Optimal regeneration and repair of critical size articular cartilage driven by endogenous CLECSF1

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Keywords: C-type lectin, recombinant lentiviral vector, stem cells, cartilage tissue engineering

One Sentence Summary:

It is the first time to propose and use endogenous lectin to repair critical size cartilage defect, which challenges the classic view of using growth factors for tissue engineering and greatly enriches the content of tissue engineering, indicating great potential application prospect in cartilage tissue engineering.

ABSTRACT

Due to the lack of blood supply and nerve in articular cartilage, it is a great challenge to repair critical size cartilage defect, to maintain the phenotype of new chondrocytes and to restore joint function. We find that the endogenous cartilage extracellular matrix specific protein C-type lectin (CLECSF1) can be applied for the repair of cartilage defect. The endogenous expression of CLECSF1 can be achieved by integrating its coding gene into the recombinant lentiviral vectors. These vectors are used to transfect into bone marrow mesenchymal stem cells (BMSCs) and then these transfected BMSCs are seeded in a scaffold. We reveal that such scaffold can promote chondrogenic differentiation, enhance cartilage extracellular matrix deposition and maintain chondrocyte phenotype. Furthermore, such scaffold can repair critical size cartilage defect and finally achieve a full recovery of both structure and function. Therefore, we believe that such scaffold can be an ideal alternative strategy for the treatment of critical size cartilage defect in future.

INTRODUCTION

The intrinsic characteristics of articular cartilage without blood supply and nerve result in its very weak endogenous repair ability[1], thus making the repair of cartilage defect face great clinical challenges, especially for critical size (diameter>4mm) cartilage defect[2,3]. The traditional repair strategies include microfracture, autologous chondrocyte or allogeneic cartilage transplantation[4-6]. However, these strategies are difficult to repair such critical size cartilage defect due to the poor integration between the new and host cartilage or the lack of hyaline cartilage formation. Relatively speaking, tissue engineering can provide a better way to solve these problems. But the low delivery efficiency and short half-life of exogenous growth factors *in vivo* make it difficult to maintain chondrocyte phenotype for a long time, eventually leading to the failure of hyaline cartilage formation and recovery of joint function[7,8]. Therefore, it is of great significance to find a efficient method for cartilage defect repair.

90% of cartilage tissue is extracellular matrix, including collagen, proteoglycan, chrondronectin and cartilage derived C-type lectin[9]. Among these, lectin can noncovalently bind to polysaccharides on the cell surface and participate in a variety of biological processes, such as cell recognition, migration, proliferation and inter-cellular signal transduction[10,11]. Mikkola et al[12] have substantiated that lectins can enhance the proliferation and pluripotent differentiation of

human pluripotent/embryonic stem cells. In recent years, many studies have proved that lectins may participate in the formation and functionalization of cartilage. Neame et al[13] confirmed that lectins play a similar role as binding protein in the formation of cartilage extracellular matrix and collagen fiber structure, while Stattin et al[14] found that C-type lectins can mediate the interaction of other proteins in cartilage extracellular matrix and have strong interaction with aggrecan (AGG). In addition, Herman et al[15] substantiated that lectins can induce chondrocytes to release proteoglycan by stimulating normal monocytes to secrete cytokines. Matsutani et al[16] revealed that lectins can enhance the chondrogenesis of mesenchymal cells and form more cartilage nodules. These studies indicate that lectins have great potential in cartilage tissue engineering applications.

As is known to all, the RNA genome of lentiviral vector can be reversely transcribed into double stranded DNA and integrated into the host cell genome[17,18]. Inspired by the application of gene-enhanced tissue engineering[19], we consider that the coding gene of CLECSF1 can be integrated into lentiviral vector and the BMSCs can be transfected by such modified lentiviral vector. Such endogenous CLECSF1 can act as a controlled and sustained biological signal, thus providing a new strategy for cartilage tissue engineering. In this study, such strategy would be used to study the behavior of its promoting cell condensation and differentiation *in vitro* and to explore the mechanism of its driving cartilage regeneration *in vivo*.

RESULTS

Safety of recombinant lentiviral vector and its triggering of strong and persistent expression of endogenous CLECSF1

Growth factors often participate in and regulate the repair and regeneration of tissues, which is one of the indispensable factors in tissue engineering[20]. However, in the absence of optimal delivery strategy and together with poor release kinetics, the dosage of exogenous growth factors is very high, which usually far exceeds the physiological level[21]. Our previous studies[22] have demonstrated that recombinant adenoviral vector is an effective in-situ controlled release system, which can avoid such problems. However, it is difficult to further improve its endogenous expression duration and intensity[23]. Theoretically, they can be improved by choosing recombinant lentiviral vector as the delivery system.

CLECSF1 is widely distributed in the resting, proliferative and hypertrophic cartilage, it can participate in the formation of cartilage extracellular matrix, cartilage collagen fiber and

cartilage[24,25]. Therefore, we designed a recombinant lentiviral vector overexpressing CLECSF1 for the repair of cartilage. As shown in Fig.1, the construction, transformation of recombinant plasmid and the packaging, collection of recombinant lentiviral vector were carried out through a series of standard methods. Then, the cultured primary cells was identified. As shown in Fig. S1 in the supplementary material, the isolated and cultured cells met the definition of stem cells from the perspective of morphology (AO fluorescence staining) and molecular biology (flow cytometry of cell surface specific markers CD29, CD90, CD105 and CD31), they would be applied in all the subsequent studies.

After the identification of BMSCs, they were transfected by the recombinant lentiviral vector overexpressing CLECSF1. Firstly, we used Live/Dead staining to verify the biological safety of recombinant lentiviral vector. As shown in Fig. S2, the morphology of BMSCs transfected with recombinant lentiviral vector was normal, which was similar to that of blank BMSCs after co-culture of 1, 4 and 7 days. These results showed that the recombinant lentiviral vector had good biological safety. Then, we screened the optimum multiplicity of infection (MOI) from a series of MOI gradients (20, 30, 40, 50 and 60). 3 days after transfection, the transfection efficiency was analyzed qualitatively and quantitatively by fluorescence microscopy and flow cytometry, respectively. As shown in Fig. 2a, many cells indicated visible fluorescence of green fluorescent protein (GFP) in each MOI group, showing strong expression of CLECSF1. As shown in Fig. 2b, the positive expression rate of CLECSF1 was 51.05%, 60.66%, 69.46%, 85.62% and 87.58% in flow cytometry, respectively, when MOI was 20, 30, 40, 50 and 60. These results showed that BMSCs could be successfully transfected by recombinant lentiviral vector. When MOI was 50, the transfection efficiency was more than 85%, such strong expression of CLECSF1 was ideal for subsequent application. To verify that the recombinant lentiviral vector could trigger the persistent expression of CLECSF1, BMSCs were transfected with MOI=50 for 7, 14, 21 and 28 days. As shown in Fig. 2c, most of the cells showed strong fluorescence of GFP at each time point, and the fluorescence intensity did not decrease with time. As shown in Fig. 2d, the positive expression rate was 90.07%, 91.82%, 92.81% and 93.66% on the 7th, 14th, 21st and 28th days after transfection, respectively, showing that the recombinant lentiviral vector triggered the strong expression of endogenous CLECSF1 at all the time points.

In our previous study[22], the endogenous growth factor triggered by the recombinant adenoviral vector could achieve persistent expression for 3 weeks *in vitro*, but its expression

intensity changed greatly during the sustained process; compared with that, endogenous CLECSF1 triggered by recombinant lentiviral vector could achieve continuous expression for at least 4 weeks *in vitro* and the amplitude of its expression intensity changed little with time, showing more lasting and stable endogenous expression. The results of PCR electrophoresis (Fig. 2e) after transfection of 7 days showed that the positive expression of CLECSF1 mRNA in BMSCs transfected with recombinant lentiviral vector carrying CLECSF1 was significantly higher than that in BMSCs transfected with recombinant lentiviral vector not carrying CLECSF1, which proved that CLECSF1 could be expressed normally in BMSCs. These results indicate that the constructed recombinant lentiviral vector has good biological safety and can trigger the sustained, stable and strong expression of endogenous CLECSF1.

