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REVIEW

Profile of biological characterizations and clinical application of corneal stem/progenitor cells

Pei-Xi Ying, Min Fu, Chang Huang, Zhi-Hong Li, Qing-Yi Mao, Sheng Fu, Xu-Hui Jia, Yu-Chen Cao, Li-Bing Hong, Li-Yang Cai, Xi Guo, Ru-Bing Liu, Fan-ke Meng, Guo-Guo Yi

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Abstract

Corneal stem/progenitor cells are typical adult stem/progenitor cells. The human cornea covers the front of the eyeball, which protects the eye from the outside environment while allowing vision. The location and function demand the cornea to maintain its transparency and to continuously renew its epithelial surface by replacing injured or aged cells through a rapid turnover process in which corneal stem/progenitor cells play an important role. Corneal stem/progenitor cells include mainly corneal epithelial stem cells, corneal endothelial cell progenitors and corneal stromal stem cells. Since the discovery of corneal epithelial stem cells (also known as limbal stem cells) in 1971, an increasing number of markers for corneal stem/progenitor cells have been proposed, but there is no consensus regarding the definitive markers for them. Therefore, the identification, isolation and cultivation of these cells remain challenging without a unified approach. In this review, we systematically introduce the profile of biological characterizations, such as anatomy, characteristics, isolation, cultivation and molecular markers, and clinical applications of the three categories of corneal stem/progenitor cells.

Key Words: Corneal epithelial stem cells; Corneal endothelium stem cells; Corneal stromal stem cells; Bioengineering; Gene markers

Core Tip: The manuscript systematically reviewed three categories of stem cells or progenitor cells in cornea (including corneal epithelial stem cells, corneal endothelial cell progenitors and corneal stromal stem cells). There are two aspects of the manuscript that will make it interesting to general readers. Not only have we systematically introduced the anatomy, characteristics, cultivation and gene markers of these corneal stem cells, but also highlighted the bioengineering in the clinical application of these corneal stem cells. In addition, this manuscript is accompanied by beautiful figures and tables.

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INTRODUCTION

The cornea acts as a structural barrier to protect the eye from the outside environment. Transparency of the cornea allows the light to enter the retina and has a very good ability to refract. The cornea is elliptical-shaped horizontally, 11.5-12.0 millimetres long horizontally and 10.5-11.0 millimetres long vertically[1]. The thickness of the cornea increases gradually from the centre (approximately 0.5 millimetres) to the periphery (approximately 1 millimetre)[2]. The cornea accounts for two-thirds of the refractive power of the eye, which is why corneal integrity is important for the maintenance of vision[3].

The cornea is composed of cellular and acellular components. Cell components include epithelial cells, keratocytes and endothelial cells as well as neural and immune cells[4]. The cell-free components include collagen and glycosaminoglycans. Corneal epithelial cells are derived from the epidermal ectoderm, while stromal cells and endothelial cells are derived from the neural crest. The corneal layer includes the epithelium, Bowman membrane, stroma, Descemet membrane and endothelium (Figure 1).

Recovery after corneal injury depends mainly on the regeneration of stem cells or progenitor cells. Corneal trauma and disease are highly associated with fibrosis, which can easily lead to severe visual impairment. In particular, the lesions of the central part of the cornea will seriously affect vision. After the corneal epithelium is damaged, it can regenerate without scarring[3]. However, when the damage affects Bowman's layer, its ability to regenerate is very limited, and Bowman's layer will be replaced by scar tissue. The stromal layer plays an important role in maintaining corneal transparency and resisting intraocular pressure and is also repaired by scar tissue after injury, resulting in the loss of transparency. The injury of Descemet's membrane can be regenerated by endothelial cells. In the past, damage to the endothelium was generally believed to be difficult to regenerate and could only cover the deficit left by injury through cell enlargement and migration. Although still controversial, in recent years, there has been increasing evidence supporting the existence of corneal endothelial progenitor cells[5-7].

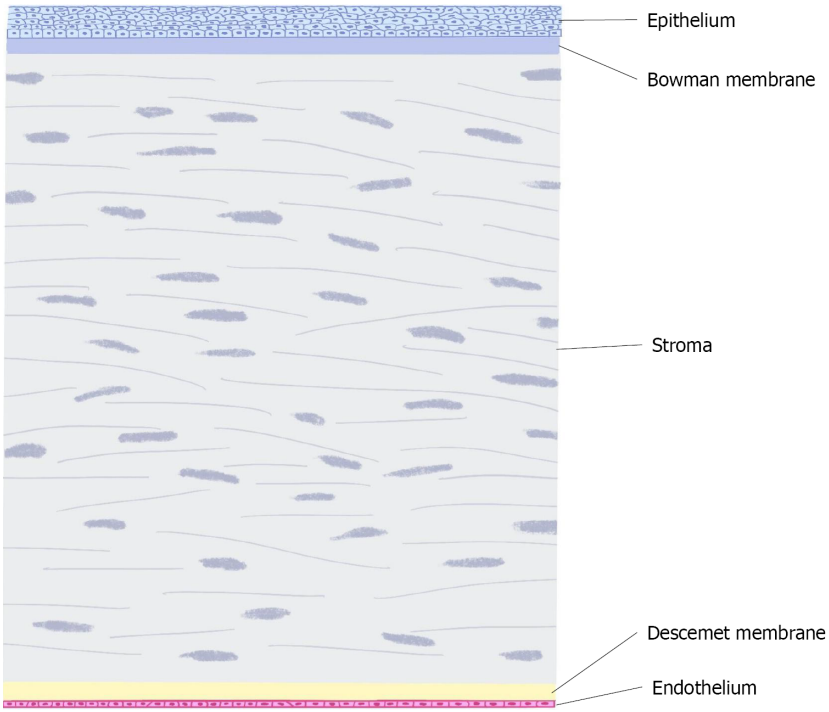

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Figure 1 Corneal stratification. The corneal layer includes the epithelium, bowman membrane, stroma, Descemet membrane and endothelium.

At present, the most commonly used method to treat corneal injury is corneal transplantation, and research on stem cells or progenitor cells will provide great help. In 1971, Wolosin *et al*[8] proposed the concept of limbal stem cells (LSCs), suggesting that epithelial corneal stem cells exist in the limbal gland. LSC deficiency (LSCD) is a serious disease that causes permanent corneal injury and visual loss due mainly to various kinds of corneal injuries and chronic immune diseases. LSCD has become a hot research direction in recent years, and new treatments are emerging. LSCs play an important role in repairing various kinds of corneal injuries and chronic immune diseases. In 2005, Du *et al*[9] found the first stem cell-like precursor cells of human corneal stromal cells. Similarly, corneal endothelial progenitors with the ability to self-renew and differentiate into mature corneal endothelial cells have also been identified, although corneal endothelial cells were widely believed not to be able to regenerate in humans. These stem cells have attracted great attention from the public, and an increasing number of people have devoted themselves to the study of their anatomy, physiology, immunology, cell culture and isolation.

It is essential to understand the anatomy, characteristics, methods of isolation and cultivation, molecular markers and therapeutic potential and applications of corneal stem/progenitor cells. We briefly discuss the three types of stem/progenitor cells of the cornea in this review.

CORNEAL EPITHELIAL STEM CELLS

Anatomy

Human corneal epithelial stem cells have been proven to be situated in the basal layer of the limbal epithelium, so they are also known as LSCs[10]. Pathologically, it is generally believed that the anterior limbus is situated in the plane connecting the end of the Bowman membrane and the Descemet membrane, while the posterior limbus is located in the plane passing through the iris root and perpendicular to the ocular surface. In addition, ophthalmic surgeons should be proficient in the gross anatomy of the limbal, which is the incision for most cataract and glaucoma surgeries. Surgically, limbus is known as the grey or blue zone because this transition zone shows a blue-grey appearance when viewed externally after the conjunctiva has been reflected away from the limbus[11].

Moreover, due to high vascularization and neuralization, the limbus is nutrient-rich and perceptive, and protected from potential ultraviolet (UV) damage by melanin pigmentation[4]. The corneal epithelial stem cell region is only 1.5-2.0 mm wide in the basal layer of the corneal epithelium, a small portion of the entire tissue, and is estimated to account for 0.5% or less to 10.0% of the total number of cells in palisades of Vogt[8,12,13] (Figure 2).

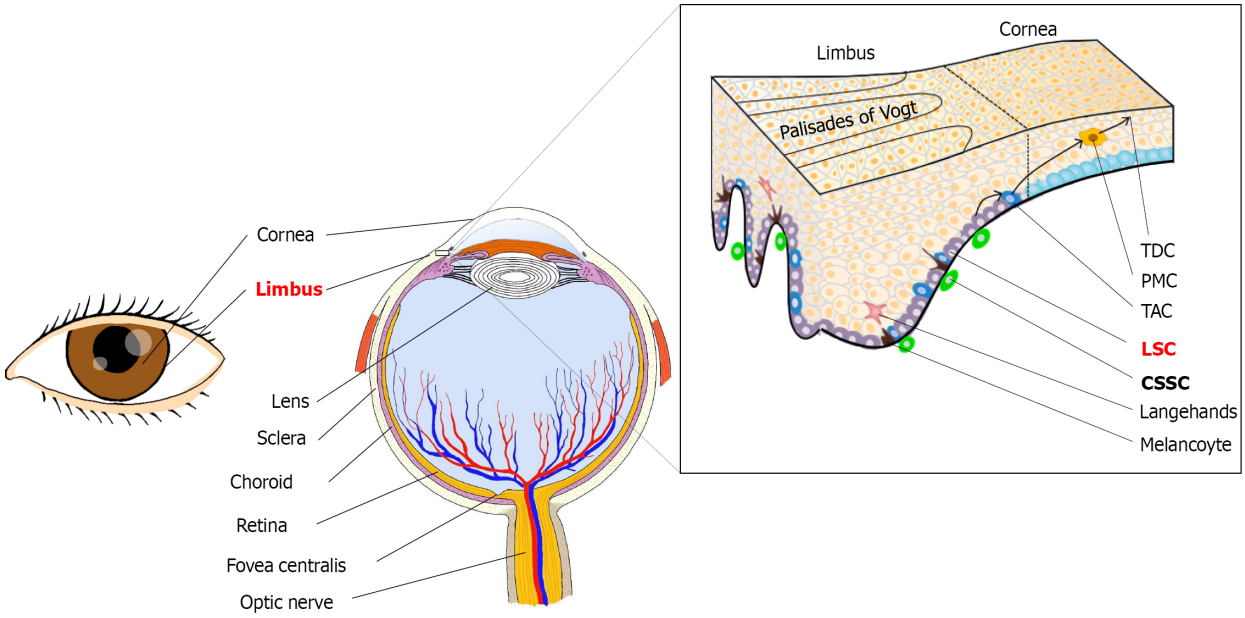

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Figure 2 Location of limbal stem cells and corneal stromal stem cells. Limbal stem cells (LSCs) are located at the base of the limbus and are in close contact with niche cells, including melanocytes. LSCs are symmetrically divided into two identical cells in the horizontal plane or asymmetrically differentiated into another LSC and a transient amplifying cell (TAC) in both vertical and horizontal planes. Then, TACs are divided into postmitotic cells (PMCs) as they migrate centripetally. The PMCs are then differentiated into terminally differentiated cells (TDCs) and shed from the corneal surface. Corneal stromal stem cells are in the anterior stroma subjacent to the epithelial basement membrane, in regions where the basement membrane has muslimah and folds termed the Palisades of Vogt. LSC: Limbal stem cell; TAC: Transient amplifying cell; PMC: Postmitotic cell; TDC: Terminal differentiated cell; CSSC: Corneal stromal stem cell.

Cell migration is one of the most basic elements of epithelial homeostasis. A mathematical analysis of the kinetics of maintenance of corneal epithelial mass confirms that the corneal epithelium can be maintained by the centripetal migration of epithelial cells of limbal origin[14,15]. Currently, it is widely accepted that LSCs generate transient amplifying cells (TACs), which then migrate centripetally and anteriorly to give rise to differentiated corneal epithelial cells that eventually fall off the surface of the cornea, as proposed in the X-Y-Z hypothesis[16,17]. LSCs are particularly important in maintaining corneal epithelial homeostasis and normal corneal wound healing. In addition, acute damage to the central cornea can be repaired by the proliferation and migration of central corneal epithelial cells[18].

Characteristics

Low differentiation with a primitive phenotype: Stem cells have long been recognized to be primitive cells with little differentiation. In 1986, Schermer *et al*[19] proposed the limbal location of corneal stem cells based on differentiation-related expression of 64K keratin. Immunohistochemical data showed that limbal basal cells were the least differentiated of all corneal epithelial cells. Moreover, cytokeratin K3 and K12, as specific markers of the corneal epithelial phenotype, were not expressed in limbal basal epithelial cells. Therefore, many studies have also confirmed the low differentiation phenotype of LSCs [20,21]. Many studies have shown that some materials or cells [such as Frizzled 7, HC-HA/PTX3 and human limbal melanocytes (hLM)] can maintain the low differentiation state of LSCs[22] (Niche regulation of limbal epithelial stem cells: HC-HA/PTX3 as a surrogate matrix niche) (Limbal melanocytes support limbal epithelial stem cells in 2D and 3D microenvironments).

Long cell cycle and high proliferative potential: A long cell cycle indicates low mitotic activity. Most stem cells are in a state of steady growth, so the proliferation rate is extremely low[23]. However, the high proliferative potential of stem cells can be activated under injury or *in vitro* culture. Taking advantage of several animal models and *ex vivo* human limbal cultures, the existence of slow-cycling and label-retaining cells in the basal layer of the limbal basal epithelium was proven by previous studies [24-27]. Multiple studies on proliferation potential in the presence of injury, absence of injury, *in vitro* culture, or differentiation-inducing agents have further supported the idea that corneal epithelial stem cells are located in the limbus[28-32]. Recent studies by Sagga *et al*[33] quantified the proliferative dynamics of LSCs during corneal wound healing. They found that the cell circulation of central corneal epithelial cells in young mice (4.97 d ± 0.50 d) was significantly slower than that in ageing mice (3.24 d ± 0.20 d). In wounded eyes, the proportion of LSCs entering S phase within 24 h in young mice increased 7 times compared with that in uninjured mice, but no significant increase was observed in aging mice. The contribution of LSCs in regenerative ophthalmology has demonstrated that LSCs can also be used to reconstruct the entire corneal epithelium in the case of severe ocular surface injury[34]. Additionally,

the location of corneal epithelial stem cells in the limbus may account for the relative superiority of limbal neoplasms and the rarity of corneal epithelial tumours[35-37].

