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ORIGINAL ARTICLE

Phenotype transition of fibroblasts incorporated into patient-derived oral carcinoma organoids

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Abstract

Objective: Cancer-associated fibroblasts (CAFs) are abundantly infiltrated in oral squamous cell carcinoma (OSCC), but the contact-dependent mechanisms that regulate CAFs phenotype in precursor cells, such as paracancerous fibroblasts (PFs), remain unclear. Here, a fibroblast-attached organoid (FAO) model was initiated to determine phenotype transition of fibroblasts triggered by contact with OSCC.

Material and methods: Organoids and fibroblasts were generated using OSCC and adjacent tissues. Cell-clusters containing fibroblasts and tumour cells were aggregated to allow for FAOs expansion. Immunoblotting assay was performed to compare expression of Notch intracellular domain (NICD) in CAFs and PFs. Colony formation assay was employed to evaluate morphological activation of fibroblasts.

Results: Compared to traditional 3D co-culture, FAOs better modulated the spatial distribution of fibroblasts with tumour nests. The presence of CAFs with multiple branches was stably observed in FAOs during serial passage. Incorporation with organoids promoted the ability of PFs to form multiple branches. Immunoblotting assay confirmed higher NICD level in CAFs than PFs. Treatment with Notch inhibitor, N-[N-(3, 5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (i.e. DAPT) blocked morphological activation of fibroblasts incorporated into FAO.

Conclusion: We developed a robust strategy to study contact-dependent mechanisms underlying tumour-stromal interaction, and suggested that Notch activity contributes to biogenesis of OSCC-associated fibroblasts.

KEYWORDS
cell-matrix interactions, fibroblasts activation, notch activity, oral squamous cell carcinoma, patient-derived organoid

1 | INTRODUCTION

The phenotype transition of fibroblasts, particularly those in the oral cavity, plays a vital role in tissue regeneration, hyperplasia of pre-malignant lesions and development of cancer (Chen & Song, 2019; Li et al., 2018; Sahai et al., 2020; Vescarelli et al., 2017). In the case of patients with oral squamous cell carcinoma (OSCC), extensive studies have reported that the crosstalk between tumour cells and activated fibroblasts in cancer (i.e. cancer-associated fibroblasts, CAFs) may contribute to tumour growth, relapse and metastasis, providing therapeutic value for targeting CAFs in the tumour microenvironment (TME) (Jiang et al., 2019; Li et al., 2018; Zhao et al., 2021). However, the mechanisms by which the CAF phenotypes in precursor cells (e.g. paracancerous fibroblasts, PFs) are triggered

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and maintained remain unclear (Sahai et al., 2020). In addition, the identification of inter- and intra-patient heterogeneity in CAF biogenesis, which implicates the diversity of tumour-stromal interplay, further challenged the studies based on traditional pre-clinical models, such as the 2D co-culture of fibroblasts with cancer cell lines (Öhlund et al., 2017; Su et al., 2018).

Organoids are novel 3D culture systems that possess patient-specific characteristics (Tuveson & Clevers, 2019). Recent advancements in organoid-based studies have boosted the use of precision medicine for the treatment of patients with cancer (Li & Izpisua Belmonte, 2019; Vlachogiannis et al., 2018). Organoid technology can largely improve the efficiency of primary culture and simultaneously preserve the genotype and phenotype of parental tumours (Drost & Clevers, 2018). Importantly, the integration of organoids with cancer-associated stromal cells, such as fibroblasts, endothelial cells and immune cells, is assumed to better modulate the human TME (Tsai et al., 2018). However, the primitive co-culture of tumour and stromal cells in Matrigel, a classical protocol to employ the organoid expansion, is not sufficient to stably model and/or manipulate the spatial distribution of stromal cells with tumour nests (Öhlund et al., 2017; Sahai et al., 2020). In latest study, of note, it is unveiled that distinct CAFs phenotype may be induced by different kinds of tumour-CAF precursor cells interaction, in which the juxtacrine signalling give rise to myofibroblastic features in pancreatic stellate cells (PSCs), while the paracrine signalling only stimulates secretory activity (Öhlund et al., 2017). To better study such intratumour heterogeneity in CAF biogenesis, modifications are still required for patient-derived organoid technologies.

Physical contact-induced pathways, such as Notch activity, have been reported to promote the abundance of CAFs in breast carcinoma (Strell et al., 2019). In this study, fibroblasts were generated from tumour (i.e. CAFs) and para-tumour specimens (i.e. PFs) of patients with OSCC. Using a fibroblasts-attached-organoid model that robustly mimics the spatial distribution of fibroblasts with tumour nests, we further investigated whether Notch activity is involved in activation of fibroblasts incorporated into OSCC organoids.

