target sites of gRNAs and binding sites of

specific primers (Pr1 and Pr2) are indicated with arrows. **(B)** The

CRISPR/Cas9-mediated knockout efficiency of ORF71 (vFLIP) gene from the KSHV

genome in KSHV-infected PDLSCs was verified by PCR. **(C)** The vFLIP expression

in ORF71 knockout cells was determined by RT-qPCR. **(D)** The effect of vFLIP

knockout (vFLIP-KO) on the generation of hybrid M/E state cells was illustrated and

quantitated by flow cytometry. **(E)** The effect of vFLIP-KO on the expression of

PDGFRA and PDPN was examined with K-PDLSC spheroids by IFA. Scale bar, 50

μm. **(F)** The effect of vFLIP-KO on malignant transformation ability was examined

using colony formation assay. Scale bar, 200 μm. **(G)** Images of WT and vFLIP-KO

KSHV-infected PDLSC spheroids showing the effect of vFLIP-KO on cell migration

capability. Scale bar, 200 μm. **(H)** The effect of vFLIP-KO on angiogenic ability of

K-PDLSCs was assayed by tubule-formation assay. Scale bar, 200 μm. **(I)**

Representative images of H&E staining of WT and vFLIP-KO KSHV-infected

PDLSC spheroid transplants in mice under kidney capsule. Scale bar, 200 μm. Higher

magnifications of the black boxes are shown on the right. Scale bar, 50 μm. (*n* = 3–4

mice for each group). **(J)** Immunofluorescence assay of WT and vFLIP-KO

KSHV-infected PDLSC transplants for LANA, PDGFRA and PDPN. Scale bar, 50

μm. **(K)** Schematic model for the role of KSHV vFLIP in promoting MEndT to

generate hybrid M/E state cells for KS tumorigenesis and aberrant angiogenesis. Error

- 1 bars represent mean $\pm$ SEM. n = 3, unless otherwise indicated. Statistical analyses
- 2 were performed using two-tailed Student's test. *p < 0.05, **p < 0.01, ***p < 0.001.

1 **Table 1.** Primers used for reverse-transcription real-time quantitative PCR

Gene	Forward (5' – 3')	Reverse (5' – 3')
TAGLN	AGTGCAGTCCAAAATCGAGAAG	CTTGCTCAGAATCACGCCAT
α -SAM	GTGTTGCCCCTGAAGAGCAT	GCTGGGACATTGAAAGTCTCA
Nestin	CAACAGCGACGGAGGTCTC	GCCTCTACGCTCTCTTCTTTGA
PDGFRA	TGGCAGTACCCCATGTCTGAA	CCAAGACCGTCACAAAAAGGC
PDPN	AACCAGCGAAGACCGCTATAA	CGAATGCCTGTTACACTGTTGA
ICAM	ATGCCCAGACATCTGTGTCC	GGGGTCTCTATGCCCAACAA
PROX1	TGACAGCACTGATTCGGAAAATG	ATAGGCCATCAAGGGAAGGG
CD31	AACAGTGTTGACATGAAGAGCC	TGTAAAACAGCACGTCATCCTT
VEGFA	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
COL1A1	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
CNN1	GAACGTGGGAGTGAAGTACGC	CAGCCCAATGATGTTCCGC
CD36	GGCTGTGACCGGAACTGTG	AGGTCTCCAACCTGGCATTAGAA
VCAM	GGGAAGATGGTCGTGATCCTT	TCTGGGGTGGTCTCGATTTTA
vFLIP	GGATGCCCTAATGTCAATGC	GGCGATAGTGTTGGGAGTGT
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

2