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Nanoscale Morphologies on the Surface of Substrates/Scaffolds Enhance Chondrogenic Differentiation of Stem Cells: A Systematic Review of the Literature

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Abstract: Nanoscale morphologies on the surface of substrates/scaffolds have gained considerable attention in cartilage tissue engineering for their potential to improve chondrogenic differentiation and cartilage regeneration outcomes by mimicking the topographical and biophysical properties of the extracellular matrix (ECM). To evaluate the influence of nanoscale surface morphologies on chondrogenic differentiation of stem cells and discuss available strategies, we systematically searched evidence according to the PRISMA guidelines on PubMed, Embase, Web of Science, and Cochrane (until April 2024) and registered on the OSF (osf.io/3kvdb). The inclusion criteria were (in vitro) studies reporting the chondrogenic differentiation outcomes of nanoscale morphologies on the surface of substrates/scaffolds. The risk of bias (RoB) was assessed using the JBI-adapted quasi-experimental study assessment tool. Out of 1530 retrieved articles, 14 studies met the inclusion criteria. The evidence suggests that nanoholes, nanogrills, nanoparticles with a diameter of 10–40nm, nanotubes with a diameter of 70–100nm, nanopillars with a height of 127–330nm, and hexagonal nanostructures with a periodicity of 302–733nm on the surface of substrates/scaffolds result in better cell adhesion, growth, and chondrogenic differentiation of stem cells compared to the smooth/unpatterned ones through increasing integrin expression. Large nanoparticles with 300–1200nm diameter promote pre-chondrogenic cellular aggregation. The synergistic effects of the surface nanoscale topography and other environmental physical characteristics, such as matrix stiffness, also play important in the chondrogenic differentiation of stem cells. The RoB was low in 86% (12/14) of studies and high in 14% (2/14). Our study demonstrates that nanomorphologies with specific controlled properties engineered on the surface of substrates/scaffolds enhance stem cells' chondrogenic differentiation, which may benefit cartilage regeneration. However, given the variability in experimental designs and lack of reporting across studies, the results should be interpreted with caution.

Keywords: nanomorphology, surface engineering, chondrogenesis, stem cell, cartilage regeneration

Introduction

Articular cartilage (AC) has limited intrinsic potential for repair due to its avascular and aneural nature and its complex hierarchical structure consisting of superficial, intermediate, and deep zones.[1–4] Currently, at the macro level, tissue engineering technologies have made progress in materials with appropriate surface chemistry, biocompatibility, and plasticity to be applied to osteochondral grafts, biomaterial scaffolds, and tissue biofabrication.[5,6] At the microscopic

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level, functional groups (ie $-CH_3$, $-OH$, $-COOH$, and $-NH_2$) regulate chondrogenic differentiation of stem cells by tuning protein adsorption and then nonspecific cell adhesion and thus cell spreading, and thus they are often modified on the surface in tissue engineering.[7] Surface charges (ie negative electric charge) promote chondrogenic differentiation by mimicking a negatively charged microenvironment in extracellular matrix (ECM), and thus they are often used as a stimulus in tissue engineering.[8] Although they provide chemical factors and electrical stimulation for chondrogenesis, imitating the topographical characteristics of microenvironment in ECM which directs cell adhesion, proliferation, and differentiation for cartilage regeneration is challenging.[6–10] In the past decades, nanostructured surface engineering, including nanoimprint lithography (NIL), electron beam lithography (EBL), microcontact printing (μCP), phase segregation of silanes, polymer demixing, electrospinning, laser etching, anodic oxidation, acid–alkali treatment, sandblasting, etc.,[11,12] has emerged with an ability to produce various types of nanoscale morphologies such as nanopillars, nanoparticles, nanopits, nanogrooves, nanotubes, and nanofibers on the surface of scaffolds/ substrates,[13–15] which direct cell orientation, geometry, and adhesion.[16–18] These technologies have also been used in cartilage tissue engineering for creating surface nanomorphologies to promote cartilage regeneration.[19]

The nanoscale surface morphology created by nanostructured surface engineering well mimics the biological, topographical, and biophysical characteristics of the pristine ECM microenvironment and thus promotes stem cell proliferation, adhesion, gene expression, and chondrogenic differentiation in vitro.[20–26] Nemeth et al[27] used ultraviolet-assisted capillary force lithography to fabricate hydrogel scaffolds with nano-linear groove characterization, which were shown to be effective in enhancing chondrogenic differentiation by gene expression and histological staining analysis. Park et al[28] used μCP to synthesize nanoparticles with different particle sizes and showed that stem cells formed poly spheres at a diameter of 300nm, diffusion occurred at a diameter of 1200nm, and large aggregates formed at a diameter of 750nm. Wu et al[9] found that nanopillars and nanoholes produced by thermal nanoimprinting enhanced mesenchymal stem cells' (MSCs) chondrogenesis and facilitated hyaline cartilage formation. MSCs on the nanogrill surface exhibited delayed chondrogenesis and formed superficial zone/fibrocartilage.

Although studies reporting the effect of nanostructured surface modification on the chondrogenic differentiation of stem cells for cartilage repair have dramatically increased in the last decade, the results of the studies are heterogeneous.[9,27,28] The relationship between the surface nanomorphology of scaffolds/substrates and chondrogenic differentiation of stem cells remains challenging to interpret, owing to the highly variable study designs and the influence of multiple experimental variables on outcome measures. This review study aims to evaluate the effect of nanoscale surface morphology on chondrogenic differentiation of stem cells systematically and summarize the strategies to pave the way of translating those from research to clinical application.

Methods

Systematic Literature Search

A comprehensive systematic review was performed independently following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines (Figure 1).[29] The focused question was "Does the nanomorphology on the surface of scaffolds/substrates promote chondrogenic differentiation of stem cells?" and was conceived according to Participants, Interventions, Control, and Outcomes (PICO) principles, as follows: (P) participant: stem cells; (I) intervention: nanoscale surface modification; (C) control group: scaffolds/substrates without nanoscale modification or polished; (O) outcome: experimental parameters related to chondrogenesis (ie, Immunocytochemistry, SEM characterization, Histological analysis, and RT-PCR analysis).

Study methods followed the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement and were documented in an open science framework (OSF) protocol with a citation: osf.io/3kvdb. (Table S1) To filter studies relevant to the focused question, relevant manuscripts were searched for published up to April 2024 using 4 electronic databases: PubMed, Embase, Cochrane, and Web of Science. We used keywords in combination with "AND" or "OR" (Boolean logic operators): ((substrate OR surface) AND (topography OR morphology) AND (stem AND cell) AND (chondrogenesis OR chondrogenic OR "chondrogenic differentiation" OR "cartilage phenotype")) to identify the relevant literature in these four databases. Three authors (YX, SY, and YS) independently inspected the titles and abstracts of the

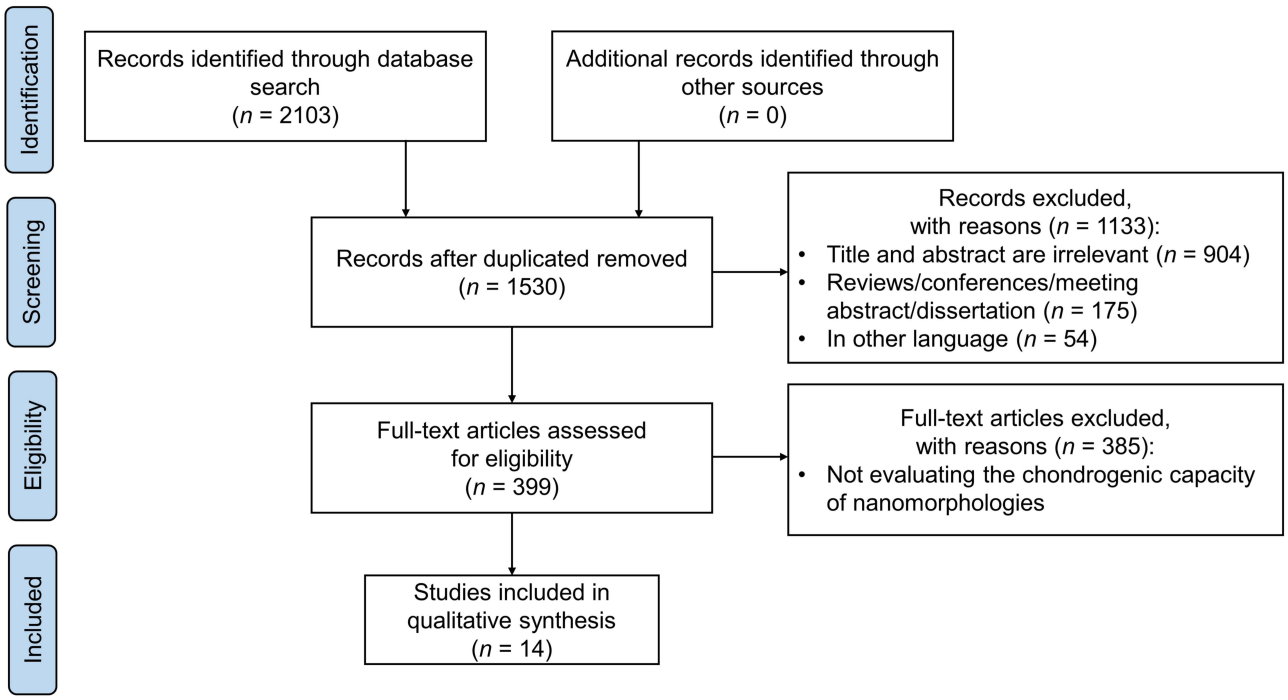

Figure 1 PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) diagram, including study algorithm.

manuscripts following the eligibility criteria. The full text of the eligible manuscripts was then browsed to select studies appropriate for this systematic review. The reference lists of relevant originals and reviews identified in the previous step were further hand-searched. The three authors discussed the selection process until a consensus was reached. The search strategy is shown in Figure 1.

Exclusion and Inclusion Criteria

In vitro peer-reviewed studies evaluating the chondrogenesis outcomes of the nanomorphologies on the surface of scaffolds/substrates were included in this systematic review. Inclusion criteria for studies were studies of surface morphology-modified scaffolds/substrates to promote chondrogenic differentiation of stem cells. The exclusion criteria for all studies were as follows: (1) titles and abstracts are irrelevant (2) articles not written in English; (3) reviews and expert opinion articles, conference proceedings, presentations, dissertations, and theses; and (4) studies that did not evaluate the chondrogenic capacity of surface nanomorphologies (Table S2). The characteristics of the included studies, the interventions, the comparisons, the measurements, and the relevant outcomes were reviewed and discussed.