Capacity for unlimited self-renewal and error-free proliferation: According to the result of division, the division of stem cells is divided into symmetric division and asymmetric division. Symmetric division occurs when a stem cell divides into two identical daughter stem cells, while asymmetric division occurs when a stem cell divides into two different cells. Lamprecht *et al*[38,39] first reported asymmetric division of mammalian corneal epithelial stem cells. Using differences in cell size, nuclear chromatin condensation, and cytoplasm density as criteria for histological analysis, they found that mitosis of corneal epithelial stem cells can be classified according to the position relationship between the spindle axis and basal lamina: Vertical mitosis (spindle axis at 60-90 degrees from basal lamina); oblique mitosis (spindle axis at 30-60 degrees from basal lamina); horizontal mitosis (spindle axis 0-30 degrees from basal lamina). Among these relationships, the daughter cells produced after horizontal mitosis were all located in the basal lamina, and their morphology and proliferation ability were similar to that before mitosis. The daughter cells produced after vertical mitosis and oblique mitosis were different in morphology and proliferation potential. The cells located in the basal lamina still had proliferation ability, while the cells located above the basal lamina developed towards the direction of terminal differentiation. The asymmetric division of corneal epithelial stem cells not only contributes to the replenishment of the stem cell pool but can also renew the corneal epithelium in time to cope with accidents or diseases such as corneal injury. This process of division does not allow errors because any genetic error at the stem cell level will continue to be transmitted to the entire cell clone, leading to abnormal differentiation and cellular dysfunction.

Morphological criteria: Both confocal microscopy and flow cytometry have shown that the smallest cells are located in the limbal basal epithelium rather than the corneal basal epithelium[40]; electron microscopy showed that limbal epithelial basal cells were characterized by immature cells, such as small cells, rich in tonofilaments in the cytoplasm, euchromatin-rich nuclei, almost undetectable nucleoli and a high nuclear-cytoplasmic ratio[41].

Isolation and cultivation

There are numerous of ways to isolate cells, one of which uses enzymes. In 2010, Yamamoto *et al*[42] cultured human corneal epithelial cells in serum-free medium by an enzyme-treated cell culture method. The corneal epithelium was separated from the limbus of the corneal peripheral area, and the corneal endothelium was removed. The limbus was then carefully separated from the underlying matrix with 0.25% collagenase and Accumax (cell aggregation disintegration medium). Finally, corneal epithelial cells were cultured in serum-free PCT corneal epithelial medium. The results showed that compared with human corneal epithelial cells (HCEC2 cells), the survival rate of the corneal epithelial cells obtained in this experiment increased significantly after being cultured on CNT-20 medium to the sixth generation, indicating that the enzyme separation method could maintain cell viability.

Recent studies have shown that flow cytometry and immunofluorescence activation can be used to isolate cells. Shaharuddin *et al*[43] isolated and identified LSCs using an optimized limbal side population (LSP) regimen, including an optimized Hoechst concentration, Hoechst incubation time and inhibitor concentration. After preoptimization, cells isolated from tissues were bound to a DNA-binding dye, Hoechst 33342, mediated by a TP-binding box (ABC) transporter. Then, two types of cells, Hoechst Blue (SP) and Hoechst Red (non-SP), were obtained by flow cytometry analysis and fluorescent-activated cell sorting. Compared with non-SP cells, the expression of the stem cell markers ABCG2, ΔNp63 and SOX2 was significantly higher in SP cells according to the immunofluorescence assay. Shaharuddin *et al*[43] demonstrated that the LSP protocol identifies and enriches LSCs by preoptimizing key parameters.

Based on previous studies, Nam *et al*[44] cultured corneal epithelial cells on the canine amniotic membrane. Under a light microscope, the cultured cells fused 100% after 7-8 d, and 5-8 Layers of epithelial cells were formed on the amniotic membrane. K3 (a corneal epithelial-specific marker) was observed in all cultures by immunofluorescence, while ABCG2, P63, and vimentin (a stem cell marker) were observed only in the basal layer. The results showed that beagle corneal epithelial cells could be cultured on the canine amniotic membrane, and the basal layer cells might be stem cells. Moriyama *et al*[45] cultured equine LSCs in standard supplemental hormone epithelial medium (SHEM). Cells isolated from LEC tissue extracted from the limbs were cultured in standard SHEM. Immunostaining showed positive p63, CK14, and negative CK3 cells, similar to the expression pattern of limbal epithelial basal cells, suggesting that LSCs could be obtained by this method.

In addition, different temperatures also affect the separation from the cells. Nam *et al*[46] compared the effects of different media (canine amniotic membrane, heterotopic collagen gel, temperature-sensitive culture dish) on corneal stem cell culture. Cells were isolated from beagle corneal epithelial cells and cultured on canine amniotic membrane, collagen gel and temperature-sensitive culture dishes. Ki-67, K3, ABCG2 and P63 were used as indices. Immunofluorescence and real-time quantitative polymerase chain reaction (RT-PCR) were used to observe the culture time and the number of cell layers

adhered to and fused. The specific results are shown in Table 1.

RT-PCR showed that the expression level of ABCG2 mRNA was 9.9 times larger on the canine amniotic membrane than on the atelocollagen gel and 7.2 times larger than on the temperature-responsive culture dish. The expression level of P63 on atelocollagen gel was 2.8 times and 3.2 times higher than that on canine amniotic membrane and temperature-responsive culture dishes, respectively. The atelocollagen gel-cultured corneal epithelium showed morphology similar to the normal corneal epithelium and retained more stem cells/progenitors than the canine amniotic membrane and temperature-responsive culture dish.

Compared to the canine amniotic membrane, the human amniotic membrane (HAM) is the most common scaffold for culture, both experimentally and clinically. Kim and Tseng[47] were the first to use HAM for clinical treatment. They transplanted HAM alone into corneas with mild LSCD that did not require LSC treatment to promote re-epithelialization. Subsequently, researchers began to study the possibility of HAM as an amplification carrier of LSCs. From the early coculture with the mouse 3T3 fibroblast feeding layer[48], animal-free amplification methods have been designed to avoid the risk of zoonosis[49,50]. With the deepening of the research, HAM was found to have increasing advantages in the aspect of corneal transplantation, including anti-inflammatory, antimicrobial, antiangiogenesis, antifibrosis, secretion of neurotransmitters and growth factors, finally reducing scar formation, stimulating the epithelialization and differentiation of corneal epithelial cells as well as enhancing adhesion and preventing apoptosis[51,52]. Today, HAM can play a role in a variety of corneal injury diseases or postoperative treatment, including refractory corneal ulcers and corneal epithelial defects, malignant tumours or pterygium resection[53].

The above studies (Table 2) provide a variety of methods for the isolation and culture of LSCs. Enzymatic dissociation is one of the most common methods to disperse tissues into single cells[54-56]. According to the differences in cell and interstitial composition between different tissues, trypsin or collagenase digestion is used to achieve cell separation. Compared with separating cells with only enzymes, the LSP protocol preoptimizes the concentration of dyes and inhibitors, staining time, *etc.*, which is beneficial to the identification and enrichment of LSCs. In the cultivation of LSCs, a variety of substances can be used as culture media for LSCs, such as media supplemented with hormones, animal materials (canine amniotic membrane), HAM, collagen gels and temperature-responsive culture dishes. Compared with animal materials and temperature-responsive culture dishes, cells cultured in collagen gel are more similar in morphology to normal corneal epithelial cells and contain more stem cells.

However, there are some disadvantages in culturing LSCs with HAM and animal materials. For example, the amniotic membrane has obvious biodegradation and immunosuppressive properties during transplantation. Its thickness and variable transparency are also disadvantages. Moreover, the use of feeder cells or a composite medium containing growth factors or animal materials may also cause biological contamination or other safety issues(such as prion transfer or unknown diseases)[57-62]. Therefore, more improvements or alternative methods are needed.

Molecular markers for LSCs

Cytoskeletal proteins: In recent years, corneal transplantation has become a research hotspot of corneal epithelial stem cell gene markers. Moriyama *et al*[45] studied the eye tissues of 12 adult thoroughbred horses. They used immunohistochemical staining for negative markers (CK3) and positive markers (P63, CK14)[63] to determine the distribution and culture of equine corneal epithelial stem cells (CESCS). The experimental results showed that P63 played an important role in the proliferation of human keratinocytes and was expressed in basal cells of various human epithelial tissues and was a stem cell marker[64]. Moreover, the results indicated that 13,14,24-26,31,33 CK14[65] is a useful indicator for the identification of epithelial progenitor cells with basal cell activity, as well as a marker of stem cells. In addition, among these stem cell markers, antihuman P63, antiCK14, and CK3 antibodies can cross-react with equine corneal epithelial cells[66]. These research results are of great significance for follow-up clinical treatment.

Cytosolic proteins: Lyngholm *et al*[67] used a proteomics method combining two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to study the difference in protein expression between human central corneal epithelium and limbal epithelial cells. A total of 25 different proteins were identified. Superoxide dismutase 2 (SOD2) is expressed in the basal limbal epithelial cell population; heat shock protein 70 protein 1 (HSP70.1) and annexin I are expressed at higher levels in the limbal epithelium, but there is also a small amount of expression in the central epithelium. They also found that SOD2 appears almost exclusively in the limbal epithelium of the basal cornea. SOD2 induces phosphorylation and activation of mitogen-activated protein kinases (MAPKs) by regulating H_2O_2 and activates signalling molecules, including extracellular regulatory kinases (ERKs) and c-Jun N-terminal kinase (JNK)[68].

In addition, the Notch-1 gene plays an important role in corneal stem cell transplantation, and related research on this gene has also been reported in recent years. For example, Thomas *et al*[69] obtained human corneoscleral tissue from the Doheny Eye & Tissue Transplant Library through explant culture and primary culture and performed cross-sectional and full-thickness analysis. Their experiment studied mainly the expression of Notch 1 in the basal epithelium of the limbus, and the results showed

Table 1 Culture conditions of limbal stem cells

Culture condition	Canine amniotic membrane	Atelocollagen gel	Temperature-responsive culture dish
Culture			
Adhesion time, d	1	1	2
Convergence time, d	5	7	14
Layers	6-8	4-5	2-3
Immunofluorescence			
Ki-67	Basal layer +	Basal layer ++	Basal layer -
K3	Each layer +	Each layer except basal layer +	Each layer +
ABCG2	Only in basal layer +		
P63	Basal layer +	2-3 layers, including the basal layer +	Basal layer +

Table 2 Methods for the isolation of limbal stem cells

Experimenter	Tissue	Isolation methods	Medium	Markers
Yamamoto *et al* [42], 2010	Human corneal epithelial stem/progenitor cells	0.25% collagenase and accumax	Serum-free PCT corneal epithelium medium	P75 ntr2, p63, integrin β-1
Albert *et al*[171], 2012	Human cornea limbal epithelial stem cells	None	Human lens capsule	KRT8, KRT14, KRT3, KRT12, KRT19, VIM, MKI67, p63α, ABCG2, CK19, Vim, Itgα9, CK8/18, CK14
Shaharuddin *et al* [43], 2014	Human limbal epithelial cell	LSP protocol, Hoechst 33342 dye, Flow Cytometry Analysis and Fluorescence Activated Cell Sorting	None	ABCG2, ΔNp63, Sox2
Nam *et al*[44], 2013	Beagle limbal epithelial cells	None	Canine amniotic membrane	ABCG2, p63, vimentin
Moriyama *et al* [45], 2014	Equine corneal epithelial stem cells	Dispase II and a 0.25% trypsin EDTA solution	Standard supplemented hormone epithelial medium	P63, CK14
Nam *et al*[46], 2015	Beagle limbal epithelial cells	Dispase II, trypsin	Canine amniotic membrane; atelocollagen gel; temperature-responsive culture dish	Ki-67, K3, ABCG2, P63
López-Paniagua *et al*[60], 2016	Human cornea limbal epithelial stem cells	0.25% trypsin-EDTA	Biosafe IOBA-HS culture medium	K14, K15, ABCG2, p63α
Shirzadeh *et al* [61], 2018	Human cornea limbal epithelial stem cells	Collagenase II and cold trypsin	Supplemental hormonal epithelial medium with husscs or 3T3 cells as feeder layers	ABCG2, ΔNP63-α, CK19, CK 3, CK12
Li *et al*[62], 2021	Human cornea limbal epithelial stem cells	0.05% trypsin/1 mm EDTA solution	Standard supplemented hormone epithelial medium	TSPAN7, SOX17

SHEM: Supplemented hormone epithelial medium.

that Notch-1 was expressed in the area of limbus stem cells. The antigenicity of Notch-1 is more obvious in the cell mass, mainly in the fence of Vogt. There is almost no Notch-1 in the centre of the cornea. In addition, ABCG2[70] and Notch1 double staining showed that some ABCG2-positive cells coexpressed Notch-1 in the limbus basal epithelium, suggesting that Notch-1-expressing cells may be a unique subgroup with stem cell characteristics. This result suggests that further research and characterization of the Notch pathway will provide valuable clues for clinical research on stem cell transplantation.

Neuronal markers: In addition, the interaction between KLF, PAX-6 and ESE-1 has important research value in human corneal development and physiology. The regulation of KLF family members by K12 is closely related to cell growth, proliferation and proliferation. GKLF/KLF4 participates in p53 transactivation of p21 WAF1/Cip1 promoter induction, cyclin D1 promoter activity inhibition and cell proliferation inhibition, which indicates that this protein may play a direct role in negative growth control[71]. A recent study found that KLF4 can block the epithelial to mesenchymal transition (EMT) of human corneal epithelial cells (HCECs) by inhibiting the canonical TGF-β signalling pathway.

Therefore, KLF4 plays a crucial role in the maintenance of HCEC homeostasis and has the potential to prevent the formation of corneal fibrosis scars[72].

Pinin: Pinin (PNN) is an exon junction component (EJC) that is important in the differentiation of corneal epithelial cells and can act as a stabilizer for the corneal epithelial phenotype. Using RNA-seq to obtain the results, PNN knockout-upregulated genes included a large number of genes related to cell migration and ECM remodelling processes, such as MMPs, ADAMs, HAS2, LAMA3, CXCRs, and UNC5C. Genes knocked down by PNN gene knockout included IGFBP5, FGD3, FGFR2, PA X6, RARG, and SOX10[73].

