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RELATIONSHIP BETWEEN CELL FUNCTION AND INITIAL CELL SEEDING DENSITY OF PRIMARY PORCINE CHONDROCYTES *IN VITRO*

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ABSTRACT

Maintenance of differentiated functional phenotype within *in vitro* chondrocyte culture requires seeding at high densities with large numbers of cells. However, optimal cell seeding numbers and densities remain elusive due to multiple varying parameters and different methodologies utilized in previous studies. In the current study, we tried to investigate the relationship between cell seeding number and differentiated functional phenotype of *in vitro* cultured chondrocytes. Varying numbers of primary porcine chondrocytes (0.25, 2.5, 25 and 250 K) were seeded in 96 well-plates and cultured for 4 weeks. Cell proliferation, glycosaminoglycan (GAG) production and gene expression levels of Sox9, aggrecan, COL II and COL I were evaluated. The results showed that GAG content was high in the 0.25 and 25 K groups, gene expression of Sox9 was high in the 2.5, 25 and 250 K groups and expression of COL II was high in the 25 K group, whereas expression of COL I was low in the 0.25, 25 and 250 K groups. It is concluded that the seeding number and density of the 25 K (78 K cells/cm^2) group achieved the optimal balance between functional phenotype of individual cells and the total ECM production for *in vitro* cultured chondrocytes.

Keywords: Cartilage; Chondrocyte; Cell number; Phenotype.

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INTRODUCTION

In the absence of timely and proper medical therapy, most cartilage defects lead to irreversible degeneration, known as osteoarthritis[1] Significant progress has been achieved in cartilage regeneration, in which chondrocytes and mesenchymal stem cells played a central role in both implantation (autologous chondrocytes implantation, mosaicplasty or tissue engineered cartilages) and recruitment *in vivo* (microfracture, bone marrow stimulation).[2,3] Appropriate cell number and cell aggregation are of great importance during both embryonic development and adult tissue regeneration. The mesoderm fails to form when the total cell number is less than 200 during mesoderm induction.[4] Aggregation of mesenchymal stem cells (MSCs) initiates MSC differentiation into mature chondrocytes, as well as chondrogenesis during embryonic development.[5] Chondrocytes also aggregate to function in pericellular matrix and microcapsules under physiological conditions.[6]

Aggregation of extra high number of cells has been widely adopted both *in vitro* (micromass and pellet culture) and *in vivo* to promote chondrogenesis, as this process partially mimics embryonic chondrogenesis.[7–9] Although initial cell seeding number is critical for chondrocyte functionality, there is still a lack of consensus on the optimal cell number, particularly when multiple parameters are involved i.e. different methodologies, biomaterials.[10–15] Raising cell number from 1 to 25 million cells/mL/alginate bead could enhance the production of glycosaminoglycan (GAG) at the expense of the functional phenotype of individual cells, but an extra-high cell number of 70 million cells/mL alginate may hinder chondrogenic differentiation within alginate beads.[10,12] In another study, 0.25 to 11 million chondrocytes were seeded onto 5 mm diameter wells to evaluate their aggregation and chondrogenesis. An optimal initial seeding number of 3.75 million cells was determined upon evaluation of gross morphology, histological, biochemical and biomechanical data.[11] Although previous studies on 3D cell culture is physiologically relevant, the multiple varying parameters involved and different methodologies limited further extrapolation of these data on cells cultured within culture plates (see Table 1). Although the use of culture plates is well established for proliferation and *in vitro* chondrogenesis, it is still challenging to balance between maintenance of differentiated functional phenotype and proliferation of the chondrocytes *in vitro*. The aim of this current study is therefore to explore optimal cell numbers for a standardized *in vitro* cell culture model without biomaterials.

Although high seeding densities of chondrocytes has been broadly adopted as models of *in vitro* chondrogenesis, there has not yet been any consensus on optimal cell numbers for functional phenotype and mechanistic studies. We hypothesize that different numbers of aggregated chondrocytes may result in varying stages of

Table 1. Summary of Published Scientific Literature on Chondrocyte Seeding Densities with Various Biomaterials.

Cell Number	Materials and Structure	Results	Conclusions	References
1−25 million cells/mL/bead	3D alginate beads	Total GAG expressed most in 10.4 million cells/mL. GAG per million cells remained falling with more cells.	There was an optimization of total ECM, but a conflict considering the efficiency of individual cells.	10
0.25−11 million cells/construct	2D agarose constructs	The self-assembling process of 3.75 million cells/construct was identified to be optimal.	There was an optimization.	11
4, 70 million cells/mL alginate	3D alginate beads	Sox9 expression was upregulated group and collagen I was downregulated in the low-cell-density. Collagen X expression was upregulated in the low-cell-density group. Only the high-cell-density group showed the expression of Collagen II.	Low cell density was better.	12
0.0125, 0.125, 0.5 million cells/sponge	3D porous alginate sponges	The higher the seeding density, the higher the final cell density and GAGs production.	High cell seeding density was better.	13
0.025−0.066 million cells/mm^3	3D type II collagen sponges	Increasing the HAC seeding density lead to higher GAG accumulation and type II collagen mRNA.	High cell seeding density was better.	14
0.2, 2, 20 million cells/mL	3D atelocollagen gel	Low cell density proliferated best. High cell-density matrixes produced the most chondroitin 6-sulfate.	There was a conflict.	15

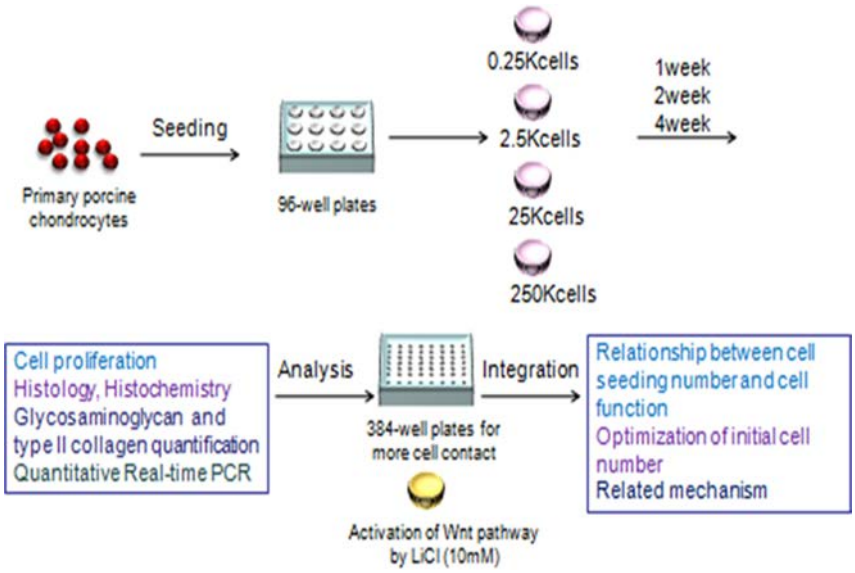


Fig. 1 Schematic diagram of experiment to explore the relationship between cell seeding density and functionality of chondrocytes.

differentiation and maturity and there is an optimized cell number for individual cells to attain differentiated functional phenotype. In this study, a simple standardized culture system was adopted without the interference of biomaterials. We investigated the relationship between individual cell phenotype and cell seeding numbers *in vitro* by evaluating cell proliferation, ECM production, gene expression and gross morphology upon culturing varying numbers of primary porcine chondrocytes at different seeding densities. Additionally, we also explored the underlying mechanism of chondrocyte functionality through activation of the Wnt pathway (see Fig. 1). The results obtained can set up a platform for subsequent experiments to explore potential therapeutic applications, as well as to characterize the underlying mechanisms of cartilage regeneration.