Risk of Bias of the Studies Included

Assessment of the selected manuscripts' risk of bias (RoB) of the selected manuscript according to the Joanna Briggs Institute-adapted quasi-experimental study assessment tool (JBI Critical Appraisal tool) with a focus on nine signaling questions to describe internal validity and statistical conclusion validity. For questions related to internal validity, these have been further separated into six domains to identify what domain of bias they are referring to, containing bias of temporal precedence, the bias of selection and allocation, bias of confounding factors, the bias of administration of intervention/exposure, the bias of assessment, detection and measurement of the outcome and bias of participant retention. The bias for each selected study was evaluated, where a "yes" judgment indicated a low risk of bias, and a "no" judgment indicated a high risk of bias. The judgment was "unclear" if insufficient details had been reported to assess the risk of bias properly. A high risk bias of the study was considered if at least one item was assessed as "high risk". A low risk of bias was judged in other cases.[30,31] All papers selected for inclusion in the systematic review were

subjected to rigorous appraisal by three critical appraisers (YX, SY, and YS), and disagreements were resolved after consensus-oriented discussions. If disagreement occurred, the senior author (CH) was consulted.

Results

Identification and Selection of Studies

Electronic database searches identified 1532 articles after removing duplicates (Figure 1). After screening titles and abstracts for relevance, 1133 articles were deemed irrelevant based on the inclusion and exclusion criteria. Out of the 399 full texts of the studies assessed for eligibility, 385 articles were excluded for not evaluating the chondrogenic capacity of nanomorphologies. Finally, 14 papers were selected and reviewed after applying the criteria. Due to the scarcity and heterogeneity of the studies, the meta-analysis was not conducted. Figure 2 shows the frequency of publications over the past three decades. There has been a substantial increase in publications over the past two decades, reflecting the growing interest in the studies of the effects of surface nanomorphology on inducing chondrogenic differentiation of stem cells (Figure 2).

In vitro Study Characteristics

The general characteristics of the selected in vitro studies are shown in Table 1. Nine studies[9,32–39] used mesenchymal stem cells, two studies[40,41] used chondrocytes, one study[28] used fetal cartilage-derived progenitor cells (FCPCs), and one study[27] used dental pulp stem cells (DPSCs). Follow-up periods ranged from 1 to 6 weeks. Histological analysis, microscopic characterization (SEM, AFM), RT-PCR analysis, Western-blot analysis, immunohistochemistry, surface wettability characterization, and DNA quantification were performed to assess the outcomes of chondrogenic differentiation comprehensively. The expression of COLII, SOX9, Cx43, and integrin β1 and β4 were analyzed by immunohistochemistry. The histological analysis included cell morphology, proteoglycan synthesis, and cellular and collagen distributions. Cell morphology and distribution were observed by SEM or AFM characterization. The expression of COLII, COLX, SOX9, and aggrecan were detected by RT-PCR analysis. Surface wettability characterization was performed to show the contact angles, and DNA quantification was performed to analyze cell proliferation.

The surface characteristics of nanostructured scaffolds/substrates in the selected studies are presented in Table 2. Three studies used PCL as the material of scaffolds/substrates,[9,37,39] two studies used PMMA (Poly (methyl methacrylate)),[39,41] and two studies used PLLA (Poly (L-lactic acid)) to construct scaffolds/substrates.[36,38] GelMA was used in two studies,[27,35]

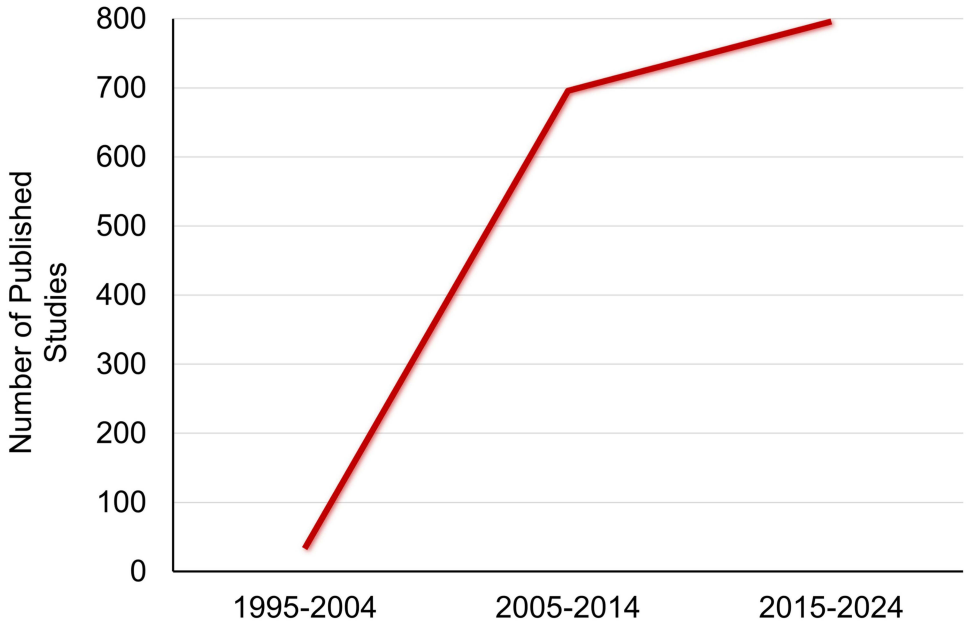

Figure 2 Frequency of studies evaluating the effects of surface nanomorphology on inducing chondrogenic differentiation of stem cells.

Table 1 General Characteristics of the Selected Studies

Authors (year)	Types of Cells	Study Groups	Follow-up	Analysis Method	Outcomes of Chondrogenic Differentiation
Ishmukhametov I et al[32]	hTERT-transduced adipose-derived MSCs (hMSCs)	Group 1: Flat glass substrate Group 2: DNA-functionalized MNPs (3mg/mL) Group 3: DNA-functionalized MNPs (6mg/mL) Group 4: MNPs (3mg/mL) Group 5: MNPs (6mg/mL)	3 weeks	Histological analysis (proteoglycans) AFM characterization	On the seventh day of incubation, Group 2 showed early condensation of cells compared to Group 1. Group 2 had a more pronounced differentiation of cells into chondrocytes compared to Group 1 and Group 3. Under the condition of the same concentration of MNPs, Group 4 and Group 5 had significantly more rough surfaces than Group 2 and Group 3.
D.Dehghan-Baniani et al[33]	Human adipose mesenchymal stem cells (hAMSCs)	Group 1: Bare silk Group 2: Silk-PIII Group 3: Silk-PIII-KGN Group 4: Silk-PIII-Nanopillar Group 5: Silk-PIII-Nanopillar-KGN	3 weeks	Immunofluorescence staining (COL II, SOX9) qRT-PCR analysis (COL2A1, SOX9, aggrecan) SEM characterization AFM characterization (elastic modulus)	Chondrogenic marker genes were significantly expressed in Groups 3 and 5 compared to Group 2. Group 5 indicates the highest cell proliferation. Group 4 had more expression of COL II (green) and SOX9 (red) by hAMSCs than Group 2.
Kim D et al[34]	Mesenchymal cells derived from the distal tips of Hamburger-Hamilton stage 22/23 embryo leg buds of fertilized White Leghorn chicken eggs	Group 1: Flat titanium Group 2: Nanotubes with an inner diameter of 30nm Group 3: Nanotubes with an inner diameter of 50nm Group 4: Nanotubes with an inner diameter of 70nm Group 5: Nanotubes with an inner diameter of 100nm	2–3 weeks	Western-blot analysis (Type I and II collagen, GAPDH, HSP70) Immunocytochemistry (vinculin, integrin β1 and β4) SEM characterization	Groups 4 and 5 showed round cell shape and organizational features of cortical actin-tissue chondrocytes compared to Group 1. Group 3 had stronger cell attachment than groups 4 and 5 and had chondrocyte-specific morphology.

(*Continued*)

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Table 1 (Continued).

Authors (year)	Types of Cells	Study Groups	Follow-up	Analysis Method	Outcomes of Chondrogenic Differentiation
Zhou X et al[35]	Adipose-derived mesenchymal stem cells (ADSCs)	Group 1: RNTK-coated three-layer GelMA-PEGDA gradient scaffold (0mg/L) Group 2: RNTK-coated three-layer GelMA-PEGDA gradient scaffold (0.005mg/L) Group 3: RNTK-coated three-layer GelMA-PEGDA gradient scaffold (0.01mg/L) Group 4: RNTK-coated three-layer GelMA-PEGDA gradient scaffold (0.05mg/L) Group 5: RNTK-coated three-layer GelMA-PEGDA gradient scaffold (0.1mg/L) Group 6: Three-layer GelMA-PEGDA gradient scaffold Group 7: RNTK-coated three-layer GelMA-PEGDA gradient scaffold	3 weeks	Histological analysis (cytoskeleton, cell nuclei) RT-PCR (Collagen IIα1, SOX 9, Aggrecan)	Cell proliferation was significantly greater in Group 5 and Group 6 than in Group 1, Group 2, and Group 3. Collagen II, GAG, and total collagen production were significantly increased in Group 7 compared to Group 6. Histochemical analysis showed that in the second and third weeks, the blue color in Group 7 was deeper than in Group 6. Group 7 had higher expression profiles of chondrogenesis-associated genes than Group 6.
Park IS et al[28]	Fetal cartilage-derived progenitor cells (FCPCs)	Group 1: Control group (coverslip) Group 2: Nanoparticle diameter of silicon dioxide nanopatterned substrates (Diameter of 300nm) Group 3: Nanoparticle diameter of silicon dioxide nanopatterned substrates (Diameter of 750nm) Group 4: Nanoparticle diameter of silicon dioxide nanopatterned substrates (Diameter of 1200nm)	1 week	SEM characterization Immunocytochemistry (Integrin β1, N-cadherin) Histological analysis (cartilage regeneration ability) Centrifuge adhesion analysis	Group 2 formed multi-spheroids, Group 4 showed spreading and Group 3 formed the mass-aggregation in the value-added differentiation of FCPCs on the substrates. Group 3 forms a large self-aggregating body that is most favorable for promoting chondrogenic differentiation. Group 4 had the highest expression of Integrin β1, and Group 2 had the highest expression of N-cadherin.