Recent research on LSC deficiency

Causes of LSC deficiency: LSCD is a pathological condition caused by dysfunction and/or insufficient quantity of LSCs, which is marked by impaired barrier function of the limbus and the replacement of corneal epithelium by conjunctival epithelial cells[74,75]. Direct damage to LSCs and/or destruction of their niche microenvironment is the causes of their pathogenesis. Loss of stem cells due to severe damage to the limbal can result in permanent corneal epithelial defects and vision loss due to the conjunctiva[76]. Moreover, although LSCD can be divided into unilateral LSCD and bilateral LSCD, their causes are similar. Burns are the most common cause, followed by Stevens-Johnson syndrome (SJS), atopic keratoconjunctivitis (AKC)/vernal keratoconjunctivitis (VKC) and mucous membrane pemphigoid (MMP). Since congenital aniridia is a disorder of two eyes caused by haploinsufficiency of the Pax6 gene, it is only the cause of bilateral LSCD[77]. Additionally, the primary burns are alkali burns. Thermal burns, acid burns, radiation burns and others account for only a small percentage[78, 79]. The conditions that lead mainly to LSCD include two main categories, hereditary LSCD and acquired LSCD, as shown in Table 3[80-115].

Diagnosis of LSCD: The main symptoms and signs of LSCD are[75,116]: (1) Nonspecific symptoms, including vision loss, photophobia, lacrimatorrhea, blepharospasm, redness associated with chronic inflammation, and recurrent episodes of pain caused by epithelial lesions; (2) signs: Under a slit-lamp biomicroscope, the corneal epithelium shows three grades of damage including mild, moderate and severe/total LSCD: Mild grade: Dull or irregular corneal surface without light reflex, opaque corneal epithelium and deterioration of limbal palisades of Vogt; moderate grade: A vortex pattern of abnormal epithelium with fluorescein staining, superficial vascularization of the cornea and peripheral pannus; and severe grade: Thick fibrous pannus formation, chronic keratitis, scarring and corneal conjunctivization; and (3) the migration of conjunctiva and goblet cells to the corneal surface was confirmed by impression cytology.

In addition, there are some new advances in the diagnosis of LSCD involving *in vivo* laser scanning confocal microscopy and anterior segment optical coherence tomography.

Basic treatment principles and methods of LSCD: Treatment strategies for LSCD can generally be divided into two categories: (1) Conservative treatment, including conservative nonsurgical options and conservative surgical options; and (2) Invasive treatment, which means transplantation aimed at repairing the structure and function of the corneal epithelium[117] (Table 4).

Conservative treatment: (1) Conservative nonsurgical options: Autologous serum drops[118], therapeutic soft contact lenses[119], therapeutic scleral lenses and eye lubrication[114]; (2) conservative surgical options: Corneal scraping[120] and amniotic membrane transplantation (AMT)[53,121]. AMT is effective in patients with partial or mild LSCD[122]. However, long-term debilitated vision remained in those with severe LSCD caused by burns[123]. In recent years, LSC deficiency has been successfully treated by direct transplantation of a portion of healthy limbal tissue or LSC, even some other alternative cells populations.

Transplantation: (1) Conjunctival limbal autograft (CLAU)[124,125]; (2) conjunctival limbal allograft (CLAL)[86,126]; (3) keratolimbal allograft (KLAL)[127,128]; (4) *ex vivo* cultivated limbal epithelial stem cells (CLET); (5) simple limbal epithelial transplantation (SLET); and (6) alternative cell population transplantation. In CLAU, CLAL and KLAL, since the long time for limbus transplantation from the donor to the stem cell deficient eye, healthy donor eyes have an increased risk of LSCD. In addition, the application of immunosuppressants in CLAL and LSCD may increase the risk of infection or cancer. CLET is a technique in which autologous or allogeneic LSCs are cultured on a carrier for transplantation, which greatly reduces the incidence of LSCD in healthy donors[129,130]. In addition, since LSCs cultured *ex vivo* do not differentiate into Langerhans cells, the incidence of immune rejection will also be decreased. SLET is a technique to evenly distribute autologous limbal tissue and attach it to HAM for transplantation. SLET not only holds the advantages of CLET in reducing the incidence of LSCD and immune rejection but also avoids the difficulties of *ex vivo* culture technology, which achieves higher economic benefits[131-133]. However, the risk of disease transmission is increased due to the application of carriers such as the HAM[134].

Emerging alternative therapies for LSCD: In addition to the above traditional treatments for LSCD performing limbal tissue or stem cell transplantation, advances in tissue engineering have led to the

Table 3 Causes of limbal stem cell deficiency

Item	Causes		
Hereditary LSCD	Congenital aniridia[80,81]		
	Dyskeratosis congenital[82,83]		
	Xeroderma pigmentosum[84]		
	Peters' anomaly[85]		
	Keratitis ichthyosis deafness syndrome[86]		
	Epidermolysis bullosa[87]		
Acquired LSCD	Acquired nonimmune-mediated	Burns	Alkali burns[88,89]
			Acid burns[90,91]
			Thermal burns[92,93]
			Radiation burns[94]
		Infection	Viral keratitis[95]
			Bacterial keratitis[96]
			Trachoma[97,98]
		Oncologic	Ocular surface tumours[99]
		Iatrogenic	Cryotherapy[84,100]
			Chemotherapeutic agents[101-103]
			Surgeries in the limbic region[103,104]
		Others	Contact lens use[105,106]
			Bullous keratopathy[107]
	Acquired primary immune-mediated	Stevens-Johnson syndrome[108]	
		Mucous membrane pemphigoid[109,110]	
		Vernal keratoconjunctivitis[111,112]	
		Atopic keratoconjunctivitis[113]	
		Graft-versus-host disease[114,115]	

LSCD: Limbal stem cells deficiency.

development of a growing number of emerging therapies, including alternative cell populations and alternative cell carriers/scaffolds.

Alternative cell populations: Compared with traditional transplants, the application of alternative cell populations avoids corneal donor shortages and the risk of disease transmission, graft rejection and tumours (due to immunosuppressant application) associated with allografts, which makes it possible to replace traditional transplants. Currently, the alternative cell populations include oral mucosal epithelial cells[135,136], human embryonic stem cells (HESCs)[137], induced pluripotent stem cells (iPSCs)[138,139], mesenchymal stem cells (MSCs)[140,141], human immature dental pulp stem cells[142,143], *etc*. Among these alternatives, MSCs and iPSCs are of vital importance and will be described in detail.

MSCs are a population of proliferative and multipotent stem cells present in various tissues throughout development. In the cornea, MSCs are natural residents of the LSC niche and can modulate immune response *via* paracrine action[144]. Additionally, MSCs have been shown to reduce neovascularization, stromal opacification, inflammation, and corneal oedema in animal models of LSCD secondary to chemical or mechanical injury, which offers advantages in corneal reconstruction[145,146]. The therapeutic effect of MSCs in regenerative corneal therapy can be attributed to direct cell replacement[147], differentiation into corneal epithelial-like cells[148] and secretion of soluble factors to regulate tissue wound repair, inflammation, angiogenesis and the immune response. Many studies have shown that MSCs have a wide range of applications, whether *in vivo* or *in vitro*, to help repair corneal epithelium[148-151], corneal stroma[152,153] and even corneal endothelium[154]. Therefore, MSCs have the potential to be differentiated into corneal cell types and can be potential candidates for regeneration of the cornea.

Table 4 Treatment strategies for limbal stem cell deficiency

Treatment	Description		
Conservative treatment	Nonsurgical options	Autologous serum drops	
		Therapeutic soft contact lens	
		Therapeutic scleral lens	
		Eye lubrication	
	Surgical options	Corneal scraping	
		Amniotic membrane transplantation	
Invasive treatment	Conjunctival limbal autograft		
	Conjunctival limbal allograft		
	Keratolimbal allograft		
	Ex vivo cultivated limbal epithelial stem cells		
	Simple limbal epithelial transplantation		
	Emerging alternative cell populations transplantation	Alternative cell populations	Oral mucosal epithelial cells
			Human embryonic stem cells
			Induced pluripotent stem cells
			Mesenchymal stem cells
			Human immature dental pulp stem cells
		Alternative cell carriers/scaffolds	Modified HAM
			Collagen
			Fibrin
			Siloxane hydrogel contact lenses
			Human lens capsule
			Poly (ε-caprolactone)
			Onion epithelial membrane scaffolds
			Carboxymethyl cellulose
			Electrospun polycaprolactone/gelatine nanocomposites

iPSCs are a population with pluripotent capacity to differentiate into many cell types and are generated from embryonic or adult body cells[155,156]. In 2006, the iPSC technique was first reported by Takahashi and Yamanaka, who dedifferentiated mouse fibroblasts into embryonic stem cell-like cells, which they named "iPSCs"[155]. iPSCs have self-renewing and multidirectional differentiation potential, which could generate corneal organoids expressing markers of adult corneal tissue[157] and displaying similar features of the developing cornea[158]. iPSCs are easy to obtain, and their autologous transplantation can avoid immune rejection, which has good prospects in the treatment of LSCD in the future.

Alternative cell carriers/scaffolds: HAM is the most commonly used cell carrier for ocular surface reconstruction. However, due to the disadvantages of HAM (such as high thickness, variable transparency, biodegradation and immunosuppression), it is urgent for us to find alternative cell carriers/scaffolds to substitute for HAM. At present, alternative cell carriers/scaffolds contain modified HAM, collagen[159,160], fibrin[161], siloxane hydrogel contact lenses, human lens capsules, poly (ε-caprolactone)[162], onion epithelial membrane scaffolds[163], carboxymethyl cellulose (CMC)[164], electrospun polycaprolactone/gelatine nanocomposites[165] and other emerging materials. The modified HAM will be described in detail below.

Modified HAM is initiated by chemical modification of HAM to avoid or mitigate the above shortcomings. Chemically modified HAMs have been developed using cross-linking agents, including glutaraldehyde[166], carbodiimide[167,168] and aluminium sulfate. Compared with glutaraldehyde, carbodiimide has lower cytotoxicity and the addition of L-lysine enhances the mechanical and thermal strength, the ability to support LESCs and the enzyme digestion resistance of HAM[169]. Additionally, aluminium sulfate cross-linked HAM remains sterile and shows increased tensile strength during 12 mo

of storage[170].

Albert *et al*[171] cultured LSCs in animal-free medium-that is, the lens capsule with human serum as the only growth supplement. The results showed that the viability of LSCs cultured on human LC was greater than 97% at the two checkpoints (day 7 and day 14), and the percentages of early apoptotic cells and late apoptotic cells were lower. Immunofluorescence showed that the resulting cells maintained their pluripotency by maintaining p63, ABCG2, CK19, Vim and Itg9 and low ck3/12 expression. The presence of differentiation characteristics (positive for CK8/18 and CK14) also indicates its potential for orthotopic differentiation into the corneal epithelium. All the above results indicated that LSCs could be cultured from lens capsules using human serum as the only growth supplement without the disadvantage of animal medium.

CORNEAL STROMAL STEM CELLS

Anatomy

The stroma, which makes up approximately 90% of the cornea, is a collagenous mesenchymal tissue composed of approximately 200 orthogonally oriented lamellae. Each lamella is made up of long uniform collagen fibrils lying parallel to one another with regular interfibril spacing, which is essential in rendering the tissue transparent[172]. Many fibroblast-like cells distributed in the matrix are commonly known as corneal stromal cells. The corneal stroma is derived from the neural crest, which is the source of mesenchymal tissue in the head and neck. Corneal stromal cells have extensive cytoplasmic processes in contact with similar processes in neighbouring cells.

By staining with ABCG2 and PAX6 proteins, corneal stromal stem cells were observed largely in the transitional zone between the cornea and sclera known as the limbus[173]. More specifically, CSSCs are in the anterior stroma subjacent to the epithelial basement membrane, in regions where the basement membrane has Muslimah and folds termed the Palisades of Vogt (Figure 2)[173,174].

Characteristics

Under normal physiological conditions, corneal stromal cells remain stationary in mitosis and maintain a highly ordered layer of collagen and proteoglycan, which are essential for providing corneal transparency. In addition, they are often characterized by molecular markers, including aldehyde dehydrogenase (ALDH), crystallins, CD133, and CD34[175]. When the cornea is injured corneal stromal cells are activated and lose the expression of cellular markers and adopt fibroblasts and myofibroblasts to form scar phenotypes.

Bioengineering corneal tissue with stromal stem cells

The highly ordered hierarchical ultrastructure of the corneal matrix, which exhibits exceptional biomechanical properties and optical transparency, makes it one of the most challenging steps in engineering human corneal tissue[176]. Fortunately, in 2005, Du *et al*[9] identified the first stem cell-like human corneal stromal cell precursors. The study found that in serum-free medium, human corneal stromal stem cells (hCSSCs) differentiate into cells expressing a gene profile similar to the profile of human keratocytes and secrete multilayered lamellae with orthogonallyoriented collagen fibrils similar to the corneal stroma, when cultured as floating pellets in the absence of rigid scaffolding or substratum [177].

Furthermore, some studies have found that CSSCs may not only have the ability of immune privilege but also have the potential to provide direct cell therapy pathways. The ability of CSSCs to exhibit immune privilege makes them excellent candidates for the generation of bioengineered corneal stromal constructs. Du *et al*[175] found that after direct injection into mouse corneas with scars, both the organization and transparency of the cornea were successfully restored without eliciting an immune T-cell response. Similarly, Ghoubay *et al*[178] developed a mouse model of corneal stromal scarring induced by liquid nitrogen (N2) application. Through direct injection of mouse or human corneal stromal stem cells in this model, they found that the transparency of the injured cornea was improved, the inflammatory response disappeared, recipient corneal epithelial cells grew collagen type III stromal content, corneal rigidity and stromal haze decreased, stromal ultrastructure was restored, and vision was improved. Their work concluded that corneal stromal stem cells could reverse the formation of mechanism scars and had the ability to promote the regeneration of transparent stromal tissue. Someone has investigated the mechanism and found that corneal stromal stem cells inhibited neutrophil infiltration on injured corneas by secreting TSG-6, thereby reversing scar formation[179].

CORNEAL ENDOTHELIAL CELLS AND CELL PROGENITORS

Anatomy

Corneal endothelial cells (CECs) are important for maintaining corneal transparency. The corneal

endothelium is derived from the neural crest and forms a monolayer of hexagonal cells[180]. In the past, the corneal endothelium has been thought to be different from the corneal epithelium in that once the mature single-cell layer is formed, corneal endothelial cells lose their ability to proliferate. Instead of regenerating into new cells to replace dead or damaged cells, the wound can only be repaired by the expansion and migration of endothelial cells around the damaged area, resulting in a decrease in the density of endothelial cells by 0.3%-0.6% per year[181].

