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Role and mechanisms of a three-dimensional bioprinted microtissue model in promoting proliferation and invasion of growth-hormone-secreting pituitary adenoma cells

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Abstract

Growth-hormone-secreting pituitary adenoma (GHSPA) is a benign tumour with a high incidence and large economic burden, which greatly affects quality of life. The aetiological factors are yet to be clarified for GHSPA. Conventional two-dimensional (2D) monolayer culture of tumour cells cannot ideally reflect the growth status of tumours in the physiological environment, and insufficiencies of *in vitro* models have severely restricted the progress of cancer research. Three-dimensional (3D) bioprinting technology is being increasingly used in various fields of biology and medicine, which allows recapitulation of the *in vivo* growth environment of tumour cells. In this study, a GHSPA microtissue model was established using 3D bioprinting. Tumour cells in the 3D environment exhibited more active cell cycle progression, secretion, proliferation, invasion, and tumourigenesis compared with those in the 2D environment. Furthermore, the molecular mechanisms of the 3D-printed microtissue model were explored. We demonstrated that the 3D-printed microtissue provides an excellent *in vitro* model at the tissue level for oncological research and may facilitate in-depth studies on the aetiology, treatment, drug resistance, and long-term prognosis of GHSPA.

1. Introduction

Pituitary adenoma is a benign tumour originating in the anterior pituitary, which accounts for 10%–15% of intracranial tumours [1] and has an incidence of 80–90 per 100 000 individuals [2]. Growth-hormone-secreting pituitary adenoma (GHSPA) accounts for 12.5% of all pituitary adenoma cases [3]. The levels of growth hormone and insulin-like growth factor are often elevated in patients with GHSPA. Such elevations can affect metabolism and lead to acromegaly, respiratory, cardiovascular, and cerebrovascular complications in patients. Furthermore, these elevations can induce malignant tumours [4, 5] and result in

extremely high mortality. Nearly half of GHSPAs exhibit the invasive growth pattern of malignant tumours and often involve important structures such as the cavernous sinus, internal carotid artery, and hypothalamus. These cases have high surgical risks and are difficult to cure by surgical treatment, prone to recurrence after surgery, and therefore difficult for clinicians. Invasive GHSPA severely impairs the physical and mental health of patients, causing social issues and economic burden. The underlying mechanisms have yet to be clarified for GHSPA.

Numerous recent studies have been conducted using GHSPA cell lines. However, a two-dimensional (2D) cell monolayer cannot fully reflect the conditions

of cell growth in three-dimensional (3D) tissues and organs. A study has shown that cells in 2D environment may lose many inherent characteristics that they possess in 3D environment, such as intercellular junctions, cell-matrix interactions, signal transduction, and metabolism [6]. These 2D cell models are unable to reflect the *in vivo* survival status of solid tumour cells, such as necrosis, proliferation, stem cell characteristics, or drug resistance [7, 8]. Additionally, cells have shown considerable differences in 2D and 3D models in terms of gene and protein expression, protein gradient distribution, signal transduction, cell migration, morphology, proliferation, and viability [9].

Among tissue engineering technologies, 3D bioprinting has received the most attention and has been applied to *in vitro* cell culture and oncological studies of cervical cancer [10, 11]. A study showed that, after 3D bioprinting, bone marrow stem cells still exhibited relatively high activity and differentiation capabilities under *in vitro* culture conditions [12]. Compared with normal cell cultures, 3D-printed models can provide intercellular junctions that more closely resemble those in a tumour and a more stable tumour microenvironment. Additionally, 3D-printed models can provide better experimental conditions to study the mutual relationship between tumour cells and the microenvironment, the effects of chemotherapeutic drugs on tumour cells, and their sensitivity.

In the present study, we established a GHSPA microtissue model by 3D bioprinting technology. We observed the effects of the model on the cell cycle, proliferation, invasion, and tumorigenesis of tumour cells, investigated the pathways and molecular mechanisms underlying the 3D-printed microtissue model, and evaluated application of the 3D-printed microtissue model for oncological studies.

2. Materials and methods

2.1. Cell culture

Rat pituitary adenoma GH₃ cells were purchased from the American Type Culture Collection. Cells were grown in F10 medium (Gibco, Grand Island, NY, USA) supplemented with 2.5% fetal bovine serum (Gibco) and 15% horse serum (Gibco) at 37 °C with 5% CO₂. 10 cm diameter cell culture dish was used. During the culture the medium was changed each 2 days, but the cells were not passaged.

2.2. Model establishment

GH₃ cells (5×10^6) were suspended in 0.5 ml culture medium, followed by addition of 0.5 ml of a gelatin-alginate blend (20% gelatin and 4% sodium alginate). Sodium alginate was purchased from WaKo (WaKo Pure Chemical Industries, Ltd, Osaka, Japan). Gelatin, fibrinogen, and thrombin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The solution was

evenly mixed and then the 3D-printed model was printed at 4 °C (Livprint-Norm, Medprin) with the size of $10 \times 10 \times 5$ mm. After printing, the model was crosslinked with an aqueous solution of 3% calcium chloride. Subsequently, the model was incubated at 37 °C with 5% CO₂. 3D non-printed model was crosslinked without printing. The number of starting cells was the same for the 2D group, the 3D-printed group and the 3D non-printed group.

2.3. Cell proliferation assay

GH₃ cell proliferation was assayed using an Alamar blue Kit (MAIBIO, Shanghai, China). The 3D-printed model was washed with phosphate-buffered saline (PBS). Then, 3 ml of Alamar blue working solution (10% Alamar blue and 90% fresh medium) was added to each well of a 6-well plate. The plate was incubated at 37 °C in an incubator with 5% CO₂ and saturated humidity for 2 h. Then, the working solution was transferred to a 96-well plate, and fluorescence was measured at excitation wavelengths of 570 and 630 nm on a microplate reader (BioTek ELX800, VT, USA). Three samples per group were analysed every 2 d.

2.4. Cell cycle analysis

The cell cycle was analysed by flow cytometry. Cells of the 2D and 3D-printed model cultured groups were collected at the same time points before and after printing. For the 3D-printed group, the scaffold material was first dissolved with a citric acid-EDTA solution before cell collection. The cells were fixed with pre-chilled 70% alcohol for 1 h and then washed with PBS. Cells were stained with a propidium iodide buffer prepared at a 25:10:1000 ratio of 40× propidium iodide stock (2 mg ml^{-1}), 100× RNase stock (10 mg ml^{-1}), and 1× PBS, and analysed by flow cytometry (Becton Dickinson, USA). Three samples per group were analysed for each time point with at least 1×10^6 cells per sample. Three experiments were performed, and the analysis was conducted once every 2 d.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Rat Growth Hormone ELISA Kit (Wuhan Colorful-Gene Biological Technology, Wuhan, China), Rat E-cadherin ELISA Kit (Wuhan Colorful-Gene Biological Technology, Wuhan, China) and Rat Nestin ELISA Kit (Wuhan Colorful-Gene Biological Technology, Wuhan, China) were used for the ELISA assay in the study. Samples were collected from each group on days 2, 4, 6, 8, 10, 12, 14, and 16. Culture supernatants were obtained for the growth hormone assay and cells for E-cadherin and nestin analyses. The scaffolds of 3D-printed and 3D non-printed groups were dissolved with the citric acid-EDTA solution, and then cells were collected by centrifugation. Cells of the 2D, 3D-P, and 3D non-printed groups were counted, and 2×10^6 cells were diluted with PBS and treated with

an ultrasonic cell disruptor. After centrifugation (4 °C, 30 min, 3000 rpm), the supernatants were carefully collected. The samples were loaded onto an ELISA plate and incubated at 37 °C for 30 min in the dark. After washes, the ELISA reagent was added, and the plate was incubated at 37 °C for another 30 min in the dark. After washes, a development reagent was added, and the plate was incubated in the dark at 37 °C for 15 min. The reaction was terminated by adding a termination solution. The fluorescence was recorded at an excitation wavelength of 450 nm on a microplate reader. Three samples per group were measured for each time point.