MATERIALS AND METHODS

Harvest and Culture of Primary Chondrocytes

Cartilage tissue was explanted from the knee joints of Wuzhishan Miniature female Pigs (1 year old, from the Institute of Animal Sciences Chinese Academy of Agricultural Sciences) not more than 4 h after the animals were culled. Chondrocytes were harvested through serial enzymatic digestion with 0.25% trypsin (27250-018, Invitrogen) for 30 min, followed with 0.1% type II Collagenase (17101-015, Gibico) at 37°C overnight. The cells were filtered and centrifuged before being cultured in a monolayer at a density of 18 K cells/cm^2 in 100 mm plates (430167, Corning) with DMEM supplemented with 10% fetal bovine serum (SV30087.02, Hyclone) 100 U/mL of penicillin/and 100 μg/mL of streptomycin at 37°C with 5% CO_2. Fresh medium was replaced at three days' interval. The cells were trypsinized at 85% confluence and cryopreserved. Thawed primary chondrocytes at passage 1 were utilized for all subsequent experiments.

Chondrocyte Culture

Varying numbers of chondrocytes (0.25, 2.5, 25, 250 K) were seeded in 96 well microplates (3628, Corning) with 200 μL (per well) of DMEM supplemented with 10% fetal bovine serum, 300 μM sodium L-ascorbate (A7631, Sigma), and 100 U/mL penicillin and 100 μg/mL streptomycin, and cultured for 4 weeks.

Proliferation of the Chondrocytes

Proliferation of chondrocytes was assayed with Hoechst 33258 Dye (H6024, Sigma) at 1, 2 and 4 weeks of culture.[16] Briefly, cells were lysed in 100 μL of sterilized water and were frozen overnight at −20°C. About 0.1 μg/mL of Hoechst 33258 Dye were mixed with equal amount of the aforementioned cell lysate sample. Fluorescence intensity was measured at excitation and emission wavelengths of 360 and 465 nm, respectively. Cell numbers were extrapolated from the calibration curve. There were three experimental replicates for each sample.

Quantification of GAG

Cells were digested with 200 μL of 50 mg/mL proteinase K (100 mg, Beijing Hua Lvyuan Biotechnology Development Center) at 56°C overnight, before being reacted with dimethylmethylene blue (DMMB, 341088, Sigma).[17] Half milliliter of working DMMB solution (16 ug/mL DMMB in 2.5% ethanol, 1 M GuHCl, 0.2 g/L sodium formate and 2% formic acid) was added to each sample and vortexed for 30 min before being centrifuged and dissolved in 0.5 mL of decomplexation solution (50 mM sodium acetate solution buffer of pH 6.8, 10% propan-1-ol, 4 M GuHCl) with vortexing for 30 min. Absorbance was measured at 630 nm. sGAG content was extrapolated from the chondroitin sulfate (27042-10G-F, Sigma) standard curve. There were three experimental replicates for each sample.

Histology, Alcian Blue and Immunohistochemical Staining

The cells were fixed with 4% formaldehyde for 30 min and washed before staining. Cells were stained with Haematoxylin & Eosin for 10 and 5 min, respectively. Alcian blue (1%, pH 0.2, 71001182, Sinopharm Chemical Reagent Co., Ltd) and Nuclear Fast Red (0.1%, 71029480, Sinopharm Chemical Reagent Co., Ltd) were incubated with cells for 30 and 5 min, respectively, to detect sulfated glycosaminoglycans (sGAG). The distribution of type II collagen was visualized by Mouse specific HRP/DAB detection kits (ab64259, Abcam), according to the manufacturer's recommended protocols and cells were counterstained with Hematoxylin. Briefly, 2 μg/mL of mouse anti-porcine monoclonal antibodies (ab3092, Abcam) against type II collagen were incubated with the fixed sample at 4°C overnight. Microscopic imaging of the distribution of GAG, Type II collagen and H&E staining was carried out with an optical microscope (DFC425, Leica). The images of type II collagen immunostaining were semi-quantified with the software Image pro plus 6.0 with integral optical density (IOD) as the units. The results were normalized to the 0.25 K group at 1 week of culture. There were three experimental replicates for each sample.

Quantitative Real-Time PCR

For PCR analysis, cells were seeded in 35 mm plate (430165, Corning) at the same cell density as in the 96-well plate. Total RNA was extracted by Trizol Reagent (206101, New Industry) according to the manufacturer's instruction after 1, 2 and 4 weeks of culture. The RNA samples were semi-quantified with Nanodrop (NanoDrop1000, Thermo) to 500 ng. Subsequently, the total RNA was transcribed into cDNA with M-MLV reverse transcriptase (C28025-011, invitrogen) and oligo-dT (FSK-201, Toyobo) using the PCR thermal cycler (MyCyclerTM, BioRad). Quantitative real-time PCR (DNA Engine Opticon 2 System, Bio-Rad Inc., CA, USA) was performed in PCR Tubes Strip (PCR-0208-C, Axygen) with RealMasterMix SYBR Green PCR master mix (FP302-02, Tiangen) following the manufacturer's instructions. The following genes were amplified for 40 cycles: glyceraldehyde-3-phosphate dehydrogenase (GAPDH, housekeeping gene as internal reference), type I collagen (COL I), type II collagen (COL II), Aggrecan (Agg) and Sox9. The results were normalized to GAPDH expression. Primer sequences are shown in Table 2. There were three experimental replicates for each sample.

Table 2. The Primer Sequences Utilized for Real-Time PCR Analysis.

Gene	Primer Sequence	Size (Basepair)
GAPDH	Forward: GTCATCCATGACAACTTCGG Reverse: GCCACAGTTTCCCAGAGG	103
Sox9	Forward: ATCAGTACCCGCACCTGCAC Reverse: CTTGTAATCCGGGTGGTCCTT	145
Type II collagen	Forward: GAGAGGTCTTCCTGGCAAAG Reverse: AAGTCCCTGGAAGCCAGAT	118
Aggrecan	Forward: CGAAACATCACCGAGGGT Reverse: GCAAATGTAAAGGGCTCCTC	107
Type I collagen	Forward: CAGAACGGCCTCAGGTACCA Reverse: CAGATCACGTCATCGCACAAC	101

Data Processing and Statistics

As current Hoechst 33258 and DMMB assays were not sensitive enough to detect trace amounts of cells and GAGs, cumulative data from 10 samples of the 0.25 and 2.5 K groups were evaluated. Statistical significance of differences in quantitative data between experimental groups were evaluated with SPSS 13.0 (One-way ANOVA, LSD, $p < 0.05$) and results were expressed as mean $\pm$ standard deviation.