2 | MATERIALS AND METHODS

2.1 | Generation of fibroblast-attached organoids (FAOs)

The clinical information of the relevant samples has been described in our previous study (Zhao et al., 2021). Primary cultures of fibroblasts and organoids were performed according to previously described protocols (Jiang et al., 2019; Zhao et al., 2021). For the generation of fibroblast-OSCC cell clusters, the single fibroblasts and OSCC cells were mixed at the indicated ratio (5:1, 3:1, 1:1, 1:3 and 1:5), and then resuspended in Dulbecco's modified Eagle medium (DMEM)/F12 (Thermo Fisher Scientific, Inc.) in an ultra-low attached plate (Corning), and after 48 h, the spheroid colonies with diameter >50 μm were defined as clusters. For the visualization of cells in clusters, the empty vector lentivirus that drives the expression of the green fluorescent protein (GFP) or mCherry (Genechem, Inc.) was stably transfected into OSCC cells or fibroblasts, and the mixture of cells in the cluster was authenticated under a fluorescence microscope (TRRFM; Olympus Corp.). For the generation of FAOs, the fibroblast-OSCC cell clusters were collected and embedded in Matrigel (BD Biosciences, USA) at the indicated intensity (200 clusters/30 μl matrigel/well), and then incubated with DMEM/F12 supplemented with 1× N2, 1× B27, 50 ng/ml human epidermal growth factor (EGF), and 500 nmol/L A83-01 (all purchased from Sigma-Aldrich); after 7–10 days, the organoid colonies attached to spindle-shaped cells were defined as FAOs, and the incorporation of fibroblasts into organoids was authenticated under a fluorescence microscope; FAOs forming efficiency (%) = scored FAOs number/total embedding fibroblast − OSCC cell clusters.

2.2 | Immunofluorescence and immunoblotting assays

Immunofluorescence staining and immunoblotting assays were performed according to previously described protocols (Jiang et al., 2019; Zhao et al., 2021). To isolate organoid-derived cell lysates for immunoblotting assay, the cell recovery solution (Corning, 354253) was used to dissociate matrigel according to the manufacture's instructions. The following antibodies were used for immunofluorescence at the indicated dilution (1:100): vimentin (VIM, CST#5741S), alpha-smooth muscle actin (α-SMA, Abcam #ab7817), fibroblast-specific protein1 (FSP1, Proteintech#16105-1-AP), fibroblast activation protein-α (FAP, ABclonal#A6349) and platelet-derived growth factor receptor α (PDGFRα, CST#3174). The following antibodies were used for immunoblotting: Notch intracellular domain (NICD, CST#4147T) and c-Myc (CST#18583).

2.3 | Matrigel-based colony formation assay

Primary CAFs or PFs were generated as previously described (Jiang et al., 2019). For the colony formation assay, single fibroblasts were seeded in Matrigel (BD Biosciences) at the indicated dosage (500 cells/30 μl matrigel/well) and incubated with DMEM (Thermo Fisher Scientific, Inc.) containing 10% foetal bovine serum and 1% penicillin/streptomycin. After 7–10 days, the cells with elongated branches (>20 μm) were counted under a bright phase microscope (TRRFM; Olympus Corp.) and defined as activated fibroblasts (Öhlund et al., 2017), and the colonies with more than 5 activated fibroblasts were scored to calculate the colony formation efficiency (number of fibroblasts/seeding cells number).

2.4 | Conditional medium experiments

Condition medium (CM) was collected after incubation with OSCC organoids and then filtered through a 0.45-μm filter to remove the cell debris for subsequent experiments. Similar dosage and ratio of

fibroblasts and tumour cells were employed for FAO generation as well as the tumour CM treatment assay. The incubation time used for tumour CM generation was based on the observation period when the multiple branches fibroblasts in FAO were presented. For preliminary identification of tumour CM, the secreted transforming growth factor-beta 1 (TGF-β1) in CM generated from OSCC organoids was determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (NeoBioscience) according to the manufacturer's instructions.

2.5 | Lentivirus transfection

To obtain cherry-labelled fibroblasts and GFP-labelled organoids, the lentiviruses containing U6-multiple cloning sites (MCS)-Ubiquitin-Cherry-internal ribosome entry site (IRES)-puromycin and hU6-MCS-Ubiquitin-enhanced green fluorescent protein (EGFP)-IRES-puromycin were purchased from Genechem company. The stable transfection of cells was conducted according to our previously described protocols (Jiang et al., 2019).

2.6 | Statistical analysis

All experiments were performed in triplicate and repeated at least three times. Data were analysed using the SPSS v.16.0 software (SPSS) and Prism v.5.0 software (GraphPad Software). The results were expressed as the mean ± standard deviation (SD). Differences were considered significant at $p < 0.05$.

3 | RESULTS

3.1 | Morphological activation of fibroblasts generated from OSCC specimens

Fresh tumour and para-tumour specimens of patients with OSCC were processed into single cells for organoid culture and fibroblast generation, according to our previously reported protocol (Zhao et al., 2021). A more invasive structure was observed in organoids derived from tumour samples than in those derived from para-tumour specimens (Figure 1a). Furthermore, immunofluorescence staining confirmed that the expression of a panel of activated fibroblasts markers, including α-SMA, FAP and PDGFRα (Sahai et al., 2020), was higher in fibroblasts generated from tumours (i.e. CAFs) than in paracancerous fibroblasts (i.e. PFs), which were both stained positive for fibroblasts specific and mesenchymal lineage markers, such as FSP1 and vimentin (Figure 1b). Consistently, results of the matrigel-based colony formation assay verified that the CAFs formed more colonies with multiple branch structures than the corresponding PFs (Figure 1c,d), demonstrating a more activated status in CAFs (Öhlund et al., 2017). Interestingly, quantificational analysis showed that the colony efficiency of CAFs gradually decreased during serial passage (Figure 1d), indicating that the interaction with tumour cells may contribute to the morphological activation of fibroblasts. To preliminarily study the paracrine mechanisms underlying tumour-fibroblast interplay, CAFs were incubated with conditional medium generated from tumour organoids (i.e. tumour CM). However, the results showed that treatment with tumour CM did not affect the colony efficiency of CAFs during serial passage (Figure 1d).