However, corneal endothelial cell progenitors, similar in part to stem cells, have been found to have the ability to self-renew and differentiate into mature effector cells. The progenitors are thought to be situated in the posterior limbus, a transitional area (also known as Schwalbe's ring region) from the periphery of the endothelium and Schwalbe's line to the anterior portion of the trabecular meshwork (TM). These hypothesized corneal endothelial cell precursors give rise to corneal endothelial cells and trabecular cells[5-7]. However, progenitor cells and stem cells are not exactly the same and cannot be substituted for each other. The self-renewal ability of progenitor cells is limited, which results in apoptosis of progenitors at the end of differentiation. Nevertheless, in contrast with the previous view that corneal endothelial cells cannot proliferate at all, the discovery of corneal endothelial progenitor cells has profound implications.

Characteristics

Different cell densities: The density distribution of corneal endothelial cells is uneven. B H Schimmelpfennig divided the collected corneas into two groups for staining. He found that in both 19 corneas stained with Orcein and 22 corneas stained with Alizarin Red, the cell density of the peripheral corneal endothelium was approximately 23.5% higher than that of the central corneal endothelium[182,183]. This difference in density suggests that the smaller peripheral endothelial cells can migrate to the centre by increasing the area, which is conducive to the repair of the corneal endothelium. Meanwhile, the possibility that progenitor cells may exist in the peripheral transition region to provide differentiated endothelial cells is also suggested[5].

The proliferative ability is related to the location: Several studies have demonstrated that the proliferative ability of peripheral endothelial cells is stronger than that of central corneal cells in terms of cell distribution, molecular expression, senescence status and mitotic activity[183-186]. In 2000, Senoo *et al*[187] found that corneal endothelial cells can enter and complete the cell cycle *in vitro* after corneal endothelial injury, regardless of donor age. However, corneal endothelial cells from older donors responded more slowly and to a lesser extent than cells from younger donors. Subsequently, Mimura *et al*[186] further investigated the replication capacity of human corneal endothelial cells (HCECs) in central and peripheral regions and between young and old donors. They divided the corneas into a younger group (donors younger than 30) and an older group (donors older than 50). Minichromosome maintenance (MCM)-2 (a marker of replication competence) and senescence-associated β-galactosidase activity (SA-β-Gal) (a marker for identifying senescent HCECs) were used for staining. They found that in corneas from elderly donors, significantly fewer HCECs migrated to the central wound than to the periphery. Compared with HCECs from the young group with little SA-β-Gal activity both in the central or peripheral regions, the SA-β-Gal activity of HCECs from the older group was easier to detect, and the SA-β-Gal activity of central HCECs was significantly higher than that of peripheral HCECs. In both the younger and older groups, there were more MCM-2-positive cells in the peripheral corneal injury area than in the central corneal injury area. *In vitro*, HCECs from the peripheral region can be shown to have a higher replication capacity than HCECs from the central region, regardless of donor age. Therefore, the peripheral cornea has been suggested to act as a physiological supply and store for corneal endothelial cells so that the central cornea can be continuously supplied[183].

Proliferation ability is related to age differences among donors: After counting the stained proliferation marker protein Ki67, Senoo *et al*[187] found that the number and peak value of Ki67-labelled cells in the old group were significantly lower than that in the young group, and the speed of the old group entering the cell proliferation cycle was significantly slower than the speed in the young group. After *in vitro* culture and staining for Ki67 count, Zhu *et al*[188] found that the density of cells with positive staining in the young group was twice as high as that in the old group, and the time required for them to enter the cell cycle was half as long as that in the old group; Konomi *et al*[189] found that the doubling time tended to be higher for cells from older donors. Some people proposed explanations for these findings. Joyce[190] believed that with increasing age, the number of corneal endothelial cells entering the proliferative and senile phases gradually increased, and the expression of CKIS also increased, leading to a decrease in cell proliferation activity and a significantly reduced response to mitotic agents. In addition, Joyce *et al*[191] found that increased concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHDG), a cell oxidative stress product, resulted in decreased proliferation.

As a result, the density and proliferation of peripheral endothelial cells are higher than those of central endothelial cells, although the proliferation of endothelial cells decreases with age. This conclusion strongly suggests that there may be corneal endothelial cell progenitors in the periphery of endothelial cells (namely Schwalbe's ring).

Molecular markers for corneal endothelial cell progenitors

As early as 2005, Whikehart *et al*[192] detected telomerase activity (a stem cell marker) in the peripheral cornea, and bromodeoxyuridine (BrdU), a marker of cell division, was observed in the trabecular meshwork (TM) and the posterior limbus. After mechanical injury to the corneal endothelium, BrdU fusion was increased and extended to the corneal endothelium. In 2007, McGowan *et al*[193] found that cells from Schwalbe's Ring expressed stem cell markers (Nestin, alkaline phosphatase, and telomerase). Additional putative stem cell markers (OCT3/4, Wnt1) and differentiation markers (Pax6, Sox2) were observed after corneal injury. In 2019, Yam *et al*[194] found that the cells expressing progenitor cell markers (*i.e.*, SOX2, Lgr5, CD34, Pitx2 and telomerase) were involved in Schwalbe's Ring on the side of the corneal endothelium. In addition, many studies have found that corneal endothelial progenitors express p75NTR, SOX9, FOXC2, Twist, Snail, and Slug[195,196].

Transplantation and therapeutic potential

Corneal endothelial cells play an important role in maintaining the stability and transparency of the corneal environment. When obvious visual impairment causes irreversible damage, the best strategy is to replace it with allogeneic corneal endothelial cells. However, severe rejection and a global shortage of donor corneal tissue have led people to seek alternative sources of transplantable tissue. Parikumar *et al*[197,198] successfully used a transparent nanocomposite sheet to transplant donor human corneal and colorectal cells into a cow's eye, and implanted HCECs within three hours after transplantation. Their experiment paved the way for further clinical research.

On the basis of previous studies, Frausto *et al*[199] used next-generation RNA sequencing technology to compare human corneal endothelial cells (evHCEnCs) cultured *in vitro* with primary human corneal endothelial cells (pHCEnCs) and the human corneal endothelial cell (HCEnC) transcription profile. Transcriptomics analysis shows that at the molecular level, pHCEnCs are the most similar to evHCEnCs and therefore represent the most feasible cell culture treatment for corneal endothelial cell dysfunction.

In addition, some studies have found that cells from other tissues may also be the source of CEC-like cells used to treat corneal endothelial diseases. Inagaki *et al*[200] successfully induced corneal endothelial cells from human skin-derived precursors (SKPs) and showed that the transplanted cornea also maintained the transparency and thickness of the cornea. Shen *et al*[201] obtained abundant CEC-like cells through the coculture of human SKPs and B4G12 cells. Similar to human CECs in morphology and characteristics, when CEC-like cells are transplanted into rabbit and monkey models of corneal endothelial dysfunction, they show excellent therapeutic effects. Shao *et al*[202] transformed human foetal bone marrow-derived colorectal progenitor cells (BEPCs) into corneal endothelial cells *in vitro*, which may be useful for repairing corneal endothelial dysfunction. Zhang *et al*[203] induced the differentiation of hESCs into periocular mesenchymal precursors (POMPs). Using lens epithelial cell conditioned medium, CEC-like cells were obtained from POMPs and successfully transplanted into the eyes of a rabbit CE dysfunction model to gradually restore corneal transparency. Chen *et al*[204] used a simpler method to generate HCEC-like cells from hESCs. This method can greatly reduce the production work of HCEC and has potential clinical application value.

CONCLUSION

In this review, we introduced the characteristics of corneal epithelial stem cells, corneal endothelial cell progenitors and corneal stromal stem cells in detail, identified their anatomical features of their location near the limbus of the cornea, discussed a variety of isolation and culture techniques and related molecular markers and summarized their application and potential in treatment (especially the treatment of LSCD). Research on corneal stem cells is of great value for corneal transplantation, regenerative medicine and bioengineered corneal grafts, especially in the era of scarce corneal donors, which will bring good news to patients with corneal diseases worldwide.

FOOTNOTES

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Jiang-Tao Wan, Xian-Shuai Qiu, Zhuo-Hang Fu, Yong-Can Huang, Shao-Xiong Min

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Abstract

BACKGROUND

Cartilage tissue engineering is a promising strategy for treating cartilage damage. Matrix formation by adipose-derived stem cells (ADSCs), which are one type of seed cell used for cartilage tissue engineering, decreases in the late stage of induced chondrogenic differentiation *in vitro*, which seriously limits research on ADSCs and their application.

AIM

To improve the chondrogenic differentiation efficiency of ADSCs *in vitro*, and optimize the existing chondrogenic induction protocol.

METHODS

Tumor necrosis factor-alpha (TNF-α) inhibitor was added to chondrogenic culture medium, and then Western blotting, enzyme linked immunosorbent assay, immunofluorescence and toluidine blue staining were used to detect the cartilage matrix secretion and the expression of key proteins of nuclear factor kappa-B (NF-κB) signaling pathway.

RESULTS

In this study, we found that the levels of TNF-α and matrix metalloproteinase 3 were increased during the chondrogenic differentiation of ADSCs. TNF-α then

bound to its receptor and activated the NF-κB pathway, leading to a decrease in cartilage matrix synthesis and secretion. Blocking TNF-α with its inhibitors etanercept (1 μg/mL) or infliximab (10 μg/mL) significantly restored matrix formation.

CONCLUSION

Therefore, this study developed a combination of ADSC therapy and targeted anti-inflammatory drugs to optimize the chondrogenesis of ADSCs, and this approach could be very beneficial for translating ADSC-based approaches to treat cartilage damage.

Key Words: Adipose-derived mesenchymal stem cells; Human adipose-derived mesenchymal stem cells; Chondrogenic differentiation; Tumor necrosis factor-alpha; Etanercept; Infliximab; Nuclear factor kappa-B

Core Tip: Adipose stem cells are important seed cells that are used in cartilage tissue engineering. However, at present, cartilage matrix secretion by adipose-derived stem cells (ADSCs) inevitably decreases during the late stage of induced chondrogenic differentiation *in vitro*, which seriously limits the further application of ADSCs. Our team found that the level of inflammation in the culture system, mainly the levels of tumor necrosis factor-alpha (TNF-α) and matrix metalloproteinase 3, continuously increased during the chondrogenic differentiation of ADSCs. To address this issue, our team added etanercept or infliximab, which are targeted inhibitors of TNF-α, to the chondrogenic differentiation induction medium and successfully restored matrix formation by human ADSCs in the late stage of chondrogenic differentiation. Further studies found that these effects were achieved by reducing NF-κB pathway activation.

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INTRODUCTION

Adipose-derived stem cells (ADSCs) are mesenchymal stem cells with multidirectional differentiation potential that are isolated from autologous adipose tissue[1]. After years of research and application since the first successful isolation of ADSCs in 2001, they have become one of the most widely used types of adult stem cells in the field of tissue regeneration. In terms of their multidifferentiation ability, a large number of reported studies have shown that these cells can differentiate into a variety of cell types, such as bone cells, cartilage cells, and muscle cells of the motor system; myocardium cells, and vascular endothelial cells of the circulatory system; and nerve cells of the nervous system[2,3]. Compared with bone marrow mesenchymal stem cells, adipose stem cells are abundant in tissue sources, are easy to extract, and exhibit higher proliferation[4]. However, similar to other tissue-derived adult stem cells, the capacity of ADSCs to synthesize and secrete cartilage matrix decreases during chondrogenic differentiation *in vitro*. There are several possible explanations for this phenomenon, including cell senescence, apoptosis[5], autophagy[6], oxidative stress[7], epigenetic inheritance[8], and abnormal cell adhesion[9]. Researchers have also proposed some signaling pathways that may participate in the process mentioned above, such as PI3K/Akt, and TGFβ/Smad3 signaling pathways. However, the specific mechanism remains to be further studied.

Tumor necrosis factor (TNF) is a serum glycoprotein that is produced by activated macrophages and other monocytes in mammals. Its functional unit is a homologous trimer that is composed of three subunits and 157 amino acids[10]. TNF, which is also known as TNF-alpha (TNF-α), exerts necrotizing effects on tumor cell lines and increases tumor transplant rejection. TNF-α is only 30% homologous to TNF-beta (lymphotoxin). Nevertheless, they share the same TNF receptors, namely, TNF-R1 and TNF-R2.11 Many studies have shown that TNF-R1 mediates most of the biological activity of TNF. The combination of these receptors triggers a series of intracellular events that ultimately lead to the activation of two major transcription factors, namely, nuclear factor kappa-B (NF-κB) and C-Jun[11]. Through these transcription factors, TNF-α induces the expression of genes that are essential for various biological processes, including cell growth and death, development, carcinogenesis, immunity, inflammation, and stress responses.

TNF-α inhibitors that are commonly used in clinical practice include etanercept, infliximab, and adalimumab. Etanercept is increasingly used because it is effective and affordable. Etanercept is a dimeric fusion protein that binds to TNF. Etanercept has been widely used in treating cartilage-related diseases, such as osteoarthritis, ankylosing spondylitis, and rheumatoid arthritis. Infliximab is a chimeric monoclonal IgG1 antibody that specifically binds to TNF-α, and it is mainly used in the study of autoimmune diseases, such as Crohn's disease, chronic inflammation and diabetic neuropathy. Both of these drugs inhibit the binding of TNF-α to its receptors on the cell surface, resulting in the biological inactivation of TNF-α, and their application is associated with few adverse reactions. In recent years, with the development of molecular biology, drugs with structures that are similar to etanercept, such as GP2015, LBEC0101, and Chs-0214, have been developed and have passed the assessments of a series of noninferiority studies[12]. In addition, some plant extracts, including saffron extract[13], resveratrol [14], nobiletin[15], *etc*., have also been reported to exert broad anti-inflammatory effects, and their effects are partly dependent on their ability to inhibit TNF-α.

In this study, we found that the expression of TNF-α increased during the early stage of the induced chondrogenic differentiation of hADSCs and resulted in a decrease in cartilage matrix secretion by hADSCs during late stages of chondrogenic differentiation; these effects could be delayed or eliminated by treatment with etanercept and Infliximab, which are TNF-α inhibitors (Figure 1A).

MATERIALS AND METHODS

Cell culture

Human ADSCs (hADSCs) were isolated from human fat tissue using the type I collagenase digestion method. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; CA, United States) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco) at 37 °C and a humidified 5% CO_2 atmosphere. The growth medium was changed every two days; third-generation cells were used for the experiments. The cells were passaged when they reached 90% confluence. Third-generation cells were used for the experiments below.

Chondrogenic differentiation and treatment

Four groups were established as follows: (1) hADSCs grown in growth medium alone (GM group); (2) hADSCs treated with chondrogenic differentiation medium (CH group); (3) hADSCs treated with chondrogenic differentiation medium and 1 μg/mL etanercept (CHE group); and (4) hADSCs treated with chondrogenic differentiation medium and 10 μg/mL infliximab (CH+Inf group).