RESULTS

Chondrocyte Culture and Cell Proliferation

The size of the chondrocytes were homogeneous, with a ellipsoid or spindle morphology [see Fig. 2(A)]. The cell surface area was approximately $1594 \pm 349\,\mu m^2$ at

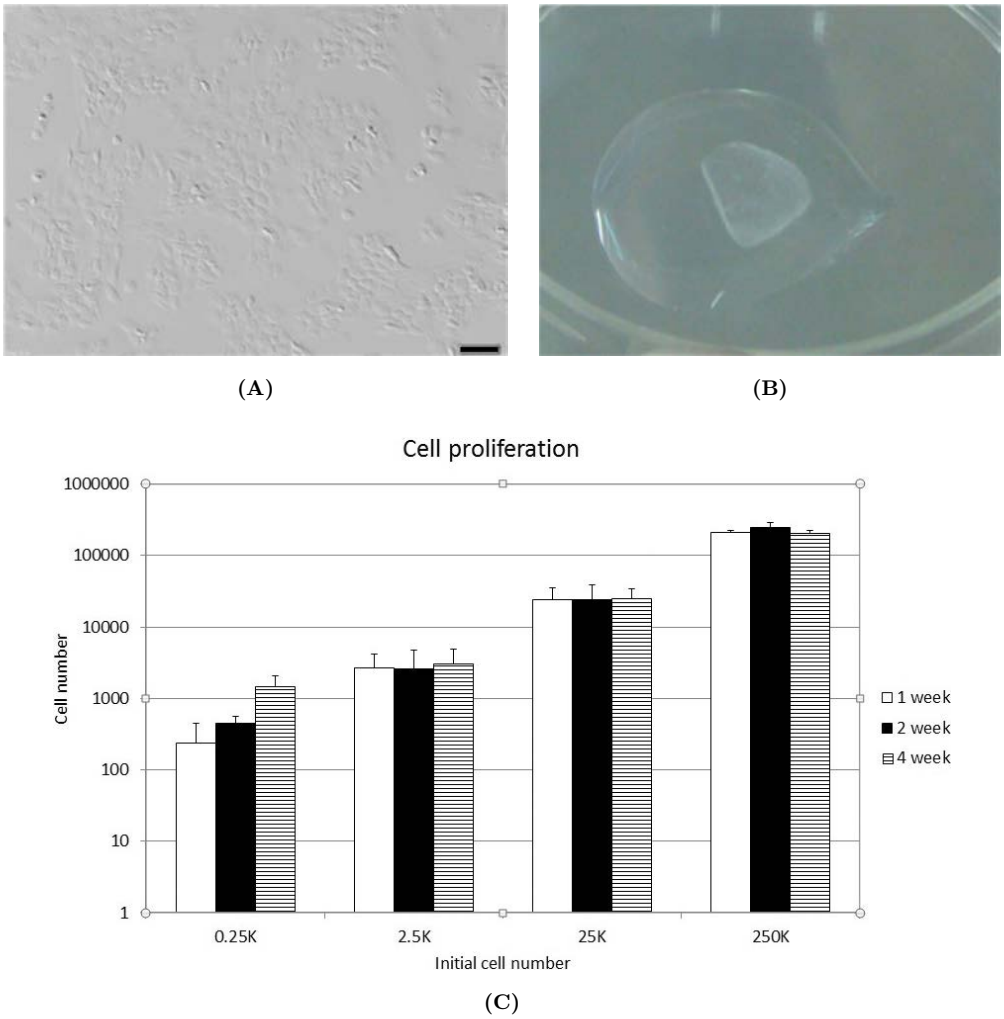
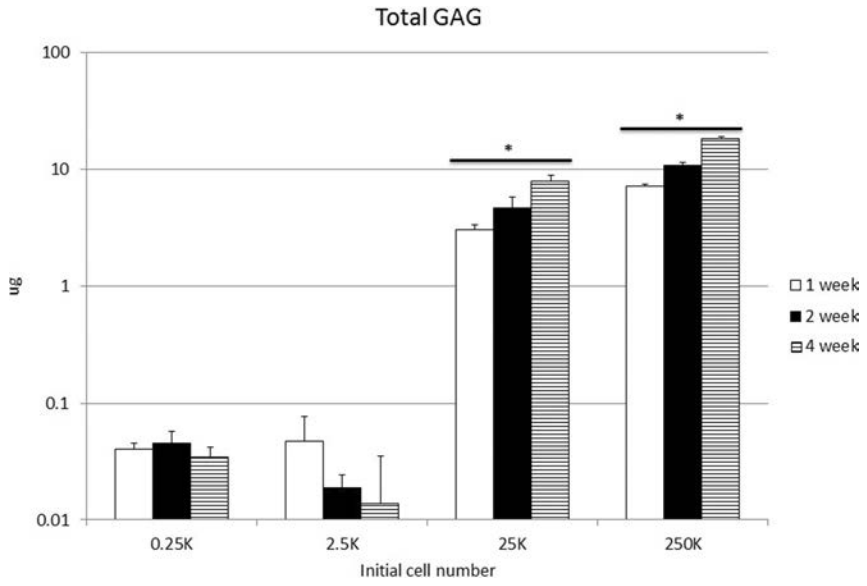


Fig. 2 Chondrocyte culture and cell proliferation. (**A**) Microscopic images of primary porcine chondrocytes. Bar = 100 um. (**B**) Neo-cartilage being formed in the 250 K group after 1 week of culture. (**C**) Chondrocytes proliferated slowly in the 0.25 K group, while the cell number was consistently maintained in the other three groups ($n = 3$, One-Way ANOVA, LSD, $p < 0.05$).

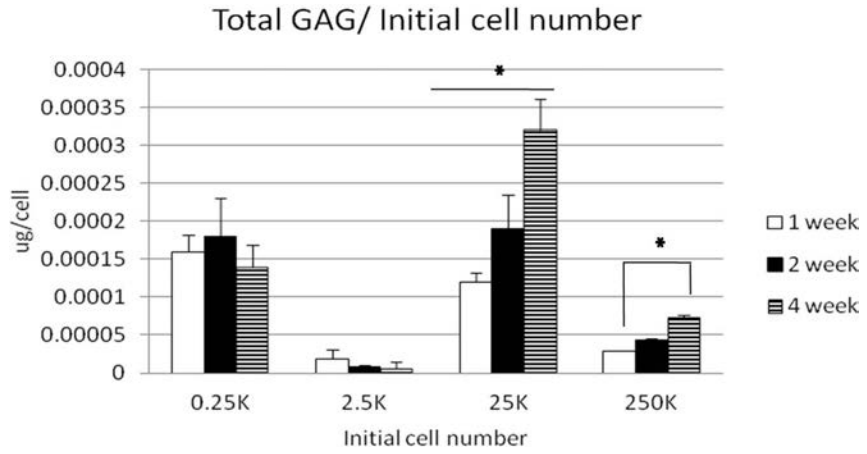
1 week, $1833 \pm 430\,\mu m^2$ at 2 weeks and $2264 \pm 523\,\mu m^2$ at 4 weeks of culture. Dozens of chondrocytes were gathered together in clusters and these aggregates were uniformly distributed. Tight cell sheets formed with thickness of about 1−2 mm with shape matching a well of the 96-well plate in the 250 K group after 1 week of culture [see Fig. 2(B)]. There was no cell sheet formation with cell number less than 250 K *in vitro*. Chondrocytes proliferated slowly in the 0.25 K group (1.8 times at 2 weeks and 6.4 times at 4 weeks), while the cell number was kept stable in the other three groups [see Fig. 2(C)], with no significant difference in cell numbers with respect to time ($n = 3$, One-Way ANOVA, LSD, $p > 0.05$).