Childs A et al[36]	Human mesenchymal stem cells (hMSCs)	Group 1: Glass reference Group 2: PLLA scaffold (control) Group 3: PLLA+TBL scaffold Group 4: PLLA+1:9 TB-RGDSK: TBL scaffold Group 5: PLLA+TB-RGDSK scaffold	4 weeks	Microscopic characterization (SEM, AFM, TEM) Immunohistochemistry (type II collagen) Histological analysis (cellular and collagen distributions)	Group 5 maximized hMSC adhesion compared to the other groups. Groups 3, 4, and 5 significantly enhanced hMSC proliferation compared to Group 2. Group 5 had the highest expression level of GAG, collagen, and total protein. Compared to Group 2, Group 5 had significantly increased matrix production in the first two weeks. Group 5 had significant type II collagen production after three weeks.
Niepel MS et al[42]	Human adipose-derived stem cells (hADSCs)	Group 1: Flat polyelectrolyte multilayer systems (F-PEM) Group 2: Hexagonally arranged nanostructures with a periodicity of 302nm and a height of 63.1nm (small, S) Group 3: Hexagonally arranged nanostructures with a periodicity of 518nm and a height of 67.3nm (medium, M) Group 4: Hexagonally arranged nanostructures with a periodicity of 733nm and a height of 55.3nm (large, L)	4 weeks	Immunocytochemistry (cell adhesion, cell differentiation) Microscopic characterization (SEM, AFM) Surface wettability characterization (water contact angles)	No cells and hence no chondrogenic differentiation were observed in Group 1. Group 2 formed the smallest clusters, while Group 4 formed the largest clusters, suggesting that cells on harder, rougher surfaces have a lower propensity for chondrogenic differentiation.
Prittinen J et al[40]	Bovine primary chondrocytes	Group 1: Smooth polypropylene Group 2: Smooth polystyrene Group 3: Patterned polypropylene Group 4: Patterned polystyrene	2 weeks	Immunocytochemistry (types I and II collagen, actin, vinculin) SEM characterization (cell morphology, distribution) RT-PCR (Sox9, aggrecan, procollagen α1(II), procollagen α1(X), procollagen α2(I))	In contrast to Group 2 and Group 3, Group 1 surface appeared to lack adhered chondrocytes. The fastest filling of the culture surface occurred in Group 2. The cell proliferation was significantly higher in Group 2 in comparison to Group 1. Group 3 and Group 4 did not differ from each other, but Group 3 had a significantly higher number of cells than Group 1. Group 3 had better three-dimensional surroundings for chondrocytes than Group 2.

(*Continued*)

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Table 1 (Continued).

Authors (year)	Types of Cells	Study Groups	Follow-up	Analysis Method	Outcomes of Chondrogenic Differentiation
Nemeth CL et al[27]	Dental pulp stem cells (DPSCs)	Group 1: Tissue culture polystyrene (TCPS) Group 2: PEG-GelMA patterned scaffolds Group 3: PEG-GelMA-HA unpatterned scaffolds Group 4: PEG-GelMA-HA patterned scaffolds	3 weeks	SEM characterization RT-PCR (Sox9, Alkaline phosphatase, Aggrecan, Procollagen type II, Procollagen type X, Twist, Snail, Slug) Immunocytochemistry (collagen type II)	Group 4 exhibited a much higher frequency of DPSCs alignment than Group 3. Group 4 expressed the highest level of chondrogenic genes and generated the largest number of 3D spheroids. Group 4 had more efficient differentiation than Group 2 and Group 3.
Wu Y et al[9]	Mesenchymal stem cells (MSCs)	Group 1: Non-pattern Group 2: Nano-pillar Group 3: Nano-hole Group 4: Nano-grill	6 weeks	SEM characterization AFM characterization (cell elasticity and substrate compressive stiffness) DNA quantification (cell proliferation) Histological analysis (F-actin) immunohistochemistry (Type I and II collagen) RT-PCR (GAPDH, aggrecan, collagen I, collagen II, collagen X)	MSCs induced by Group 2 were round and had filipodial extrusion, those induced by Group 3 were polygonal morphology, and those induced by Group 4 and Group 1 were similar in morphology and were spindle shape. MSCs in Group 2 expressed the highest amount of type II collagen, Group 3 expressed type II collagen at three times the level of Group 1, and Group 4 expressed similar levels to those in Group 1. Compared to other groups, Group 4 had the highest type I collagen deposition. The highest expression of PRG4 was found in Group 4. Collagen X mRNA expression was significantly increased in Group 2 and Group 3, and the expression of Group 3 was higher than that of Group 2

Wu Y et al[37]	Mesenchymal stem cell (MSCs)	Group 1: Nano-pillar-soft (PCL) Group 2: Nano-grating-soft (PCL) Group 3: Nano-pillar-medium (PLA) Group 4: Nano-grating-medium (PLA) Group 5: Nano-pillar-stiff (PGA) Group 6: Nano-grating-stiff (PGA)	6 weeks	SEM characterization AFM characterization (substrate compressive stiffness) Histological analysis (F-actin) RT-PCR (GAPDH, aggrecan, collagen I, collagen II and collagen X)	MSCs in Groups 2, 4, and 6 adopted fibroblastic morphology with extensive stress fibers, with the formation of aligned cells. Morphology of MSCs on nano-pillars, however, was more sensitive to the substratum stiffness, adopting round morphology on the softer materials (Group 1) and subsequent cell aggregation, while forming polygonal morphology on the stiff material (Group 5), with increased stress fiber length. Group 5 formed transitional osteochondral cartilage, while had fibro and hypertrophic cartilage characteristics. Group 2 formed fibro/superficial-like cartilage and Group 6 formed non-cartilaginous tissue.
		Group 7: Nano-grating (PCL, PLA, PGA) Group 8: Nano-pillar (PCL, PLA, PGA)	1 week	DNA quantification (cell proliferation)	Group 7 had a higher cell proliferation rate than Group 8 and proliferation rate remained similar across all three polymers of the same topography.

(*Continued*)

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Table 1 (Continued).

Authors (year)	Types of Cells	Study Groups	Follow-up	Analysis Method	Outcomes of Chondrogenic Differentiation
Rodriguez-Pereira C et al[38]	Osteoarthritic bone marrow-mesenchymal stromal cells (OA-BM-MSCs) and Healthy bone marrow-mesenchymal stromal cells (H-BM-MSCs)	Group 1: Fibronectin-coated PLLA Healthy Group 2: RGD-Cys-D1 PLLA nanopatterned substrates (10^-2) Healthy Group 3: RGD-Cys-D1 PLLA nanopatterned substrates (2.5×10^-8) Healthy Group 4: RGD-Cys-D1 PLLA nanopatterned substrates (10^-8) Healthy Group 5: RGD-Cys-D1 PLLA nanopatterned substrates (4×10^-9) Healthy Group 6: Untreated PLLA Healthy Group 7: Fibronectin-coated PLLA OA Group 8: RGD-Cys-D1 PLLA nanopatterned substrates (10^-2) OA Group 9: RGD-Cys-D1 PLLA nanopatterned substrates (2.5×10^-8) OA Group 10: RGD-Cys-D1 PLLA nanopatterned substrates (10^-8) OA Group 11: RGD-Cys-D1 PLLA nanopatterned substrates (4×10^-9) OA Group 12: Untreated PLLA OA (Healthy, Healthy bone marrow-mesenchymal stromal cells; OA, Osteoarthritic bone marrow-mesenchymal stromal cells)	Less than 1 week	Immunofluorescence staining (collagen type-II, Cx43) Histology analysis (cell morphology, proteoglycan synthesis) Molecular Analysis (CX43, COL2A1, SOX9, COL1A1, TNC, RPL13A)	Group 12 induced a higher degree of cell aggregation than Group 6. Early chondrogenic markers gene expression was significantly upregulated in Group 3 compared to Group 1 and Group 6, the same is true for Group 8 compared to Group 7 and Group 12. Cell aggregation and chondrogenic differentiation were best in Group 3 and Group 9, and were more pronounced in Group 9 than in Group 3.

Khattak M et al[39]	Human mesenchymal stem cells (hMSCs)	Group 1: PCL Group 2: PCL: PMMA = 75: 25 Group 3: PCL: PMMA = 50: 50 Group 4: PCL: PMMA = 25: 75 Group 5: PMMA Group 6: Tissue cultured polystyrene (TCPS)	4 weeks	AFM characterization Immunofluorescence staining (adiponectin, collagen II, osteocalcin)	Cell counts showed a decline only in Group 3. Only Group 4 had the potential to differentiate towards chondrogenesis. Group 4 had lower numbers of cells than Group 6.
Huethorst E et al[41]	Chondrocytes	Group 1: Flat Group 2: Nanopillar-height-62nm Group 3: Nanopillar-height-77nm Group 4: Nanopillar-height -127nm Group 5: Nanopillar-height -190nm	4 weeks	Immunofluorescence staining (actin, nucleus of cell) qRNA (SOX 9, COL2A/ COL1A ratio, ACAN, COL10A1) SEM characterization	Compared to Group 1, Group 4 significantly enhanced matrix deposition, chondrogenic gene expression, and chondrogenic maintenance.

Abbreviations: MNP: Magnetic iron oxide nanoparticle; PIII: Plasma immersion ion implantation technique; KGN: Kartogenin, a biomolecule can promote chondrogenesis of hAMSCs; GelMA: Gelatin methacrylate; PEGDA: Poly (ethylene glycol diacrylate); RNTK: Lysine functionalized rosette nanotube; PLLA: Poly (L-lactic acid); TBL: Aminobutane linker molecule; TB-RGDSK: Twin guanine/cytosine DNA base hybrids-based rosette nanotubes functionalized with arginine–glycine–aspartic acid–serine–lysine integrin binding peptide; RGD, arginine–glycine–aspartate; PCL: Polycaprolactone; PLA: Polylactide; PGA: Polyglycolide; GAG: Glycosaminoglycan; PMMA: Poly (methyl methacrylate).