2.6. SEM

The 3D-printed and 3D non-printed groups of models and the 2D group of cells growing on coverslips were fixed with 2.5% glutaraldehyde at 4 °C for 24 h. The samples were dehydrated in graded ethanol solutions (30%, 50%, 60%, 70%, 80%, 90%, 5%, and 100%; 30 min at each concentration). After critical point drying, the samples were coated with gold by an ionizer (5 nm thickness) and then observed under an ULTRA 55 scanning electron microscope (ZEISS, Germany).

2.7. TEM

Cells of the 3D-printed group were collected by centrifugation after the scaffold material was dissolved with the citric acid-EDTA solution. The cells of both 3D-printed and 2D groups were washed with PBS and fixed with 2.5% glutaraldehyde at 4 °C for 4 h. After centrifugation, the cells were collected and rinsed, followed by fixation in 1% osmic acid, rinsing, dehydration, soaking, and embedding in Epon 812. Ultrathin sections were prepared using a LKB-V microtome. Staining was conducted using lead citrate and uranyl acetate. The samples were then observed under a transmission electron microscope (TECNAI 10, Philips, Netherlands).

2.8. Transwell invasion assay

Transwells (COSTAR, Corning, Kennebunk ME, USA) with the pore size of 8 µm were placed in a 24-well plate. The upper chamber was coated with Matrigel at a 1:8 dilution and allowed to air dry at room temperature. Cells of the 3D-printed group were harvested by centrifugation after the scaffold material was dissolved with the citric acid-EDTA solution. The collected cells of 3D-printed and 2D groups were counted to prepare a cell suspension in serum-free medium. The cell density was adjusted to 1×10^5 cells ml⁻¹, and 200 µl of cell suspension was added to the upper chamber of Matrigel-coated Transwells. Subsequently, 600 µl serum-containing medium was added to the lower chamber, followed by incubation at 37 °C with 5% CO₂. The Transwells were collected after 1, 2, 3, 4, 5, 6, and 7 d. Cells in the upper chamber of Transwells were removed with a cotton swab. The remaining cells were then fixed in

4% paraformaldehyde for 15 min and stained with a 0.5% crystal violet-methanol solution for 15 min. After PBS washes, samples were observed under a microscope. Five fields of view on the bottom surface of the Transwell were selected at random to count cells.

2.9. Nude mouse tumorigenesis experiment

12 Balb/c nude mice (3–4 weeks old, 18–25 g; six males and six females) were purchased from the Experimental Animal Center, Sun Yat-sen University of Medical Sciences (Guangzhou, China). Mice were randomly and equally divided into two groups. Cells of the 3D-printed and 2D groups were collected for nude mouse tumorigenesis experiments at 12 d after 3D printing. The scaffold material of the 3D-printed group was dissolved with the citric acid-EDTA solution before the cells were collected by centrifugation. The collected cells of 3D-printed and 2D groups were counted to prepare cell suspensions. Each nude mouse was subcutaneously inoculated with 0.2 ml suspension (5×10^6 cells) in the left hip on the back. After the appearance of the tumour, the length and diameter of the tumour were measured with a Vernier calliper, and the tumour volume was calculated. The mean tumour volume was determined to construct a growth curve. Nude mice were sacrificed by cervical dislocation after 40 d, and the tumours were collected.

2.10. GeneChip assay and analysis

Cells were collected from 3D-printed ($n = 3$) and 2D ($n = 3$) groups, and total RNA was extracted by Trizol reagent using a single step protocol. The quantity and quality of RNA extracts were evaluated using the NanoDrop 6000 (NanoDrop, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) with Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). Gene expression profiles were determined using rat OneArray gene expression microarrays following the manufacturer's instructions. Affymetrix GeneChip 3'IVT Express Kit (Thermo Fisher, Waltham, MA, USA) was used for reverse transcription, conversion to a double-stranded DNA template, synthesis of mRNA, and labelling of RNA by *in vitro* transcription. Microarrays were hybridized, washed, and stained using a Affymetrix GeneChip Hybridization Wash and Stain Kit (Thermo Fisher, Waltham, MA, USA). Arrays were scanned using an Agilent Microarray Scanner (G2505C) to collect raw data. Differentially expressed genes between 3D-printed and 2D groups were selected according to the criteria $p < 0.05$ and $|FC| \geq 1$. GO and KEGG pathway analyses were applied to the differentially expressed genes at $p < 0.05$. Two significantly enriched pathways, cell proliferation and ubiquitin-mediated proteolysis, were imported into Cytoscape software to construct co-expression networks.

2.11. Statistical methods

Data were analysed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Results are expressed as the mean $\pm$ standard deviation ($\bar{x} \pm s$). Comparisons between groups were conducted by analysis of variance and t-tests. A *p*-value of less than 0.05 was considered as statistically significant.

3. Results

3.1. 3D-bioprinted microtissue model

To simulate and recapitulate the *in vivo* growth status of GHSPA GH3 cells in an *in vitro* environment, we applied 3D bioprinting to establish a 3D-printed microtissue model (figure 1(A)). The 3D-printed model had a grid-like structure arranged in multiple layers, and cells were uniformly distributed in the scaffold material (figures 1(B)–(D)). The cells grew into spheroids of various sizes after 6–12 d (figures 1(E)–(G)). Compared with the membrane-like structure in the 2D environment, the spheroid morphology was more biomimetic to the solid tumour morphology. After the scaffold material was dissolved to prepare cells on coverslips, cell aggregates can still be observed by scanning electron microscopy (SEM; figure 1(H)). In the 3D non-printed group, cells on the surface of the model grew well. However, observation of a cross-section revealed cell atrophy and necrosis inside the model (figure 1(I)). Multiple cells with tight junctions formed spheroid aggregates in the 3D-printed model, which was in sharp contrast to the independently growing cells in the 2D group, as observed by transmission electron microscopy (TEM; figures 1(J) and (K)). These results indicated that the 3D-printed model was more conducive for the growth and survival of tumour cells than the 3D non-printed model. The former also showed morphological and structural advantages over 2D-cultured cells, thus providing a better *in vitro* model for basic and clinical research.

3.2. Cell cycle and proliferation

To investigate the effect of the 3D-printed microtissue model on the growth and proliferation of tumour cells, we performed cell cycle analysis and Alamar blue assays of cells cultured in 3D-printed model and 2D groups. The cells showed S phase arrest in the 3D-printed model from day 2, and the percentage of cells in S phase was significantly higher compared with that in the 2D group (figures 2(A)–(C)). Alamar blue assay revealed that cell proliferation was faster in the 2D group at day 4, whereas the 3D-printed group showed a significantly higher proliferation rate of cells after day 6 (figure 2(D)). These results indicated that tumour cells in the 3D-printed model exhibited more active cell division compared with the 2D group.

3.3. Cell secretory function

To investigate the effect of the 3D-printed model on the secretory function of tumour cells, we assayed the

level of growth hormone secretion from tumour cells of the 3D-printed, 3D non-printed, and 2D groups. We found that secretion of growth hormone from cells in the 3D-printed group was significantly higher than that in the 3D non-printed group throughout the experimental period from day 4. The 3D-printed group showed significantly higher secretion of growth hormone than the 2D group from day 10, and the difference gradually became pronounced over time. Growth hormone secretion reached a peak in the 3D-printed group at day 12 (figure 3(A)). SEM observations revealed the presence of numerous secretory granules on the surface of cells in the 3D-printed group (figure 3(B)) [13–15], whereas fewer secretory granules were found on the surface of cells in the 2D group (figure 3(C)) [13–15]. TEM observations revealed numerous mitochondria, rough endoplasmic reticulum, and secretory granules in cells of the 3D-printed group. The numbers of mitochondria, rough endoplasmic reticulum, and secretory granules in cells of the 2D group were lower than those in the 3D-printed group (figures 3(D) and (E)). These results indicated that the secretory function of the tumour cells was significantly higher in the 3D-printed model compared with 2D and 3D non-printed groups.