Quantification of GAG

Total cumulative GAG content remained unchanged in the 0.25 K group, while it dropped to 40% at 2 weeks culture and 30% at 4 weeks culture in the 2.5 K group. However, there was no significant difference between these two time-points ($n = 3$, One-Way ANOVA, LSD, $p = 0.991$) [see Fig. 3(A)]. Total GAG content increased significantly in the 25 and 250 K groups with time (1.5 times at 2 weeks and 2.6 times at 4 weeks) ($n = 3$, One-Way ANOVA, LSD, $p < 0.05$) [see Fig. 3(A)]. On the other hand, cumulative GAG production per cell was significantly higher in the 0.25 K group and the 25 K group, as compared to the other two groups, while cumulative GAG production per cell in the 25 K group was highest after 4 weeks of culture. Cumulative GAG production per cell significantly increased with time in both the 25 and 250 K groups, while they remained unchanged with time in both the 0.25 and 2.5 K groups. ($n = 3$, One-Way ANOVA, LSD, $p < 0.05$) [see Fig. 3(B)].



(A)



(B)

Fig. 3 Quantization of GAG. (**A**) Total GAG production was quantified during 4 weeks of culture. (**B**) Cellular production of GAG was presented as "Total GAG/ Initial cell seeding number" from 1 to 4 weeks of culture. Significant difference was indicated by an asterisk ($n = 3$, One-Way ANOVA, LSD, $p < 0.05$).

Histology and Immunohistochemical Staining

The cells in the 250 K group displayed round-shaped morphology which was not observed in the other groups (see Fig. 4). GAG and COL II staining were strongly expressed after 1 week of culture in the 250 K group, and continue to increase with time. For the 25 K group, the seeded cells initially adopted a fibroblastic morphology after 1 week of culture, and only regained round-shaped mophology when cell density increased after 2 and 4 weeks of culture. GAG and COL II expression were much weaker, and were intially distributed non-homogenously but increased with time. In the 0.25 and 2.5 K seeding groups, cells retained fibroblastic morphology throughout the entire 4 weeks culture period with slight expression of GAG and a few COL II positively-stained cells [see Figs. 5(A)–5(C)].

Semi-quantitative analysis of type II collagen (COL II) expression indicated significant increase with both time and initial seeding cell number in the 25 and 250 K groups. Significant differences were detected between 2 weeks (1.4 times) and 4 weeks (5.0 times) for the 25 K group, and between 1 week and 2 and 4 weeks (2.0 times) for the 250 K group [see Fig. 5(D), $n = 3$, One-Way ANOVA, LSD, $p < 0.05$]. No significant increase in COL II expression with time was detected for the two lower seeding densities.

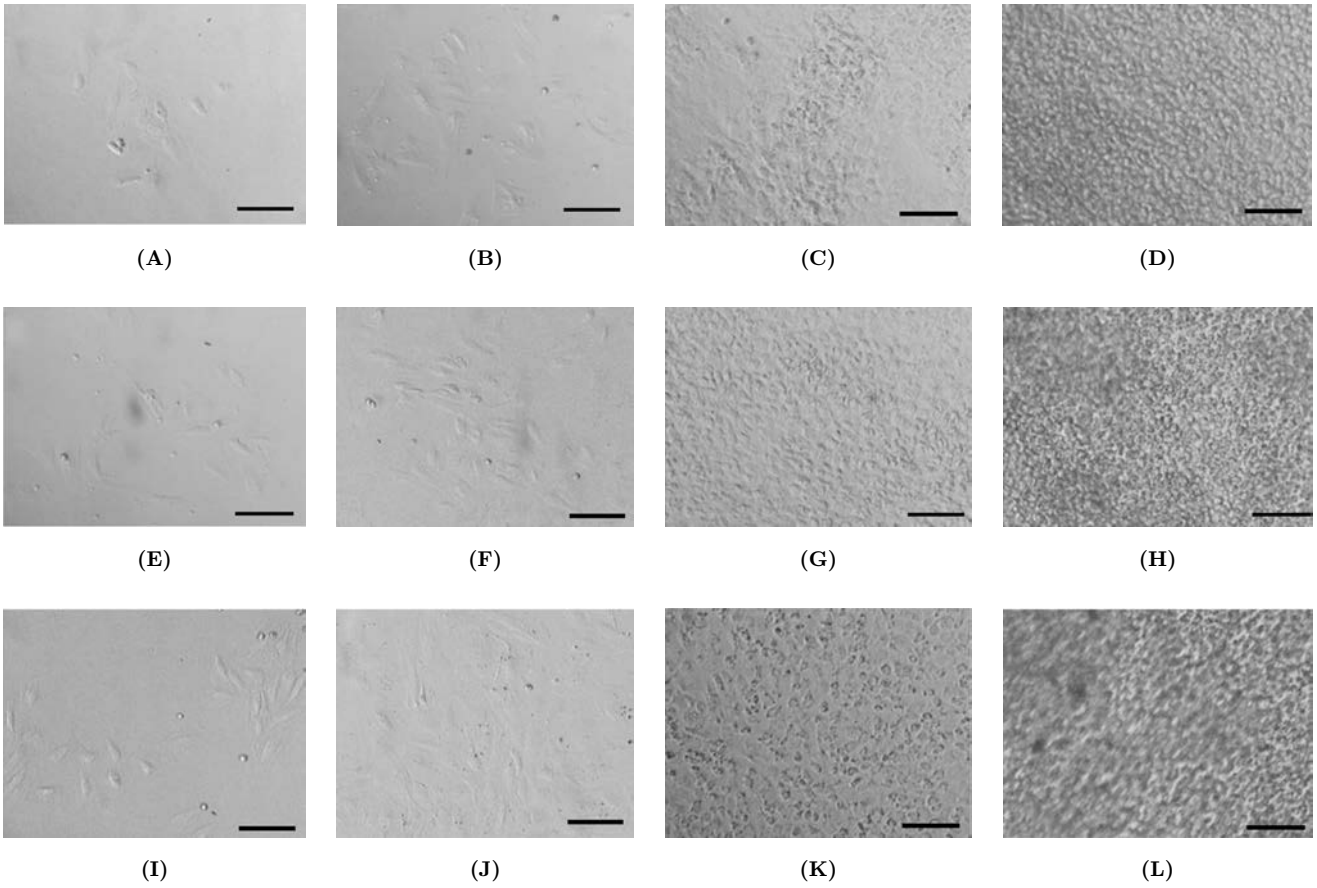
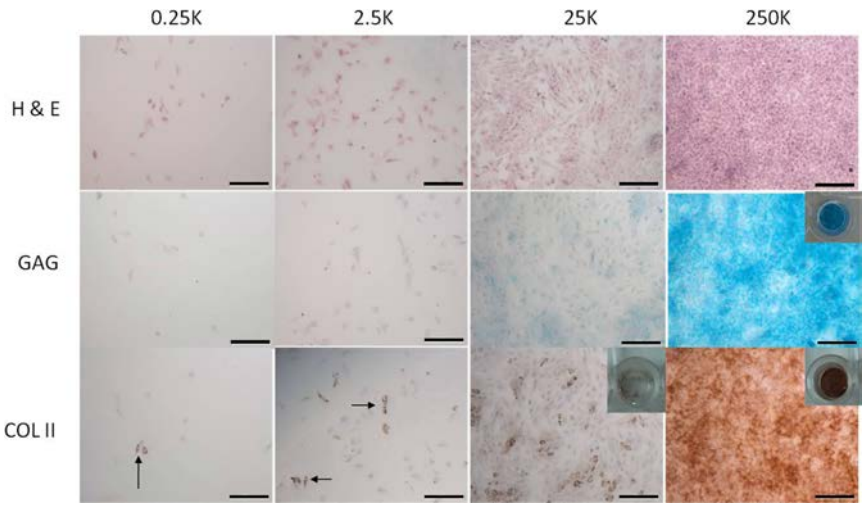
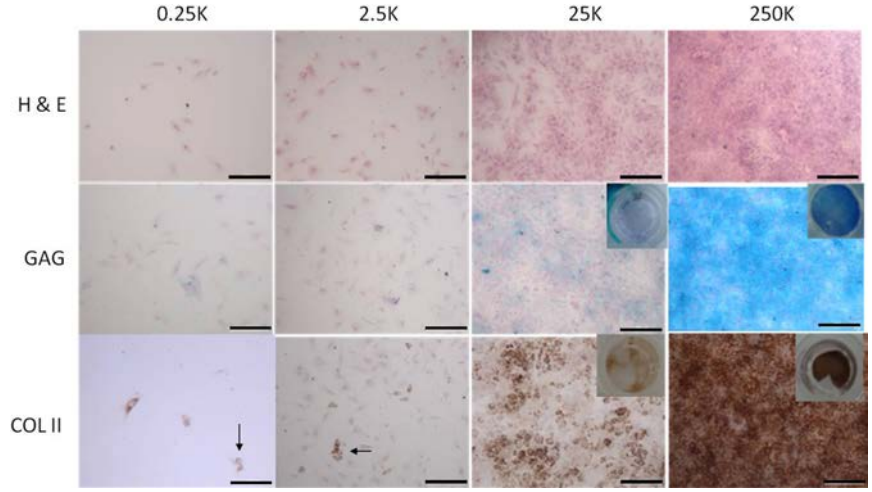