3.2 | Generation and characterization of the FAO model

To study the contact-dependent mechanisms, we modified our previously described 3D co-culture system of CAFs with OSCC organoids (Zhao et al., 2021; Figure 2a). Briefly, single CAFs (labelled with mCherry) and OSCC cells (labelled with GFP) were mixed at a 1:1 ratio and suspended in ultra-low attached plates (ULA) for 48 h to generate CAF-OSCC clusters (see methods for more details), which were then embedded in Matrigel to allow for expansion (Figure 2a). Interestingly, a mixture of CAFs and OSCC cells in clusters, as well as the morphological transition of CAFs incorporated into OSCC organoids, were observed according to the bright phase and fluorescence imaging (Figure 2b). We termed these CAF-OSCC cluster-derived organoid colonies as FAOs, as the FAOs stably modelled the attachment relationship of CAFs with tumour organoids when compared to the traditional 3D co-culture system (Zhao et al., 2021). More importantly, the morphological analysis of growing FAOs, combining with the immunohistochemical analysis of α-SMA in corresponding OSCC specimens, partly showed that the FAOs resembled the spatial distributions of CAFs with tumour nests, including but not limited to the surrounding, embedding, dragging and concomitant relationship of them (Figure 2c), which was not observed in the preliminary 3D co-culture system at all (Zhao et al., 2021).

3.3 | Phenotype transition of fibroblasts incorporated into the FAO model

To better study the phenotype transition of fibroblasts during tumour-stromal interplay, the CAFs and OSCC cells were mixed at a 1:1 ratio to perform the serial passage assay either through the co-culture system or the FAO model. As a result, the colony efficiency of CAFs gradually decreased in the co-culture system (Figure 3a,b). Although the CAFs with multiple branches disappeared in the co-culture system at 3rd passage, their presence was stably observed in the FAO model during long-term passage (Figure 3a,c). To test the biological mechanisms that regulate the trigger of the CAF phenotype in precursor cells, the PFs were subjected to several manipulations, including treatment of tumour CM and incorporation into OSCC organoids. The results demonstrated that the treatment of tumour CM did not affect