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Table 2 Implant-Related Characteristics of the Selected Studies

Authors (year)	Material of Scaffolds/ substrates	Substrate Stiffness	Surface Manufacturing	Surface Nanopatterns	Nanopattern Dimensions D(Ø) × L (nm)	In Vitro Chondrogenic Activity	Mechanisms
Ishmukhametov I et al[32]	Glass	N/A	Chemical co-precipitation method	Nanoparticles	AFM: Ø 14–40 TEM: Ø 10	MNPs@DNA coatings induced chondrogenesis and osteogenesis in hTERT-transduced MSCs.	N/A
D. Dehghan-Baniani et al[33]	Bombyx mori silk	Dynamic storage shear modulus of Silk-PIII-Nanopillar-KGN: 10.2 ± 0.4kPa; Compressive elastic modulus of Silk-PIII-Nanopillar-KGN: 38.8 ± 1.5Pa.	Colloidal lithography, Plasma etching	Nanopillars	Height: 330 ± 35 Center-to-center: 260 ± 30	While the nanofeatures positively affect the chondrogenesis of hAMSCs, immobilization of a small biomolecule (KGN) on the nanopillars additionally enhances cartilage regeneration.	This implantable smart platform which mimics the SZ of articular cartilage not only delivers topographical cues, but also releases biomolecules to sequentially recruit, organize, and differentiate hAMSCs to chondrocytes.
Kim D et al[34]	Ti	N/A	Anodization	Nanotubes	Group 2: Ø 30 Group 3: Ø 50 Group 4: Ø 70 Group 5: Ø 100	A titanium-based nanotube surface can support chondrocytic functions among chondroprogenitors.	Nanotubes enhance chondrogenic differentiation of chondrogenic cells into chondrocytes by down-regulating Erk signaling.
Zhou X et al[35]	GelMA–PEGDA (gelatin methacrylate and poly (ethylene glycol) diacrylate)	N/A	Self-assembly	Nanotubes	Ø 3–4	RNTKs can promote the chondrogenic differentiation of ADSCs.	N/A
Park IS et al[28]	Silicone	N/A	μ-contact printing	Nanoparticles	Group 2: Ø 300 Group 3: Ø 750 Group 4: Ø 1200	FCPCs aggregation using Nano-patterned substrate of tissue-engineered constructs has a beneficial effect on 3D culture for cartilage regeneration.	Nanoparticles contract through cell-substrate forces followed by cell-cell contact forces to form cell monolayers and thus self-aggregations.
Childs A et al[36]	PLLA, TBL, Glass	N/A	Self-assembly	Nanotubes	Ø 4.6 ± 0.1	Increased cell adhesion, proliferation, and differentiation were observed in all RNTs poly (L-lactic acid) scaffolds, with the TB-RGDSK scaffolds having the best performance.	Novel RNTs may improve chondrogenic differentiation of hMSCs through their bionic nanoscale morphology, increased surface roughness, and stem cell-favorable surface chemistry.
Niepel MS et al[27]	Silicon	Elastic modulus of the native PLL/HA multilayers: 6.6kPa; Cross-linking with different EDC concentrations: 20.9kPa	Laser interference lithography and the layer-by-layer technique	Hexagonally arranged nanostructures	Group 2: 63.1 (height) × 302 (periodicity) Group 3: 67.3 (height) × 518 (periodicity) Group 4: 55.3 (height) × 733 (periodicity)	hADSC differentiation into chondrogenic and osteogenic lineages is superior to adipogenic lineages on nanostructures modified with multilayers.	The multilayered cross-linked nanostructures form cell-cell contacts by upregulating N-calmodulin, and subsequently intracellular signaling begins to shift from progenitor cells to fully committed chondrocytes.
Prittinen J et al[40]	Polypropylene or Polystyrene	N/A	Anodization	Nano-sized bumps (random)	N/A	The bovine primary chondrocytes could be grown on patterned PS and PP surfaces, and they produced an extracellular matrix network around the adhered cells. However, neither the patterned PS nor PP could prevent the dedifferentiation of chondrocytes.	N/A

Nemeth CL et al[27]	PEG-GelMA-HA	N/A	Ultraviolet-assisted capillary force lithography	Nano-linear grooves	Group 2 and Group 4: 800 (ridge) × 800 (width) × 500 (height)	Nanotopography and HA provide important cues for promoting the chondrogenic differentiation of DPSCs.	N/A
Wu Y et al[9]	PCL	N/A	Thermal nanoimprinting	Nanopillars, Nanogrills, Nanoholes	Group 2: Nanopillars: 250 (diameter) × 500 (pitch) × 250 (height) Group 3: Nanoholes: 225 (diameter) × 400 (pitch) × 300 (depth) Group 4: Nanogrills: 250 (line) × 250 (space) × 150 (height)	Compared to non-patterned PCL surface, nano-pillar and nano-hole topography enhanced MSC chondrogenesis and facilitated hyaline cartilage formation. MSCs experienced delayed chondrogenesis on nano-grill topography and were induced to fibro/superficial zone cartilage formation.	Specific nanopatterns can facilitate the initiation of chondrogenic differentiation of MSCs by making them acquire stiffer membranes upon interaction with them.
Wu Y et al[37]	PCL, PLA, PGA	The compression modulus of nonpatterned PGA: 204mPa; The compression modulus of nonpatterned PLA: 128mPa; The compression modulus of nonpatterned PCL: 62mPa.	Thermal nanoimprinting	Nanopillars, Nanogratings	Nanogratings: 250 (line) × 250 (space) × 150 (height) Nanopillars: 225 (diameter) × 400 (pitch) × 300 (height)	MSC morphology and aggregation were highly sensitive to both the substratum stiffness and topographical cues, and has the potential to influence the early differentiation direction of MSCs towards the specific cartilage phenotype.	N/A
Rodriguez-Pereira C et al[38]	PLLA	N/A	Dendrimer nanopatterning	N/A	N/A	RGD-Cys-D1 PLLA nanopatterned substrates supported the formation of pre-chondrogenic condensates from OA- and H-derived MSCs. OA-derived MSCs cultured in nanopatterned substrates formed bigger and more compact aggregates.	N/A
Khattak M et al[39]	PCL, PMMA	N/A	Polymer demixing	Nanoislands, Nanopits (random)	Group 2: Nanopits 200 (depth) × 400–700 (diameter) Group 3: Combinations of Nanopits and Nanoislands Group 4: Nanoislands 200 (height)	The substrate with nanoisland topography has the potential to differentiate into osteogenic, chondrogenic, and adipogenic lineages.	N/A
Huethorst E et al[41]	PMMA	Shear rigidity: 9, 16, 70, 87, 93kPa	Electron beam lithography	Nanopillars	Group 2: Ø 100×62 (height) × 300 (pitch) Group 3: Ø 100×77 (height) × 300 (pitch) Group 4: Ø 100×127 (height) × 300 (pitch) Group 5: Ø 100×190 (height) × 300 (pitch)	Group 4 significantly improved the maintenance of chondrogenesis in primary isolated chondrocytes compared to Group 1.	N/A

Abbreviations: TiO2: Titanium dioxide; G^C: Guanine/Cytosine DNA base hybrids; PLL: Poly-L-lysine; HA: Hyaluronic acid.

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polypropylene or polystyrene was used in one study,[40] and glass was used in two studies.[32,36] One study used Bombyx mori silk, Ti, silicone, and silicon to construct scaffolds/substrates.[28,33,34,42] The shear modulus (G) of scaffolds/substrates included ranging from 9 to 93kPa, and elastic modulus (E) varies from 38.8 ± 1.5Pa to 20.9kPa. The compression modulus of nonpatterned PGA, PLA, and PCL is 204, 128, and 62kPa, respectively.[37] Lithography technology was utilized to develop the nanopatterns in four studies,[27,33,40,41] thermal nanoimprinting was used in two studies,[9,37] anodization was utilized in two studies,[34,40] self-assembly was used in three studies.[35,36,38] Chemical co-precipitation method,[32] μ-contact printing,[28] and polymer demixing[39] were used separately in other studies. Two studies[28,32] constructed nanoparticles with a diameter ranging from 10 to 1200nm on the surface of the scaffolds. Four studies[9,33,37,41] created nanopillars with a height ranging from 62 to 330 nm and a diameter from 100 to 250nm on the surface. Three studies[34–36] developed nanotubes with a diameter ranging from 3 to 100nm on the surface of scaffolds; nanogrills were reported in two studies.[9,37] The remaining five studies respectively reported hexagonally arranged nanostructures,[42] nano-sized bumps,[40] nano-linear grooves,[27] nanoislands,[39] nanopits,[39] and irregular nanopattern.[38]

Risk of Bias of the Studies Included

The studies included were critically appraised using the Joanna Briggs Institute (JBI) checklist for quasi-experimental studies[30] (Figure 3). A higher total score on the JBI checklist for quasi-experimental studies indicates a lower RoB. A score of one was assigned to a "Yes" response for other questions and a "No" for Question 4, while a score of zero was given for "No" and "Unclear" responses each for other questions and a "Yes" for Question 4. Eleven studies had an overall score of 8, representing a low risk of bias. The reviewers were in complete agreement on 13 of the 14 studies selected, 89% agreement on 1 of the 14 articles included. Disagreements arose regarding JBI's item 2 in one study,

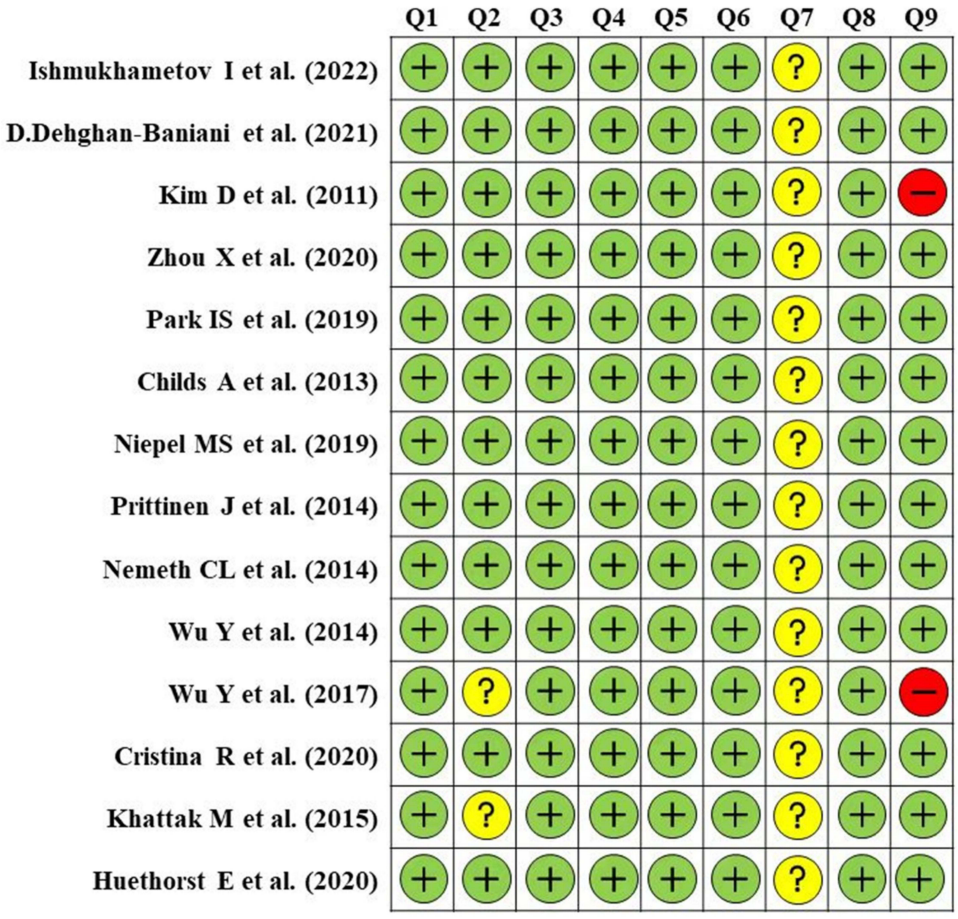

Figure 3 Risk of bias of the included studies based on the Joanna Briggs Institute (JBI) checklist for quasi-experimental studies.