3.4. E-cadherin expression and intercellular junctions

To study the effect of the 3D-printed model on intercellular junctions and the invasive ability of tumour cells, we analysed the E-cadherin levels in cells of the various groups. We found that E-cadherin expression in cells of the 3D-printed group was higher than that in the 2D group (figure 4(A)). E-cadherin expression in cells of the 3D-printed group was higher than that in the 2D group from day 2. These groups generally had equal E-cadherin expression at day 4. The 3D-printed group showed significantly higher E-cadherin expression than the 2D group after day 6. E-cadherin expression of cells in the 3D non-printed group was higher than that in the 2D group at day 2 and lower than that in the 3D-printed group at day 6. No significant differences were found at the remaining time points compared with the other two groups. SEM observations revealed abundant intercellular junctions between cells in the 3D-printed group with multiple layers densely interwoven in a reticular pattern (figure 4(B)) [13–15]. In the 2D group, there were sparse intercellular and cell-coverslip junctions (figure 4(C)) [13–15]. Denser intercellular junctions were also observed by TEM between cells in the 3D-printed group compared with the 2D group (figures 4(D) and (E)). E-cadherin is an intercellular adhesion molecule that participates in the formation of intercellular adhesion junctions [16]. The results indicated that the intercellular junctions between tumour cells were more robust in the 3D-printed model compared with the 2D group.

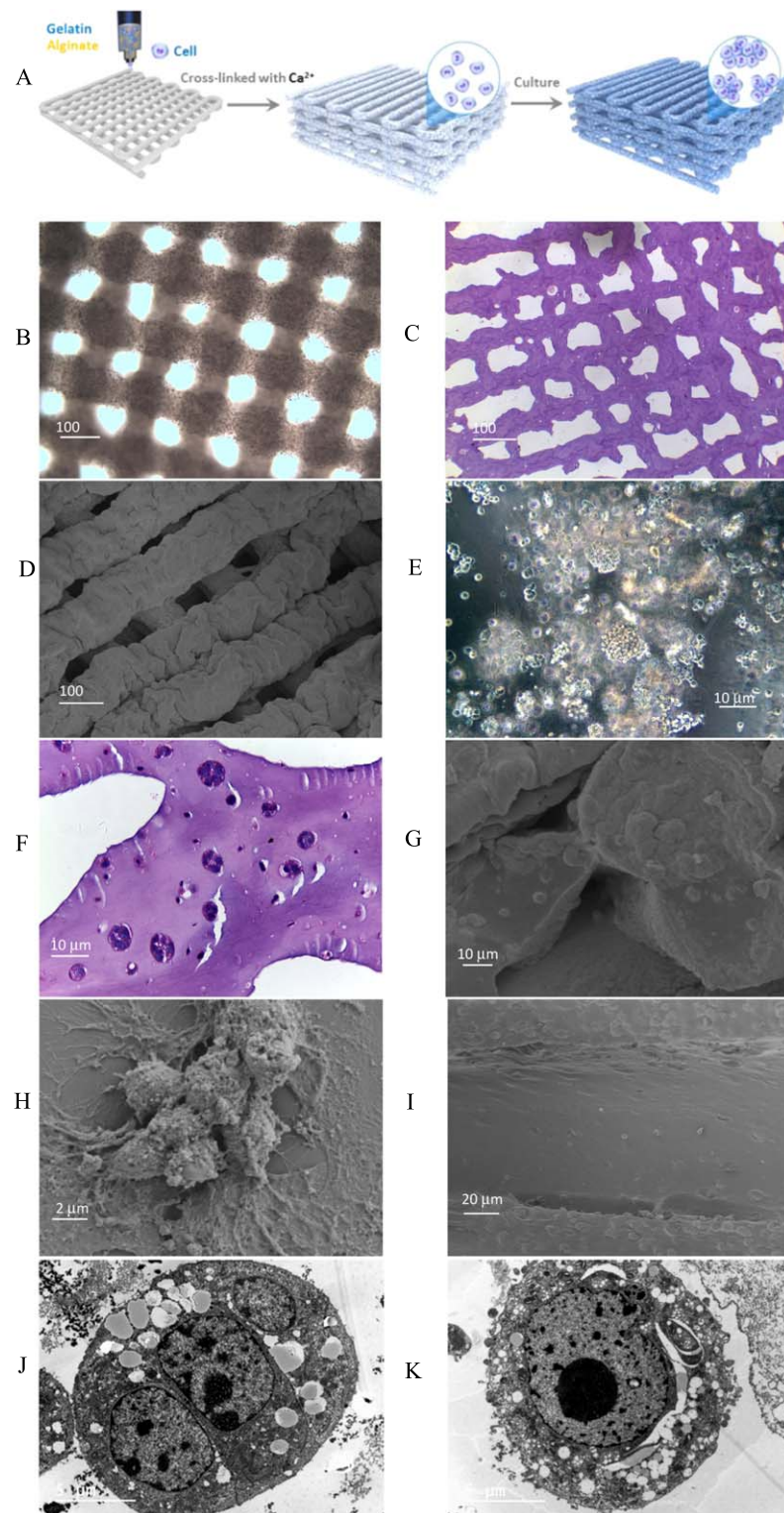


Figure 1. 3D-bioprinted microtissue model. (A) Schematic diagram for construction, printing, crosslinking, and culture of the 3D-printed model. (B) Structure of the 3D-printed model (8 d) observed by microscopy ($\times 100$ magnification). (C) HE-stained section of the 3D-printed model (4 d) observed by microscopy ($\times 100$ magnification). (D) Structure of the 3D-printed model (6 d) observed by SEM ($\times 100$ magnification). (E) Cell spheroid aggregates of various sizes inside the 3D-printed model (16 d) observed by microscopy ($\times 400$ magnification). (F) Cell spheroid aggregates in a HE-stained section of the 3D-printed model (12 d) observed by microscopy ($\times 400$ magnification). (G) Structure of cell spheroid aggregates in a cross-section of the 3D-printed model (16 d) observed by SEM ($\times 500$ magnification). (H) Cell spheroid aggregates on a coverslip after the scaffold material of the 3D-printed model (16 d) was dissolved as observed by SEM ($\times 200$ magnification). (I) Cross-section of the 3D non-printed model (16 d) observed by SEM ($\times 250$ magnification), showing that cells inside the model were mostly atrophic and necrotic, while cells on the model surface had a good growth status. (J) Cell spheroid aggregates in the 3D-printed model observed by TEM ($\times 5800$ magnification). (K) Independent tumour cells in the 2D group observed by TEM ($\times 5800$ magnification).

