Electronic Supplementary Material (ESI) for Biomaterials Science.

Supplementary Data

Maintenance of the spheroid organization and properties of glandular progenitor cells by chitosan fabrication

Figures and figure legends

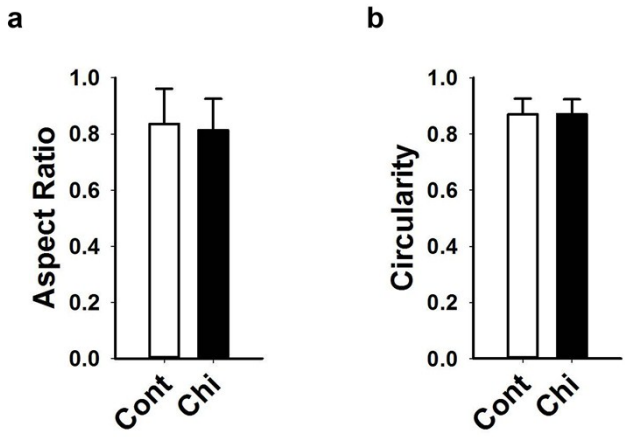

Fig. S1. Quantitative analyses of the phenotypic parameters of 9-day salispheres cultured in the control and chitosan groups. (A) Aspect ratio, and (B) circularity were measured and compared between the control and chitosan groups. Bar charts indicated mean±SD, N≧100 for each condition, $p>0.05$ (*t-test*).

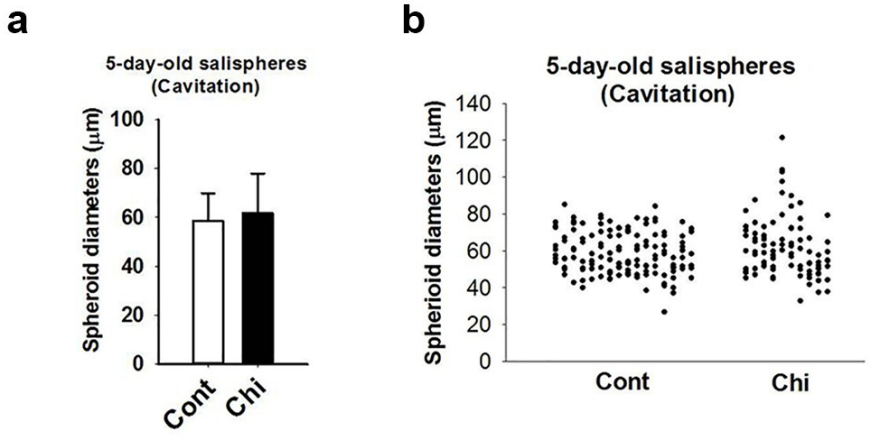

Fig. S2. Analyses of the diameters of the salispheres with cavitation after 5 day culture (a) Quantitative analyses of the average diameters on 5d salispheres cultured from the control (Cont) and chitosan (Chi) groups. (b) Distribution of the diameters of 5d salispheres with cavitation were shown in both groups. ($n \geqq 50$ for each group, $p>0.05$ (*t-test*).