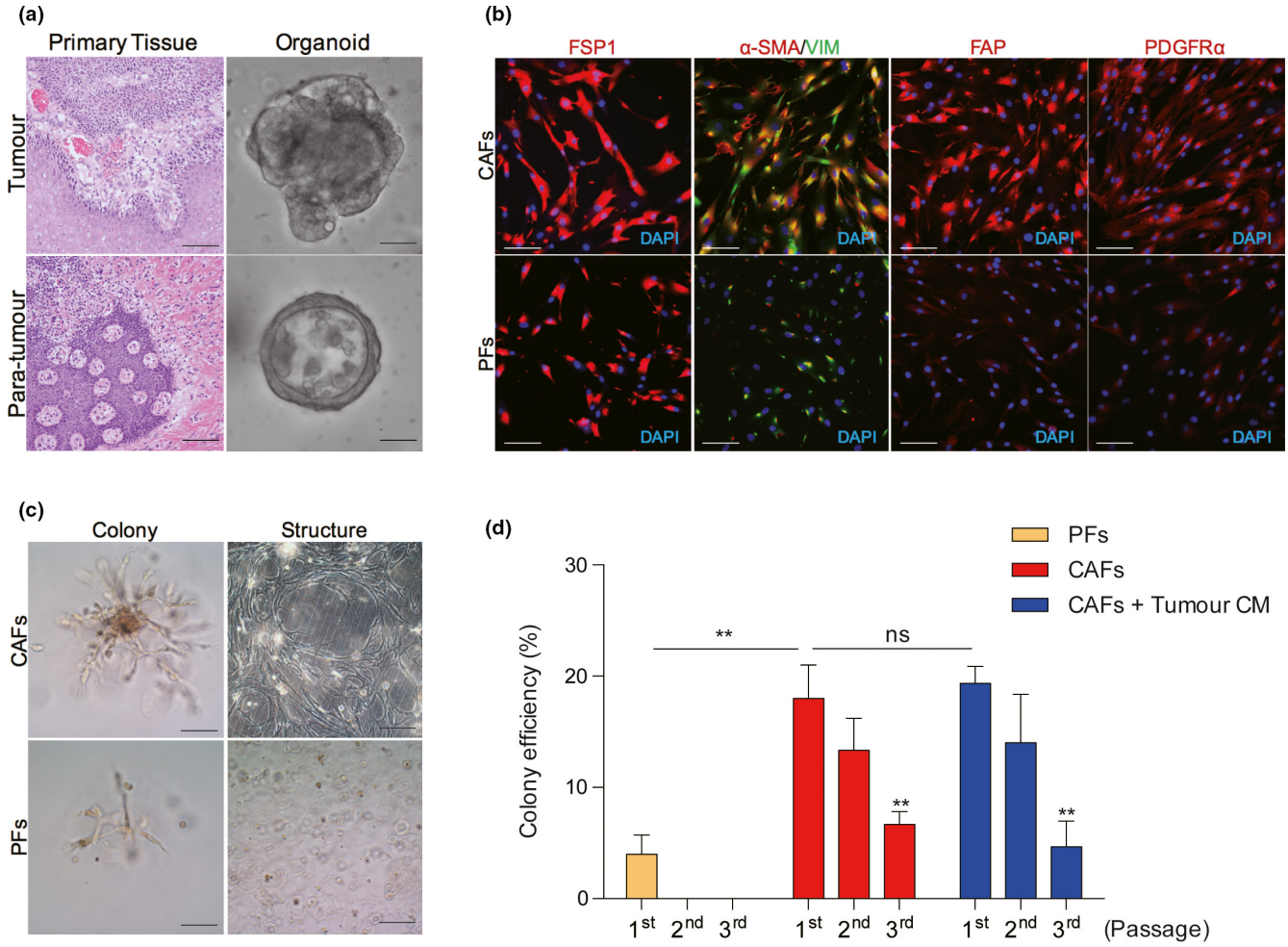
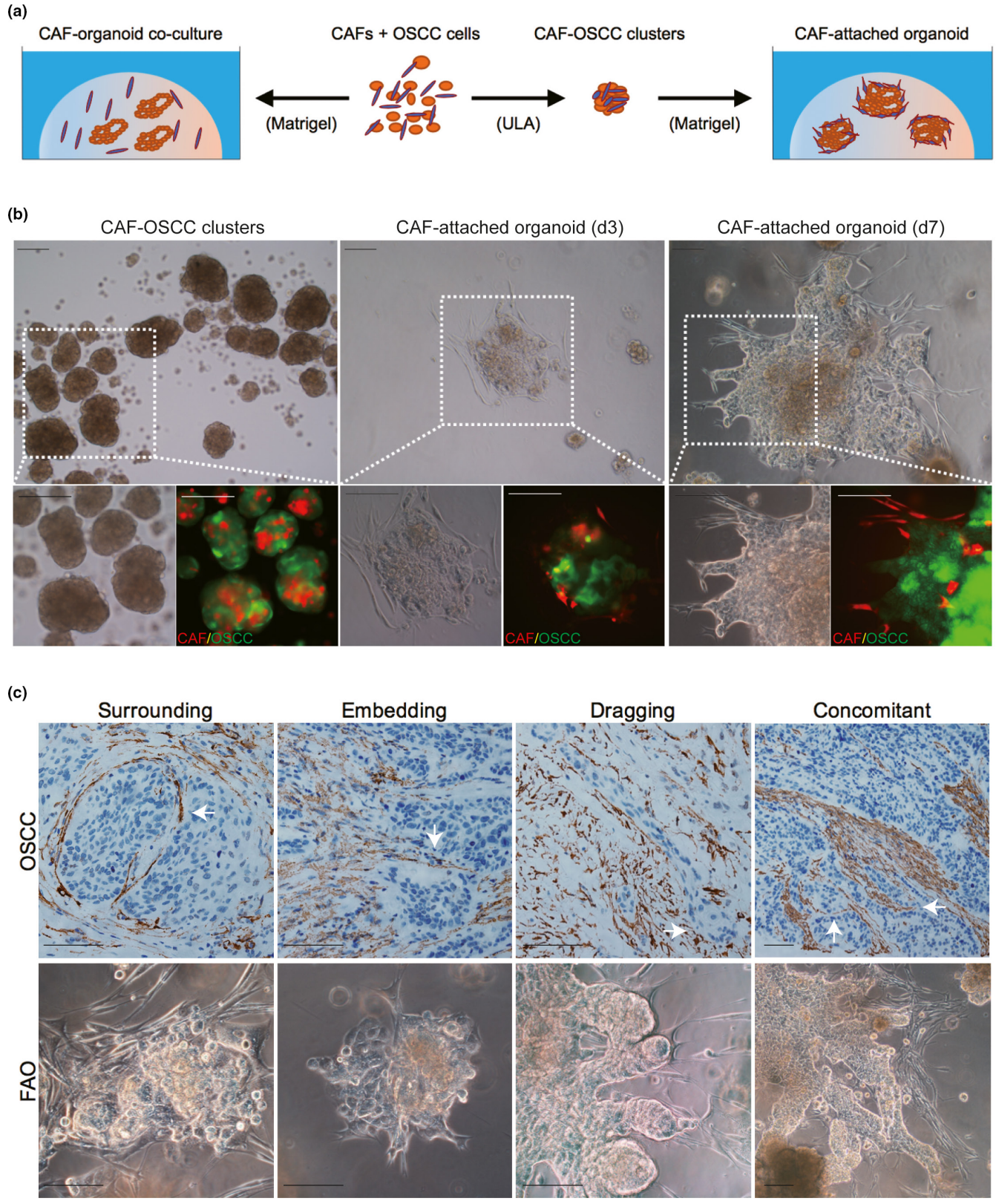


FIGURE 1 Morphological activation of fibroblasts generated from oral squamous cell carcinoma (OSCC) specimens. (a) Representative images show the morphology of tumour and para-tumour specimen-derived organoids. Scale bars = 50 μm. (b) Immunofluorescence staining of alpha-smooth muscle actin (α-SMA), vimentin (VIM), fibroblast activation protein (FAP), fibroblast-specific protein 1 (FSP1) and PDGFRα in fibroblasts generated from tumour (i.e. cancer-associated fibroblasts, CAFs) and para-tumour (i.e. paracancerous fibroblasts, PF) specimens. Scale bars = 50 μm. (c) Representative images show the morphology of CAFs and PFs cultured in the matrigel. Colony (left), single fibroblast-derived colonies were imaged at day 7 after embedding; Scale bars = 50 μm. Structure (right), the multiple branches of fibroblasts were observed and captured at day 14 after embedding; Scale bars = 50 μm. (d) Quantificational analysis of single fibroblast-derived colonies shows the colony formation abilities of PFs and CAFs, as well as CAFs, incubated with the tumour organoid-derived conditional medium (Tumour CM) harvested at day 7 since 5,000 tumour cells were embedded, in which the concentration of TGF-β1 was 40.27 ± 8.85 pg/ml ($n = 3$). $^{**}p < 0.01$; ns, not significant

the morphological transition of PFs (Figure 3d). Otherwise, a multiple branch structure was observed in the PFs incorporated into the OSCC organoid (Figure 3d). Consistently, the expression of α-SMA was increased in PFs incorporated into the OSCC organoid (Figure 3e). Together, these results suggest that, in addition to paracrine signals, some contact-dependent mechanisms may be also involved in the phenotype transition of fibroblasts incorporated into OSCC organoids.