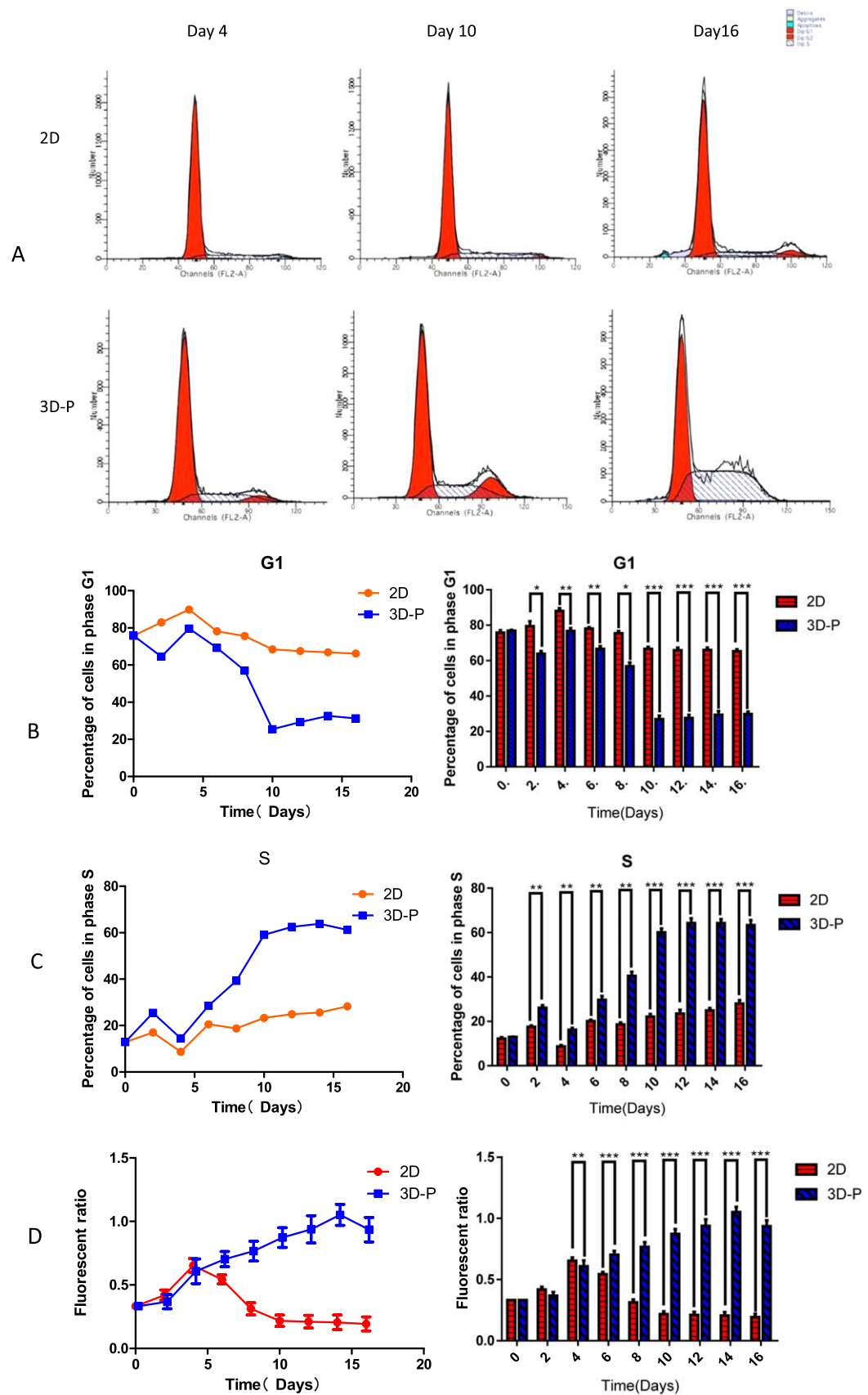
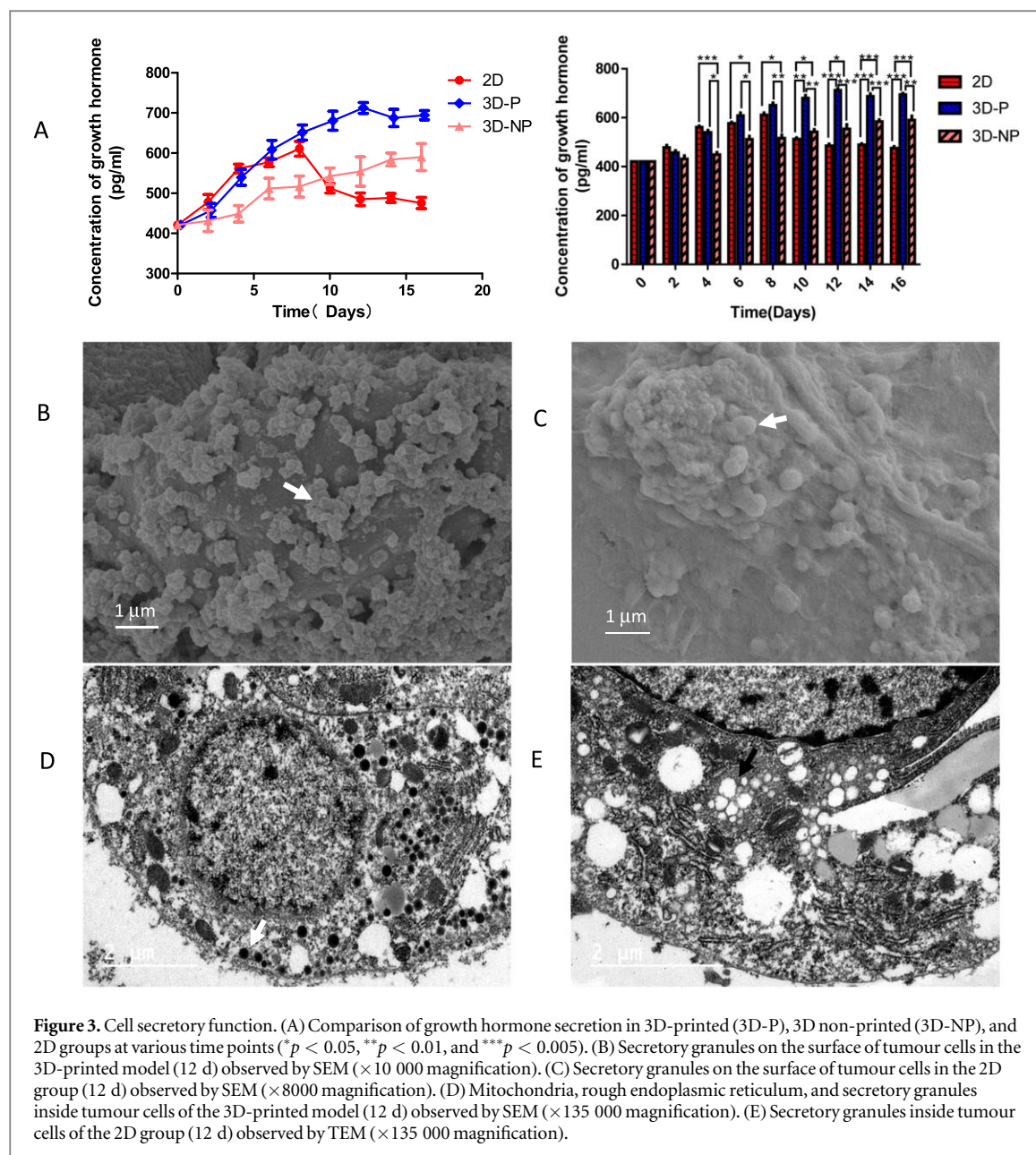


Figure 2. Cell cycle and proliferation analyses. (A) Cell cycle distribution of 3D-printed (3D-P) and 2D groups. (B) Percentage of cells in G1 phase of 3D-printed and 2D groups ($*p < 0.05$, $**p < 0.01$, and $***p < 0.005$). (C) Percentage of cells in S phase of 3D-printed and 2D groups ($**p < 0.01$ and $***p < 0.005$). (D) Comparison of fluorescence ratios between 3D-printed and 2D groups after Alamar blue staining ($**p < 0.01$ and $***p < 0.005$).



3.5. Tumour stem cell (TSC) activity and invasive ability

To investigate the effect of the 3D-printed model on TSC activity and the invasive ability of tumour cells, we measured nestin expression levels in the various groups and evaluated the invasive ability of cells in 3D-printed and 2D groups by Transwell assays. Nestin expression was significantly higher in cells of the 3D-printed group compared with that in 2D and 3D non-printed groups. The nestin expression peaked in cells of the 3D-printed group at days 4 and 12 (figure 5(A)). Nestin expression in cells of the 3D-printed group was significantly higher than that in the 2D group from day 2 and significantly higher than that in the 3D non-printed group from day 4. The 3D non-printed group showed higher nestin expression than the 2D group at day 2, whereas the latter showed higher expression than the former at

day 4. There were no significant differences in nestin expression between these two groups from day 4 to 14. Nestin expression in cells of the 3D non-printed group became higher than that in the 2D group after day 14. Transwell assays revealed that the number of invaded cells in the 3D-printed group was significantly higher than that in the 2D group from day 3 (figures 5(B) and (C)). Nestin is a type VI intermediate neurofilament protein and marker of stem cells, which is related to signalling pathways of tumour cell proliferation, such as p53, Wnt/ β -catenin, and Akt-GSK3 β -Rb [17, 18]. The above results indicated that the TSC activity and proliferative capacity of tumour cells in the 3D-printed group were higher than those in 2D and 3D non-printed groups. Additionally, the invasive ability of tumour cells in the 3D-printed group was stronger than that in the 2D group.