Fig. 4 Chondrocytes cultured at varying densities from 1 to 4 weeks. (**A**–**D**) 0.25 K to 250 K groups at 1 week, (**E**–**H**) 2 weeks, (**I**–**L**) 4 weeks Bar = 100 um.



(**A**)

Fig. 5 H&E staining, GAG staining with Alcian Blue, and Immunohistochemical staining for COL II at 1 and 4 weeks of culture. (**A**) 1 week; (**B**) 2 weeks; (**C**) 4 weeks. The black arrows indicate the aggregated cells with type II collagen expression. Bar = 200 um. (**D**) Semi-quantization of COL II expression by immunohistochemical staining. Total COL II production was recorded during 4 weeks of culture. Significant difference is indicated by an asterisk ($n = 3$, One-Way ANOVA, LSD, $p < 0.05$). "IOD" = integral optical density.



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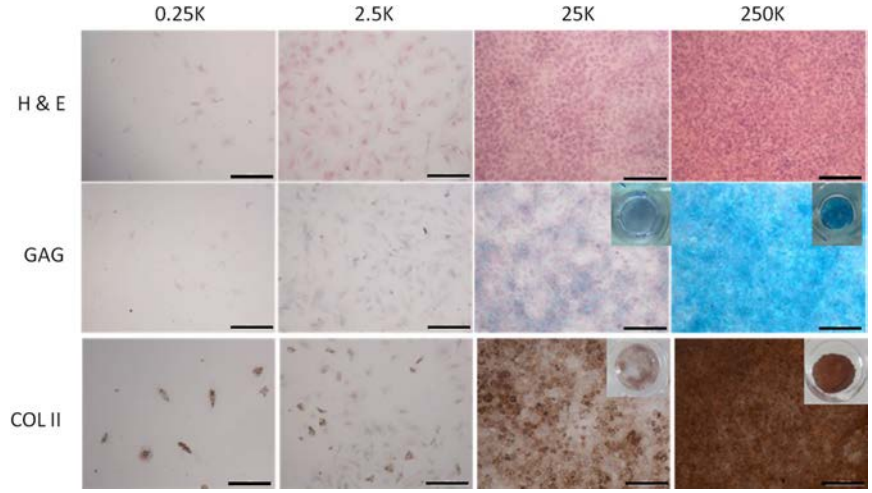
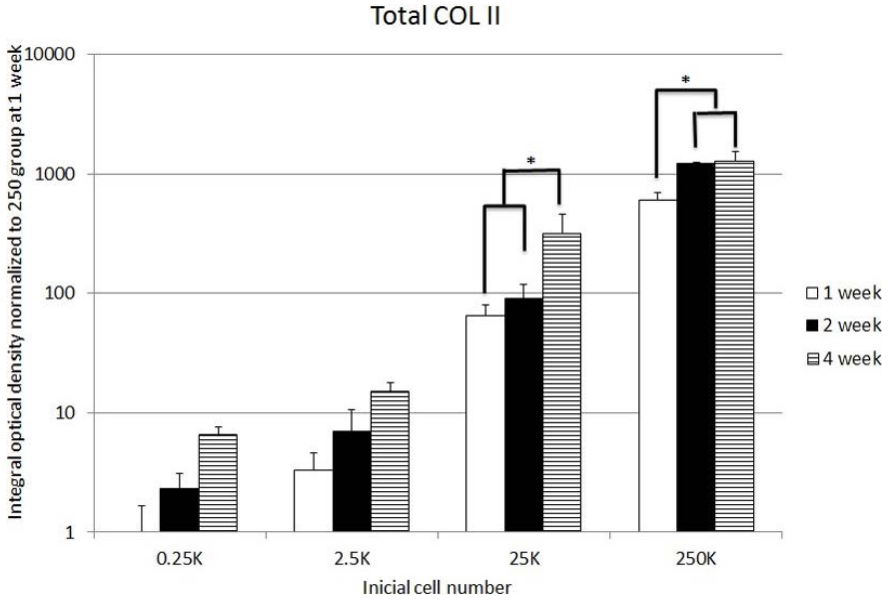


(C)



(D)

Fig. 5 (*Continued*)

Gene Expression

The expression of Sox9 increased after 2 weeks of culture, but subsequently decreased significantly in all groups at 4 weeks, except for the 0.25 K group which displayed low Sox9 expression levels that remained unchanged throughout the entire cuture period [see Fig. 6(A), $n = 3$, One-Way ANOVA, LSD, $p < 0.05$]. The other three groups from 2.5 to 250 K displayed incresed Sox9 expression levels of 4.7, 3.9 and 2.7 folds, respectively at 2 weeks compared to 1 week, while decreasing to 2.0, 1.2 and 0.12 folds, respectively at 4 weeks compared to 1 week.

The expression of aggrecan increased after 2 weeks of culture and subsequently decreased at 4 weeks for all groups, except for the 0.25 K group. The significant difference was found between 2 weeks and 1, 4 weeks in the 250 K group [see Fig. 6(B), $n = 3$, One-Way ANOVA, LSD, $p < 0.05$].