resolved by a fourth reviewer (CH). The included studies presented heterogeneous levels of risk of bias, and the overall score ranged between 6 and 8. Of the 14 studies analyzed, 79% of the accepted studies scored 8, 14% of the accepted studies scored 7, and 7% scored 6. Two studies were considered as high risk of bias because the criterion "Was appropriate statistical analysis used?" was assessed as a high risk of bias. The risk of bias was low in 86% of studies and high in 14%. With regard to the criterion "Were outcomes measured reliable?" where none of the studies had a low risk of bias. The reliability of the measurements used, such as the number of raters, the training of raters, and the reliability of the intra-rater and the inter-rater within the study, were unclear in those studies. Two studies[37,39] had insufficient details to access the risk of bias for the criterion "Was there a control group?" for not clearly presenting the control group. Most studies presented a low risk of bias for the criterion "Was appropriate statistical analysis used?". The exception was the study by Kim et al[34] and Wu et al,[37] which did not clearly present the details of the statistical procedures or methods used.

Discussion

Nanostructured Surface Modifications for Cartilage Tissue Engineering

Over the past decades, tissue engineering has evolved in cartilage repair and regeneration, aiming to produce biomaterials comprising scaffolds, cell sources, and growth or differentiation factors suitable for implanting and repairing of significant cartilage defects on the macroscale.[43,44] Scaffolds provide a 3D environment desirable for the regeneration and differentiation of cartilaginous tissue, and numerous natural and synthetic materials are used as scaffolding for cartilage repair.[45–49] Natural polymers, including alginate, agarose, fibrin, HA, collagen, gelatin, chitosan, chondroitin sulfate, and cellulose, have been investigated as bioactive scaffolds for cartilage engineering, and synthetic polymers, including poly(α-hydroxy esters), PEG, poly(NiPAAm), poly(propylene fumarates), and polyurethanes. The optimal cell source for cartilage repair is still being identified.[50] Chondrocytes, fibroblasts, stem cells, and genetically modified cells have all been explored for their potential as viable cell sources.[6,51,52] The recent focus has been on stem cells for their multi-lineage potential, including MSCs,[53] adipose-derived mesenchymal stem cells (ADSCs),[54] and fetal cartilage-derived progenitor cells (FCPCs).[55] Differentiation factors mainly include members of the transforming growth factor-β (TGF- β) superfamily,[56] insulin-like growth factor-1 (IGF-1),[57] and fibroblast growth factor (FGF) family-specific members.[58] However, despite advances in biomaterial synthesis, scaffold fabrication, and development of growth factor delivery systems, cartilage tissue engineering is still not well equipped to modulate cell behavior by mimicking the environmental stimuli (cell signaling molecules, extracellular matrices, and biologically active macromolecules), which are subjected at the nanoscale.[59,60]

Scaffold/Substrate surface modified with micro/nanoscale features could improve the ECM microenvironment to provide topographical, biochemical, and mechanical factors for cellular responses, such as differentiation, adhesion, and migration.[61,62] Colloidal lithography, plasma etching, self-assembly, anodizing, thermal nanoimprinting, and many other nanostructured surface engineering technologies have been explored to fabricate different forms of nanopatterns such as nanopores, nanopillars, nanogrids, nanotubes, nanofibers, and nanoparticles mimicking native biological system to promote cell growth and tissue regeneration.[63] In neural tissue engineering, Zha et al[64] construct nanoparticles and nanofibers on the surface of electrospun cellulose (EC) fiber mats by an in-situ polymerization to provide more protein contact sites for cells, promoting neural cell adhesion and growth. In bone tissue engineering, Sun et al[65] fabricated a composite artificial periosteum containing carbon nanotubes (CNTs) on the surface, which proximally induced the directional alignment, expression of osteogenic genes, and protein production of human bone marrow mesenchymal stem cells (hBMSCs). In skin tissue engineering, Zhao et al[66] fabricated nanofiber-rich chitosan/silk protein (CS/SF) cryogel scaffolds by electrostatic spinning and freeze/freeze-drying, and promoted smooth muscle cell proliferation, infiltration, and contraction-related gene expression. Nanostructured surface modifications of scaffolds/substrates also provide a new perspective for the future development of cartilage tissue engineering.

Mechanism

Nanoscale morphology on the surface of scaffolds/substrates promotes cell adhesion, growth, and chondrogenic differentiation through a series of pathways.[59] Cell adhesion is a crucial prerequisite for cell functions, such as

synthesizing ECM proteins, forming focal adhesion complexes, and cell proliferation.[5] Topographical factors induce mechanotransduction pathways and cell adhesion on the surface nanomorphology by forming focal adhesion complexes on the cell surface.[67] Precartilage condensation is an essential step for chondrogenic differentiation, which is also associated with increased cell-to-cell contacts and interactions through cell-cell adhesion molecules such as N-cadherin and N-CAM.[68–71] Adhesive interactions on synthetic nanoscale surfaces/substrates are mediated by the interactions between cell-surface integrins and extracellular proteins such as collagen, fibronectin, and laminin that have been secreted to the nanostructured surface.[72] The interaction between integrin and cadherin also directs the localization of forces within cell aggregates via the tensional changes in the actin cytoskeleton.[73]

ECM topography regulates cellular behavior at the nanoscale, and cellular receptors, particularly integrins, are the most well-defined cell adhesion receptors and are responsible for sensing and transmitting information about micro-environmental topography.[74] The intracellular part of the integrin extends into the cytoplasm and connects to the cell's cytoskeleton, and the extracellular part connects to the ligand and triggers cell activation. Signaling through integrins leads to the bidirectional transmission of signaling molecules.[75] The integrin receptor plays a vital role in the localization of cytoplasmic focal adhesion protein-tyrosine kinase (FAK) at the cell-ECM interfaces (Figure 4).[76] Kim et al[34] found that surfaces bearing nanotubes of 70 to 100 nm in diameter could trigger the morphological transition to a cortical actin pattern and rounded cell shape (both indicative of chondrocyte differentiation), as well as the up-regulation of type II collagen and integrin β4 protein expression through the down-regulation of Erk activity. The results of immunocyto-chemical staining and cell adhesion experiments showed that the expression of integrins on the nanomorphic surface was significantly higher than that on the smooth surface.[28,34,42,77] More importantly, cell differentiation experiments showed that stem cells on scaffolds/substrates with nanoscale surface morphology increased the expressions of cartilaginous genes like SOX9, aggrecan, collagen I, collagen II, and collagen X (Figure 5).[9,33] These results confirm that suitable nanoscale surface morphology can trigger the integrin-mediated cell adhesion pathway to improve stem cell differentiation and maturation, enhance cell adhesion, and promote chondrogenesis. However, most current studies focus on the general effects of nanomorphology on integrin-mediated signaling pathways within the cell; future research should focus more on how specific nanomorphology parameters interact with the cellular receptors and the detailed mechanisms.

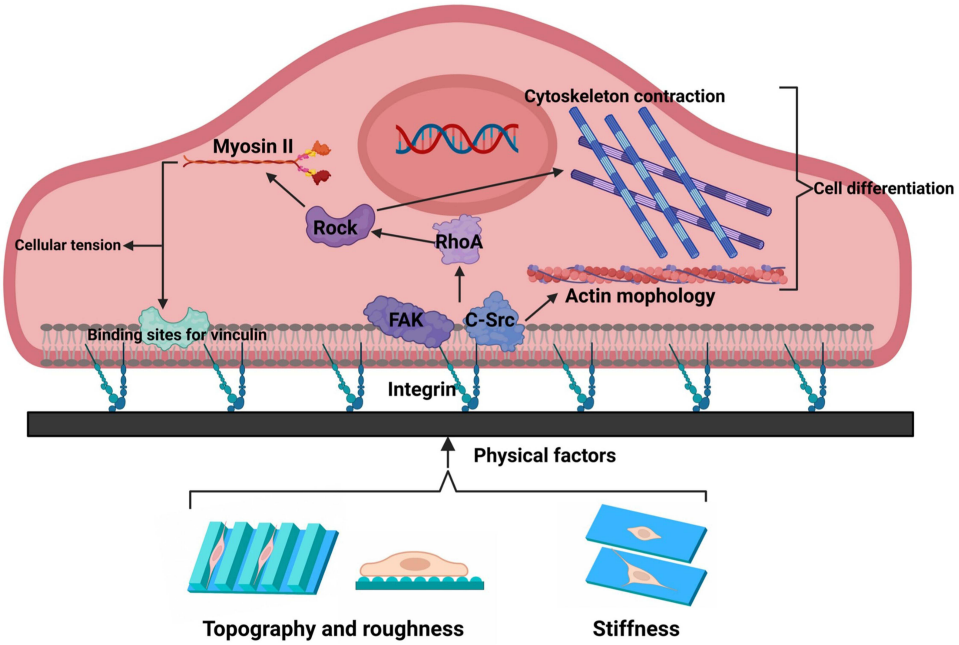

Figure 4 Transduction of stem cell differentiation induced by physical factors. Substrate morphology, roughness, and stiffness are sensed by integrins, and activated integrins activate FAK and tyrosine kinase Src, transmiting information about physical factors to the subsequent RhoA/ROCK signaling pathway. Activated RhoA stimulates actin phosphorylation and further activates ROCK, thereby increasing the stability of actin polymeric filaments. Myosin II, a downstream effector of ROCK, is essential for increasing cellular tension and binding sites for adhesion plaque proteins. In addition, Src activation primarily regulates actin morphology, and RhoA/ROCK significantly affects fiber formation. Ultimately, it affects cell differentiation by regulating cytoskeleton formation and actin morphology. Created in BioRender. Xiao, Y. (2024) https://BioRender.com/q28g769.