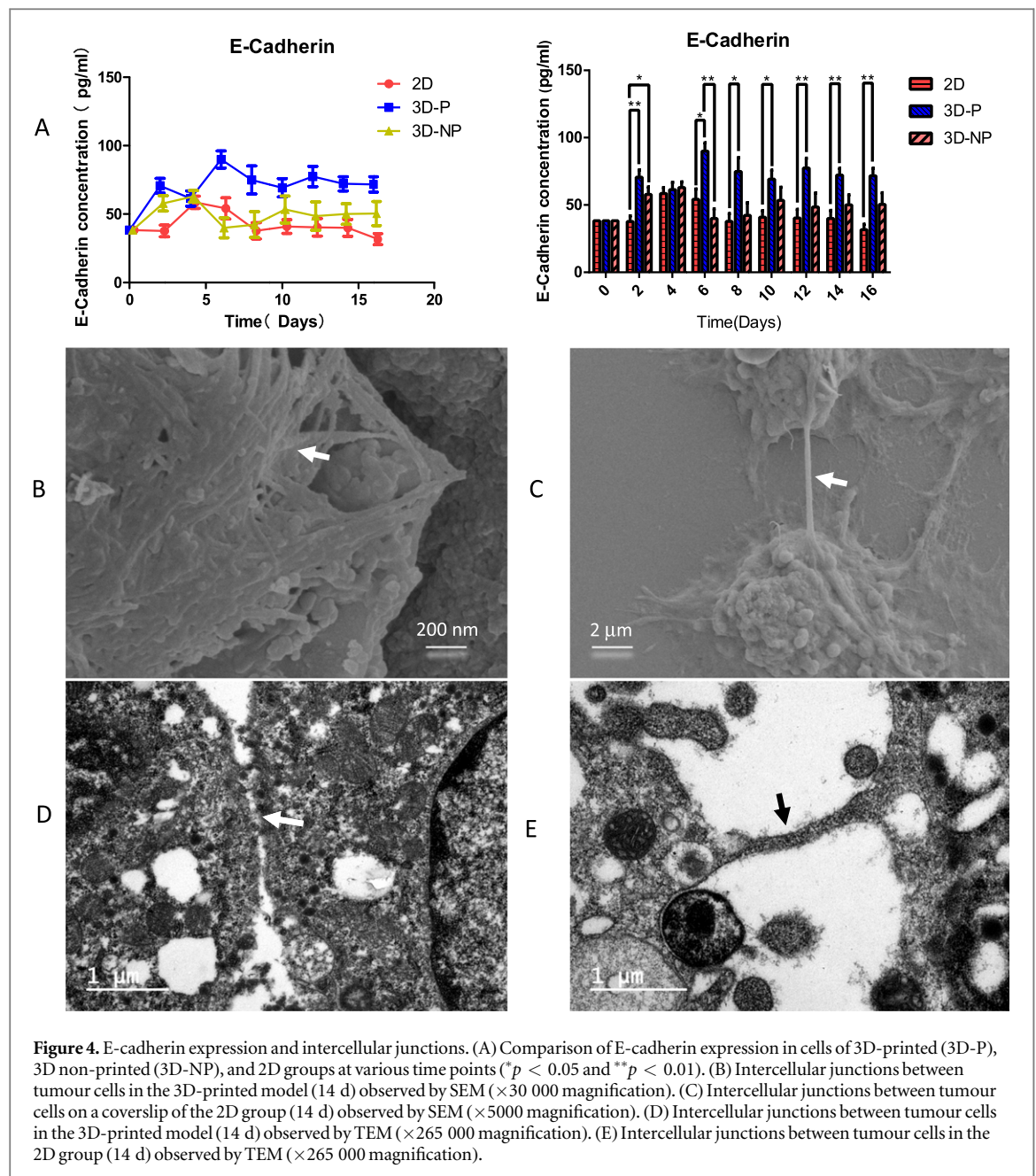


Figure 4. E-cadherin expression and intercellular junctions. (A) Comparison of E-cadherin expression in cells of 3D-printed (3D-P), 3D non-printed (3D-NP), and 2D groups at various time points (* $p < 0.05$ and ** $p < 0.01$). (B) Intercellular junctions between tumour cells in the 3D-printed model (14 d) observed by SEM ($\times 30\,000$ magnification). (C) Intercellular junctions between tumour cells on a coverslip of the 2D group (14 d) observed by SEM ($\times 5000$ magnification). (D) Intercellular junctions between tumour cells in the 3D-printed model (14 d) observed by TEM ($\times 265\,000$ magnification). (E) Intercellular junctions between tumour cells in the 2D group (14 d) observed by TEM ($\times 265\,000$ magnification).

3.6. Tumourigenic ability

To study the effect of the 3D-printed model on the tumourigenicity of tumour cells, we performed a tumourigenesis experiment in nude mice using the cells of 3D-printed and 2D groups. Tumours visible to the naked eyes were formed in five mice of the 3D-printed group and two mice of the 2D group at 10 days post-inoculation. All mice of the 3D-printed group and only three of the 2D group showed visible tumours at day 15. All mice of the 2D group showed visible tumours by day 20. The tumours appeared as a subcutaneous nodule visible to the naked eyes at the site of inoculation, which were initially round and then gradually enlarged and showed an irregular or lobulated morphology at the end of the experiment (figures 6(A) and (B)). The growth rate of tumours in the 3D-printed group was markedly higher than that

in the 2D group after 15 days, and the difference was significant between the two groups ($p < 0.005$; figure 6(C)). These results indicated that tumour cells in the 3D-printed model had markedly higher tumourigenicity than those in the 2D group.

3.7. Mechanisms of the 3D-printed model affecting tumour cells

The above results indicated that the 3D-printed model had a significant effect on tumour progression. Next, we systematically elucidated the mechanisms underlying the role of the 3D-printed model affecting the proliferation and development of pituitary tumours and their related pathways. To this end, we analysed gene expression profiles in 3D-printed and 2D groups using GeneChip technology. The samples showed high correlations between the 3D-printed and 2D groups with