Expression of endogenous CLECSF1 and its promotion on proliferation, condensation and chondrogenic differentiation of BMSCs *in vitro*

BMSCs and BMSCs transfected by recombinant lentiviral vector with MOI=50 were cultured in high glucose medium containing 10% newborn calf serum. From the fluorescence images of GFP (Fig. 3a), no GFP was observed at the first two days while it was observed at the third day, indicating that the expression of endogenous CLECSF1 requires a period of 3 days. As shown in Fig. 3c, the absorbance value of BMSCs overexpressing CLECSF1 was almost equal to that of BMSCs at days 1 and 4. This is because the expression of endogenous CLECSF1 required 3 days as described above. At days 7 and 10, the absorbance values of BMSCs overexpressing CLECSF1 were 1.48±0.21 and 1.89±0.13 times of those of BMSCs. These biological activities were also identified by AO/PI staining in Fig. 3b. It can be concluded that endogenous CLECSF1 can promote the proliferation of BMSCs *in vitro*.

BMSCs, chondrocytes and BMSCs overexpressing CLECSF1 were cultured in high glucose medium containing 10% newborn calf serum. After 1, 2, 3 and 4 weeks of culture, the morphology and condensed state of these cells was observed via AO staining and Gimesa staining, respectively. As shown in Fig. 4a, the morphology of BMSCs did not change significantly during the whole culture process and remained a typical spindle shape; while the morphology of chondrocytes gradually began to change from the initial spindle shape to oval or triangular shape after 2 weeks, which was similar to those in natural articular cartilage. The BMSCs overexpressing CLECSF1 have changed from typical spindle shape to irregular shape similar to ellipse at the first week. As shown in Fig. 4b, the condensation of BMSCs overexpressing CLECSF1 was more obvious than

that of BMSCs and chondrocytes during the whole culture process. It is clear that endogenous CLECSF1 can enhance the cell condensation to a certain extent. After 28 days of culture, RNA was extracted. The mRNA of chondrogenic differentiation markers *Sox9*, *Col2α1* and C-type lectin ligand *Fibulin-1* were detected by fluorescence quantitative RT-PCR. From the quantitative histogram (Fig. 5a) and PCR electrophoresis images (Fig. 5b), it can be seen that, compared with BMSCs group, the mRNA expression level of *Sox9*, *Col2α1* and *Fibulin-1* in BMSCs overexpressing CLECSF1 was significantly up-regulated (4.59±0.42, 2.55±0.23, and 3.34±0.31 times, respectively). The mRNA expression level of *Sox9*, *Col2α1* and *Fibulin-1* in chondrocytes group was 4.67±0.51, 2.89±0.33 and 3.39±0.38 times of that in BMSCs group, respectively. It is clear to see that the endogenous CLECSF1 can promote the chondrogenic differentiation of BMSCs *in vitro.*

Generally speaking, these results suggest that endogenous CLECSF1 can very effectively promote the proliferation, condensation and chondrogenic differentiation of BMSCs *in vitro*.

Endogenous CLECSF1 promoting cartilage repair and regeneration

We constructed a critical size cartilage defect (5mm diameter) model in the femoral trochlear groove of rabbit knee joint, most of the traditional repair strategies are difficult to repair such defect within 12 weeks. As shown in Fig. S3, both the blank hydrogel and the cell-hydrogel composite could fill the defect completely, and both material can adhere closely to the defect cavity. At 2 weeks postoperatively, the defect was still visible in the blank hydrogel group due to the complete degradation of the hydrogel, while it was completely filled with the newly formed tissue in the cell-hydrogel composite group. At 12 weeks postoperatively, the defect was filled with rough and discontinuous fibrous tissue in the blank hydrogel group, and there was a visible gap between the surrounding normal cartilage and the defect. It is worth noting that the defect has been completely filled with new tissue in the cell-hydrogel composite group. These tissues had not only smooth and translucent surface but also no boundaries with adjacent normal cartilage, showing good cartilage integration.

HE staining images (Fig. 6a) showed that the defect in the blank hydrogel group was filled with new regenerated tissue at 12 weeks after operation, but the boundary between the regenerated and host area was clearly visible. The outer edge of the regeneration tissue was not smooth, most of which were fibrocartilage tissue, and its thickness was significantly different from that of the adjacent normal cartilage. However, the defect in the cell-hydrogel composite group was

completely filled with regenerated cartilage tissue, and there was no boundary between the regenerated and host area. The outer edge of the regenerated cartilage tissue was smooth and its thickness was similar to that of the adjacent normal cartilage, and there was obvious cartilage lacuna in the regenerated cartilage. The number of chondrocytes in some areas was slightly lower than that in the normal cartilage tissue. Masson staining images (Fig. 6b) showed a significant difference in the density of collagen fibers and the pattern of cell arrangement between the regenerated and adjacent normal tissue in the blank hydrogel group at 12 weeks after surgery. However, in the cell-hydrogel composite group, they were basically the same as those in the adjacent normal tissue.

These results clearly show that the cell-hydrogel composite can effectively promote the healing of critical size cartilage defect and the integration between newly formed and host cartilage.

We used specific histochemical staining and histological scoring to further evaluate the repair of critical size cartilage defect. Cartilage-specific toluidine blue staining (Fig. 6c) showed that there was a significant difference in the amount of proteoglycan between the regenerated and normal tissue in the blank hydrogel group at 12 weeks after operation. The amount of proteoglycan in the defect area of the cell-hydrogel composite group was only slightly lower than that in the adjacent normal tissue, which was much higher than that in the blank hydrogel group. From the cartilage-specific alcian blue staining images (Fig. 6d), it was also observed that the amount of extracellular matrix secreted by the regenerated tissue was slightly lower than that of the adjacent normal tissue in the cell-hydrogel composite group, but much higher than that in the blank hydrogel group at 12 weeks after surgery. Obviously, the cell-hydrogel composite effectively enhanced the deposition of cartilage-specific extracellular matrix in the defect area.

Based on the staining results mentioned above and the O'Driscoll scoring system previously reported by Wakitani et al[26], the repaired cartilage was comprehensively evaluated. At 12 weeks after operation, the O'Driscoll histological score in the cell-hydrogel composite group was 23.2 ± 0.8, which was significantly higher than 12.6 ± 1.5 in the blank hydrogel group. This indicates that the regenerated cartilage in the cell-hydrogel composite group has been very close to natural cartilage in terms of physiological structure.

To further evaluate the functional recovery of the repaired joints, the expression of cartilage-specific collagen (Col II, Col IX and Col XI, three components of fine collagen fibers) and matrix protein (AGG and Matrilin-1) were analyzed by immunofluorescence staining. The fluorescence

intensity of Col II, Col IX and Col XI in the cell-hydrogel composite group was much higher than that in the blank hydrogel group, indicating that more Col II, Col IX and Col XI was secreted in the defect area of the cell-hydrogel composite group (Fig. 7a, 7b and 7c), respectively. The similar results were observed in the immunofluorescence staining of AGG and Matrilin-1 (Fig. 7d and 7e). These results demonstrate that cell-hydrogel composite can effectively stimulate the synthesis of cartilage-specific collagen and matrix proteins.

We also evaluated the recovery of the biomechanical properties of the regenerated joint by biomechanical testing. As shown in Fig. 8, the mechanical compressive strength and the compression modulus of the natural joints were 53MPa and 3.64MPa, respectively. The mechanical strength of the repaired joints in the blank hydrogel group was only 28MPa at 12 weeks postoperatively, being 54% of that of the natural joint while the compression modulus was as high as 7.89MPa, much higher than that of the natural joint. In sharp contrast, the mechanical compressive strength of the cell-hydrogel composite group was about 50MPa at 12 weeks after surgery, being 98% of that of the natural joint while the compression modulus was 4.07MPa, very close to that of natural joint.

The overall results showed that the cell-hydrogel composite greatly promoted the healing of cartilage defect, the integration between new and host cartilage, the secretion of extracellular matrix, the synthesis of cartilage-specific collagen and matrix proteins, and the recovery of biomechanical function.