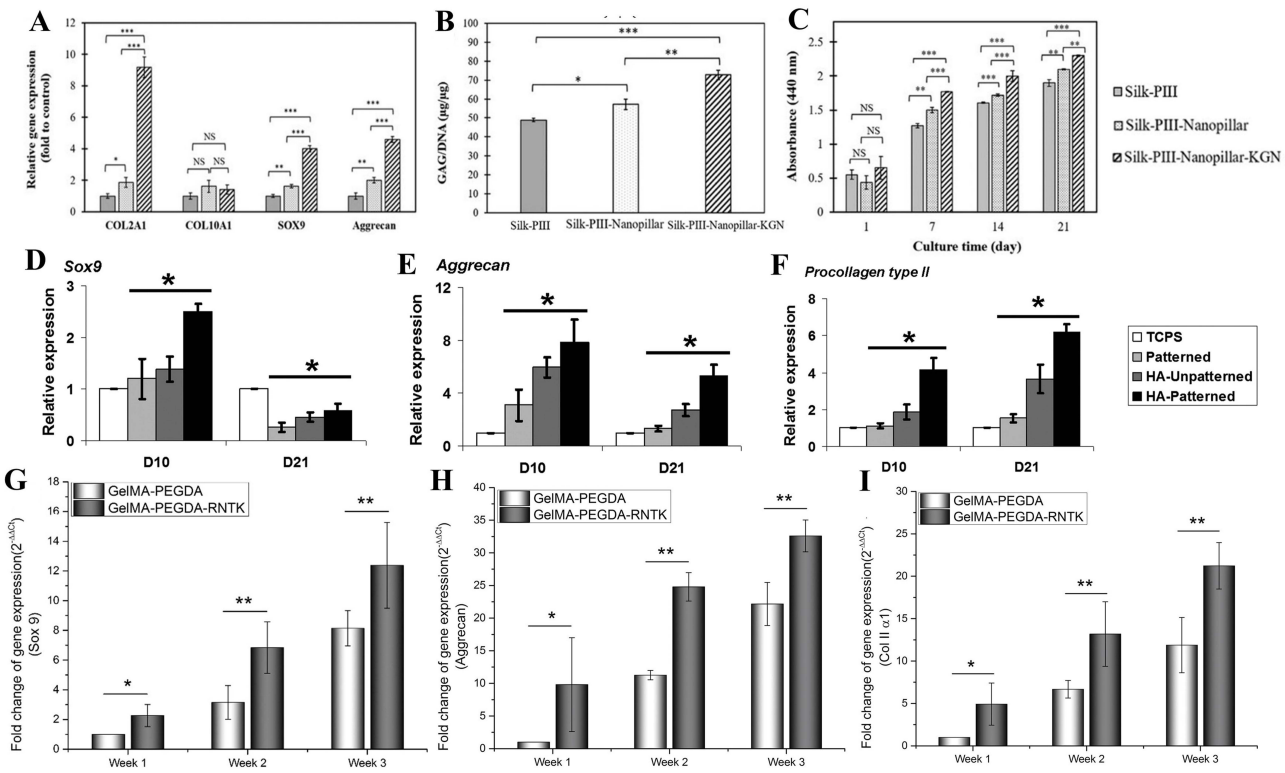

Figure 5 Evidence of stem cells on scaffolds/substrates with nanoscale surface morphology increased the expressions of cartilaginous genes. (**A-C**) Quantitative analysis of nanopillars: (**A**) RT-qPCR results for genes of COL2A1, aggrecan, SOX9, and COL10A1 (results shown in fold-change relative to control (Silk-PIII)); (**B**) GAG per DNA content after 21 days of culture; (**C**) cell proliferation results after 1, 7, 14, and 21 days for hAMSCs. (*p < 0.05, **p < 0.01, ***p < 0.001). Reproduced with permission from Dehghan-Baniani D, Mehrjou B, Chu PK, Wu H. A biomimetic nano-engineered platform for functional tissue engineering of cartilage superficial zone. Adv Healthc Mater. 2021;10(4):e2001018 from John Wiley and Sons Ltd.[33] (**D-F**) Chondrogenic gene expression of DPSCs cultured on different scaffolds for 10 days (D10) and 21 days (D21): (**D**) Sox9 relative expression; (**E**) Aggrecan relative expression; (**F**) Procollagen type II relative expression (Values are represented as mean –SD from three independent experiments (n = 3), *p < 0.05 with respect to indicated groups and analyzed by Student's *t*-test or one-way ANOVA). (Tissue Eng Part A, Vol 20(21–22), Nemeth CL, Janebodin K, Yuan AE et al,[27] Enhanced chondrogenic differentiation of dental pulp stem cells using nanopatterned PEG-GelMA-HA hydrogels, 2817–2829, Copyright © 2014, The publisher for this copyrighted material is Mary Ann Liebert, Inc. publishers.). (**G-I**) Normalized gene expression of ADSCs after chondrogenic differentiation on gradient GelMA−PEGDA scaffolds without and with RNTK over 3 weeks: (**G**) Col II α1; (**H**) Sox-9; (**I**) Aggrecan (Data are presented as the mean ± standard deviation, n =8. *p < 0.05, and **p < 0.01 when compared to control groups at each week). Reproduced with permission from Zhou X, Tenaglio S, Esworthy T, et al. Three-dimensional printing biologically inspired DNA-based gradient scaffolds for cartilage tissue regeneration. ACS Appl Mater Interfaces. 2020;12(29):33219–33228. Copyright © 2020, American Chemisty Society).[35]

Effect of Nanoscale Surface Morphologies on Chondrogenesis

Nanoscale morphologies on the surface of substrates/scaffolds could effectively promote chondrogenic differentiation of stem cells compared to smooth/unpatterned surfaces. Among the studies included, four showed that surfaces with nanopillars improved the maintenance of chondrogenesis and promoted chondrogenic differentiation of stem cells compared to unpatterned surfaces.[9,33,37,41] Huethorst et al[41] designed nanopillars with heights ranging from 27–205nm and showed that nanopillars with a height of 127nm significantly enhanced matrix deposition, chondrogenic gene expression, and maintenance of chondrogenesis compared to unpatterned surfaces. Wu et al[37] showed that surface morphology with nanopillars promotes cell proliferation and chondrogenic differentiation. In the quantitative analysis of nanopillars, D. Dehghan-Baniani et al[33] observed that the nanopillars significantly increased the expression of chondrogenic genes, GAG per DNA content, and cell proliferation results compared to the unpatterned surface, which proved that nanopillars significantly promoted chondrogenic differentiation of stem cells, cell proliferation, and improved cartilage tissue functions (Figure 5A-C). Besides, the study by Wu et al[9] also showed that nanopillars could promote hyaline cartilage formation, suggesting that nanopatterns can lead to specific chondrogenic differentiation outcomes. One study showed that hexagonally arranged nanostructures promote the chondrogenic differentiation of stem cells. Niepel et al[42] designed hexagonally arranged nanostructures with different periodicities showed that cells formed the largest clusters of cells on hexagonal nanopillars with a periodicity of 733nm and then differentiated into chondrogenic lineages.

Three studies showed that nanotubes promoted stem cell proliferation, adhesion, and chondrogenic differentiation compared to unpatterned surfaces.[34–36] Zhou et al[35] and Childs et al[36] fabricated scaffolds with rosetted nanotubes (RNTs), which created an environment similar to the natural cartilage ECM and could be used as a potentially improved material for future cartilage repair and regeneration. In normalized gene expression analysis, Zhou et al[35] observed that scaffolds with RNTs all had higher normalized gene expression than unpatterned scaffolds after 3 weeks of chondrogenic differentiation, which proved that RNTs could effectively promote chondrogenic differentiation (Figure 5G-I). Kim et al[34] designed nanotubes with a diameter of 30–100nm and found that nanotubes with diameters of 70–100nm significantly triggered the transformation of cellular morphology and the upregulation of type II collagen expression, which clearly showed that they could promote chondrogenesis. Two studies used surface morphology with nanoparticles to probe their ability to promote chondrogenic differentiation of stem cells.[28,32] Park et al[28] designed nanoparticles with diameters of 300–1200 nm that could promote the self-aggregation of stem cells. Their nanoparticles with diameters of 750nm are most useful for this, but whether the nanoparticles had the regenerative potential of cartilage tissue remains to be verified. A three-dimensional sphere culture system that helps optimize stem cell differentiation was developed. Ishmukhametov et al[32] coated the glass surface with different concentrations of 10nm diameters' magnetic nanoparticles (MNPs), finding that the surface roughness increased, and the cell adhesion decreased with the increase of the concentration of MNPs. They concluded that the concentration of 3mg/mL could effectively promote the chondrogenic differentiation of stem cells. Three studies investigating the effect of surface nanogrills on chondrogenesis.[9,27,37] In chondrogenic gene expression analysis, Nemeth et al[27] observed a significant increase in chondrogenic gene expression on patterned scaffolds at days 10 and 21 of cell differentiation culture, which proved that scaffolds with nano-linear grooves (800nm (ridge) × 800nm (width) × 500nm (height)) (Figure 6A) effectively induced chondrogenic differentiation compared to unpatterned scaffolds by chondrogenic gene expression analysis (Figure 5D-E). Wu et al[9,37] showed that stem cells experienced delayed chondrogenic differentiation on the surface of the nanogrills (250nm (line) × 250nm (sapce) × 150nm (height)), eventually differentiating into superficial zone cartilage,[9] and nanogratings (250nm (line) × 250nm (space) × 150nm (height)) promoted the chondrogenic differentiation of stem cells.[37] However, this contradicts previous studies showing that anisotropic topographical scaffolds for tissue engineering of superficial cartilage explored in the 500–1000nm range could successfully induce cell alignment.[78,79] Therefore, the size of the grating-like nano-morphologies that better promote chondrogenic differentiation remains to be explored. Three articles investigated the effect of irregular nanopatterns on chondrogenic differentiation and showed that suitable nanopatterns had the potential for chondrogenic differentiation, but not all nanopatterns could improve chondrogenic differentiation.[38–40]