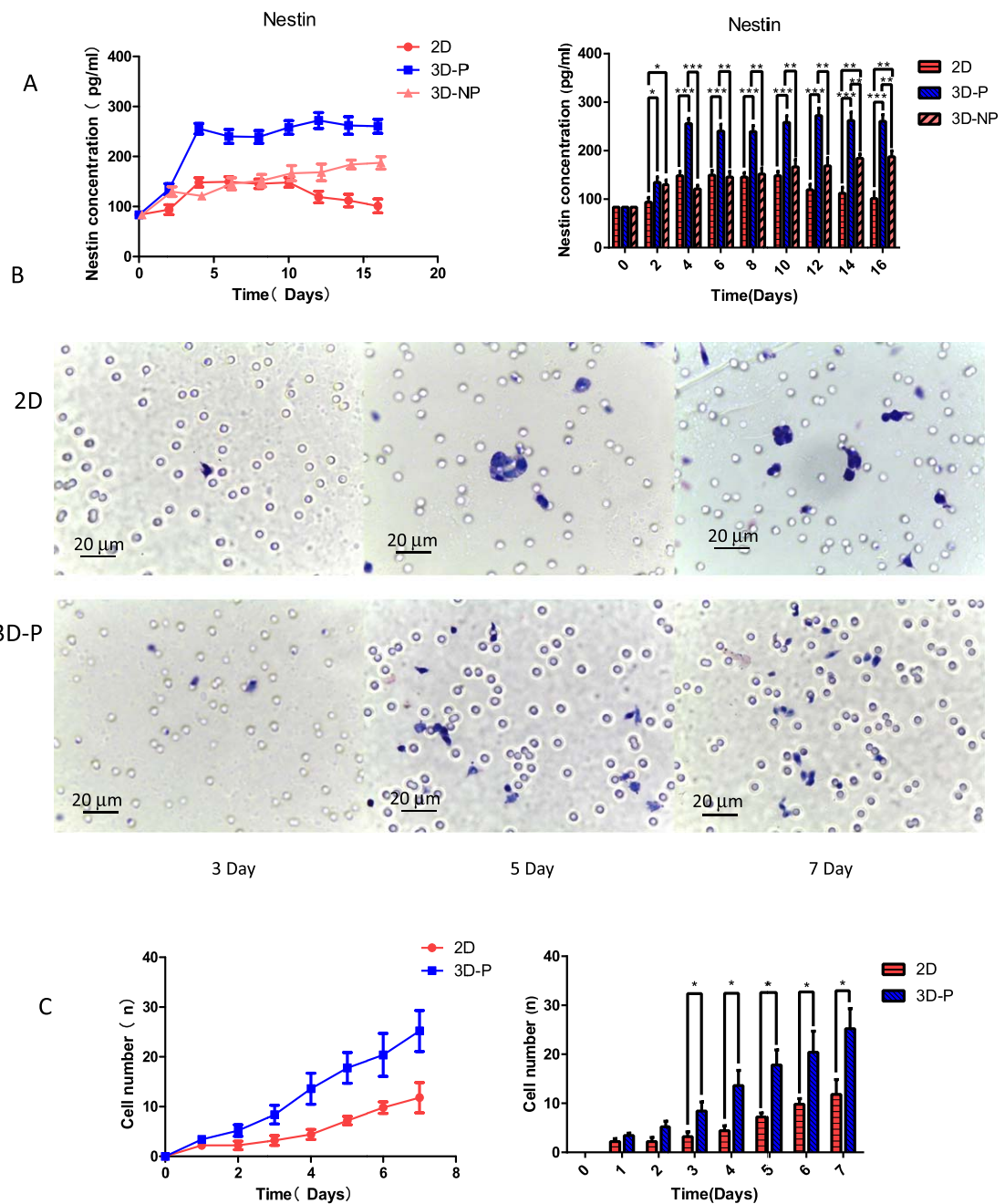


Figure 5. Cancer stem cell activity and invasive ability. (A) Comparison of nestin expression in cells of 3D-printed (3D-P), 3D non-printed (3D-NP), and 2D groups at various time points (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$). (B) Tumour cells that invaded through the membrane at various time points in 3D-printed and 2D groups observed by microscopy ($\times 200$ magnification). (C) Comparison of the number of cells that invaded through the membrane of 3D-printed and 2D groups at various time points (* $p < 0.05$).

correlation coefficients of >0.94 (figure 7(A)). Significant differences were found in expression of 1069 genes ($p < 0.05$, $|FC| \geq 1$) of which 656 were upregulated and 413 were downregulated (figures 7(B) and (C)). In Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, the differentially expressed genes were enriched in 10 pathways ($p < 0.05$) including cell cycle regulation, protein ubiquitination, G2/M transition of mitotic cell cycle, mitotic cytokinesis, stem cell population maintenance, cell proliferation, microtubule cytoskeleton organization,

positive regulation of apoptotic process, cell cycle, and ubiquitin-mediated proteolysis (figure 7(D)). These pathways play crucial roles in the development and progression of tumours.

Furthermore, we analysed the relationship between the 3D-printed model and genes related to the significantly enriched pathways (i.e. cell cycle and ubiquitin-mediated proteolysis) by functional interaction network analysis. The genes involved in the two pathways, cell cycle ($n = 19$) and ubiquitin-mediated proteolysis ($n = 15$), were all upregulated in the

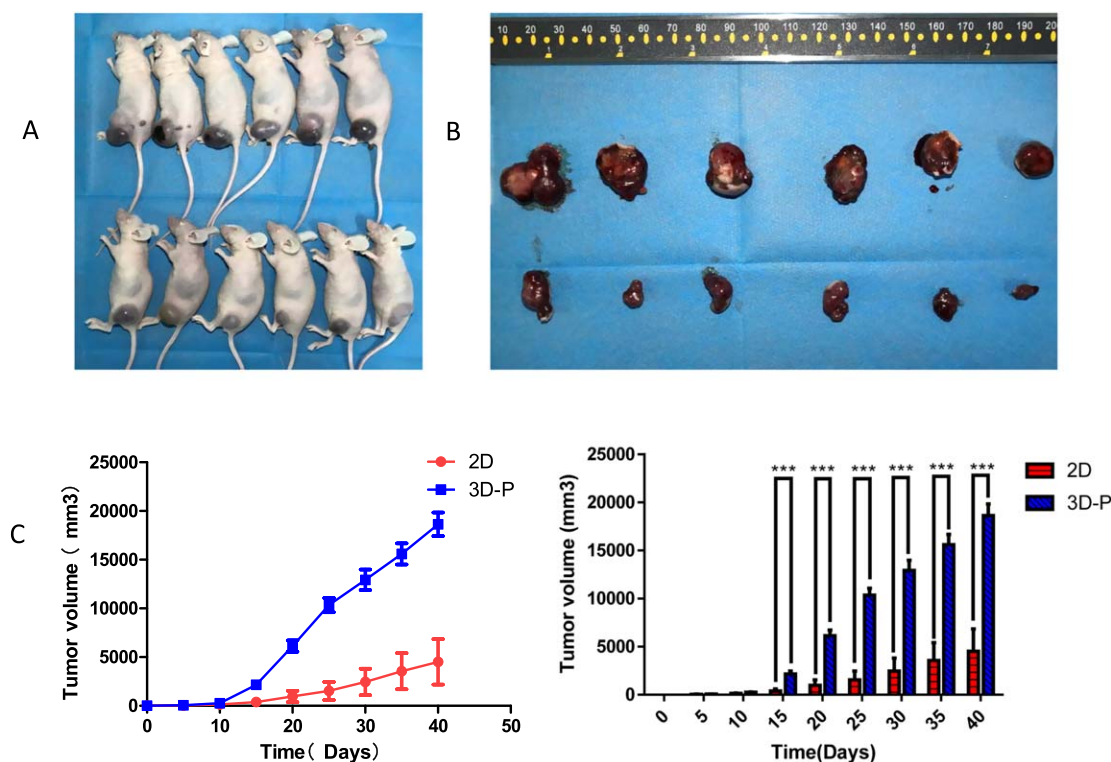


Figure 6. Tumorigenesis in nude mice. (A) Growth condition of tumours in nude mice at 40 d post-inoculation with cells of 3D-printed (3D-P, top) and 2D (bottom) groups. (B) Observation of tumours, after removing from the body of a nude mouse, at 40 d post-inoculation with cells of 3D-printed (top) and 2D (bottom) groups. (C) Comparison of tumour volumes between 3D-printed and 2D groups at various time points (***) $p < 0.005$.

3D-printed group (figures 7(E) and (F)). Among the upregulated genes, *CDK-1* (FCAbsolute = 0.9251, $p = 0.001\,468$) and *SKP-2* (FCAbsolute = 1.3327, $p = 0.000\,314$) are recognized as major oncogenes. *CDK-1* is an important kinase that controls cell cycle and proliferation processes [19]. *SKP-2* promotes the invasion and metastasis of tumour cells by regulating the expression and activity of RhoA, matrix metalloproteinase-2 and -9, as well as E-cadherin degradation [20]. These results indicated that the 3D-printed model upregulated *CDK-1* and *SKP-2* expression by impacting the tumour microenvironment, thereby facilitating the growth and proliferation of GHSPA cells.

4. Discussion

GHSPA is a benign tumour that often manifests with invasive growth and is a serious health problem. Extensive basic and clinical research has used cells cultured in 2D environment and animal xenografts as *in vitro* models, which have difficulty to simulate the real growth environment and status of tumour cells, and thus greatly limit in-depth research. 3D bioprinting technology is increasingly being applied to various fields of biology and medicine. Despite their unclear mechanism-of-action, evidence shows that 3D culture systems promote cell proliferation, migration, and stem cell differentiation [21–23]. We have

demonstrated this phenomenon through previous studies on glioma cells, but the research on its mechanisms of role is not deep enough [24]. In this study, we constructed a three-dimensional bioprinted microtissue model of GHSPA, and observed the differences of cell proliferation, differentiation, secretion, cell structure, invasion, tumorigenicity and genomics in different experimental groups, in order to reveal the mechanism of the role of the three-dimensional bioprinted microtissue model. We hypothesized that a 3D-printed GHSPA microtissue model established using 3D bioprinting might provide a better *in vitro* model and facilitate research.

In this study, we applied 3D bioprinting to establish a GHSPA microtissue model. Then, we examined the differences in the growth, development, secretion, intercellular junctions, and TSC characteristics of tumours cells in three environments, namely the 3D-printed model, 3D non-printed model, and 2D culture. We found that cells cultured in the 3D-printed model had superior cellular functions compared with those in 3D non-printed and 2D groups. Moreover, a 3D-printed model has design advantages by printing pre-designed shapes based on specific experimental needs. Because of structural limitations, 3D non-printed model led to non-uniform growth and apoptosis of the cultured cells, which cannot provide a good 3D culture environment for tumour cells and thus does not apply to clinical research as an *in vitro* model.