FIGURE 2 Generation and characterization of the fibroblasts-attached-organoid (FAO) model. (a) Schematic shows the key steps to generate the CAF-organoid co-culture system, as well as the CAF-attached organoids. ULA, ultra-low attachment plate. (b) Representative images show the mixture of CAFs and OSCC cells in the CAF-OSCC clusters, and the morphological transition of CAFs and organoids in the CAF-attached organoids at different time points (i.e. day 3 and 7). The CAFs or OSCC cells were transfected with an empty vector lentivirus that drives the expression of mCherry or the green fluorescent protein (GFP). Scale bars = 50 μm. (c) Representative images show the spatial distribution relationship of fibroblasts with organoids in the CAF-attached organoid model (bottom). The immunohistochemical staining images of α-SMA in corresponding OSCC specimens were shown to symbolize the spatial distribution of fibroblasts with tumour nest *in vivo* (top), as indicated by the white arrows; concomitant relationship means that more than two kinds of spatial distribution relationship, including but not limited to the surrounding, embedding and dragging, were presented in the field of observation. Scale bars = 50 μm



3.4 | Notch activity is involved in the activation of fibroblasts attached to OSCC organoid

The contact-induced NOTCH activity is assumed to be correlated with the CAF abundance in tumours (Strell et al., 2019). To evaluate the NOTCH activity in tumour and stromal cells, immunoblotting assays were employed to test the protein levels of the NICD for them (Majumder et al., 2021). The results showed that the expression of NICD was higher in CAFs compared to that in PFs, tumours and para-tumour organoids (Figure 4a). To