DISCUSSION

Bioactive factors are essential for tissue regeneration, especially for tissue defects[27]. One of the main limitations of regenerative therapy based on bioactive factors is the very short half-life *in vivo*[28]. Khosravi et al[29] have confirmed that the half-life of fibroblast growth factor-23 is only 46 to 58 min, which leading to a short-time therapeutic effect. To prolong the half-life, Lewitt et al[30] extend the half-life of insulin-like growth factor from 1.2 to 5.3 min, but it is still very short. Mochizuki et al[31] improve the bioavailability of exogenous bioactive factors, however, this still could not fundamentally solve the problems caused by high doseage. In addition, it is still difficult to achieve the effective and accurate release of exogenous bioactive factors *in vitro*[32].

Endogenous in situ expression of bioactive factors may be a feasible alternative strategy. So far, viral vectors have become the most effective vectors to achieve such endogenous expression.

The transfection efficiency of the recombinant lentiviral vector constructed by Li et al[33] was about 60%. Mao et al[34] confirmed that the transposase/integrase can integrate DNA into host cell genome through non-homologous end-joining. Therefore, we chose lentivirus as the vector to express the endogenous bioactive factors. However, the lentivirus also faces many challenges due to its biosafety and the risk of recovering into wild-type virus. Luciw et al[35] demonstrated that Tat and Rev are essential for viral replication, accessory genes Nef, Vif, Vpr and Vpu are essential for the pathogenesis *in vivo*..In this study, the envelope plasmid was only containing VSV-G gene, which was different from that of the core protein of the virus, it reduced the possibility of recovering to wild-type virus. The knockout of Nef, Vif, Vpr and Vpu increased the biological safety of the recombinant lentiviral vector.Tat was replaced by strong promoter sequence, which did not affect the transfection efficiency of the vetcor. Only Rev reaction element was retained in Rev, which did not affect the production of viral RNA and its entry into the nucleus. The cell experiment *in vitro* showed that there was no significant difference between blank BMSCs and BMSCs overexpressing CLECSF1. The strong expression of CLECSF1 was triggered as long as 28 days, and its expression intensity did not decrease significantly during the whole period, thus fully guaranteeing the demand for bioactive factors in the early stage of tissue repair. Our *in vivo* animal experiments also demonstrated that those experimental animals in cell-hydrogel composite group grow healthily and move freely and freely, thus showing good biosafety.

Chondrogenic differentiation has been widely studied because of its important clinical application value[36,37]. From the point of view of growth and development, during embryogenesis, the pre-mesenchymal cells in the extracellular matrix of limb buds rearrange to form condensed cell aggregates, which eventually differentiate into chondrocytes (Tacchetti et al)[38]. Therefore, cell condensation is a prerequisite for chondrogenic differentiation (Bobick et al; Delise et al)[39,40]. In the process of condensation, the most obvious phenomenon is the change of cell shape (from spreading to round) and the deposition of extracellular matrix of cartilage (Delise et al)[41]. Based on this, we speculate that it is feasible to promote chondrogenic differentiation by promoting cell condensation, cell morphology roundness and cartilage extracellular matrix deposition. The cell experiments *in vitro* revealed that the endogenous CLECSF1 significantly promoted cell condensation, such cell condensation mode is obviously conducive to promote chondrogenic differentiation. Haas et al[42] have demonstrated that the pluripotent mouse embryonic mesenchymal cell line C3H10T1/2 can be induced into chondrocytes by BMP-2 or TGF-β1 only

under high-density culture. Gao et al[43] induced the chondrogenic differentiation of mesenchymal stem cells through the synthesis of matrix, which limit the spread of cells and control the shape of cells. Twal et al[44] demonstarted that Fibulin-1 can significantly inhibit the cell spreading to a certain extent.The cell experiments *in vitro* showed that endogenous CLECSF1 increased the cell density and up-regulated the mRNA expression level of Fibulin-1,which was obviously conducive to further promoting chondrogenic differentiation. Kamiya et al, White et al and Wilson et al revealed that extracellular matrix components, such as fibronectin, versican and collagen, can promote chondrogenesis[45-47]. Oda et al[48] demonstrated that lectin can induce morphological changes (from fibroblasts to spherical cells), and enhance proteoglycan synthesis, cartilage characteristic gene expression and chondrogenic differentiation of human BMSCs. The cell experiments *in vitro* showed that the mRNA expression levels of *Sox9*, *Col2α1* and *Fibulin-1* in BMSCs overexpressing CLECSF1 were much higer than those in natural BMSCs at 28 days, which was obviously beneficial to further promoting chondrogenic differentiation.

Matsumoto et al[49] and Betka et al[50] also demonstrated that C-type lectin can contribute to chondrogenic differentiation, but there is no further report on the application of C-type lectin in animal experiments *in vivo*. For this reason, we proposed a strategy to construct the genetically enhanced tissue engineering cartilage by loading CLECSF1 encoding gene into the recombinant lentiviral vector to transfect BMSCs. Up to now, the reported maximum diameter of defects in osteochondral defect models was not more than 5 mm. For example, the diameter of defects constructed by Deng et al[51], Marmotti et al[52] and Duan et al[53] was 4 mm, 4.5mm and 4mm, respectively. These repair strategies have achieved a certain effect, but there are still some obvious shortcomings, including incomplete subchondral bone reconstruction and slightly rough cartilage surface. If the defect size is larger, it will take longer time to finish cartilage repair. It took 24 weeks to produce white hyaline cartilage when the diameter of defect constructed by Maehara et al[54] was 5mm. Moreover, the defect size in the reported cartilage defect model was relatively smaller (diameter$\leqslant$4mm). For example, the diameter of defects constructed by Guo et al[55] and Man et al[56] was 3 mm and 4 mm, respectively. However, the integration between the new and host cartilage layer was not complete enough, and the surface of the new cartilage layer was not smooth enough. The animal experiments *in vivo* corroborated that endogenous CLECSF1 could significantly enhance the deposition of extracellular matrix and the synthesis of cartilage specific collagen and matrix proteins. Moreover, it could maintain the stability of cartilage phenotype and

restore the biomechanical function of repaired joint, which has not been achieved by using a single known bioactive factor yet.

In addition, we verified the safety, effectiveness and long-term effect of the proposed strategy through animal experiments *in vivo*. Compared with the reported cartilage tissue engineering strategies, our strategy has the following advantages: 1) Repairing defects of larger sizes. The diameter of the created defect size was 5 mm, it is bigger than the critical size of 4 mm. 2) Shorter repair time. At 12 weeks after operation, hyaline cartilage was regenerated, which was almost the same as the adjacent cartilage. 3) More stable phenotype maintenance. At 12 weeks after operation, the cartilage extracellular matrix deposition and cartilage specific collagen and matrix protein synthesis were almost consistent with the adjacent normal cartilage tissue. 4) Faster recovery of the biomechanical function. The mechanical compressive strength of the repaired joint at 12 weeks after operation was about 50 MPa, which is close to 98% of the natural joint. This is even equivalent to the mechanical compressive strength of the gene enhanced osteochondral double-layer scaffold used for repairing 5 mm diameter osteochondral defect at 24 weeks after operation[22]. Based on these facts, we believe that this strategy can become an ideal alternative strategy for cartilage repair.

CONCLUSIONS

In summary, we revealed that CLECSF1 can play an active role like growth factor in cell differentiation and tissue regeneration. And we also found its multi-functional synergistic effect and sequential synergistic effect of cell condensation, cell proliferation, inhibition of cell spreading, cell rounding and extracellular matrix deposition caused by type II collagen mRNA expression that greatly stimulate chondrogenic differentiation, maintain the phenotype of chondrocytes very well, and fully restore the physiological function of regenerated cartilage. Our study results demonstrated that the *in vivo* use of endogenous CLECSF1 is safe and effective, which can provide a promising strategy for the treatment of critical size cartilage lesions and even other potential biological applications.