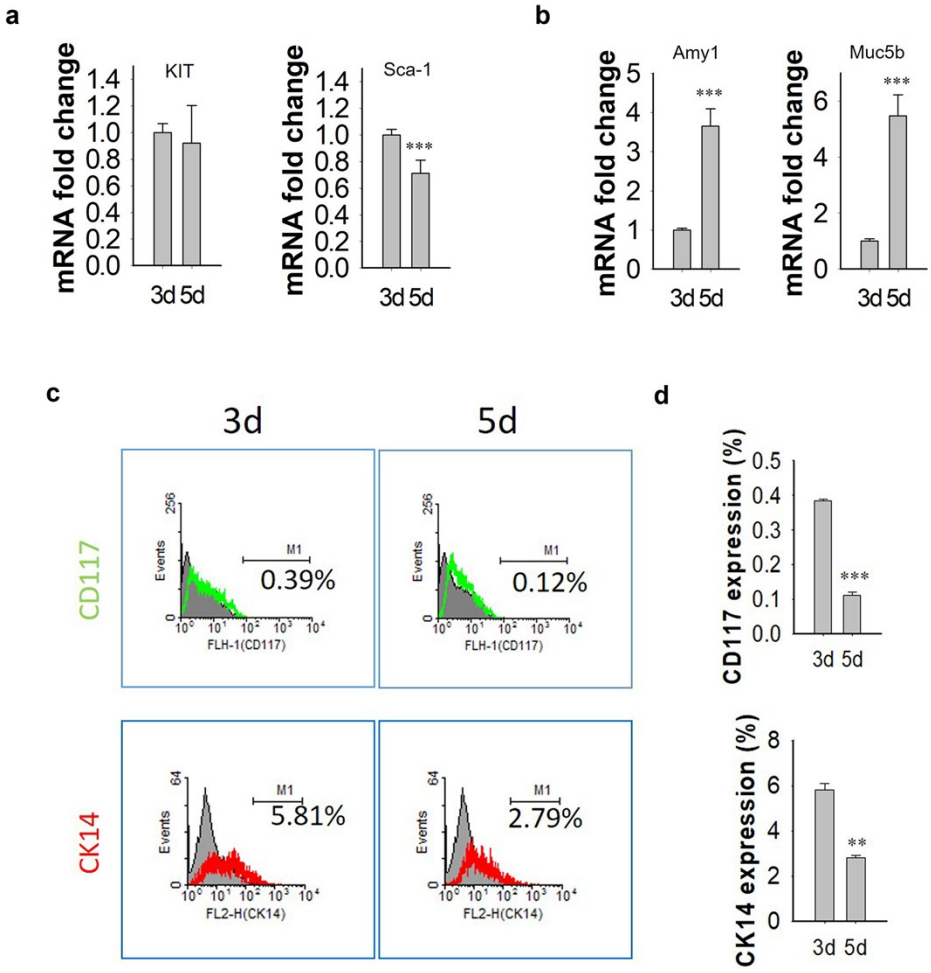

Fig. S3. Comparative analysis of the properties of 3d and 5d salispheres. qPCR analyses of (a) the genes associated with stemness, and (b) the gene associated with differentiation. (c) The representative histograms of flow-cytometry analysis of 3-day-old (3d) and 5-day-old (5d) salispheres by showing expression of CD117 (green), and CK14 (red). (d) Quantification of the flow-cytometry results. (*p<0.05; **p<0.01; ***p<0.001, *t-test*)

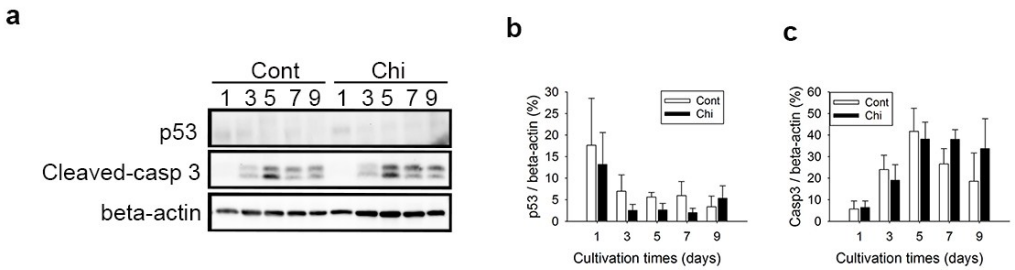

Fig. S4. Analyses of protein expression of p53 and cleaved caspase-3 by immunoblotting.

(a) Immunoblotting of p53 and cleaved caspase-3 of both groups were shown with beta-actin as internal controls. Semi-quantitative analyses of immunoblotting of (b) p53 and (c) cleaved caspase-3 of both groups at indicated time points. The asterisks indicated significant differences (*t-test, *p<0.05; **p<0.01;***p<0.001*).