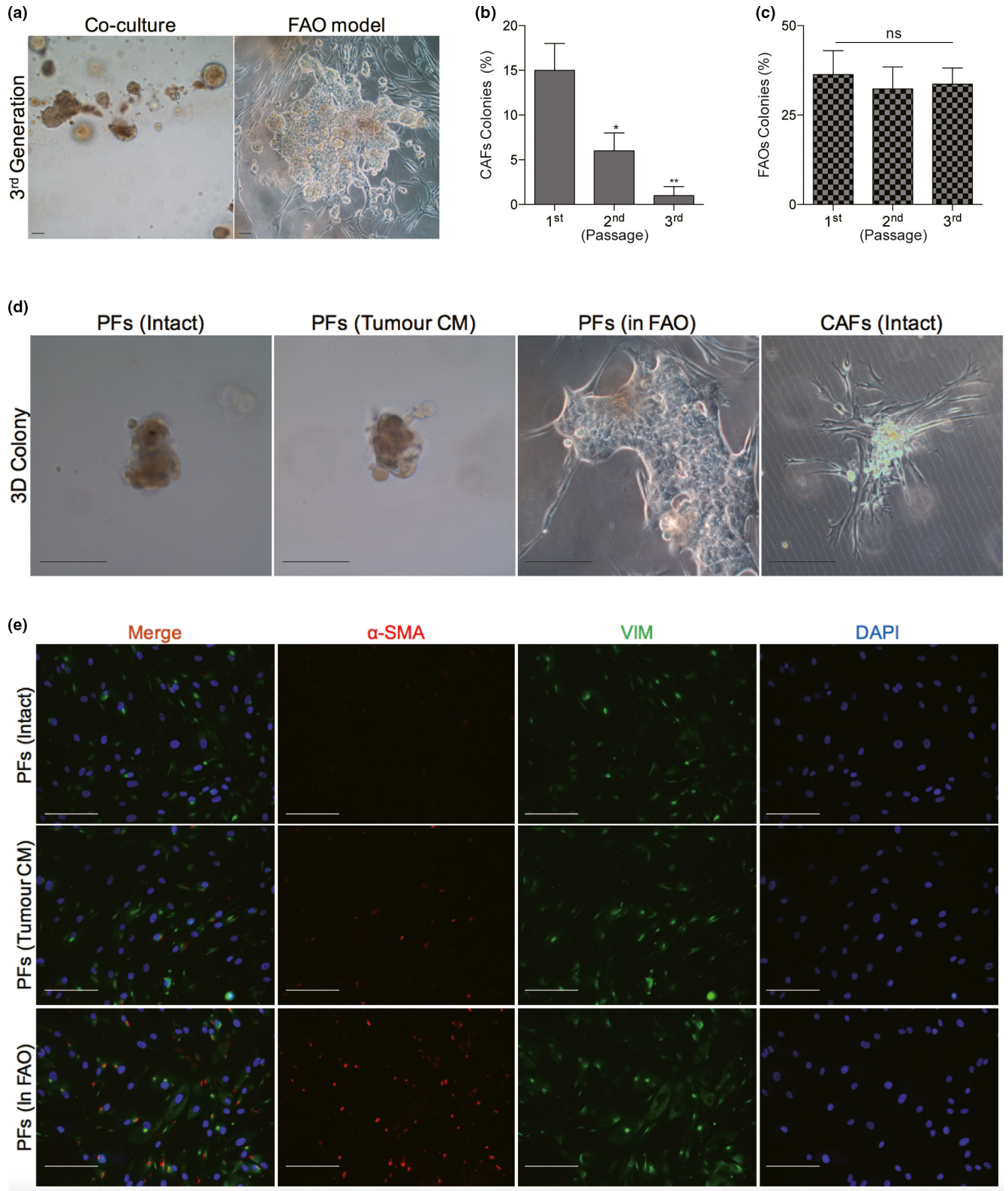


FIGURE 3 Phenotype transition of fibroblasts incorporated into the FAO model. (a) Representative images show the absence (left) or presence (right) of CAFs with multiple branches structure in co-culture system (left) or FAO model (right). Scale bars = 50 μm. (b–c) Quantificational analysis shows the colony formation ability of CAFs either co-cultured with organoids (b) or incorporated into the FAO model (c). $^{*}p < 0.05$; $^{**}p < 0.01$; ns, not significant. (d) Representative images show the structure of PFs cultured intactly, or incubated with tumour CM, in which the concentration of TGF-β1 was 35.22 ± 2.27 pg/ml ($n = 3$), or incorporated into the FAO model. The morphology of CAFs cultured intactly was shown as the positive control of fibroblasts with multiple branches structure. Scale bars = 50 μm. (e) Immunofluorescence staining of α-SMA and VIM in PFs cultured intactly, or incubated with tumour CM, or incorporated into the FAO model. Scale bars = 50 μm