The types and sizes of surface nanomorphology have different effects on the chondrogenic differentiation of stem cells.[80] Three studies investigated the effect of different nanopatterns on chondrogenesis.[9,37,39] Khattak et al[39] reported that nanoisland morphology (PCL: PMMA=25:75) promotes cell proliferation and has the potential for chondrogenic lineages more than nanopit morphology (PCL: PMMA=75:25). Wu et al[37] reported that stem cells grew significantly faster on the surface of nanogratings than on nanopillars during the early stages of chondrogenic differentiation and that proliferation rates remained similar for all three polymers (PCL, PLA, PGA) of the same topography. However, this conflicts with Li et al's study[81] on the interaction of substrate hardness, morphology, and size, which showed that both hardness and size, but not morphology, influence cell proliferation. The current explanation is that adapting different cellular morphologies to specific nanopatterns may be a determining factor in regulating proliferation.[37] Moreover, Wu et al[9] reported that nanopillar and nanohole morphology induced the formation of hyaline cartilage and nanogrill morphology induced the formation of fibro/superficial zone cartilage from cells compared to non-patterned surfaces, which means different nanomorphic patterns lead to different chondrogenic differentiation results. Future studies should focus on the effect of different nanopatterns at similar sizes on the chondrogenic differentiation of stem cells. In summary, nanoholes, nanogrills, nanoparticles with a diameter of 10–40nm, nanotubes with a diameter of 70–100nm, nanopillars with a height of 127–330nm, and hexagonal nanostructures with a periodicity of 302–733nm on the surface of substrates/scaffolds significantly enhance cell adhesion, growth, and chondrogenic differentiation of stem cells compared to the smooth/unpatterned ones through increasing integrin expression. Large nanoparticles with a diameter of 300–1200nm promote pre-chondrogenic cellular aggregation. However, further studies are needed to investigate their suitable sizes, which could effectively promote chondrogenic differentiation.

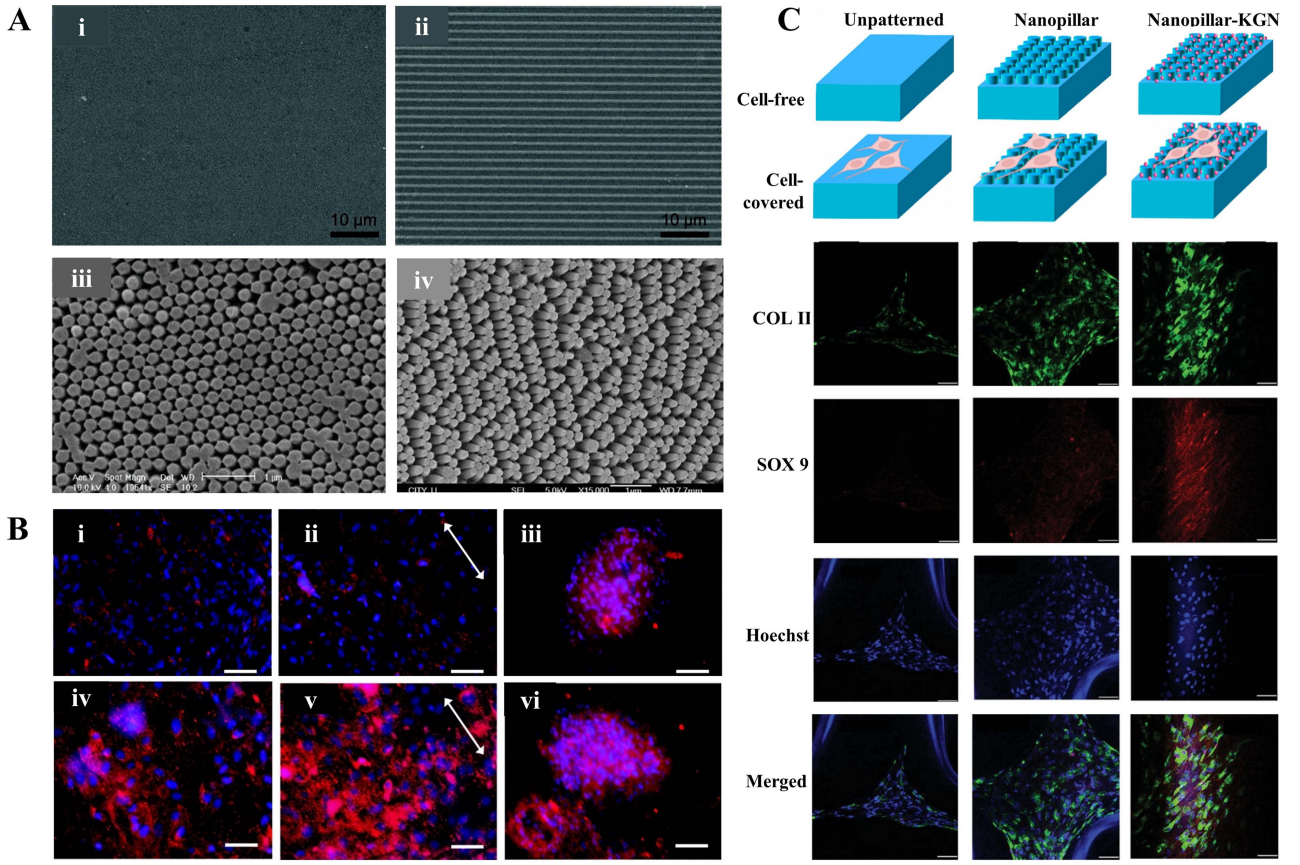

Figure 6 Evidence of the types and sizes of surface nanomorphology have different effects on the chondrogenic differentiation of stem cells. (**A**) Scanning electron micrographs of surfaces: (i, ii) images of PEG-GelMA-HA unpatterned and patterned hydrogels, Scale bars = 10 mm; The publisher for this copyrighted material is Mary Ann Liebert, Inc. publishers.[27] . Reproduced with permission from Dehghan-Baniani D, Mehrjou B, Chu PK, Wu H. A biomimetic nano-engineered platform for functional tissue engineering of cartilage superficial zone. Adv Healthc Mater. 2021;10(4):e2001018 from John Wiley and Sons Ltd.[33] (**B**) Positive collagen type II staining (in red) was observed in DPSCs cultured on TCPS (i), patterned (ii, iii), HA unpatterned (iv), and HA patterned (v, vi), c and f are stained images of the spheroid areas; b, d, and e are stained images of the monolayer areas, Arrows indicate the direction of pattern, Scale bars = 50mm. (Tissue Eng Part A, Vol 20(21–22), Nemeth CL, Janebodin K, Yuan AE et al,[27] Enhanced chondrogenic differentiation of dental pulp stem cells using nanopatterned PEG-GelMA-HA hydrogels, 2817–2829, Copyright © 2014, The publisher for this copyrighted material is Mary Ann Liebert, Inc. publishers.). (**C**) Confocal laser scanning microscopy and three-dimensional mold images of COL II, SOX9, and Hoechst staining of cell-seeded Silk-PIII, Silk-PIII-Nanopillar, and Silk-PIII-Nanopillar-KGN after culturing for 14 days, Scale bar = 100μm. Reproduced with permission from Dehghan-Baniani D, Mehrjou B, Chu PK, Wu H. A biomimetic nano-engineered platform for functional tissue engineering of cartilage superficial zone. Adv Healthc Mater. 2021;10(4):e2001018 from John Wiley and Sons Ltd.[33]

Combination of Topographical and Other Factors

In recent years, many studies have combined topographical and other factors to investigate the effects on chondrogenic differentiation of stem cells.[82] Among the studies we included, five combined topographical, chemical, biochemical, and biological factors to explore the effects on chondrogenic differentiation of stem cells.[27,32,33,35,36,38] Three studies combined topographic and chemical factors to investigate the effect on chondrogenesis. Zhou et al[35] and Childs et al[36] fabricated RNTs combining topographical factors and chemical factors by self-assembly technology, which effectively promoted chondrogenic differentiation; RNT is a promising nanomaterial due to its unique nanostructure, high density of functionalizable surface group, and collagen-like soft properties. Nemeth et al[27] fabricated scaffolds containing hyaluronic acid (HA) and linear nano-grooves as HA provides chemical signals to promote stem cell differentiation toward the chondrogenic lineage. Immunofluorescence staining of collagen type II revealed that stem cells cultured on HA patterns exhibited the significantly most robust staining among all scaffolds, proving that scaffolds containing HA and linear nano-grooves promoted chondrogenic differentiation (Figure 6B). They also hypothesized that HA might signal by binding to CD44, which regulates the EMT gene's function. Future studies should determine the mechanism and focus on the role of HA combined with other nanopatterns on chondrogenesis. The natural materials suitable for cartilage tissue engineering include polysaccharides, such as chitosan, hyaluronan, and alginate; polyesters, such as

polyhydroxybutyrate; proteins, such as collagen, elastin, keratin and fibroin. These materials are processed into scaffolds that impart features of pore structure, elasticity, and surface energy parameters.[83] Chitosan has cationic properties, biocompatibility, stability, the ability to assume a variety of physical forms, and promotes the expression of cartilage matrix components and reduces the production of inflammatory and catabolic mediators by chondrocytes in vitro.[84,85] Therefore, it could be used as a material of scaffold in cartilage tissue engineering. Alginate is suitable for the redifferentiation of chondrocytes after monolayer expansion and can be used as a carrier for mesenchymal stem cells undergoing chondrogenesis.[86,87] Cellulose can be used as a material of scaffold in cartilage tissue engineering due to its ability to mimic in vivo cellular architecture to support biological functions, similarity to the natural ECM, and can be adjusted in assembly of scaffold.[88] Future studies should combine chemical factors including synthetic polymers and naturally occurring polysaccharides with nanostructured surface modifications to investigate their effects on chondrogenic differentiation of stem cells.