MATERIALS AND METHODS

Preparation of hydrogel scaffold

The hydrogel was prepared according to the following processes. In brief, 1g of polyglutamic acid was dissolved in 4mL of deionized water and then 0.4g of polylysine was added and continued

to stir until it was completely dissolved. After that, 0.4g EDC-HCl was added into it to yield aqueous solution A. 0.4g NHS was dissolved in 2mL of deionized water to form solution B, then the solution A was mixed with solution B under the condition of vigorously stirring. Then the homogeneous solution was poured into the mold to obtain the hydrogel. The hydrogel was placed in -20℃ for pre-freezing and the hydrogel scaffolds were produced by freeze-drying.

Construction of recombinant lentiviral vector

The procedures of constructing recombinant lentiviral vector were as follows: 1) the preparation of target gene: the corresponding primer was designed and synthesized according to the coding DNA of C type lectin from the NCBI website and it was amplified by polymerase chain reaction (PCR). The primers were annealed to 4℃ for preservation. 2) the construction of recombinant plasmid: the bacterial solution containing vector plasmids of pLV-CMV-MCS-EF1-ZsGreen1-T2A-Puro was cultured overnight and the vector plasmids were extracted from the bacterial solution. The extracted fresh vector plasmids were digested by restriction enzyme Age I and EcoR I at 37℃ for 3h and then the products were recovered via agarose gel electrophoresis. The preserved primers were connected to the recovered plasmids to yield the recombinant plasmids via homologous recombination. 3) the transformation of recombinant plasmids: the recombinant plasmids were added into the centrifuge tubes containing the suspension of the sensitive cells DH5α at 4 ℃ for 30 minutes. After that, the centrifuge tubes were placed in water bath at 42℃ for 90s then placed them at 4 ℃ for 2-3 minutes. The super optimal broth with catabolite repression (SOC) medium without antibiotics was added into the centrifuge tubes and they were cultured at 37 ℃, 225 rpm for 45 minutes. After that, the supernatant was discarded after centrifugation at 3000 rpm for 2 minutes, the sensitive cells DH5α were added to the culture plate containing ampicillin (AMP) and they were cultured at 37℃, 225 rpm for 12-16 h. The plasmids of several positive monoclonal colonies from the culture plate were extracted via Plasmid Extraction Kit and then were sequenced. The monoclonal colonies whose plasmids were proved to be correct were inoculated into SOC medium for expanding culture to obtain enough recombinant plasmids. 4) the packing of recombinant lentiviral vector: the 293T cells were cultured in a 6-well plate with DMEM containing 10% fetal bovine serum (FBS) for 12-16 h, then the medium was discarded and the cells were washed 3 times with sterile PBS, 2 mL of DMEM containing 10% FBS were added into the 6-well plate again. 200 μL of serum-free DMEM and 6 μL of lipo 2000 were added into an EP tube to yield solution A, then 200 μL of recombinant plasmids solution was added into

solution A to form the mixture solution. Then the mixture solution was added into the 6-well plate dropwise, the cells were incubated in a humidified atmosphere of 5% CO_2 and 37℃. 10-16 h later, the medium was renewed with DMEM containing 10% FBS and the cells were incubated for another 48h. After that, the supernatant containing packed recombinant lentiviral vector was collected via centrifugation at 1500 rpm for 5 minutes and filtration using a 0.45 μm filter.

Isolation and culture of rabbit BMSCs

Rabbit BMSCs were isolated and cultured as previously described. Briefly, the femur and tibia were aseptically harvested from the 3-month-old New Zealand white rabbit. Subsequently, they were washed with sterile PBS and were cut via a bone rongeur to expose the marrow cavity. The marrow cavity was washed repeatedly with DMEM medium to collect the bone marrow cells. The collected cells were evenly dispersed and inoculated into a T25 cell culture flask, the cells were incubated in a humidified atmosphere of 5% CO_2 and 37℃. After 12 h, the medium was changed with DMEM containing 10% FBS, and then the medium was renewed every 2-3 days. The BMSCs at the 3^{rd} passage were used in all the following study.

Characterization of BMSCs

Stem cell surface specific marker CD29, CD90, CD105 and endothelial cell surface specific marker CD31 were used to characterize these isolated and cultured BMSCs via a flow cytometry. Briefly, the BMSCs were rinsed three times with PBS and then harvested with 0.25% trypsin, these harvested cells were resuspended in sterile PBS after centrifugation at 1200 rpm for 5minutes. These resuspended cells were incubated with fluorescein isothiocyanate (FITC)-conjugated CD29, CD90, CD105 and CD31 antibodies (Bio legend, USA) at room temperature for 30 minutes. These incubated cells were rinsed twice with PBS and then were resuspended in 0.5 mL of PBS and evaluated by using a FACS Aria instrument (BD Biosciences, USA). The cells that had adequate size and granularity were used for the following statistical analysis.

Transfection of BMSCs by recombinant lentiviral vector

After BMSCs were rinsed, digested, centrifuged, resuspended and counted, they were inoculated into a 6-well plate and were incubated in a humidified atmosphere of 5% CO_2 and 37℃ for 12-24h. Serum-free DMEM medium was used to dilute the recombinant lentiviral vector to the required MOI according to the titer of recombinant lentiviral vector and the quantity of BMSCs. After the diluted recombinant lentiviral vector was added into the 6-well plate dropwise, the cells were incubated in a humidified atmosphere of 5% CO_2 and 37℃. The medium was renewed with

DMEM containing 10% FBS 24 h later, subsequently, the medium was replaced with fresh medium every 2 days.

Transfected BMSCs encapsulation and culture

When the transfected BMSCs became confluent, the cells were rinsed three times with sterile PBS and were quickly isolated with 0.25% trypsin-EDTA. Subsequently, after the cells were centrifuged, resuspended and counted, they were mixed with DMEM medium containing 10% FBS to yield a cell suspension with a final concentration of cells: 2-3*10^6/mL. The cell suspension was directly injected into the hydrogel scaffolds, and the cell-hydrogel composites were incubated in a humidified atmosphere of 5% CO_2 and 37℃ for the following study.

Cell viability

BMSCs and BMSCs overexpressing CLECSF1 were cultured in high glucose medium containing 10% newborn calf serum. After 1, 4, 7 and 10 days of culture, cell proliferation of these BMSCs was assessed by using MTT assay and cell viability was assessed by using Live/Dead assay according to the manufacturer's instructions. The cells were rinsed three times with sterile PBS and then were incubated in 0.1mL PBS containing 1% acridine orange/propidium iodide (AO/PI) at room temperature for 3 minutes. Microscopy observation was performed by using an Olympus fluorescence microscope equipped with a digital camera.

Cell condensation

BMSCs, chondrocytes and BMSCs overexpressing CLECSF1 were cultured in high glucose medium containing 10% newborn calf serum. After 7, 14, 21 and 28 days of culture, cell morphology of these cells were observed via AO staining and the condensed state of these BMSCs was assessed by Gimesa staining. The procedures were carried out according to the manufacturer's instructions. Microscopy observation was performed by using an Olympus fluorescence microscope equipped with a digital camera.

Chondrogenic differentiation

BMSCs, chondrocytes and BMSCs overexpressing CLECSF1 were cultured in high glucose medium containing 10% newborn calf serum. After 28 days of culture, the cells were collected to quantify the mRNA expression of GAPDH, chondrogenic differentiation markers *Sox9*, *Col2α1* and C-type lectin ligand *Fibulin-1*. GAPDH was used as a reference gene. The details of primers were showed in Table 1.

RT-PCR

The isolation of total RNA was performed as following: 1mL Trizol was added to each sample, after homogenization, the sample was allowed to stand at room temperature for 5min. The mixture of 0.2mL chloroform/1mL Trizol was added, then it was shaken violently for 15 seconds and was allowed to stand at room temperature for 2-3min. The supernatant was collected after centrifuge for 15min (10000rpm, 2-8 ℃). The equal volume of isopropanol was added and was allowed to stand at room temperature, then the supernatant was discarded after centrifuge for 10min (10000rpm, 2-8 ℃). 1mL of 75% alcohol was added for precipitation and it was allowed to stand, then the supernatant was discarded after centrifuge for 5min (5000rpm, 2-8 ℃). DEPC water was added to dissolve the RNA, then it was packed into EP tube.