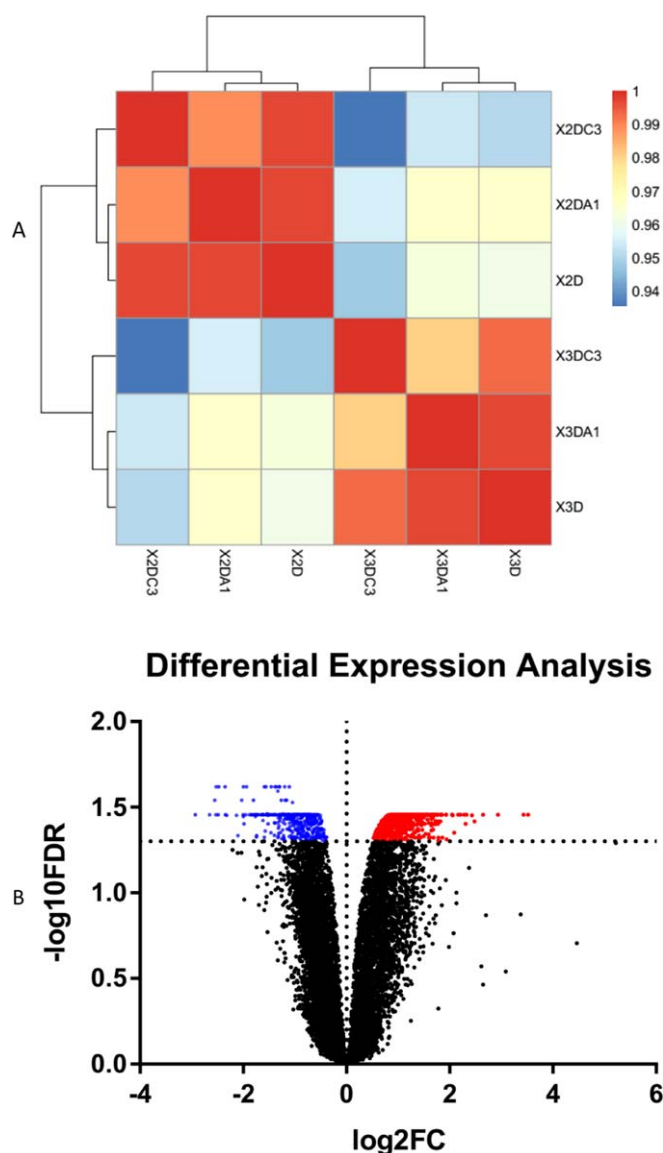
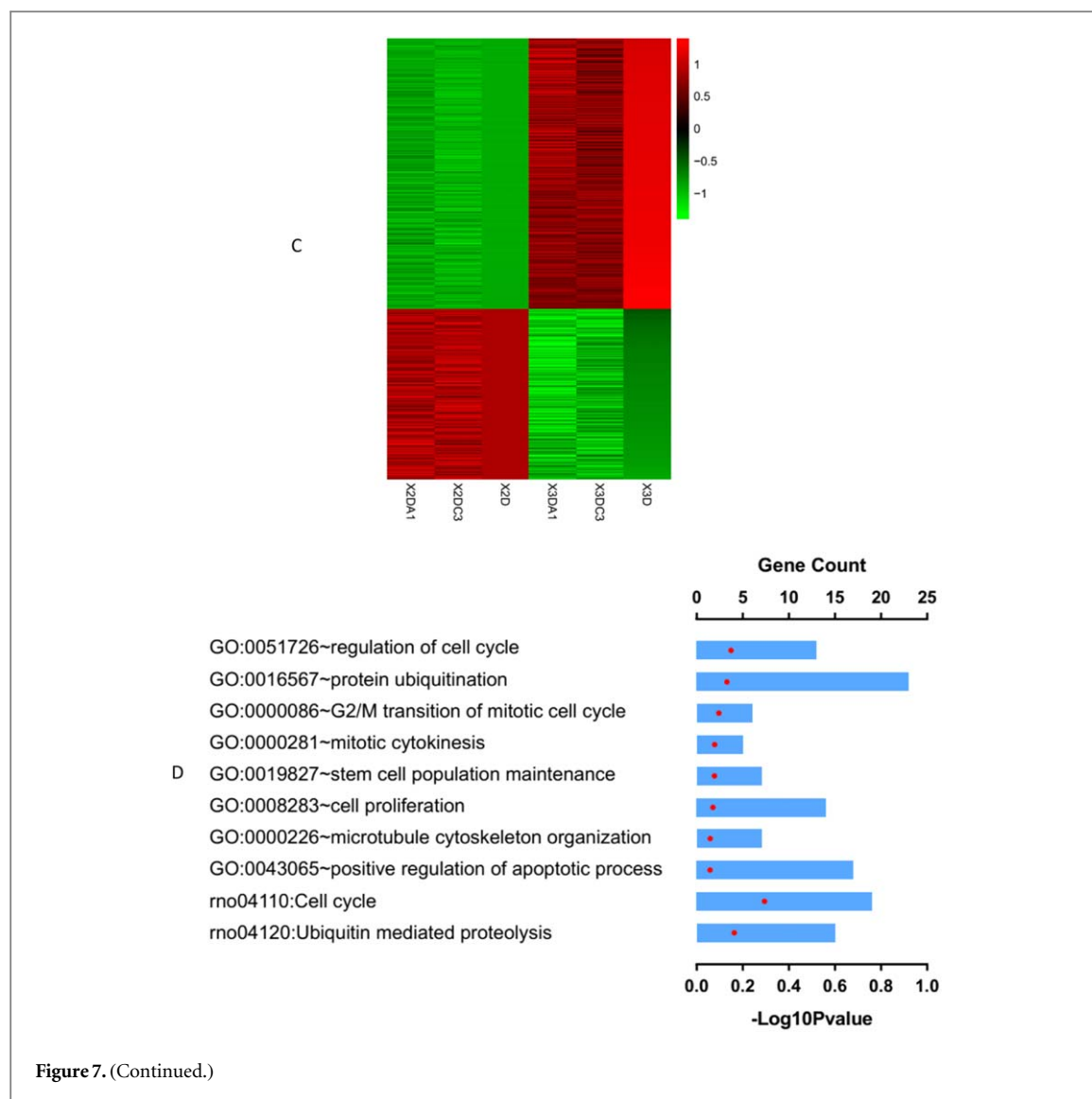


Figure 7. GeneChip assay and analysis. (A) Correlation analysis of samples from 3D-printed (3D-P) and 2D groups by Pearson's correlation coefficient. A coefficient of 1 indicates a completely positive correlation. The closer the coefficient is to 1, the higher the correlation. (B) Volcano plot of differential gene expression in 3D-printed and 2D groups with blue representing downregulated genes and red representing upregulated genes. (C) Cluster analysis of 1069 genes with significant differences ($p < 0.05$, $|FC| \geq 1$), of which 656 were upregulated and 413 were downregulated. (D) GO and KEGG enrichment analyses of the differentially expressed genes in 10 pathways ($p < 0.05$) with the anti-logarithm of p -values to indicate statistical significance on the x -axis. (E) Co-expression network of the differential genes involved in the KEGG cell cycle pathway. (F) Co-expression network of the differentially expressed genes involved in the KEGG ubiquitin-mediated proteolysis pathway.

As observed in sections and SEM images of the 3D-printed group at an early stage, the tumour cells appeared as single cells and were uniformly distributed in the scaffold material after printing. Each tumour cell had an identical initial state. However, at days 14 and 16, tumour cells in the 3D-printed model had developed into spheroid aggregates of various sizes. This observation indicates that the 3D-printed model can well reflect the heterogeneity of tumour cells and maintains relative independence between different cell aggregates. Heterogeneity of tumour cells occurs because progeny cells exhibit changes at the gene level during tumour growth, thereby resulting in differences in tumour proliferation, invasion, and drug

sensitivity. Tumour cell heterogeneity is closely related to the microenvironment.