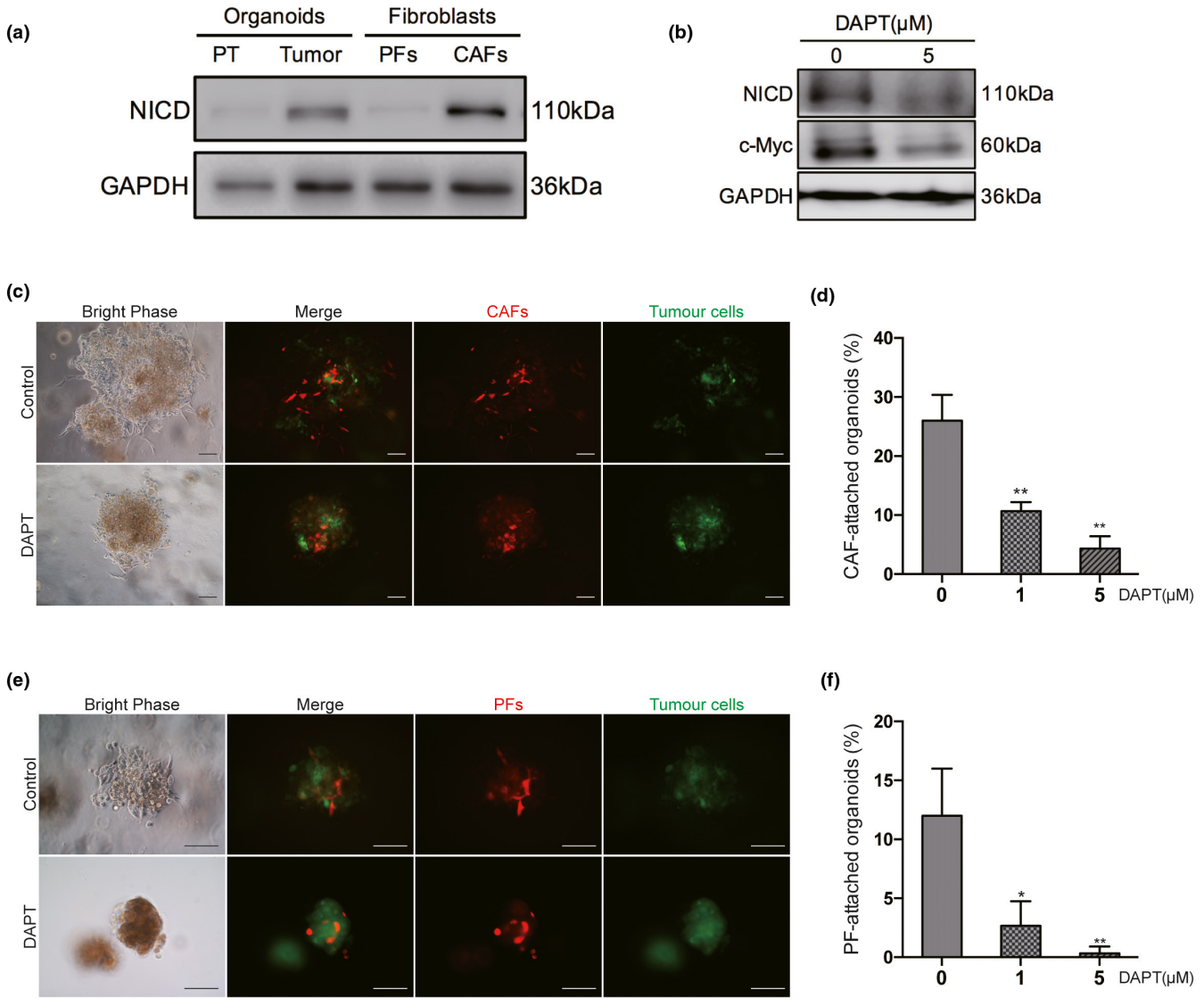


FIGURE 4 Notch activity is involved in the activation of the FAO model. (a) Immunoblotting assay showed the expression of the Notch intracellular domain (NICD) in organoids and fibroblasts generated from tumour or para-tumour specimens; PT, para-tumour; PFs, paracancerous fibroblasts; CAFs, cancer-associated fibroblasts. (b) Immunoblotting assay showed the expression of NICD and c-Myc, a representative NICD targeted genes, in CAFs pre-treated with the NOTCH inhibitor (i.e. DAPT; purchased from MedChemExpress, China) at 5 μM for 48 h. (c) Representative images show the morphology of CAFs pre-treated with DAPT (5 μM) and then incorporated into the OSCC organoids. Scale bars = 50 μm. (d) Quantificational analysis shows that the treatment of DAPT decreased the formation efficiency of CAF-attached organoids. $^{*}p < 0.05$; $^{**}p < 0.01$. (e) Representative images show the morphology of PFs pre-treated with DAPT (5 μM) and then incorporated into the OSCC organoids. Scale bars = 50 μm. (f) Quantificational analysis shows that the treatment of DAPT decreased the formation efficiency of PF-attached organoids. $^{*}p < 0.05$; $^{**}p < 0.01$

verify the role of NOTCH activity in the phenotype transition of fibroblasts, CAFs were pre-treated with a Notch inhibitor, DAPT (Saito et al., 2014; Singh et al., 2021). Results of the immunoblotting assays confirmed that the expression of NICD and c-Myc, a representative NICD-targeted genes, was reduced in fibroblasts treated with DAPT at 5 μM (Figure 4b), which were then incorporated into OSCC organoids. Further morphological and quantificational analysis demonstrated that treatment with DAPT reduced the frequency of CAF with multiple branches in the FAO model (Figure 4c,d). Also, treatment with DAPT impaired the colony efficiency of PF-attached organoids (Figure 4e,f). Collectively, these data suggest that Notch activity is upregulated in CAFs and contributes to their morphological activation.

4 | DISCUSSION

Fibroblasts in and surrounding cancer are increasingly recognized as versatile master regulators that determine the prognosis of patients with tumours, particularly OSCC (Li et al., 2018; Sahai et al., 2020). Coupled with the exploitation of novel experimental technologies, it is presumed that a better understanding of the phenotype transition

in fibroblasts may benefit the development of stromal targeting therapies (Öhlund et al., 2017; Su et al., 2018). In the present study, a fibroblast-attached organoid (FAO) model was initiated, based on the modification of our previously reported 3D co-culture system (Zhao et al., 2021), to study the trigger and maintenance of CAF phenotype in fibroblasts generated from paracancerous (i.e. PFs) and tumour (i.e. CAFs) specimens. Of note, we found that contact-dependent Notch activity may contribute to the activation of fibroblasts incorporated into OSCC organoids.

The characterization of CAFs, even with promising therapeutic value, is surprisingly tricky (Kalluri, 2016). During a long periods of time, the fibroblasts are defined by a combination of their morphology with specific tissue location, lack of lineage markers for other cells, and achievement of mesenchymal cells markers (e.g. vimentin) (Kalluri, 2016; Li et al., 2018; Sahai et al., 2020). In patients with cancer, the precursor cells of CAFs are recruited and infiltrated into the tumour bulk (Sahai et al., 2020). Studies have reported that the interactions with cancer cells via either secretion- or contact-dependent mechanisms may confer a more activated status on fibroblasts, which is accompanied by the ability to form multiple branch structures, increased expression of CAF markers (e.g. α-SMA and the fibroblast activation protein [FAP]), and the dysregulation of oncogenic cytokines and matrix genesis (Eckert et al., 2019; Öhlund et al., 2017; Sahai et al., 2020). Similar to other findings, our results confirmed that the expression of a panel of activated fibroblasts markers, including α-SMA, FAP and PDGFRα (Sahai et al., 2020), was higher in OSCC-associated fibroblasts in comparison with corresponding PFs. Using a matrigel-based 3D colony formation assay, we found that the ability to form multiple branches was enhanced in CAFs compared to PFs. Moreover, we observed that the presence of multiple branches CAFs gradually decreased during serial passage, indicating that the tumour-stromal interaction may contribute to the morphological activation of fibroblasts in cancer.

Emerging evidence has shown that the organoid technology can model many aspects of parental tumours, and the proposal to incorporate organoids with cancer-associated stromal cells is providing a new window to better study the cellular crosstalk in TME (Dijkstra et al., 2018; Seino et al., 2018). In previous studies, 3D co-culture systems were established to investigate the tumour-fibroblast interplay in pancreatic cancer and OSCC (Öhlund et al., 2017; Zhao et al., 2021). However, the traditional protocol to co-embed tumour and stromal cells in Matrigel only allows for single-cell colonies, rather than their intergrowth situation, challenging the discovery of mechanisms other than secretion-dependent signals (Zhao et al., 2021). To address this question, we have recently developed a 3D bio-printing system to artificially manipulate the spatial distribution of tumour cells and fibroblasts, in which their physical contact was stably recapitulated (Chen et al., 2021). Nonetheless, the cost of time and labour makes it difficult to arrange this bio-printing system into large-panel pre-clinical studies. In the present study, we further initiated the FAO model to ask for a more convenient strategy. We found that compared with the traditional 3D co-culture system, the FAO better mimics the *in vivo* spatial distribution of fibroblasts with tumour nests, such as the imbedding, surrounding, dragging and concomitant relationship of them. More interestingly, the presence of CAFs with multiple branches was stably observed in the FAO model, rather than in the primitive co-culture system, when they were performed for serial passage. These results suggested that, apart from the pathways that were modulated in traditional 3D co-culture system, other mechanisms may be involved in the morphological activation of fibroblasts incorporated into FAO.