The chondrogenic differentiation medium was composed of basic high-glucose DMEM (1X), 5% FBS, 1% penicillin/streptomycin, 1% insulin-transferrin-selenium-sodium-pyruvate solution (ITS-A; Gibco), 100 nmol/L dexamethasone (Theremofisher; Massachusetts, USA) 50 μg/mL, 40 mg/mL L-proline (Macklin; Shanghai, China), and 10 ng/mL TGF-β3 (Peprotech; NJ, United States). For the CHE and CH+Inf groups, 1 μg/mL etanercept (MCE, NJ, United States) and 10 μg/mL infliximab (MCE) were added to the chondrogenic differentiation medium to inhibit the bioactivity of TNF-α. For every group, the GM was changed every other day, and all the treatments were applied when the cells reached confluence in the culture dishes.

The cells were harvested and assessed on days 7, 14, 21, and 28. The results presented are mainly those of Western blotting analysis, enzyme linked immunosorbent assay (ELISA), fluorescence imaging, and toluidine blue staining.

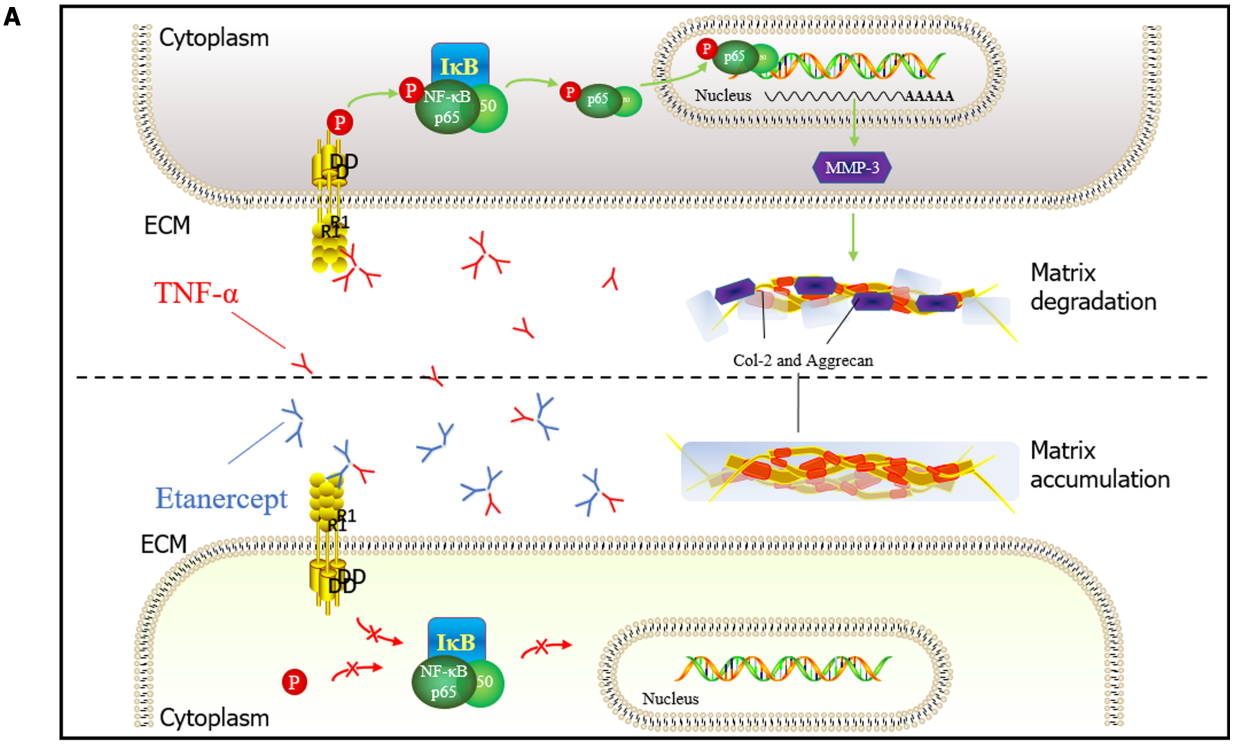
Flow cytometry

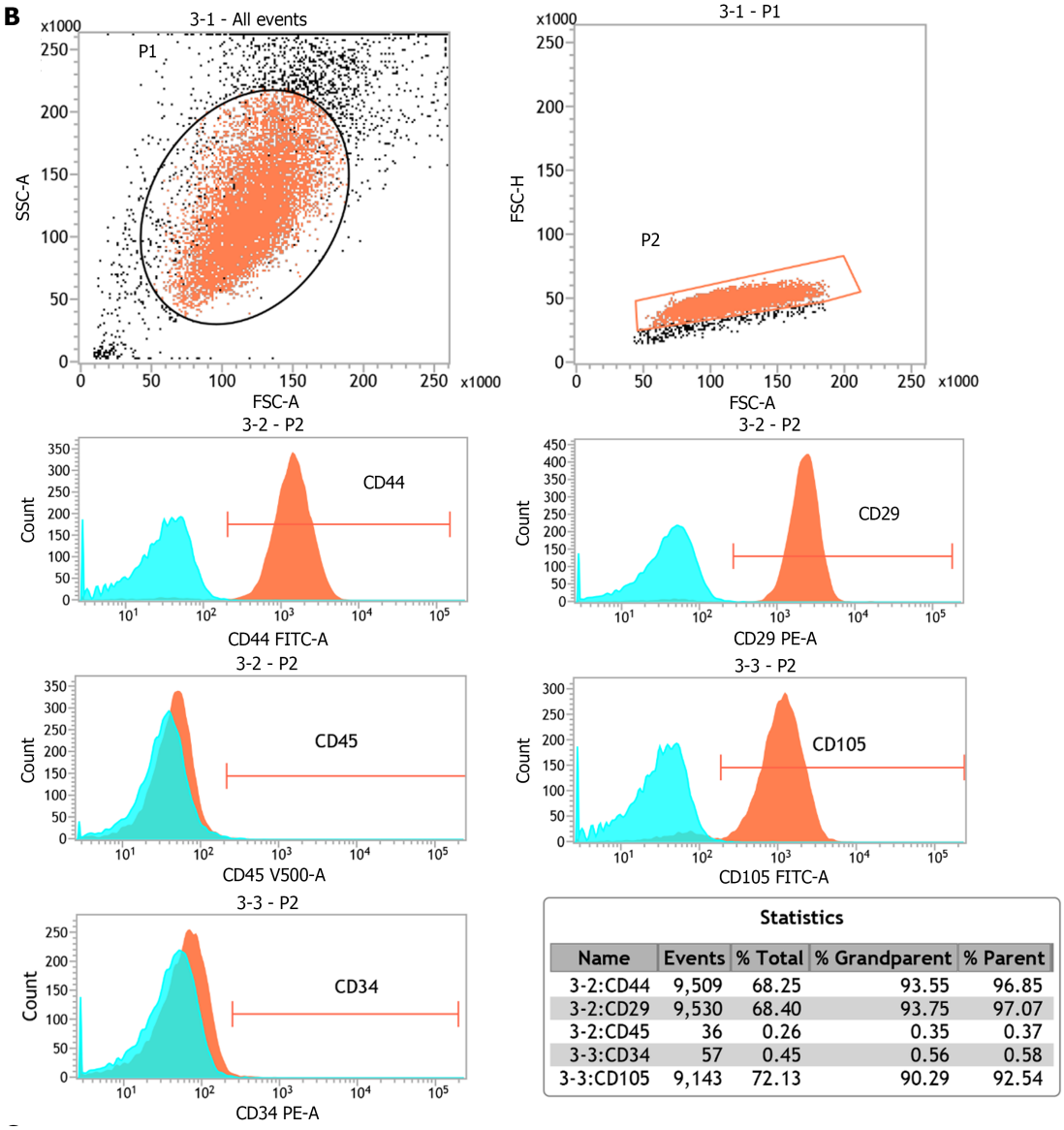
To identify the types of cells used in the experiment, passage 3 hADSCs were removed from the culture dish by digestion with 0.1% trypsin and centrifuged. The cells were suspended (10^6/mL), and the solution was aliquoted into EP tubes after washing twice with phosphate-buffered saline (PBS). Diluted antibodies, namely, FITC-labeled CD29, CD34, CD44, CD45, and CD105 antibodies, were added according to the antibody instructions. The mixtures were incubated at 4 °C for 30 min, and then, the supernatants were discarded after centrifugation. The remaining antibodies that had bound to cell surface molecules were removed by washing with PBS. The cells in each tube were suspended in 400 μL of PBS and placed into the flow tube. The processed cells were stored at 4°C in the dark or analyzed by flow cytometry.

Cell proliferation measurement

Passage 3 hADSCs were harvested and seeded in a 96-well culture plate. For the experimental group, on the second day, the GM was removed and replaced with chondrogenic differentiation medium containing etanercept. For the control group, the hADSCs were cultured in GM. Every 12 h, the CCK8 kit was used to measure the proliferation of the cells in these groups.

A premixed CCK-8 detection solution was added to the samples and incubated for 2 h. Finally, the absorbance of the samples was measured at 450 nm by a spectrophotometer.





Statistics

Name	Events	% Total	% Grandparent	% Parent
3-2:CD44	9,509	68.25	93.55	96.85
3-2:CD29	9,530	68.40	93.75	97.07
3-2:CD45	36	0.26	0.35	0.37
3-3:CD34	57	0.45	0.56	0.58
3-3:CD105	9,143	72.13	90.29	92.54

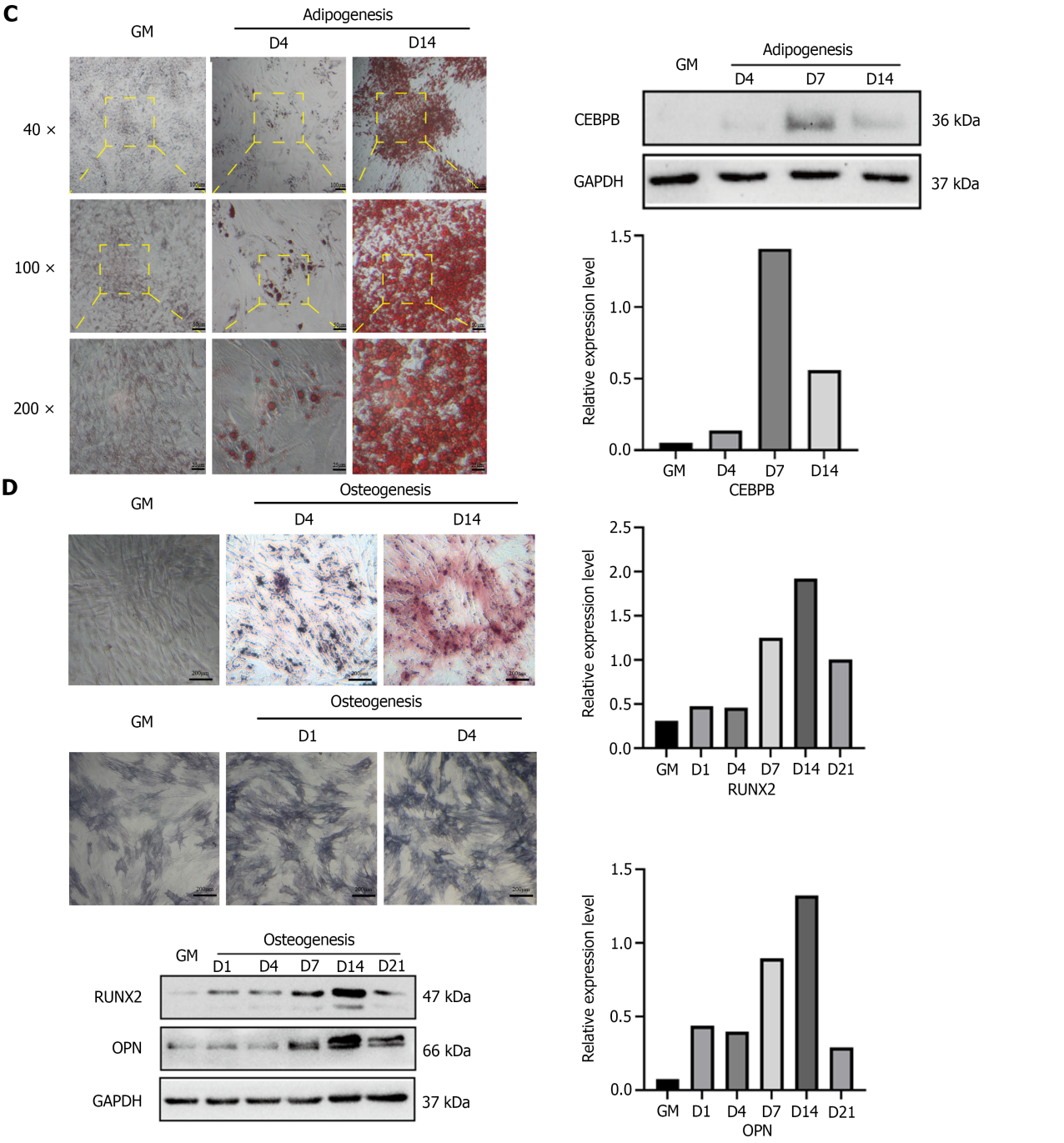


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Figure 1 Human adipose-derived stem cells flow cytometry analysis results and differentiation ability identification. A: Schematic diagram of TNF-α interferes with Human adipose-derived stem cells (hADSCs) chondrogenic differentiation; B: Flow cytometer identification of surface molecules of hADSCs: CD44, CD29, CD45, CD34, and CD105; C: Oil red O staining and Western blot detection of marker proteins performed after lipogenic differentiation induction of hADSCs; D: Alizarin red, alkaline phosphatase staining, and Western blot detection of osteogenic differentiation marker proteins performed after osteogenic differentiation of hADSCs. MMP-3: Matrix metalloproteinase 3; NF-κB: Nuclear factor kappa-B; ECM: Extracellular matrix; GM: Human adipose-derived stem cells grown in growth medium alone.

Scratch test

Cell migration ability was test through scratch test. The tip of a 200 μL pipette was used to create several scratches of the same width in the Petri dishes. The cells in the dishes were cultured with GM and chondrogenic differentiation medium without FBS. The width of the scratches was observed and recorded under a microscope every 12 h.