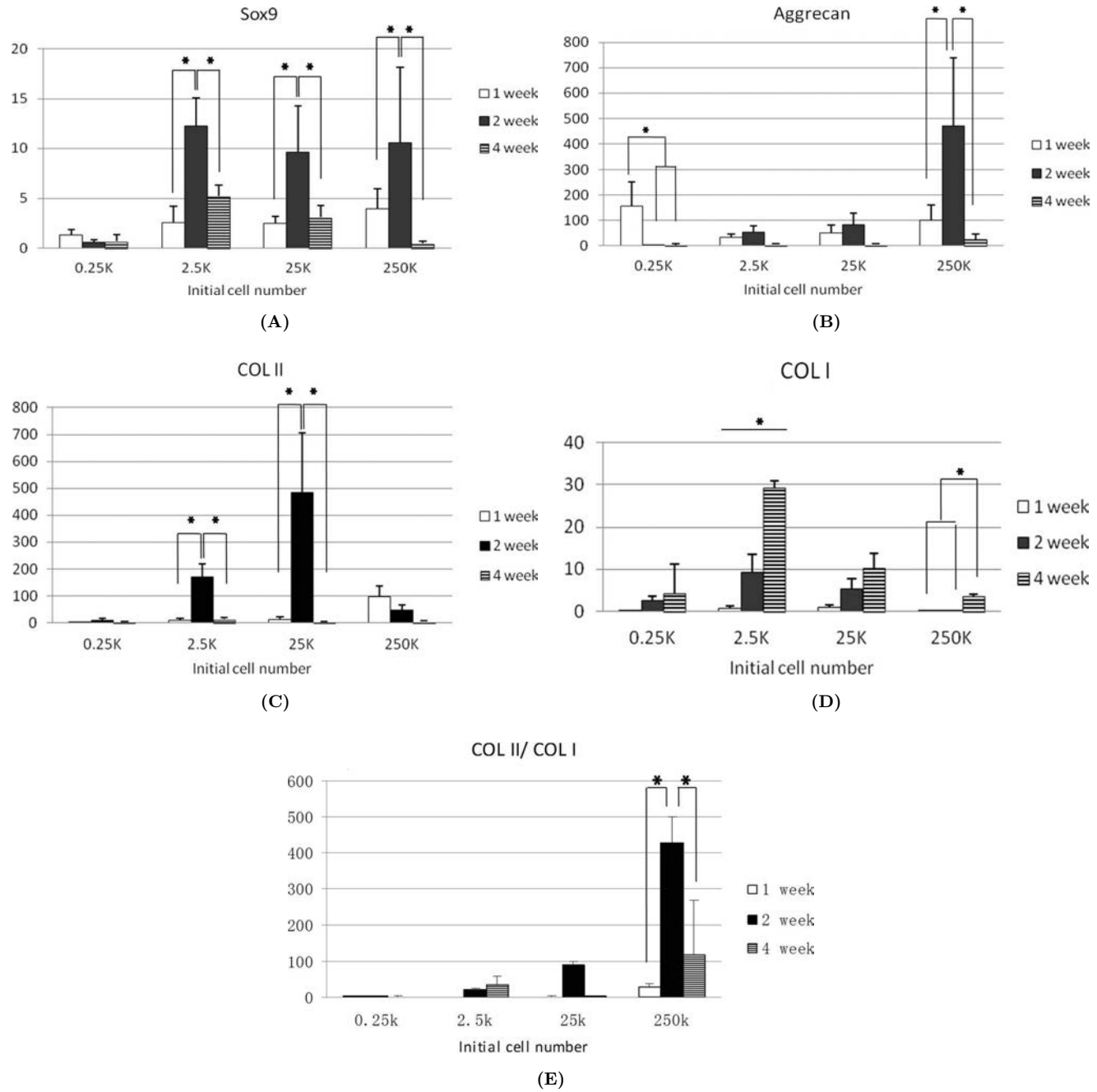


Fig. 6 Expression of cartilage specific genes by the 0.25 to 250 K seeding density groups during 4 weeks of culture. (**A**) Sox9; (**B**) Aggrecan; (**C**) COL II; (**D**) COL I; (**E**) Ratios of COL II/ COL I. Significant difference is indicated by an asterisk ($n = 3$, One-Way ANOVA, LSD, $p < 0.05$).

The gene expression of COL II increased after 2 weeks of culture but decreased at 4 weeks for all groups, except for the 250 K group (47% at 2 weeks and 3.5% at 4 weeks). There was a significant difference between the 2.5 and 25 K groups at 2 weeks and 1 or 4 weeks (17 and 39 times at 2 weeks, 1.3 and 0.2 times at 4 weeks) [see Fig. 6(C), $n = 3$, One-Way ANOVA, LSD, $p < 0.05$].

The gene expression of COL I increased with time for all groups, except for the 250 K group (32% at 2 weeks and 12-folds at 4 weeks). Significant increase was seen in the 2.5 K group at each progressive time point, as well as in the 250 K group between 4 weeks and other time points ($n = 3$, One-Way ANOVA, LSD, $p < 0.05$) [see Fig. 6(D)]. Upon comparing across seeding densities of 2.5, 25 and 250 K, it was observed that COL I expression level was inversely proportional to the seeding density, with higher COL I expression being detected at lower seeding densities.

The expression ratio of COL II/COL I increased with increasing cell density across the same time point. However, for all seeding density groups, the highest ratio of COL II/COL I was at 2 weeks and subsequently decreased at 4 weeks, while significant differences only existed between 2 weeks and other time points for the 25 and 250 K groups [see Fig. 6(E), $n = 3$, One-Way ANOVA, LSD, $p = 0$].

DISCUSSION

Proper cell−cell contact and cell aggregation are of utmost importance to chondrocyte differentiation and maturation. Increased cell density should lead to enhanced cell−cell contact, according to the hypothesis that formation of cell aggregates is guided by the diminution of adhesive-free energy as cells tend to maximize their mutual binding.[18] In this study, the optimal cell seeding number to maintain the differentiated functional phenotype of chondrocytes was determined by examining a range of increasing chondrocyte seeding densities within *in vitro* culture. In the normal functional chondrocyte phenotype, GAG content and gene expression of Sox9 and COL II should be maintained at high levels while COL I expression level should be low. The results showed that the total GAG content and COL II gene expression increased significantly in both the 25 and 250 K groups. The total GAG content and COL II is positively correlated to increasing initial cell seeding number, and it is expected that there would be more total ECM deposition with more seeded cells.[10,11] Optimal cell numbers to achieve the best functional phenotype of individual chondrocytes remained elusive, mainly due to varying methodologies and different ranges of cell numbers utilized in previous studies.[11] Aggregation mediated through cell−cell interaction is crucial in modulating the phenotype of individual chondrocytes.[19] GAG production per cell was highest in the 25 K group and increased with time. The consequence of low cell seeding number is the loss of functional chondrocyte phenotype in the 0.25 and 2.5 K groups. Subsequent GAG production was inadequately maintained when cell seeding number was low.[20] Interestingly, GAG content per cell in the 0.25 K group was higher than in the 2.5 K group. This difference was likely related to decreased cell−cell contact with extremely low seeding density versus other culture systems with high seeding density and dimensionality.[10,11] Although the 250 K group was better than the 0.25 and 2.5 K groups, with respect to maintainance of differentiated chondrocyte phenotype, it was less functional compared to the 25 K group. The extremely high cell seeding density of the 250 K group resulted in the formation of tight cell sheets, and this affected oxygen transport and nutrient exchange, leading to cellular apoptosis and death and limiting the ability of cells to produce matrix.[10,21] Transferring 0.25 and 2.5 K cells from a 96 to 384 well plate to increase seeding density also demonstrated that cell aggregation played an important role in modulating the functional phenotype of chondrocytes (see Fig. S1).