Physical contact-induced signals are presumed to be critical for cellular crosstalk in solid tumours (Cox, 2021; Dawson et al., 2021; Nia et al., 2020). However, it was not until recently that specific contact-dependent mechanisms such as Notch activity were identified for fibroblast activation (Strell et al., 2019). The mature Notch receptors in responding cells are heterodimers consisting of extracellular and transmembrane subunits, and the binding of Notch ligands from signal-sending cells can induce the proteolytic cleavage of the NICD, a key secondary messenger that regulates the transcription of Notch target genes, such as the *Hes1*, *c-Myc*, *cyclin D1* and *p21*, which can potentially modulate a variety of cellular behaviours, such as the cell cycle arrest (Borggrefe & Oswald, 2009; Majumder et al., 2021). Although Notch plays a context-dependent role in cancer display tissue-specific manners (Procopio et al., 2015; Sahai et al., 2020), Strell et al. found that the epithelial-stromal interaction in breast carcinoma was affected by the expression of the ligand (i.e. Jagged-1 [Jag1]) and receptor (i.e. Notch2) of Notch signalling in cancer cells and fibroblasts, which was also correlated with poor prognosis (Strell et al., 2019). Consistent with this, we found that the expression of NICD was higher in OSCC-associated fibroblasts than in PFs. More importantly, results based on a novel FAO model confirmed that treatment with Notch inhibitor, such as DAPT (Saito et al., 2014; Singh et al., 2021), reduced the morphological activation of CAFs/PFs incorporated into OSCC organoids, indicating that contact-dependent Notch activity may contribute to the triggering and maintenance of the malignant phenotype in fibroblasts surrounding OSCC. Future studies upon this FAO model may provide a brand new window to clarify the involvement of Notch pathways, as well as other contact-dependent signals in cancer progression.

An open question for the present study is the unexpected colony efficiency data showing no effect of tumour CM, a classical strategy to determine the paracrine signals in cell-cell interaction, upon morphological activation of fibroblasts (Sahai et al., 2020). Although the existence of TGF-β1 in tumour CM was confirmed by ELISA analysis, further evidences supporting the role of paracrine TGF-β1 signals in phenotype transition of fibroblasts, or the analysis of other components in tumour CM, which may display antagonism role against TGF-β stimulation were not examined clearly here. Apart from the morphological activation, more importantly, there are also other CAFs phenotypes, such as the dysregulated secretory features (Öhlund et al., 2017) that were not interrogated for the fibroblasts incubated with tumour CM. Therefore, findings of the present data set are not at all against with the presumption that

secretion-dependent activity may contribute to tumour-stromal interaction even in our experimental model.

Current studies focusing on the systematic understanding of tumour-stromal interplay are largely dependent on *in vivo* xenograft models via co-injection of cancer and stromal cells, as the xenograft model can faithfully modulate not only the paracrine signals, but also their physical contact effects (Sahai et al., 2020). However, the incorporation of host-derived stromal cells into xenograft tumours has challenged the findings based on this model (Sahai et al., 2020). Here, we developed a robust strategy to model the spatial distribution of stromal cells with tumour nests *in vitro*, providing an alternative approach to comprehensively study the human TME. Of note, previous study has already reported the intra-tumour heterogeneity regarding to the spatial distribution of fibroblasts with tumour nest *in vivo*, where the CAFs most proximal to the cancer cells exhibit a myofibroblasts phenotype, while the more distal CAFs is characterized by higher levels of secretory feature (Öhlund et al., 2017). It is believed that the upcoming studies based on this personalized FAO system may also benefit to understanding the molecular insight and predictive value of this spatial heterogeneity of fibroblast-tumour interaction *in vivo*.

5 | CONCLUSION

In this study, we developed a novel strategy to study the contact-dependent mechanisms underlying tumour-stromal interactions. Based on the FAO model, we confirmed that Notch activity contributes to the biogenesis of OSCC-associated fibroblasts.

ETHICS STATEMENT

Collection of tumour and para-tumour specimens of patients with OSCC was processed according to IRB-approved guidelines at the Ethics Committee of School and Hospital of Stomatology at Wuhan University (IRB-ID:2021A18).

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CONFLICTS OF INTEREST

The authors declare that there are no potential conflicts of interest. Xu Chen, Rui Li, Hui Zhao, Xinmiao Wang, Zhe Shao, Zhengjun Shang.

AUTHOR CONTRIBUTIONS

Xu Chen: Conceptualization; Data curation; Formal analysis; Writing-original draft. **Rui Li:** Conceptualization; Data curation; Formal analysis; Writing-original draft. **Hui Zhao:** Conceptualization; Formal analysis; Methodology; Writing-review & editing. **Xinmiao Wang:** Conceptualization; Data curation; Validation. **Zhe Shao:** Conceptualization; Methodology; Resources; Supervision. **Zhengjun Shang:** Conceptualization; Funding acquisition; Methodology; Supervision; Writing-review & editing.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/odi.14071.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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