Western blotting

Collagen type II (Col-2) and Aggrecan (ACAN) expression was measured with the Col-2 antibody

(NB600-844, Novus, US.) and ACAN antibody (DF7561, Affinity, China) to prove chondrogenic differentiation. An anti-TNF-α antibody (AF7014, Affinity, China) was used to measure TNF-α expression. TNF-α functions mainly by activating the NF-κB pathway and upregulating the expression level of matrix metalloproteinase 3 (MMP-3). Therefore, we measured the expression of MMP-3, NF-κB p65, and pNF-κB p65 (phosphorylated NF-κB p65) with the corresponding antibodies (AF0217, AF5006, AF2006, respectively, Affinity, China).

The cells were lysed and the proteins were extracted with a protein extraction kit (KeyGEN BioTECH; Nanjing, China), and the lysis buffer was prepared according to the instructions. A spatula was used to scrape the cells off the bottom of the dish, and the cell fragments were mixed with lysis buffer. The mixtures were placed on ice, and the lysates were incubated for 30 min. The samples were centrifuged at 14,000 rpm at 4 °C for 15 min and boiled at 99.8 °C for 5 min.

The protein samples were loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and the separated proteins were transferred to polyvinylidene fluoride (PVDF) (Millipore, United States) membranes. Nonspecific binding sites were blocked with 5% bovine serum albumin (BSA) dissolved in Tris-buffered saline with Tween 20 (TBST). The membranes were then incubated with the antibodies mentioned above overnight at 4°C. The antibodies were then diluted strictly according to the instructions. The secondary antibody [Goat Anti-rabbit IgG (H+L) HRP, S0001, Affinity, China; Goat Anti-Mouse IgG (H+L) HRP, S0002, Affinity, China] was selected according to the species and origin of the primary antibody; the secondary antibody was added and incubated with the membranes at room temperature for 1 h. Signal detection was performed using the FDbio-Dura ECL kit (fdbio science, Hangzhou, China). ImageJ (software) was used to perform a semiquantitative analysis of the Western blotting bands.

Immunofluorescence microscopy analysis

The cells were seeded on cell slides and fixed with 4% paraformaldehyde for 10 min. The membranes of the cells were disrupted by incubation with 0.1% Triton-X 100 for 5 min. The samples were then blocked with 2% BSA dissolved in PBS for 1 h at room temperature. The samples were stained with the following primary antibodies: anti-MMP-3 antibody (1:200, AF0217) and anti-TNFα antibody (1:200, AF7014). Actin was stained with Phalloidin-iFluor 594 Reagent (ab176757). A goat anti-rabbit IgG/Alexa Fluor 555 antibody (Bioss, Beijing, China) was used as the secondary antibody. After these steps were complete, DAPI (4',6-diamidino-2-phenylindole) was used to label the nuclear DNA.

ELISA

The cell supernatants were collected when the culture medium was changed on days 7, 14, 21, and 28. A proteinase inhibitor was added (1 μg/mL) to the samples, and the samples were stored at -80 °C. The cells were starved for 24 h in medium without FBS before the cell supernatants were collected. The concentrations of TNF-α and MMP-3 in the supernatants were measured using specific ELISA Kits (SEA133 Hu, SEA101Hu, Clone Cloud, Wuhan, China) according to the instructions.

Toluidine blue staining

Toluidine blue (0.1 g) was dissolved in 100 mL of 0.2 mol/L acetate buffer and stirred with magnetic stirrers for 1 h. The solution was then filtered; the pondus hydrogenii (pH) of the final solution was approximately 3.72 to 4.25. The hADSCs were fixed with 4% paraformaldehyde for 15 min. Then, toluidine blue and stain were added to the shaker for 30 min. Finally, cytoplasmic staining was observed under a microscope.

Statistical analysis

All the experiments were repeated more than three times. A one-way analysis of variance (ANOVA) or *t* test (GraphPad Prism 9.0 software, La Jolla, CA, United States) was used to identify significant differences. $P < 0.05$ was considered statistically significant.

RESULTS

The cells that were isolated from adipose tissue exhibited properties of mesenchymal stem cells

Flow cytometry showed that CD44, CD29, and CD105 were highly expressed, while CD34 and CD45 were hardly expressed, on the cell surfaces (Figure 1B), which was consistent with the reported molecular expression pattern of mesenchymal stem cells. The cells were treated with adipogenic induction medium and osteogenic induction medium. The expression of marker proteins of differentiation was measured, and characteristic staining was performed. The results showed that the expression of CCAAT-enhancer-binding protein-beta (CEBPB), which is the transcription factor that is characteristic of adipogenic differentiation, increased after 7 d of treatment with adipogenic induction medium. The cells were also positive for Oil red O staining (Figure 1C). In addition, the expression of Runt-related transcription factor 2 (RUNX2) and osteopontin (OPN), which are markers of osteogenic

differentiation, increased and peaked on day 14 after treatment with osteogenic induction medium; alkaline phosphatase staining and alizarin red staining were also positive (Figure 1D).

A low concentration of etanercept did not reduce the proliferation of hADSCs

In the CCK-8 experiments, hADSCs that were treated with chondrogenic differentiation medium containing 1 μg/mL etanercept showed similar growth curves to those that were treated with GM (Figure 2A). In addition, the migration ability of the hADSCs that were grown in these two culture media was assessed with a scratch experiment. The curve of scratch width with time showed that there was no significant difference in the migration ability of hADSCs grown in GM and CHE medium (Figure 2B). After being cultured for 48 h, the scratches disappeared in both groups.

The expression of cartilage matrix decreased during the late stage of hADSC chondrogenic differentiation

One week after the induction of chondrogenic differentiation, toluidine blue staining showed darker blue staining in the cytoplasm and the surrounding region of the cells (Figure 2D), suggesting the accumulation of cartilage matrix and the differentiation of hADSCs into chondrocytes. Western blotting analysis showed that compared with GM, chondrogenic differentiation medium increased the expression of Col-2 and Aggrecan by hADSCs from weeks 1 to 4, and the expression peaked from days 14 to 21 (Figure 2D). The expression of the chondrogenic marker proteins showed a declining trend on day 28 (Figure 2D).

The expression of TNF-α was increased in the early chondrogenic differentiation of hADSCs, and MMP-3 expression was upregulated in late differentiation

The WB results showed that TNF-α expression was increased in the early stage of chondrogenic differentiation (specifically, it increased by 3.7 times on day 7, $P < 0.05$), and its expression level was not significantly affected by the presence of interleukin (IL)-1β. Compared with the control group, the expression level of MMP-3 was not significantly increased at week 1 of hADSC chondrogenic differentiation, but it was significantly increased at week 4 (it increased by nearly 15 times on day 28, $P < 0.1$) (Figure 3A). The ELISA results were consistent with the Western blotting results. The TNF-α concentration in the cell supernatants increased by 2.4 times on day 7 ($P < 0.1$), and the concentration of MMP-3 in the cell supernatants increased by 10.8 times on day 28 ($P < 0.1$) (Figure 3B).

At 1 and 4 wk of chondrogenic differentiation, the cellular localization of TNF-α and MMP-3 in hADSCs was analyzed, and their concentrations were semiquantitatively measured, using immunofluorescence staining. The results were consistent with the Western blotting results. TNF-α expression was increased in the early stage of chondrogenic induction, while MMP-3 expression was significantly increased in the late stage. The fluorescence of these two proteins was primarily concentrated in the cytoplasmic region, which was identified by the red fluorescence of the microfilaments (Figure 3C).

IL-1β (10 ng/mL) was added to the culture medium, and chondrogenic differentiation of hADSCs was observed. Western blotting analysis showed that treatment with IL-1β alone did not affect the expression of TNF-α or MMP-3 in hADSCs. However, it upregulated the expression of MMP-3 in the presence of chondrogenic differentiation medium. Surprisingly, the expression level of TNF-α was slightly downregulated by chondrogenic differentiation medium containing IL-1β. Toluidine blue staining showed that the staining was lighter in the group treated with IL-1β and chondrogenic differentiation medium than in the group treated with chondrogenic differentiation medium alone on day 7. The staining of hADSCs with IL-1β alone without chondrogenic differentiation medium showed no significant difference from the control group (Figure 3D). As the viability of ADSCs was affected by IL-1β, a large number of ADSCs died after 7 d of treatment, so the long-term effects of IL-1β could not be observed. These research results need to be further explored.

Etanercept and infliximab alleviated the reduced secretion of cartilage matrix by hADSCs during chondrogenic induction

hADSCs treated with chondrogenic differentiation medium containing 1 μg/mL etanercept maintained their ability to secrete cartilage matrix for 28 d (Figure 4A, Supplementary Figure 1A). Toluidine blue staining showed that after 28 d of treatment with etanercept combined with chondrogenic induction medium, the staining intensity of the group treated with etanercept combined with chondrogenic induction medium was higher than that of the group treated with chondrogenic differentiation medium alone, and the intensities of both groups were higher than that of the control group (Figure 4B). ELISA showed that the concentrations of TNF-α and MMP-3 in the cell supernatants decreased after etanercept treatment (Figure 4C).

Toluidine blue staining showed that the cartilage matrix accumulation of CH+Inf group was more than that of CH group and GM group but not as much as CHE group (Figure 5A). hADSCs treated with chondrogenic differentiation medium containing 10 μg/mL infliximab maintained their ability to secret cartilage matrix capacity 28 d (Figure 5B and Supplementary Figure 1B). Western blot showed that the expression of TNF-α and MMP-3 in CH+Inf group was lower than CH group on both Day 14 and Day

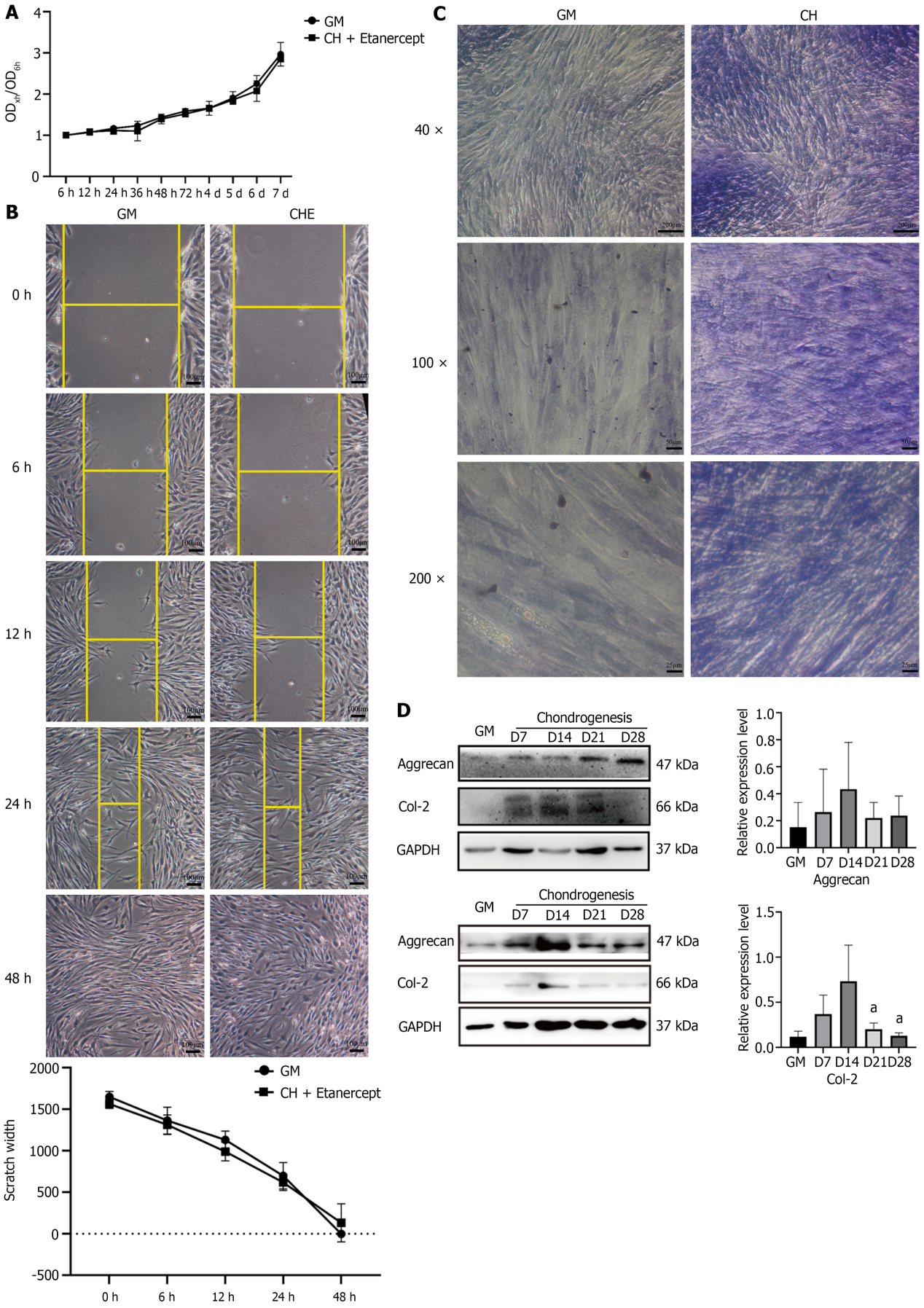

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Figure 2 Etanercept cytotoxicity analysis and human adipose-derived stem cells chondrogenic differentiation ability identification. A: The

growth curves of human adipose-derived stem cells (hADSCs) treated with chondrogenic medium containing etanercept were measured using the CCK-8 method; B: Scratch test performed to determine the migration of hADSCs treated with chondrogenic medium containing etanercept; C and D: Toluidine blue staining and Western blot detection of chondrogenic differentiation marker proteins. $^{a}P < 0.05$. GM: Human adipose-derived stem cells (hADSCs) grown in growth medium alone; CHE: hADSCs treated with chondrogenic differentiation medium and 1 μg/mL etanercept.

28 (Figure 5C).

Etanercept and infliximab inhibited the binding of TNF-α to its receptor and delayed the decrease in the secretion of cartilage matrix by hADSCs via the NF-κB pathway

The levels of total and phosphorylated NF-κB p65 in hADSCs were increased during the induction of chondrogenic differentiation. After treatment with 1 μg/mL etanercept and 10 μg/mL infliximab, the expression level of NF-κB p65 did not significantly change, but the level of phosphorylated NF-κB p65 decreased on day 28 (Figures 4D, 5D and Supplementary Figure 1C). In addition, the expression level of MMP-3 decreased on day 28 in the CHE group.