Besides GAG content, analysis of gene expression levels is also important for characterizing the functional phenotype of chondrocytes. The gene expression of Sox9 was higher in the 2.5, 25 and 250 K groups, COL II expression was high in the 25 K group, while COL I expression was high in the 2.5 K group. The results reaffirm that the 25 K group in the 96-well plate (78 K cells/cm^2) is optimal for maintaining the functional phenotype of chondrocytes and also for GAG production. Sox9 is an important factor in chondrogenic differentiation, and has been shown to directly upregulate expression of COL II.[22] The expression of Sox9 increased during the first 2 weeks of culture and then declined thereafter, which is consistent with previous studies.[12] As an upstream regulator, the gene expression of COL II was similar to Sox9 in the 0.25, 2.5 and 25 K groups.[23] However, the expression of COL II was not proportional to Sox9 in the 250 K group. The 250 K group with high density formed cell sheets within 96-well plates, reinforcing the previous observation that Sox9 expression under high cell density inhibited expression of COL II.[24] This effect might arise from a Sox9-sensitive gene aggrecan.[12]

Cell−cell contact and interaction between chondrocytes are directly regulated and controlled by cadherins. Beta-catenin is linked to cadherin-based adhesive junctions, which are in turn influenced by the wnt signaling pathway.[25] The wnt signaling pathway is known to be critical for chondrocyte function, while both activation and disruption of the wnt/beta-catenin pathway resulted in cartilage breakdown.[26] Proper cell−cell contact and round morphology of the chondrocytes in the high-density (25 and 250 K) groups maintained functional phenotype of the chondrocytes, whereas spindle morphology of the cells cultured in the low-density groups (0.25 and 2.5 K) hinted at dedifferentiated phenotypes. Activation of the wnt pathway with LiCl changed the morphology of chondrocytes in both the 25 and 250 K groups from round to spindle-shaped morphology (see Fig. S2). This was accompanied with decreased expression of Sox9, aggrecan and COL II in the 25 and 250 K groups, and increased expression of collagen I (see Fig. S3), suggesting a rapid loss of hyaline phenotype in the presence of beta-catenin activation despite high cell seeding density. Given the close relationship between the wnt signaling pathway and cell-adhesion, it is possible that beta-catenin activation has a role in cell aggregation-related phenotype maintenance of chondrocytes in this study.[25]

Future studies to examine the expression and localization of beta-catenin and cadherins during cell aggregation would further confirm the role of wnt. Proper cell aggregation will certainly be beneficial for chondrocyte functionality. However, optimal parameters such as aggregation force, mode, duration, effects of biomaterials etc., still needs to be investigated. The current study has established a simple cell−cell contact research model, which can be utilized as a platform for investigating cell−cell and cell−matrix interaction. Future investigations on the role of wnt should be focused on chondrocyte functionality.

CONCLUSIONS

Approximately 25 K (78 K cells/cm^2) cells has been determined as the appropriate cell number for attaining balance between chondrocyte proliferation, GAG production and functional gene expression. The current study has optimized the cell number and seeding density for maintaining functional chondrocyte phenotype within a standardized *in vitro* culture model. Furthermore, it has established a simplified research model for cell-aggregation study, which could advance both basic research and clinical applications.

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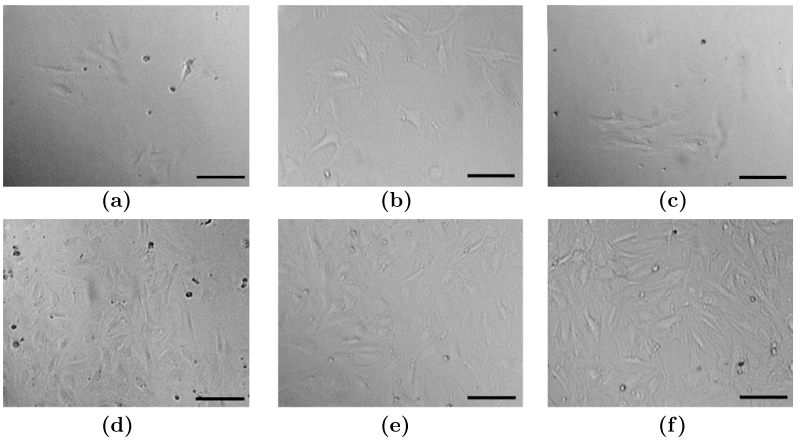
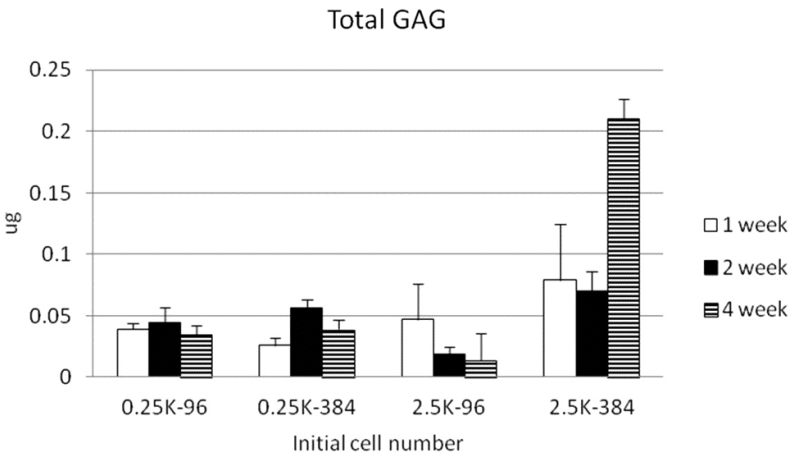


(A)



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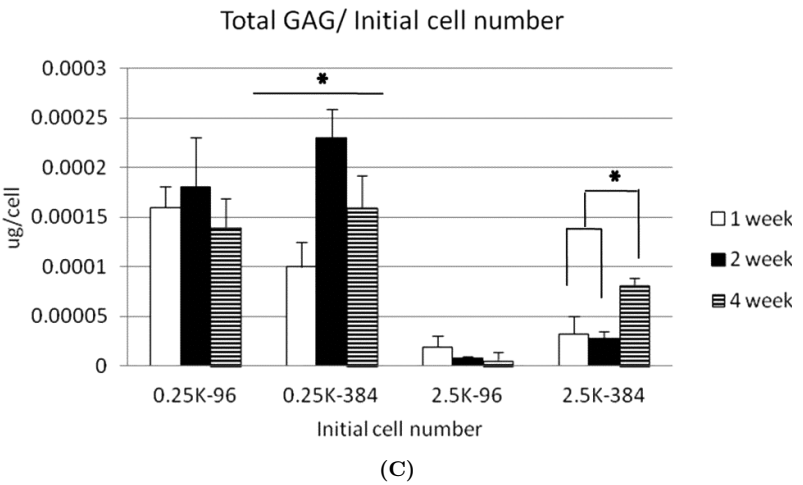

(C)

Figure S1. Chondrocytes cultured within the 0.25 K and 2.5 K groups in 384-well plates. (**A**) Microscopy images of 0.25 K and 2.5 K chondrocytes cultured within 384-well plates from 1 week to 4 weeks. (a)−(c) 0.25 K group at 1, 2 and 4 weeks, (d)−(f) 2.5 K group at 1, 2 and 4 weeks. (**B**) Total GAG production by the 0.25 K and 2.5 K groups within 96-well and 384-well plates were assayed. (**C**) GAG production normalized to cell numbers for the 0.25 K and 2.5 K groups in 96-well and 384-well plates. Significant difference was indicated by an asterisk ($n = 3$, One-Way ANOVA, LSD, $P < 0.05$).