In the present study, cell cycle analysis and Alamar blue assay suggested that the proliferation and growth activities of pituitary adenoma cells in the 3D-printed model were more active than cells cultured in the 2D environment. E-cadherin expression in cells growing in the 3D environment indicated stronger cell junctions and invasiveness than cells in the 2D environment. The same results were obtained from the Transwell invasion assay. The above results indicate that there might be a greater number of TSCs among tumour cells in the 3D-printed model compared with the 2D culture environment. Nestin expression also



suggested that tumour cells in the 3D-printed model had stronger TSC characteristics. Furthermore, the *in vivo* experiments in nude mice demonstrated that tumour cells in the 3D-printed model had a stronger tumourigenic ability than those in the 2D environment. TSCs have a self-renewal capacity and produce heterogeneous cells in tumours [25]. Thus, they possess stronger invasive and tumourigenic abilities than ordinary tumour cells [26]. TSCs have been found to present in leukaemia, breast cancer, prostate cancer, and glioma. Despite their small numbers, TSCs play a major role in the development, progression, recurrence, and metastasis of tumours [27, 28]. TSCs have a close relationship with the microenvironment of tumour cells. A suitable microenvironment for TSCs has features such as hypoxia, adjacent to blood vessels, inflammatory reactions, and promotion of epithelial-mesenchymal transition, which jointly participate in the regulation of TSC functions [29].

With regard to morphology, the 3D-printed microtissue model had a grid-like structure arranged in layers. In terms of the microscopic structure, the

cells were uniformly distributed in a column of scaffold material with a diameter of 0.2 mm. The scaffold material had good permeability and ductility, playing dual roles for tumour cells. The first role was to support and protect cells with space for survival and development. The 0.2 mm fibre diameter ensured that nutrients were transported from the culture medium to cells in a timely manner. The second role was sealing. The scaffold material encapsulated tumour cells to form a relatively closed small elastic enveloped chamber, which in turn resulted in a biomolecular gradient between the independent microenvironment of tumour cells within the small chamber and the external environment [30]. Thus, it could better recapitulate the survival status of the invasive growth of tumour tissues. As a result, tumour cells in the 3D-printed model exhibited more obvious heterogeneity and TSC characteristics compared with the 2D group.

The 3D environment provided by the 3D-printed microtissue model can affect the heterogeneity and TSC characteristics of tumour cells by altering the microenvironment of tumour cell growth, playing a

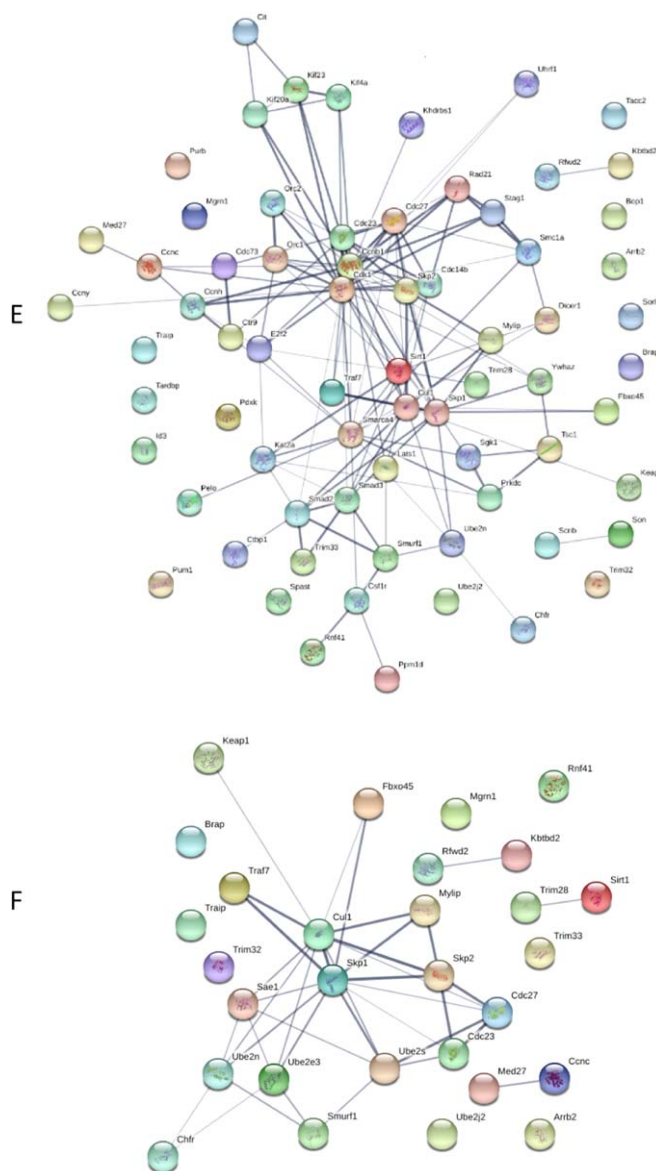


Figure 7. (Continued.)

further role in promoting the biological processes of GHSPA cells, such as secretion, proliferation, invasion, and tumourigenesis. However, the related molecular mechanisms have not been reported. To further understand the mechanisms by which the 3D-printed microtissue model affects the development and progression of tumours, we analysed cells in the 3D-printed model by microarray. We found more than one thousand genes with significant differential expression.

We also analysed the characteristics of the differentially expressed genes by functional pathway analysis and elucidated multiple biological pathways involved in tumour growth and development. For example, the cell cycle pathway is an important pathway involved in regulating mitosis, which affects cell proliferation and apoptosis. Abnormal expression of factors related to the regulation network of the cell cycle pathway results in tumour development [31, 32]. Many related genes, such

as *cyclinD1*, *P53*, *CHEK2*, and *Rb1*, have been shown to be closely associated with the development and progression of tumours [33, 34]. The ubiquitin-mediated proteolysis pathway participates in regulation of multiple biological functions such as the cell cycle, inflammatory reactions, apoptosis, and immune responses [35]. It has a close relationship with tumour development and progression, and is currently one of the main targets for tumour treatment [36, 37].

All other signalling pathways were closely associated with tumour development and progression, including cell cycle regulation, protein ubiquitination, G2/M transition of mitotic cell cycle, mitotic cytokinesis, microtubule cytoskeleton organization, and positive regulation of apoptotic process. Stem cell population maintenance and cell proliferation are two pathways that are clearly associated with tumours. More importantly, the well-known proto-oncogenes *CDK-1* and *SKP-2* were significantly upregulated in

cells of the 3D-printed model. CDK-1 and SKP-2 are involved in the signalling pathways of the cell cycle and ubiquitin-mediated proteolysis. CDK-1, a core molecule among cell cycle-dependent kinases [38], is highly expressed in various malignant tumours and associated with tumour prognosis [39, 40]. Studies have shown that CDK-1 is involved in regulating the self-renewal processes of TSCs [41, 42]. SKP-2 is an essential protein for ubiquitination and subsequent degradation of P27. SKP-2 participates in multiple biological processes such as regulation of cell cycle transition, glycolysis of tumour cells, apoptosis, and regulation of TSCs [43, 44]. In addition, SKP-2 is closely associated with the development, progression, proliferation, invasion, metastasis, malignancy, and prognosis of tumours [45, 46]. SKP-2 is currently a research focus for anti-tumour therapy [47].

Our study showed that the microtissue model established using 3D bioprinting promoted the secretion, proliferation, invasion, and tumorigenesis of GHSPA cells via two pathways (i.e. the cell cycle and ubiquitin-mediated proteolysis regulated by CDK-1 and SKP-2) by changing the microenvironment. These results provide reliable evidence for revealing the mechanism by which 3D-printed microtissue models promote tumour cells proliferation and invasion.

In summary, our research confirms that 3D printing microtissue model can simulate the actual living environment of cancer cells *in vivo*, create near-real living conditions for cancer cells, and is more conducive to restore the real characteristics of cancer cells *in vitro*. This study shows that the 3D-bioprinted microtissue model simulates human tumour tissue *in vitro* to provide a better research platform for studies of tumour cell proliferation, development, invasion, tumorigenesis, drug susceptibility, and prognosis. It fills gaps in the model at the tissue level and may effectively advance in-depth study of GHSPA.

5. Conclusions

This study applied 3D bioprinting to establish a GHSPA microtissue model. The experiments show that the 3D-printed microtissue model promotes the proliferation and invasion of tumour cells more than 2D and 3D non-printed model. It can simulate the microenvironment and the double molecular gradient of the survival of the tumour cells, so that the heterogeneity of the tumor cells and the characteristics of stem cells can be reflected. The 3D-printed microtissue model acted on the tumour cell microenvironment and exerts its promotion on GHSPA cells mainly through two cell pathways, cell cycle and ubiquitin-mediated proteolysis which regulated by CDK-1 and SKP-2, respectively. The 3D-printed microtissue model is expected to provide a significant *in vitro* model for oncology scientific research.

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