The complete sequence of genes was searched from NCBI database, and Primer Premier software was used to design and screen gene specific primers. All primers were designed and synthesized by Shanghai Shenggong Bioengineering Technology Service Co., Ltd. and purified by ULTRAPAGE. The primers and base sequences used in this study are shown in Table 1. *GAPDH* Gene was used as internal reference gene, the gene expression levels of *Sox9*, *Col2α1* and *Fibulin-1* were standardized by using ΔΔ CT method and the results were presented in multiples.

Cartilage repair in a rabbit articular cartilage defect model

The healthy New Zealand white rabbit weighing 2.2-2.5 kg was used in *in vivo* experiments. The rabbits were anesthetized with 10% chloral hydrate (2 mL/kg) via auricular vein injection. The right hind knee joint area was shaved and sterilized with 0.5% povidone-iodine. An arthrotomy was performed by a longitudinal incision at the knee joint, the skin and joint capsule were cut in turn, the patella was everted. The cartilage defect (5 mm in diameter and 1 mm in depth) was created by an electric hand drill in the trochlear groove of the distal femur, and the drill bit was rinsed with 0.9% sterile normal saline during the drilling process. The rabbits were randomly divided into 2 groups, the cartilage defect was implanted with blank hydrogel and cell-hydrogel composite in the control group and experimental group, respectively. After implantation, the joint capsule and skin were sutured in turn, and the knee joint area was sterilized with 0.5% povidone-iodine again. Subsequently, the rabbits were injected with penicillin (100000 U/kg) for three days, they were raised in a single cage and could move freely, and the state of the rabbits was observed and recorded regularly.

Histological analysis and score

The rabbits were sacrificed via auricular vein injection of over doseage of 10% chloral hydrate, the joint samples of the distal femurs were harvested, fixed in 10% formaldehyde solution, decalcified in 10% EDTA solution. The decalcified specimens were trimmed, embedded with paraffin and cut into 5μm slices, then these sections were stained with HE, Masson, Toluidine blue and Alcian blue. Immunofluorescence staining was performed with antibodies against Col II, Col IX, Col XI, AGG and Matrilin-1. Finally, the histological scores of cartilage repair were quantitatively evaluated with the grading scale described by Wakitani et al[26].

Biomechanical properties of the repaired joints

Biomechanical properties of the repaired joints were analyzed using a universal mechanical testing machine at 12 weeks post-surgery. Briefly, the repaired joints were pushed with a 4.7 mm dimeter cylindrical plunger, load was then applied to the joint at a speed of 0.5mm/min until the joint was ruptured. The natural joint from the left hind was used as the positive control group.

Statistical analysis

Statistical analysis for the quantification of experimental data was performed with Statistical Product and Service Solutions Software version 22.0. The data were transformed and standardized. All the data were presented as the mean ± standard deviation and Student's t-test was used to determine the statistical significance. Three independent biological replicates were carried out for all experiments and "n" denotes the number of independent biological replicates performed. If the p-value is lower than 0.05, it will be considered to be statistically significant.

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Funding:

National Key Technologies R&D Program of China 2016YFC110201 (JXW)

National Natural Science Foundation of China 31370966 (JXW)

Author contributions:

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Synthetise: TJC, RW

Cell experiment: TJC, WZP

Animal experiment: TJC, FC, QZX

Writing-original draft:TJC, JXW

Writing-review & editing: All the authors

Competing interests:

The authors declare no competing interests.

Data and materials availability:

All data are available in the main text or the supplementary materials.

Figures

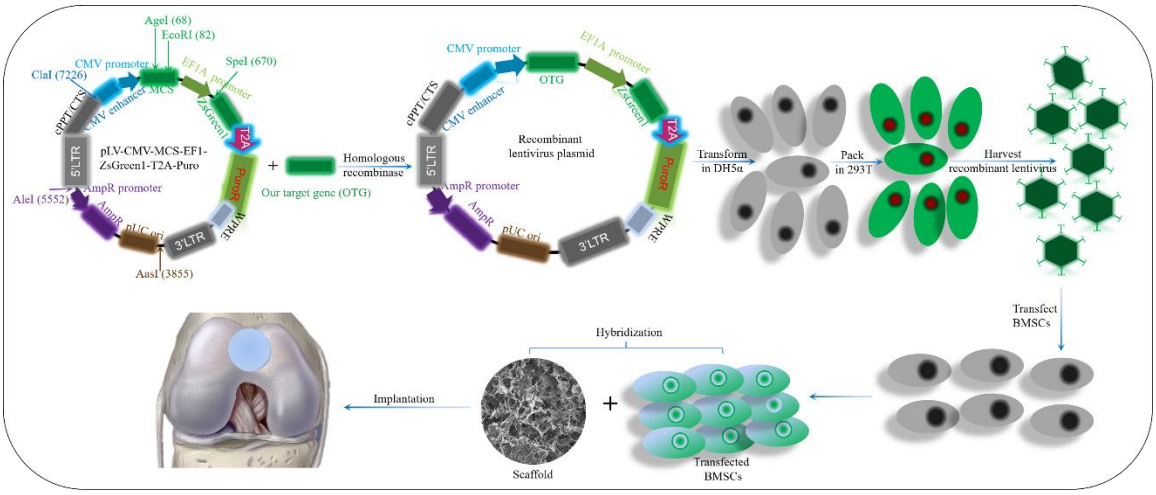

Fig. 1. Construction of target gene-loaded recombinant lentiviral vector, transfection of BMSCs by recombinant lentiviral vector, and construction and application of cell-hydrogel composite scaffold for the repair of critical size cartilage defect.

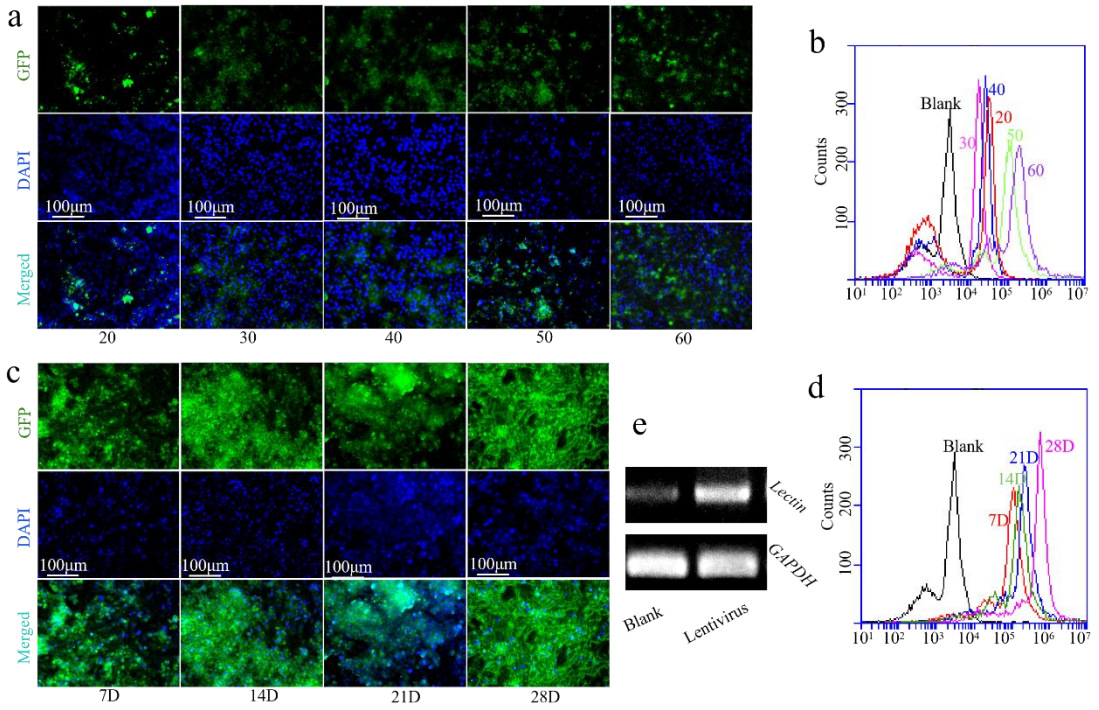

Fig. 2. Strong and persistent expression of endogenous CLECSF1. a) Fluorescence images of GFP in different MOI group after 3 days of transfection, b) Flow cytometry result in different MOI

groups after 3 days of transfection, c) Fluorescence images of GFP in MOI=50 group after 7, 14, 21 and 28 days of transfection, d) Flow cytometry result in MOI=50 group after 7, 14, 21 and 28 days of transfection, e) Result of PCR electrophoresis for the expression of CLECSF1 in BMSCs transfected or not transfected with recombinant lentiviral vector after 7 days of transfection.