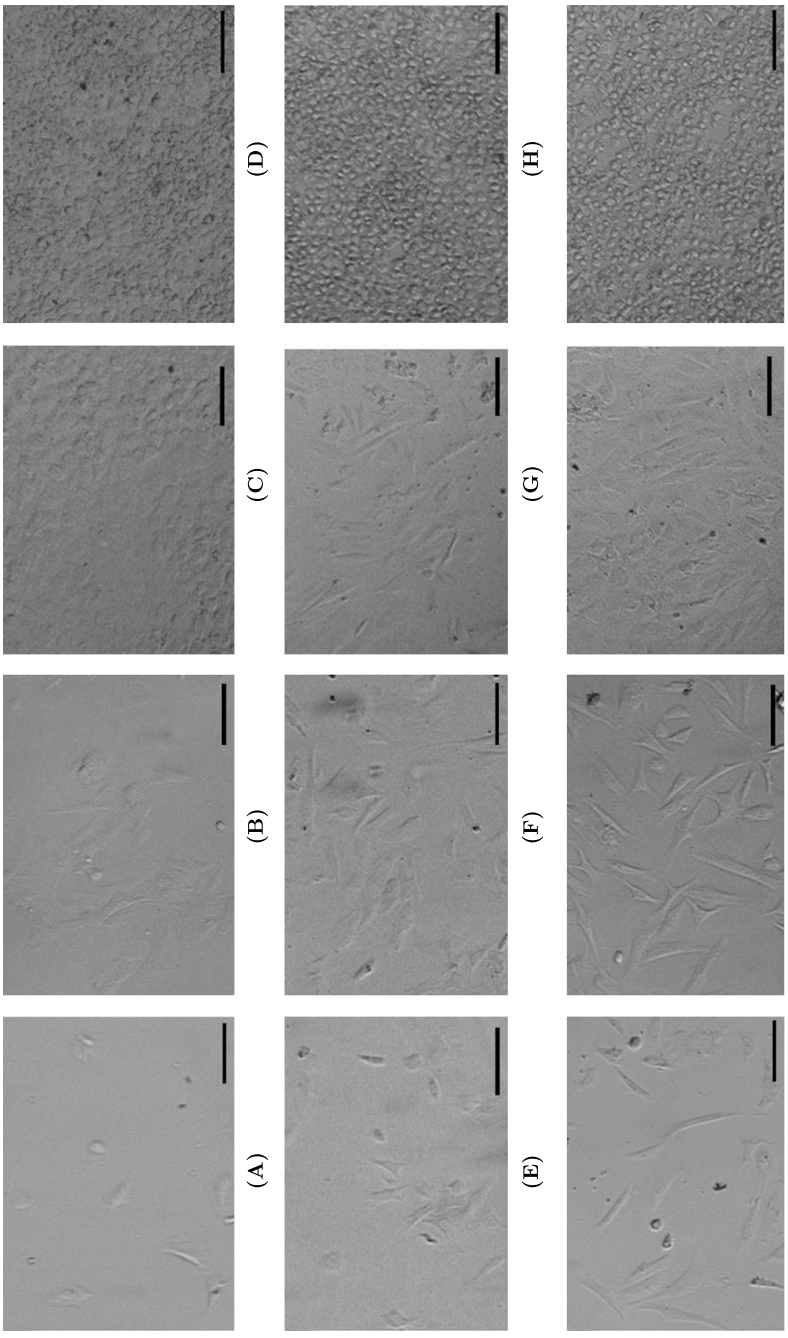

Figure S2. Microscopy images of chondrocytes after treatment with LiCl, from 1 to 4 weeks of culture. (**A**)–(**D**) 0.25 K to 250 K groups at 1 week, (**E**)–(**H**) 2 weeks, (**I**)–(**L**) 4 weeks. Bar = 100 μm.

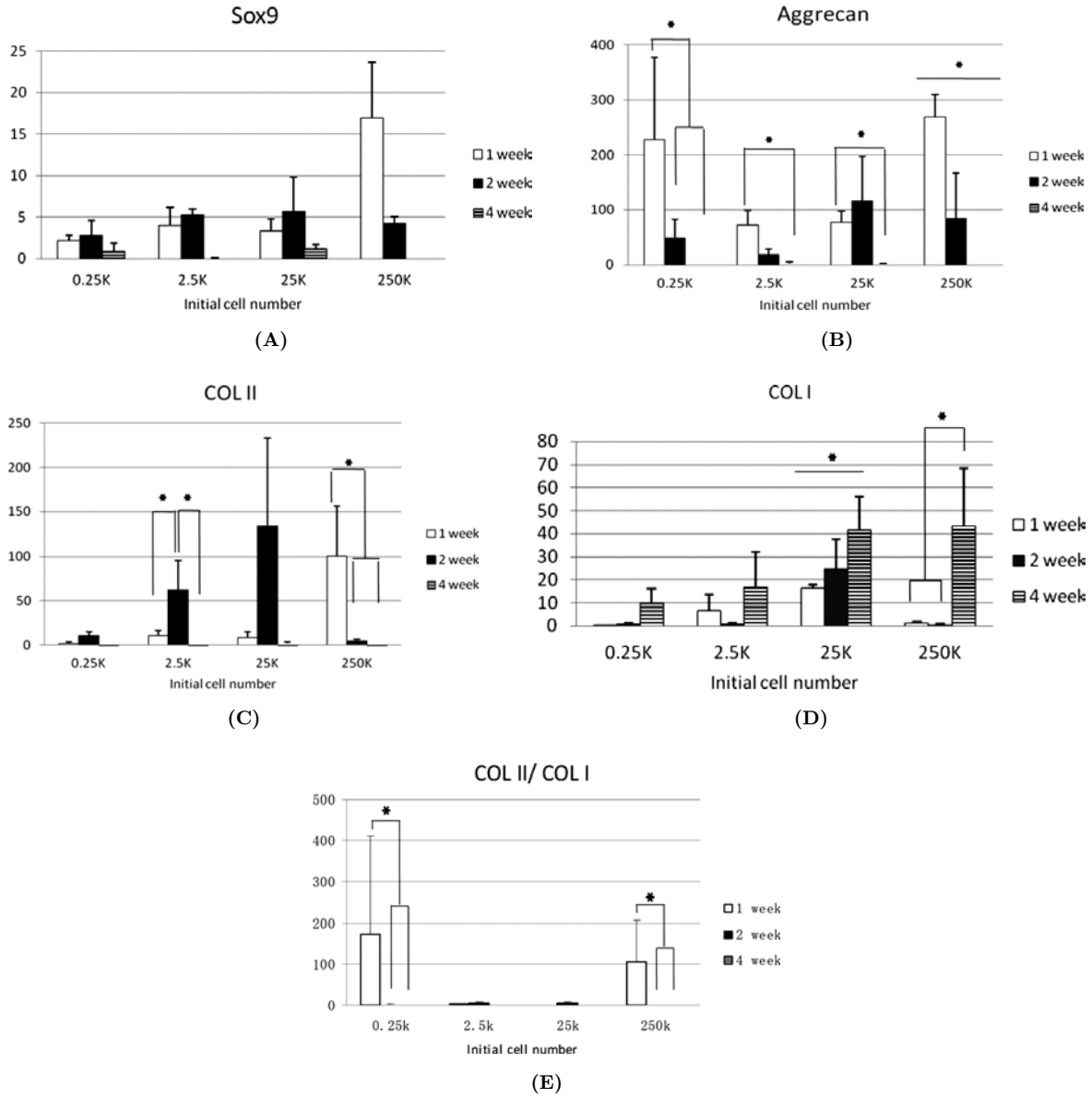

Figure S3. Expression of cartilage specific genes after activation of the Wnt signaling pathway by LiCl. (**A**) Sox9; (**B**) Aggrecan; (**C**) COL II; (**D**) COL I; (**E**) COL II/ COL I. Significant difference was indicated by an asterisk ($n = 3$, One-Way ANOVA, LSD, $P < 0.05$).