DISCUSSION

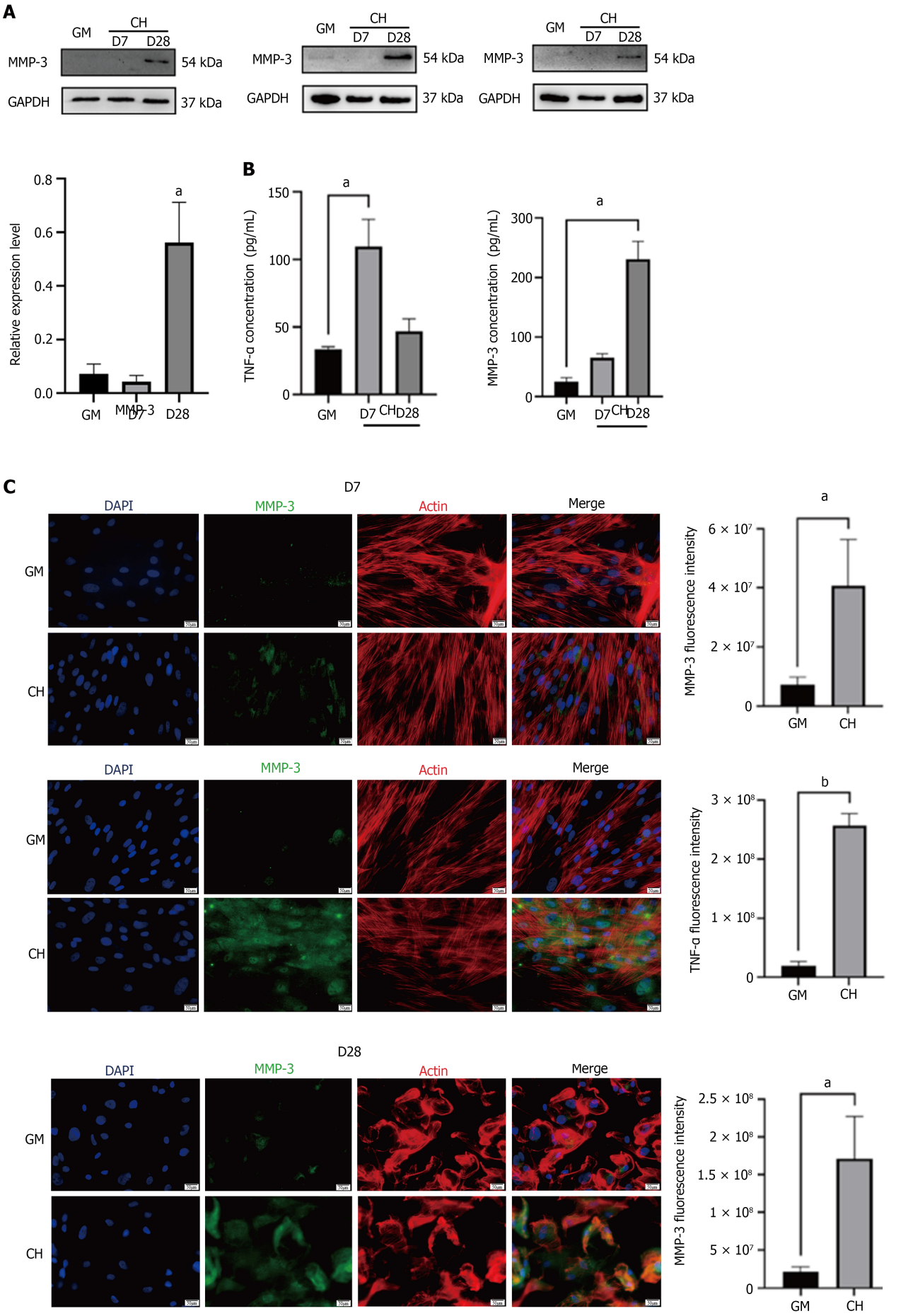
In this study, we explored the possibility that the inflammatory environment caused by the elevated levels of TNF-α in the ADSC culture microenvironment at the early stage of chondrogenic differentiation induction affected the synthesis and secretion of cartilage matrix at the late stage of chondrogenic differentiation. Therefore, we used etanercept to inhibit TNF-α throughout the entire process of chondrogenic differentiation *in vitro* and observed changes in the cartilage matrix synthesis and secretion of ADSCs.

The induction of hADSCs to form cell lines that are capable of continuously secreting cartilage matrix *in vitro* is one approach for treating diseases that involve damaged cartilage. However, the decrease in cartilage matrix secretion in the late stage of chondrogenic differentiation is a major limitation to this research. Researchers have tried to solve this problem in several ways. Examples include: (1) 5-Aza cytidine (5-AZAC) was used to reduce the DNA methylation level[8]; (2) The expression level of cartilage matrix proteins was upregulated using noncoding RNA[16]; (3) A variety of cytokines, such as BMP and TGF-β, were added to culture[17]; and (4) A three-dimensional culture system was generated based on biomaterial scaffolds[18,19].

However, none of these methods could completely solve the problem that the production of cartilage matrix decreases during the late stage of the induced chondrogenic differentiation of stem cells *in vitro*. In this study, during the induced chondrogenic differentiation of hADSCs *in vitro*, the inflammatory factors TNF-α and MMP-3 accumulated in the culture system. Moreover, the ability of the cells to secrete cartilage matrix decreased. Therefore, we conducted a series of studies to determine whether there was a link between these two phenomena.

Long-term cartilage damage, which is characterized by local damage that is caused by the implant surgery, the immune reaction to implants, and the inflammatory factors that are inherent to the implants, can create an inflammatory microenvironment that is not conducive to stem cell therapy[20]. Improving the inflammatory state of the microenvironment can enhance many therapeutic responses, as has been reported in stem cell transplantation therapy, rheumatoid arthritis and psoriasis treatment, and even depression treatment[21]. In a study on hematopoietic stem cell transplantation, the direct binding of donor-derived TNF-α to TNF-R1 impaired the survival and division of transplanted hematopoietic stem cells and progenitor cells[22]. Anti-TNF therapy has also been shown to increase the success of hematopoietic stem cell therapy in treating human adenosine deaminase deficiency. The response to anti-TNF therapy can be considered one of the indications of whether the patient is a candidate HSCT[23]. Under physiological conditions, nearby stem cells are the first to differentiate and repair the damage when the body is injured[24,25]. Therefore, a microenvironment that is similar to that of the target tissue can greatly facilitate stem cell-induced differentiation *in vitro*. In this study, we found that TNF-α in the culture system upregulated the expression of MMP-3, which degraded the extracellular cartilage matrix, resulting in a lack of external support for the induced differentiation of hADSCs.

The NF-κB pathway is the primary signaling pathway that is activated when TNF-α binds to TNFR [11,26]. This pathway is often associated with inflammation by researchers[27] and is widely involved in the homeostatic regulation of the musculoskeletal system[28]. This pathway has been described in many studies to block chondrogenic differentiation[29,30]. In this study, etanercept and infliximab successfully reduced the level of phosphorylated NF-κB p65 and the expression level of MMP-3. This study confirmed that the NF-κB pathway is one of the signaling pathways by which TNF-α blocks the chondrogenic differentiation of hADSCs. It is worth considering whether drugs that inhibit TNF-α can be used to improve the protocol of ADSC chondrogenic induction *in vitro*. It has been reported that some substances, such as hawthorn[31], saffron extract[13] and resveratrol[14], can not only inhibit the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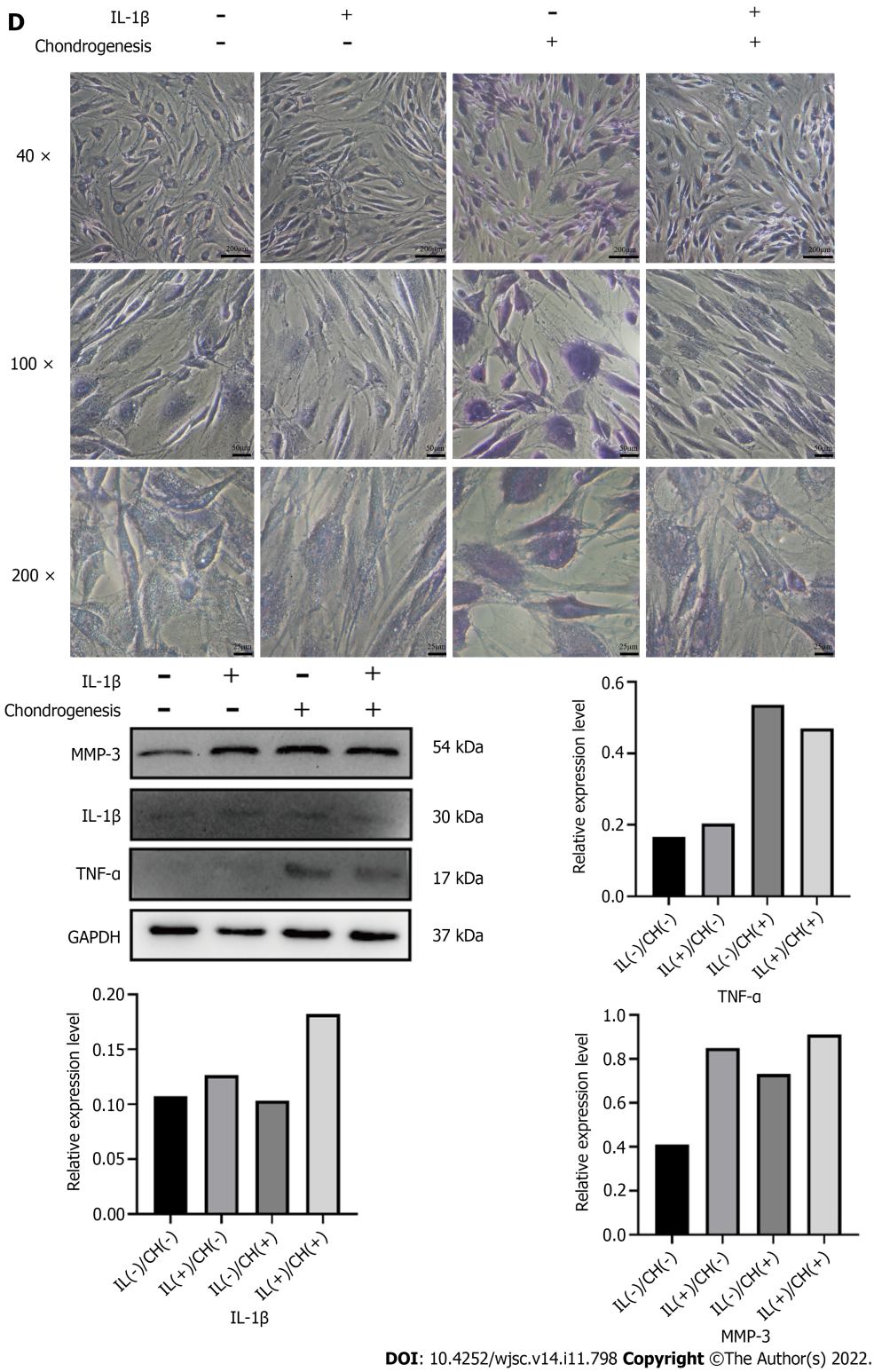

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Figure 3 Expression levels of tumor necrosis factor-alpha and matrix metalloproteinase 3 were detected during chondrogenic differentiation of human adipose-derived stem cells. A: Western blot detection of MMP-3; B: Elisa detection of the concentrations of tumor necrosis factor-alpha (TNF-α) and matrix metalloproteinase 3 (MMP-3) in the cell supernatant; C: Immunofluorescent staining of TNF-α and MMP-3; D: Toluidine blue staining and the Western blot detection of TNF-α and MMP-3 of human adipose-derived stem cells treated with chondrogenic medium containing interleukin-1β. $^{a}P < 0.05$; $^{b}P < 0.01$. TNF-α: Tumor necrosis factor-alpha; MMP-3: Matrix metalloproteinase 3; IL-1β: Interleukin-1β; GM: Human adipose-derived stem cells (hADSCs) grown in growth medium alone; CH: hADSCs treated with chondrogenic differentiation medium.

biological effects of TNF-α but also have antioxidant and antifibrosis abilities. Could these substances be more effective components for improving the chondrogenic induction medium? In addition, our study found that the continuous accumulation of inflammation level may be one of the reasons for the decreased cartilage matrix secretion. Are there other substances besides TNF-α involved in the inflam-