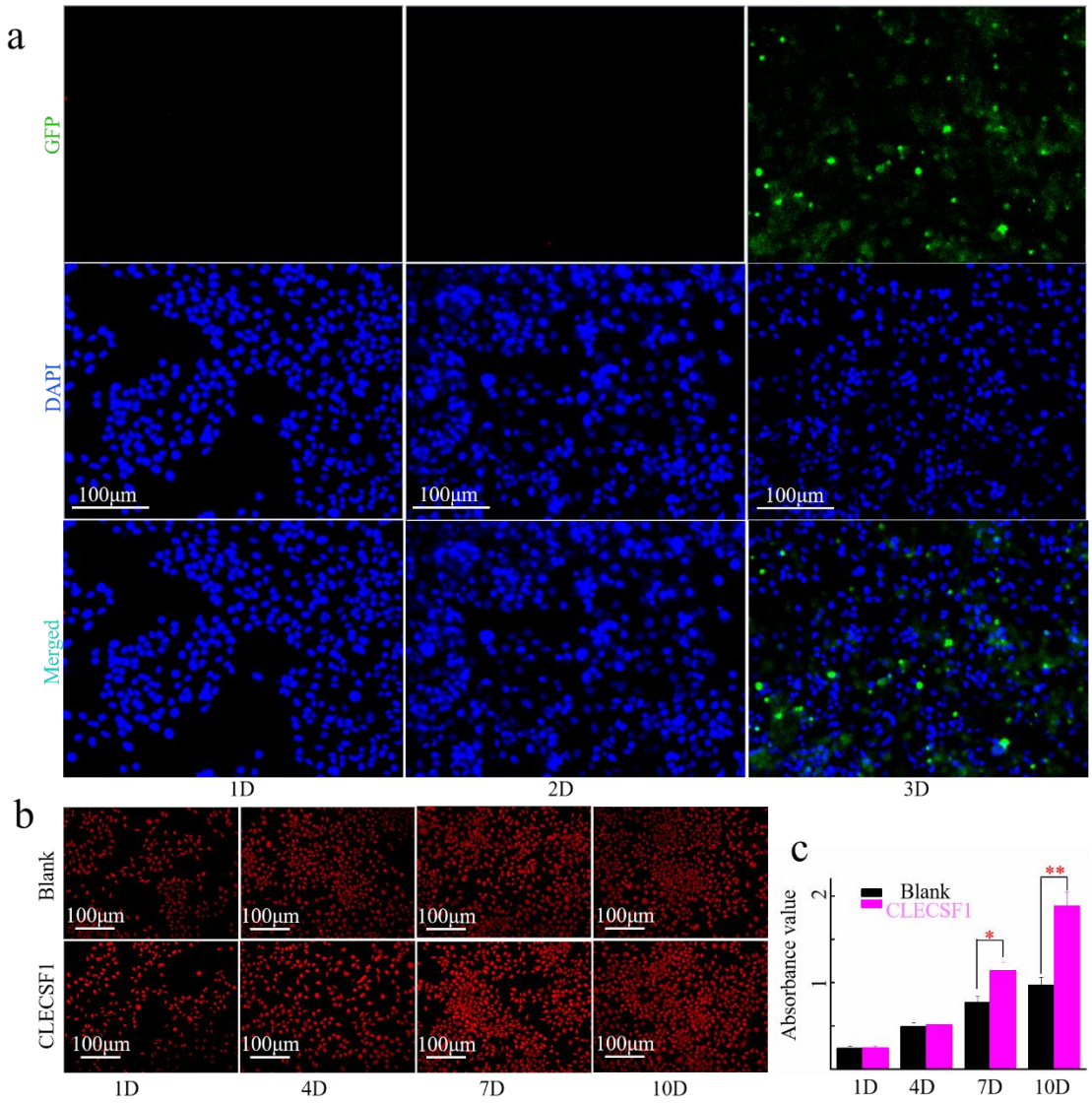

Fig. 3. Expression of endogenous CLECSF1 and its effect on proliferation of BMSCs *in vitro.* a) Fluorescence images in MOI=50 after 1, 2 and 3 days of transfection, b) Fluorescence images of BMSCs and BMSCs overexpressing CLECSF1 after 1, 4, 7 and 10 days of culture, c) Absorbance values of BMSCs and BMSCs overexpressing CLECSF1 after 1, 4, 7 and 10 days of culture.

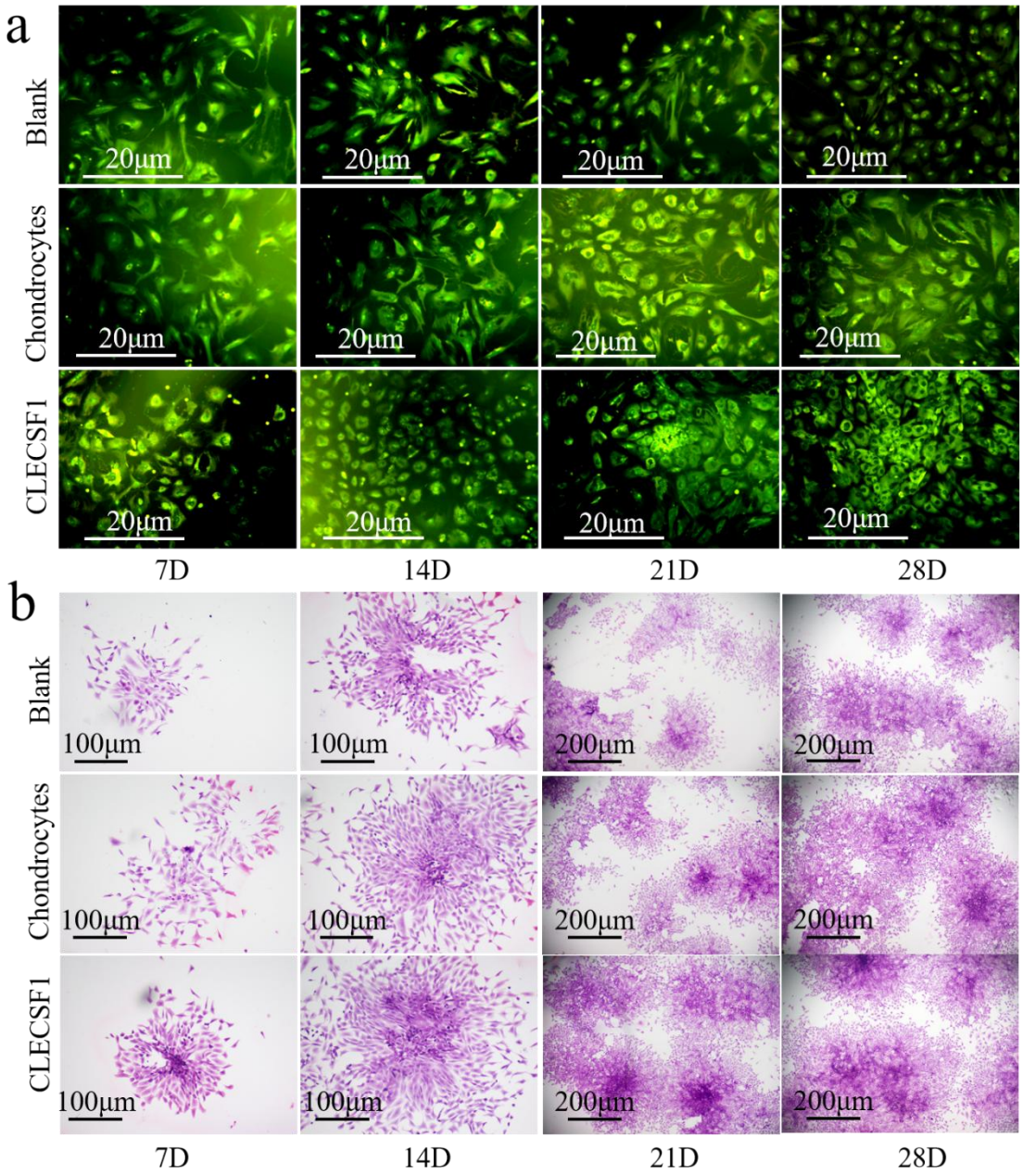

Fig. 4. Effect of endogenous CLECSF1 on condensation of BMSCs *in vitro*. a) Fluorescence images of BMSCs, chondrocytes and BMSCs overexpressing CLECSF1 after 7, 14, 21 and 28 days of culture, b) Condensed state of BMSCs, chondrocytes and BMSCs overexpressing CLECSF1 after 7, 14, 21 and 28 days of culture.