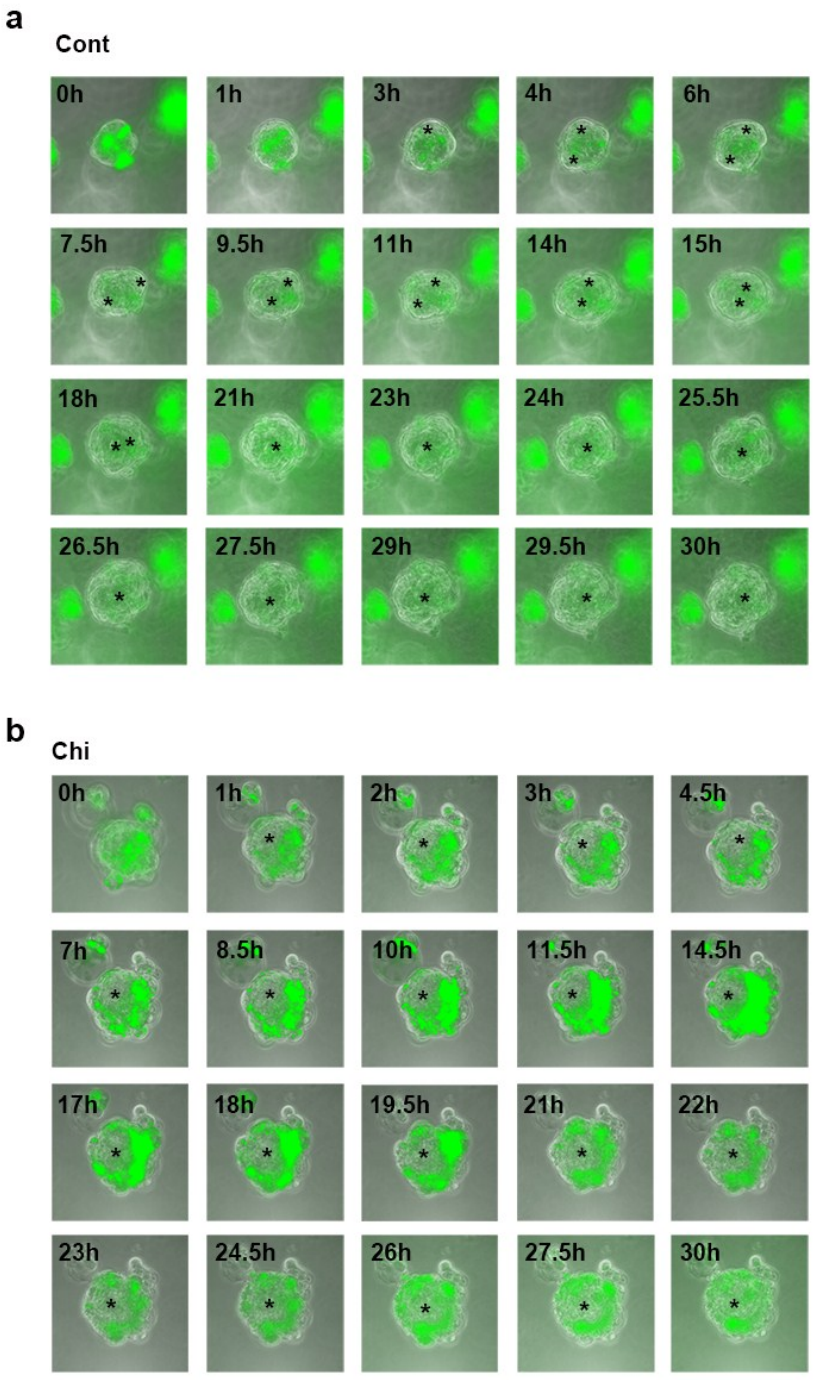

Fig. S5. Time-lapse recording of cavitation formation. Cavitation formation of cultured salispheres in (a) the control group, and (b) the chitosan-containing group**.** The asterisks (*) indicated the location of cavitation. (Green: calcein AM; h: hours)

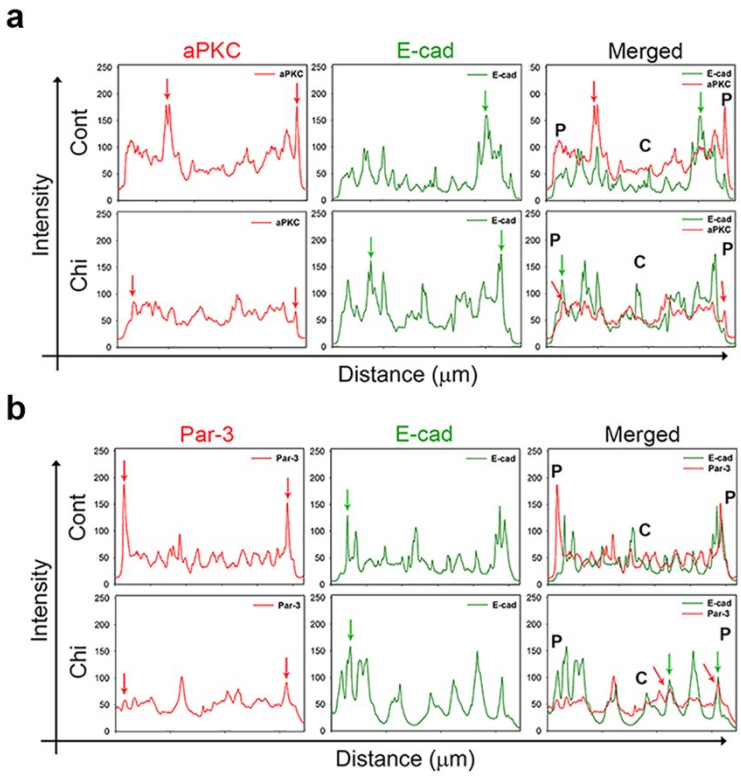

Fig. S6. Analyses of the fluorescence intensity line profiles of the salispheres. (a) The profiles of aPKC (red) and E-cadherin (green) on the salispheres of the control (Cont) and chitosan (Chi) groups. (b) The profiles of Par-3 (red) and E-cadherin (green) on the salispheres of both groups.