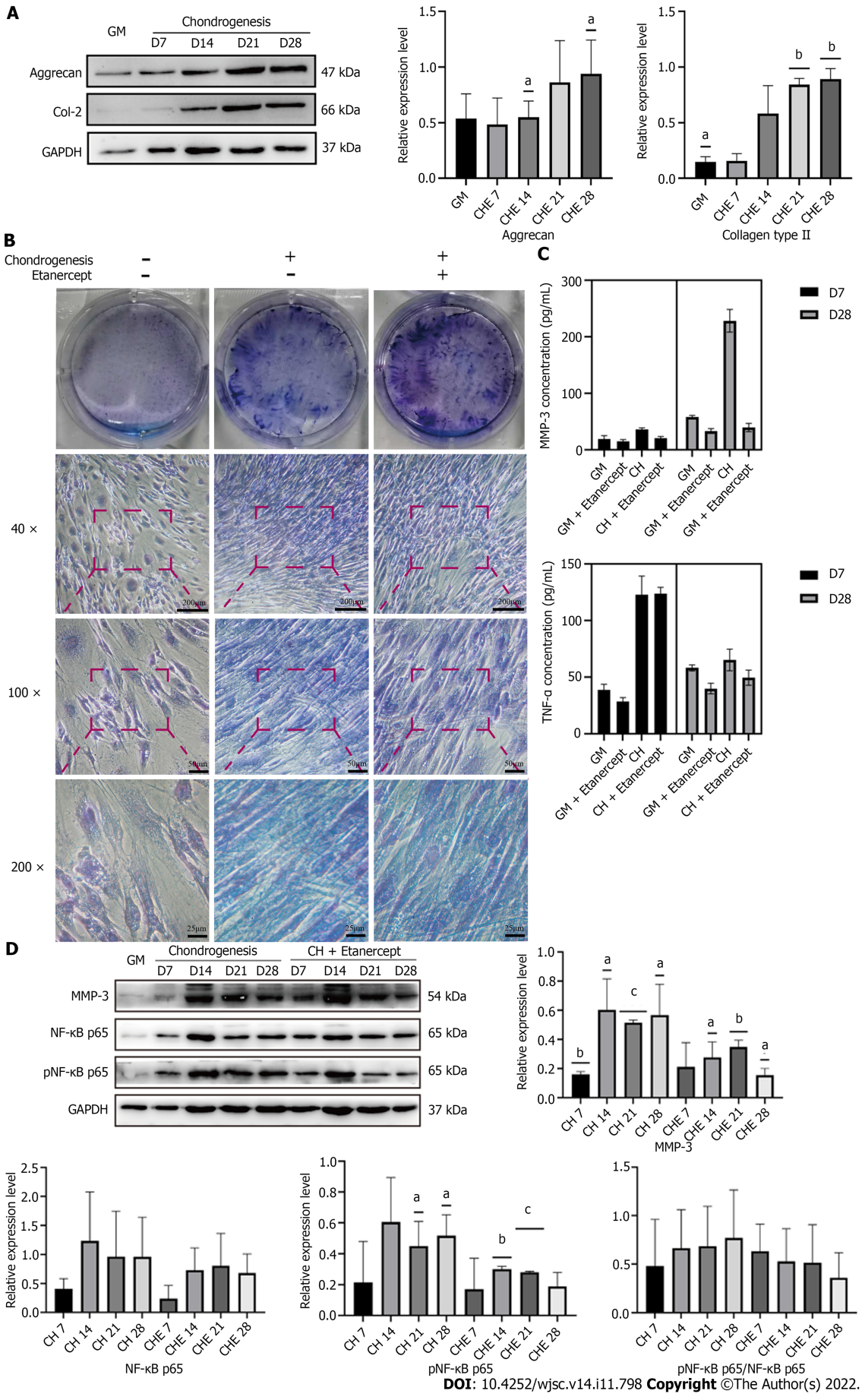

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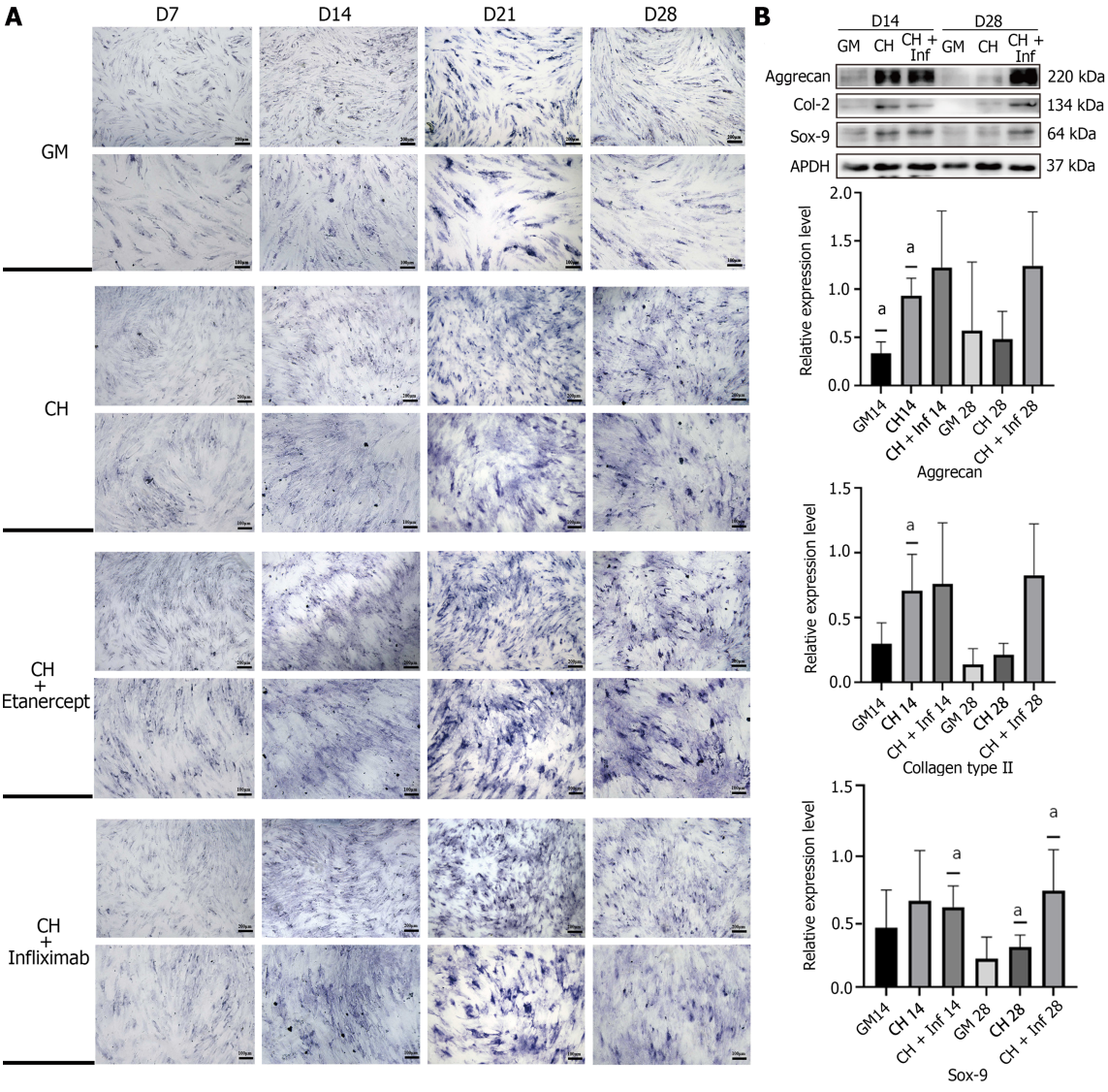
Figure 4 Detection of human adipose-derived stem cells chondrogenic differentiation marker proteins and tumor necrosis factor-alpha

and matrix metalloproteinase 3 performed after treatment with chondrogenic differentiation medium containing etanercept. A: Western blot detection of Col-2 and Aggrecan of adipose-derived stem cells treated with chondrogenic differentiation medium containing etanercept; B: Toluidine blue staining; C: Elisa detection of the tumor necrosis factor-alpha and matrix metalloproteinase 3 (MMP-3) concentration in cell supernatant; D: Western blot detection of MMP-3 and NF-κB pathway. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. MMP-3: Matrix metalloproteinase 3; NF-κB: Nuclear factor kappa-B; GM: Human adipose-derived stem cells (hADSCs) grown in growth medium alone; CH: hADSCs treated with chondrogenic differentiation medium; CHE: hADSCs treated with chondrogenic differentiation medium and 1 μg/mL etanercept.

matory response of the culture system? Can therapies targeting other inflammatory targets, such as TIMPs and NSAIDs, also be used to assist the chondrogenic differentiation of ADSCs *in vitro*? Further research is needed to find out the answers.

CONCLUSION

Through this study, we confirmed that TNF-α increased and inhibits the secretion of cartilage matrix by activating the NF-κB pathway. The use of TNF-A inhibitors, such as etanercept and infliximab, can maintain the cartilage matrix secretion of ADSCs at the late stage of chondrogenic differentiation. We hope to highlight an approach that combines stem cell therapy and targeted anti-inflammatory drugs to treat diseases that involve cartilage damage. The adverse effects associated with *in vitro*-induced chondrogenic differentiation can be eliminated and the therapeutic effect of hADSCs can be maximized with specific targeted drugs. Animal and *in vivo* studies remain to be conducted, which is the future direction of our team.



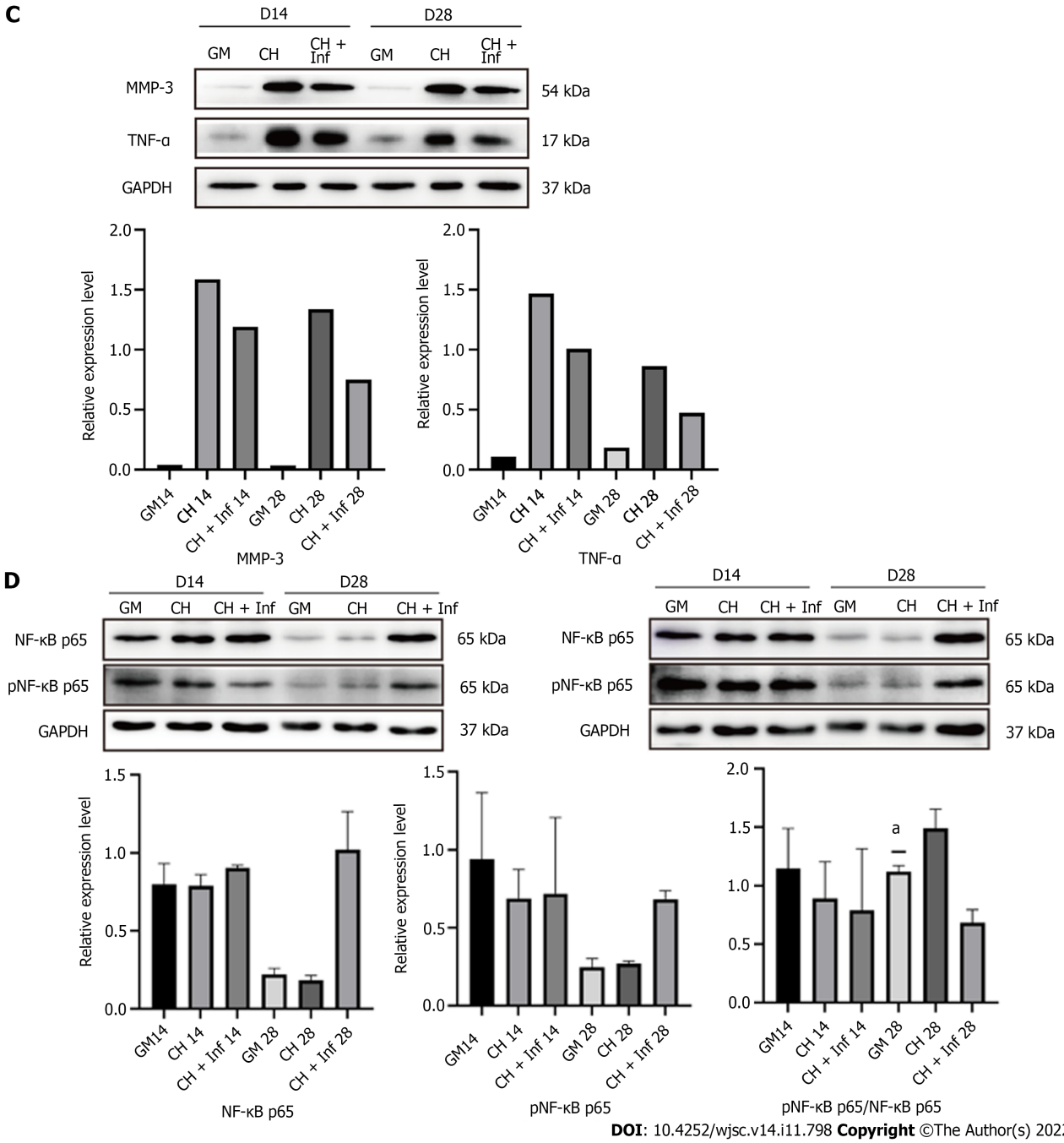


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Figure 5 Detection of human adipose-derived stem cells chondrogenic differentiation marker proteins and tumor necrosis factor-alpha and matrix metalloproteinase 3 performed after treatment with chondrogenic differentiation medium containing Infliximab. A: Toluidine blue staining; B: Western blot detection of Col-2, Aggrecan and Sox-9 of adipose-derived stem cells treated with chondrogenic differentiation medium containing Infliximab; C: Western blot detection of matrix metalloproteinase 3 and tumor necrosis factor-alpha pathway; D: Western blot detection of nuclear factor kappa-B pathway. $^{a}P < 0.05$. TNF-α: Tumor necrosis factor-alpha; MMP-3: Matrix metalloproteinase 3; IL-1β: Interleukin-1β; GM: Human adipose-derived stem cells (hADSCs) grown in growth medium alone; CH: hADSCs treated with chondrogenic differentiation medium; CHE: hADSCs treated with chondrogenic differentiation medium and 1 μg/mL etanercept; CH+Inf: hADSCs treated with chondrogenic differentiation medium and 10 μg/mL infliximab.

ARTICLE HIGHLIGHTS

Research background

During the induced differentiation, the inability of adipose-derived mesenchymal stem cells (ADSCs) to secret cartilage matrix durably has been one of the difficulties in cartilage tissue engineering. Therefore, understanding the changes in the culture system before and after ADSCs differentiation and improving the induction program can help improve the chondrogenic differentiation efficiency of ADSCs.

Research motivation

In the previous study, we conducted single-cell sequencing of ADSCs before and after chondrogenic differentiation and found that the expression levels of Matrix metalloproteinase 3 (MMP-3) and Tumor necrosis factor receptor superfamily member 12A (TNFRSF12A) were significantly increased. Therefore, we hypothesized that the accumulation of inflammatory levels in THE culture system resulted in decreased cartilage matrix secretion at the late stage of differentiation and designed this study for this reason.

Research objectives

To investigate the changes of tumor necrosis factor-α (TNF-α) and MMP-3 Levels in the culture system of ADSCs before and after chondrogenic differentiation. To confirm that TNF-α increased and decreased cartilage matrix secretion of ADSCs by activating the NF-KB pathway. To confirm that adding TNF-α inhibitor to chondrogenic medium could improve the chondrogenic differentiation efficiency of ADSCs.

Research methods

Treat ADSCs with chondrogenic medium containing TNF- α inhibitors, such as etanercept and infliximab. Then observe the changes of cartilage matrix secretion and the level of inflammation in the culture system through western blot, Elisa, immunofluorescence and toluidine blue staining.

Research results

During the differentiation of ADSCs, the expression levels of TNF-α and MMP-3 increased gradually, and the activation of NF-κB signaling pathway increased. Adding TNF-α inhibitors, etanercept (1 μg/mL) or inflixib (10 μg/mL), to the chondrogenic medium can reduce the activation of NF-κB pathway, alleviate the inflammation and preserve the secretion of cartilage matrix of ADSCs.

Research conclusions

When TNF-α increases and binds to its receptor, activates NF-κB pathway and reduces cartilage matrix secretion of ADSCs. TNF-α inhibitors can block the above process and improve the chondrogenic differentiation efficiency of ADSCs *in vitro*.

Research perspectives

In future studies, the TNF-α inhibitor etanercept or infliximab used in this study could be combined with the scaffold material to optimize the growth environment of ADSCs and make the drug release more durable and gentle, so as to achieve higher chondrogenic differentiation efficiency of ADSCs. And obtain a transplantable cartilage engineering material with similar properties to the natural cartilage tissue for the repair and treatment of cartilage defects, eventually.

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FOOTNOTES

Author contributions: Min SX and Wan JT conceived and designed the experiments; Wan JT, Qiu XS, and Fu ZH performed the experiments; Wan JT and Qiu XS analyzed the data; Wan JT and Huang YC composed the manuscript.

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