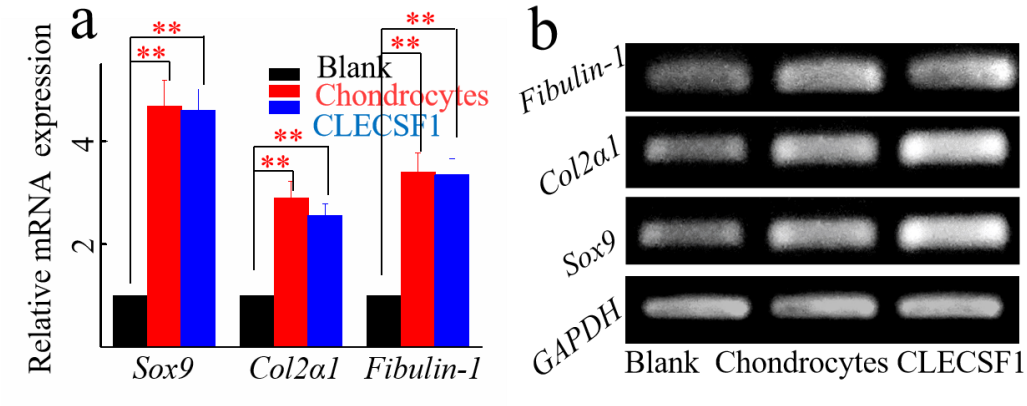

Fig. 5. Effect of endogenous CLECSF1 on chondrogenic differentiation of BMSCs *in vitro*. a) Quantitative PCR of the mRNA expression level of *Sox9*, *Col2α1* and *Fibulin-1* in BMSCs, chondrocytes and BMSCs overexpressing CLECSF1 after 28 days of culture, b) PCR electrophoresis for the mRNA expression level of *Sox9*, *Col2α1* and *Fibulin-1* in BMSCs, chondrocytes and BMSCs overexpressing CLECSF1 after 28 days of culture.

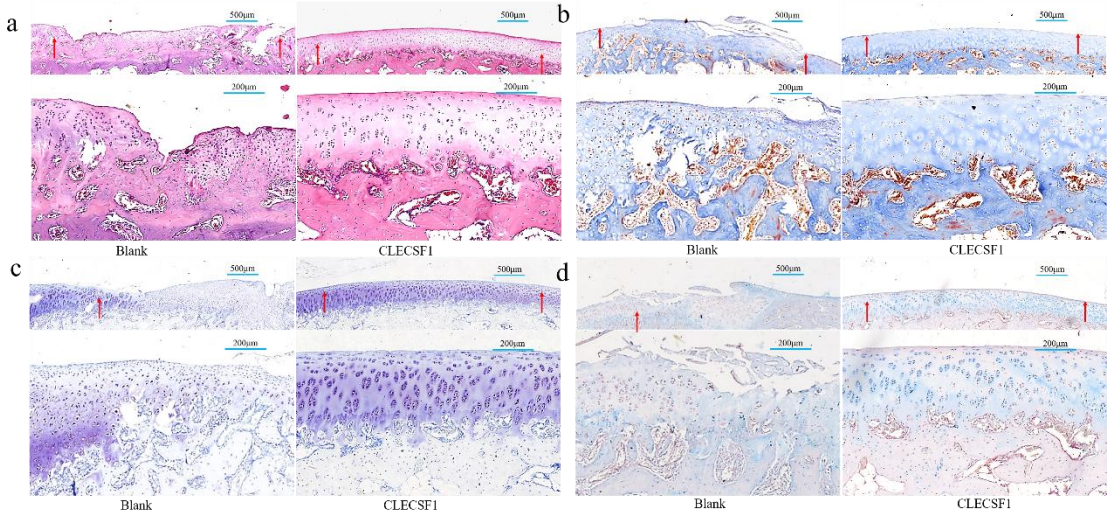

Fig. 6. The effect of endogenous CLECSF1 on cartilage repair and regeneration *in vivo*. a) HE staining images, b) Masson staining images, c) Cartilage-specific toluidine blue staining images and d) Cartilage-specific alcian blue staining images of repair joint after 12 weeks of implantation of blank hydrogel and cell-hydrogel composite. Arrow shows the interface of host cartilage and new cartilage while the area between two arrows is the defect.