Table S1. Primers for qPCR analysis

Table-S1	Primers for qPCR analysis		
Gene symbol	Primer sequence	Base pairs	Sequence accession number
Gapdh	F: CTGGCATTGCTCTCAATGAC	122	NM_008084.2
	R: CAGGGTTTCTTACTCCTTGG		
Kit (cd117)	F: GCACCAAGCACATTTACTCC	213	NM_001122733.1
	R: GTAACCATCACAGAAGCCAG		
Krt5	F: GAAGGCCAAGCAGGACATGG	197	NM_027011.2
	R: TGCCTCCTCCGTAGCCAGAA		
Krt14	F: GAGAGGACGCCCACCTTTCA	151	NM_016958
	R: TCGTGGGTGGAGACCACCTT		
Ly6a (Sca-1)	F: TGCCCCTACCCTGATGGAGT	104	NM_001271416
	R: GGAGGGCAGATGGGTAAGCA		

Materials and Methods

Aspect Ratio (AR)

For phenotypic analyses of the salispheres, the long-axis and short-axis of selected salispheres were measured by ImageJ software. More than 50 salispheres of each condition were quantified. The AR was defined as: AR = the length of short-axis / the length of long-axis. The values of AR close to 1 suggested roundness; while the AR close to 0 suggested elongated phenotypes.

Circularity

The circularity of salispheres were measured and calculated by ImageJ software. More than 50 salispheres of each condition were quantified. The circularity was defined as: Circularity = $4\pi A / P^2$; A: the area of spheres; P: the perimeters of spheres.

Flow-cytometry analysis

Salispheres were harvested at defined time-points from the control and chitosan groups. To harvest single cells the cells were re-suspended by trypsin-EDTA (0.025%). Digested spheres were then re-suspended in cold PBS buffer with 0.1% BSA, and then centrifuged. Single cell suspensions were then incubated with primary antibodies including FITC-conjugated anti-mouse CD117 (Abcam), anti-mouse Ly-6A/E (Abcam), anti-cytokeratin 5 antibody (Abcam) and anti-cytokeratin 14 antibody (Abcam). Secondary antibodies were then applied. The cells were further filtrated by 40mm mesh before analyzed by FACS caliber flow-cytometry (BD). All the acquired data were analyzed by NIH WinMDI 2.9 software.

Time-lapse imaging

The 3-day-old salispheres were harvested after only 3-5% primary salivary cells labelled with calcein AM dye (Invitrogen). The salispheres were then re-suspended in a 0.8% low melting point agarose gel for facilitating time-lapse recording. The time-lapse imaging was recorded by Leica DMi8 inverse microscopy with Andor Zyla 5.5 high resolution CCD, all images were captured under 400x high powered view with 30 minutes interval.

Self-renewal assay of salispheres

All the protocols of salispheres self-renewal assays followed previously published

protocols [1]. Briefly, cultured salispheres were dissociated by 0.025% trypsin-EDTA Isolated cells were re-suspended in the growth factor-reduced Matrigel Matrix (BD) with culture medium, or chitosan-containing medium (Chi-M) in a 6:4 ratio, with around 1000 cells in each drop. Matrigel drops were plated in 24-well plates and incubated at 37℃ for 45-50 minutes for gelation. The media were replenished every 3 days. For subsequent passage, the gel-drops were dissociated with dispase (Corning, 10U dispase/mL culture medium) for 1h, followed by digestion medium (1.5 mg type I collagenase / mL culture medium) for another 45 minutes at 37℃ humidified 5% CO_2. The pellets were further digested by 0.025% trypsin-EDTA for 20 minutes. The cells were counted prior to re-plate into another new Matrigel drop following the steps mentioned above.

Quantitation of salispheres formation

The methodology of analyzing salisphere formation reported in the previous study were followed.[2] Salispheres were counted after 7 days culture in gelated Matrigel. The efficiency of salispheres formation was defined as: Salispheres formation (%) = Amounts of salispheres / total plated cells. Each drop was calculated independently. At least 15 drops were analyzed in both control and chitosan groups for comparison.

References

1. J. Feng, M. van der Zwaag, M. A. Stokman, R. van Os and R. P. Coppes, *Radiother Oncol*, 2009, **92**, 466-471.
2. A. Banh, N. Xiao, H. Cao, C. H. Chen, P. Kuo, T. Krakow, B. Bavan, B. Khong, M. Yao, C. Ha, M. J. Kaplan, D. Sirjani, K. Jensen, C. S. Kong, D. Mochly-Rosen, A. C. Koong and Q. T. Le, *Clin Cancer Res*, 2011, **17**, 7265-7272.