Two studies used a combination of biochemical, biological, and topographical factors to explore the effects of chondrogenesis. D. Dehghan-Baniani et al[33] immobilized the small biological molecule kartogenin (KGN) on the surface of nanopillars, and immunofluorescence images confirmed the most significant expression of chondrogenesis-related proteins on the surface of KGN-loaded nanopillars, demonstrating that the combination of KGN and nanopillars could effectively promote the chondrogenic differentiation of stem cells (Figure 6C). In addition, Wei et al[89] showed that a biomimetic structure with KGN as the only biochemical inducer could promote the recovery of cartilage and subchondral bone in osteochondral defects in vivo. Rodriguez-Pereira et al[38] investigated the promotion of pre-chondrogenic aggregation of stem cells by coating nanopatterned surfaces with different concentrations of arginine–glycine–aspartate (RGD). They concluded that 2.5×10^{-8}w/w was the optimal concentration. Furthermore, Prittinen et al[40] showed that nanopatterned surfaces alone could not prevent the dedifferentiation of primary chondrocytes, demonstrating the importance of the biochemical factor. In conclusion, future studies should focus on the role of nanomorphology on chondrogenic differentiation and the effect of topographic factors combined with biochemical factors on chondrogenic differentiation.

Influence of Cell Sources

Although there are currently various options for cell sources in cartilage tissue engineering, choosing the right one is critical and challenging.[90] Applying stem cells to cartilage tissue engineering requires transforming stem cells into chondrocytes capable of producing a functional cartilage matrix, which is also the key to the successful use of stem cells.[50] Currently, mesenchymal stem cells (MSCs) are the predominant cell source for cartilage tissue engineering. MSCs are adult stromal cells with a wide range of differentiation potential when cultured in vitro.[91] The self-renewal ability and relatively low immunogenicity of MSCs favor their use in cartilage tissue engineering.[92,93] MSCs come from many sources, including fat and bone tissue, umbilical cord blood, placenta, dental pulp, and many other tissues in adults.[94,95] Different sources of MSCs have different properties and unique advantages, which is also demonstrated in the study by Rodriguez-Pereira et al[38] Bone marrow and adipose tissue are the primary sources of therapeutic MSC.[96] Bone marrow-derived MSCs (BM-MSCs) have been heavily used in cartilage tissue engineering due to their easy accessibility, rapid cell proliferation, maintenance of differentiation capacity, and low immune rejection. Monaco et al[97,98] used BM-MSCs to generate cartilage tissue in a medium that combined specific growth factors and oxygen tension. Adipose-derived MSCs (AD-MSCs) possess various properties for cartilage tissue engineering, such as abundant availability, ease of collection, minimal trauma, low complication rates, and high proliferative potential.[99] Zhou et al[35] and Ishmukhametov et al[32] promoted chondrogenic differentiation of AD-MSCs by culturing them on surfaces with specific nanopatterns. Besides, compared to BM-MSCs, AD-MSCs are relatively more abundant and have a lower harvesting risk.[54,100] Tigli et al[101] compared the chondrogenesis potential of human articular chondrocytes, human embryonic stem cells, and MSCs derived from three different sources (adipose tissue, embryonic stem cells, and bone marrow), finding that human embryonic stem cell-derived MSCs had the best potential for chondrogenesis. Meanwhile, Park et al[102] reported that fetal chondrogenic progenitor cells (FCPCs) have the potential for high yield, proliferation, pluripotent differentiation, and maintenance of the chondrogenic phenotype in chondrogenesis, which means FCPCs could act as a novel cell source for cartilage regeneration. Although some progress has been made in cell sources for cartilage tissue engineering, there are still problems, such as the low structural similarity of stem cell-based regenerated cartilage tissue

to natural cartilage tissue. In the future, focusing on the cell source and the effect of combining stem cells from different sources on cartilage regeneration is essential.

Influence of Matrix Stiffness

Environmental physical characteristics also play an important role in stem cells' chondrogenic differentiation. In addition to topographical factors, matrix stiffness has been shown to influence chondrogenic differentiation of stem cells.[103] Among the selected studies, three investigated the effect of the combination of nanomorphology and substrate stiffness on the chondrogenic differentiation of stem cells.[33,37,42] D. Dehghan-Baniani et al[33] used the viscoelasticity of a thermosensitive hydrogel to improve the mechanical properties of the substrate and increase the adhesion strength at the graft-host interface, which in turn improved chondrogenesis. Niepel et al[42] reported a lower propensity for chondrogenic differentiation of cells on harder, smoother surfaces. Wu et al[37] found that hyaline-like cartilage exhibiting characteristics of the middle/deep zone was generated on a softer pillar surface, while stiffer nanopillar material MSCs demonstrated the potential to generate constituents of hyaline/fibro/hypertrophic cartilage. Additionally, fibro/superficial zone-like cartilage could be derived from nanogratings with a softer stiffness, whereas stiffer nanogratings led to negligible chondrogenesis, which means that different nanopatterns combined with different stiffness could lead to differentiation of stem cells into different types of cartilage. These three results demonstrate the tendency of stem cells grown on substrates with low stiffness to chondrogenic differentiation compared to substrates with high stiffness, consistent with previous research findings.[103] In addition, the culture systems used in the current study vary widely in their stiffness values, and there is yet to be a relatively precise range of stiffness values that could promote chondrogenic differentiation of stem cells well. Current evidence suggests that matrix stiffness-mediated differentiation of stem cells to chondrocytes might involve several signaling pathways, including integrin-mediated focal adhesion signaling, tension-sensitive protein pathway, stretch-activated channel pathway, TWIST1 signaling, and YAP/TAZ signaling.[104] However, the exact mechanism by which matrix stiffness affects chondrogenic differentiation of stem cells remains unclear. Although the effects of mechanical stiffness or topographical factors on cell fate and chondrogenic differentiation have been studied in the literature, how these factors work together to influence stem cell chondrogenic differentiation remains unclear.[37,79,105,106] Future studies could focus on designing systems that combine topographical and mechanical stiffness factors to promote chondrogenic differentiation.

Limitations

Due to methodological and statistical heterogeneity in trial protocol design and efficacy evaluation in the selected studies, this may ultimately affect the accuracy of analysis results.[107] No studies have qualitatively compared the characteristics of different nanofabrication techniques currently. Cartilage development consists of four main stages: anterior cartilage coalescence, interstitial band formation, cavitation, and articular cartilage stabilization. In humans, anterior cartilage coalescence can be observed in the 5th-6th weeks of embryogenesis. Morphologically dense and minimal cartilage matrix-containing structures can be observed in the 11th-12th weeks.[108] Scaffolds/substrates with nanostructured surface morphology appeared to accelerate cartilage aggregation within 1 week and cartilage tissue formation in 6 weeks. Besides, the cell sources were different in all selected studies. Since different cells perceive the extracellular microenvironment differently, this can lead to differences in cell proliferation, adhesion, and chondrogenic differentiation. Therefore, choosing the right cell source is challenging. The analysis methods of the included studies were similar and helpful in evaluating chondrogenesis, but the physical and chemical factors of the biomaterials are non-negligible variables that affect chondrogenesis.

There are limitations to this systematic review study. Although a systematic literature search was conducted and enrollment in this systematic review was completed prior to the completion of data extraction, the limited number of studies and the heterogeneity of experimental designs and observational metrics may prevent the comparison of results and affect the outcomes. Due to these reasons, we cannot determine the optimal nanoscale surface morphology for promoting chondrogenic differentiation of stem cells in vitro.

Conclusion

In vitro studies suggest that nanoholes, nanogrills, nanoparticles with a diameter of 10–40nm, nanotubes with a diameter of 70–100nm, nanopillars with a height of 127–330nm, and hexagonal nanostructures with a periodicity of 302–733nm on the surface of substrates/scaffolds significantly improve cell adhesion, growth, and chondrogenic differentiation of stem cells

compared to the smooth/unpatterned ones through increasing integrin expression. Large nanoparticles in 300–1200nm diameter promote pre-chondrogenic cellular aggregation. However, the limited number and heterogeneity of current studies hinder the determination of the optimal nanoscale surface morphology to promote chondrogenic differentiation of stem cells. Moreover, due to the need for studies on the interaction between different nanomorphology parameters and cellular receptors, future research should focus more on this area to investigate the detailed mechanisms. The application of surface topographic modifications, piggybacking small biological molecules, coatings, or novel materials opens new opportunities for the design of scaffolds/substrates in cartilage tissue engineering. Future work needs to focus on the precise control of the sizes and types of nanoscale morphologies, the synergistic effects of the composite treatment of nanomorphologies and other factors, and the evaluation of the mechanical properties, degradability, durability, and biocompatibility of the fabricated nanostructures. More standardized and comprehensive reports of nanoscale surface morphology are needed to better understand the topographical influences on chondrogenesis by scaffolds/substrates for cartilage regeneration, with in vivo studies required for translating findings to clinical applications.

Abbreviations

ECM, Extracellular matrix; AC, Articular cartilage; NIL, Nanoimprint lithography; EBL, Electron beam lithography; μCP, microcontact printing; LBL, Layer-by-layer; NSL, Nanosphere lithography; MSCs, Mesenchymal stem cells; FCPCs, Fetal cartilage-derived progenitor cells; DPSCs, Dental pulp stem cells; MNP, Magnetic iron oxide nanoparticle; PIII, Plasma immersion ion implantation technique; KGN, Kartogenin, a biomolecule can promote chondrogenesis of hAMSCs; GelMA, Gelatin methacrylate; PEGDA, Poly (ethylene glycol diacrylate); RNTK, Lysine functionalized rosette nanotube; PLLA, Poly (L-lactic acid); TBL, Aminobutane linker molecule; TB-RGDSK, Twin guanine/cytosine DNA base hybrids-based rosette nanotubes functionalized with arginine–glycine–aspartic acid–serine–lysine integrin binding peptide; RGD, arginine–glycine–aspartate; PCL, Polycaprolactone; PLA, Polylactide; PGA, Polyglycolide; PMMA, Poly (methyl methacrylate); ADSC, Adipose-derived mesenchymal stem cell; FGF, Fibroblast growth factor; TiO2, Titanium dioxide; G^C, Guanine/Cytosine DNA base hybrids; PLL, Poly-L-lysine; HA, Hyaluronic acid; EC, Electrospun cellulose; CNTs, Carbon nanotubes; hBMSCs, human bone marrow mesenchymal stem cells; RNTs, Rosetted nanotubes; MNPs, Magnetic nanoparticles; BM-MSCs, Bone marrow-derived MSCs; AD-MSCs, Adipose-derived MSCs; TGF- β, Transforming growth factor-β; IGF-1, Insulin-like growth factor-1; FAK, Focal adhesion protein-tyrosine kinase.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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