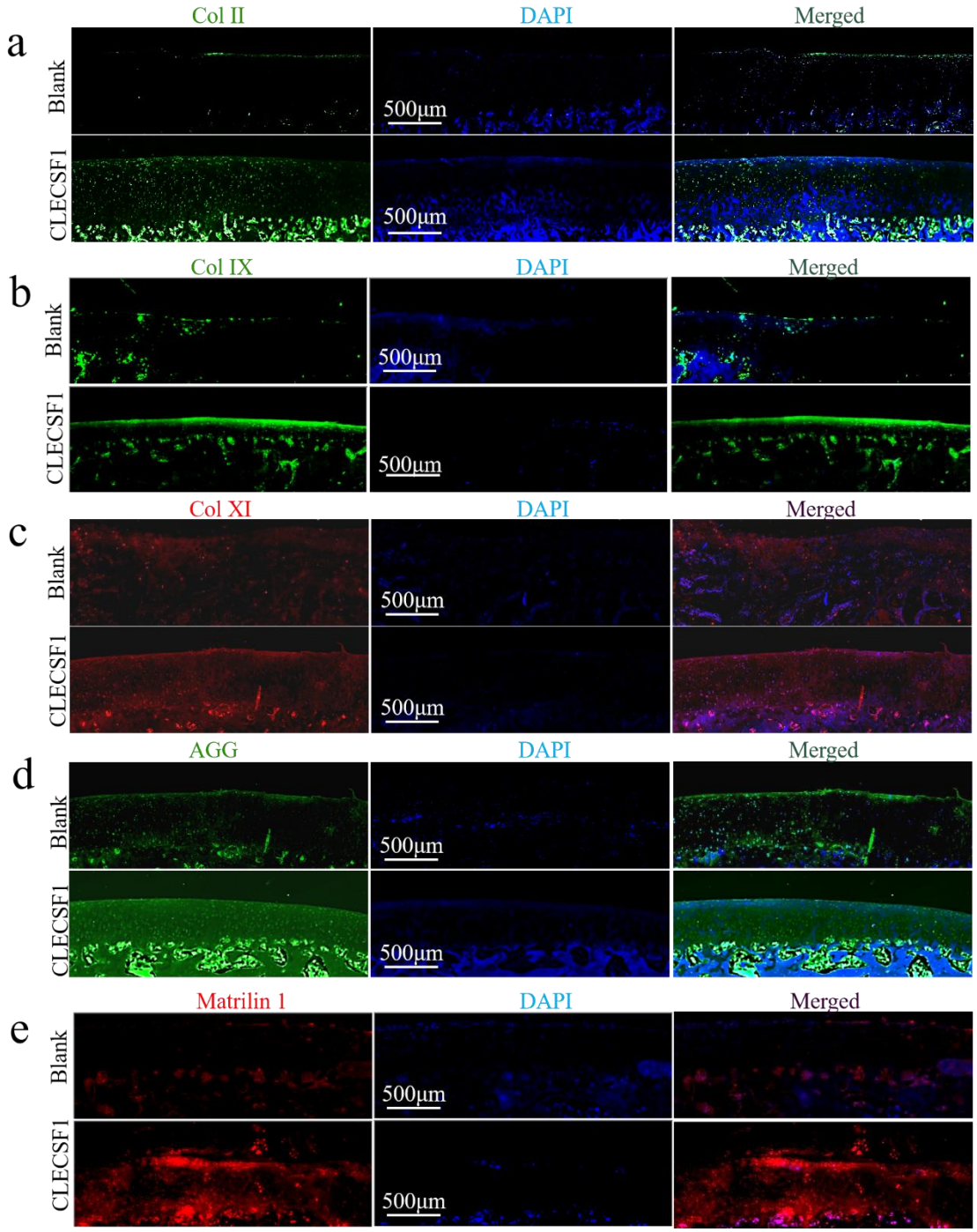

Fig. 7. The effect of endogenous CLECSF1 on cartilage repair and regeneration *in vivo*. Immunofluorescence staining images of cartilage-specific collagen a) Col II, b) Col IX, c) Col XI and matrix protein d) AGG, e) Matrilin-1 of repair joint after 12 weeks of implantation of blank hydrogel and cell-hydrogel composite.

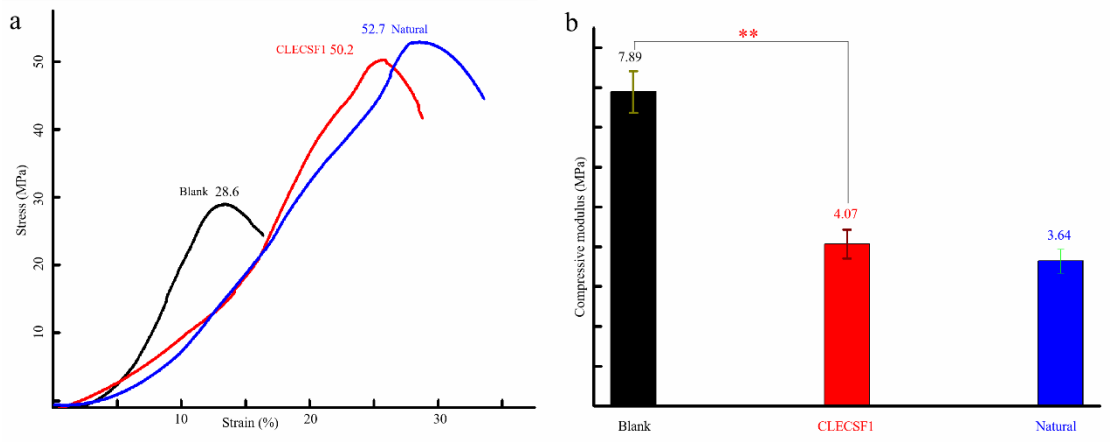

Fig. 8. a) Stress strain curve and b) compression modulus of natural joint and repaired joint after 12 weeks of implantation of blank hydrogel and cell-hydrogel composite.

Table1. The details of primers for PCR

Genes	Primer sequences F	Primer sequences R
GAPDH	gatgctggtgccgagtac	gctgagatgatgacccttttgg
Sox9	gcgtcaacggctccagcaagaacaag	gcctgcccattcttcaccgacttcct
Col2α1	cgccacgctcaagtccctcaacaac	ggctcttgctgctccaccagttcttc
Fibulin-1	aaagcccagcggtgcagagtct	cctcttctggagtttattgggagcagc

Supplementary Material

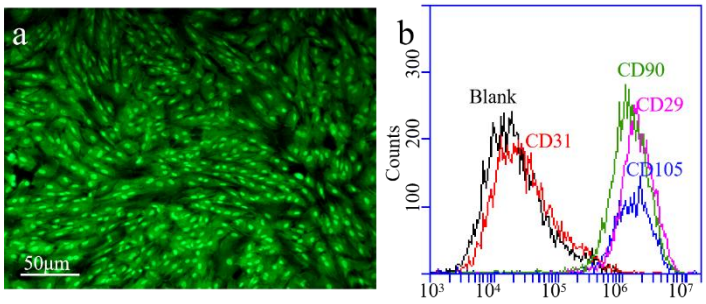

Fig. S1. Identification of the cultured primary BMSCs. a) Fluorescent images of the BMSCs, b) Flow cytometry results of stem cell surface specific markers CD 29, CD 90, CD 105 and endothelial cell surface specific marker CD31 expressed in the cells.

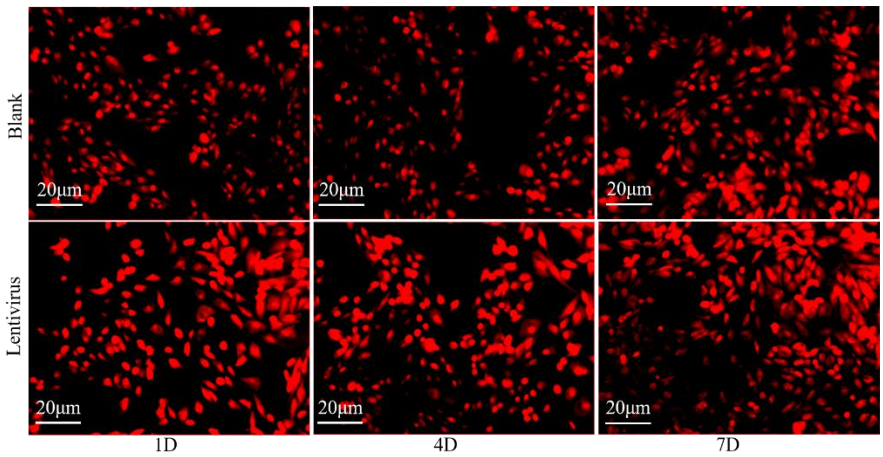

Fig. S2. Morphology of BMSCs transfected with or without recombinant lentiviral vector after 1, 4 and 7 days of culture.

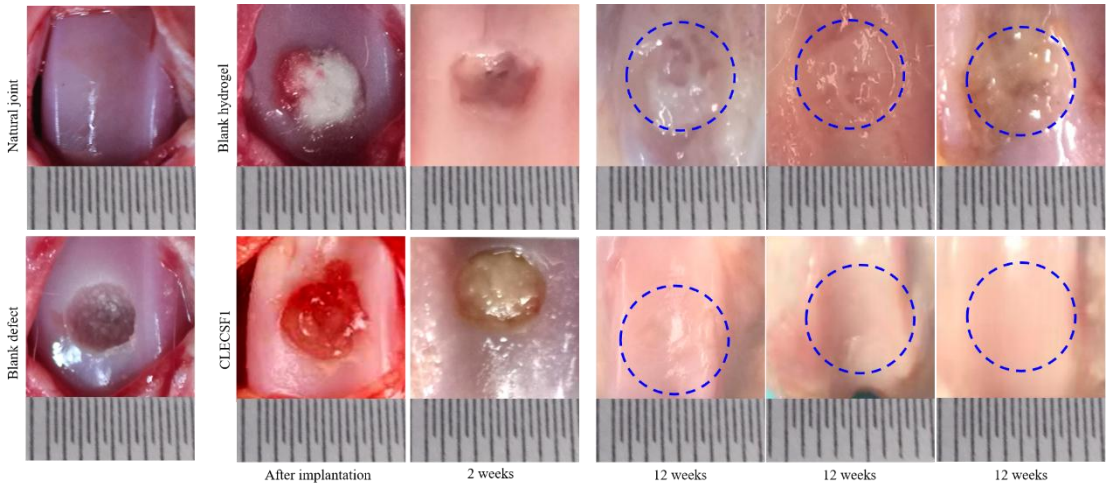

Fig. S3. Gross observation of cartilage repair. Images of cartilage defects in femoral trochlear grooves before and after implantation of blank hydrogel and cell-hydrogel composite, and images of repaired joint after 2 weeks and 12 weeks of implantation.