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Environmental preconditioning rejuvenates adult stem cells' proliferation and chondrogenic potential

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Abstract

Adult stem cells are a promising cell source for cartilage regeneration. Unfortunately, due to donor age and *ex vivo* expansion, stem cell senescence becomes a huge hurdle for these cells to be used clinically. Increasing evidence indicates that environmental preconditioning is a powerful approach in promoting stem cells' ability to resist a harsh environment post-engraftment, such as hypoxia and inflammation. However, few reports organize and evaluate the literature regarding the rejuvenation effect of environmental preconditioning on stem cell proliferation and chondrogenic differentiation capacity, which are important variables for stem cell based tissue regeneration. This report aims to identify several critical environmental factors such as oxygen concentration, growth factors, and extracellular matrix and to discuss their preconditioning influence on stem cells' rejuvenation including proliferation and chondrogenic potential as well as underlying molecular mechanisms. We believe that environmental preconditioning based rejuvenation is a simpler and safer strategy to program pre-engraftment stem cells for better survival and enhanced proliferation and differentiation capacity without the undesired effects of some treatments, such as genetic manipulation.

Keywords

Preconditioning; Extracellular matrix; Fibroblast growth factor 2; Hypoxia; Adult stem cell; Chondrogenesis

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Disclosure Statement

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Introduction

Due to the hostile environment in damaged tissue, such as inflammation, immune compromise, hypoxic stress, and insufficient blood supply [1], the survival rate of transplanted cells *in vivo* is low, only 1–3% [2,3], which is a huge hurdle for cell-based therapy [4,5]. The concept of "preconditioning-induced protection", first raised by Murry in 1986, is a process by which myocardial stem cells exposed to a sub-lethal ischemic condition could promote the heart's tolerance to severe ischemia [6]. Since then, the preconditioning concept has been used as the most effective means of cytoprotection, especially for cell-based treatment of ischemic myocardium and stroke [7]. Despite the fact that cell death in musculoskeletal transplantation, such as cartilage [8] or intervertebral disc (IVD) repair [9], is not as robust as in the heart and brain, it is still crucial for cells to survive before a sufficient repair response is induced.

Common preconditioning approaches include hypoxia, cytokines and growth factors, and genetic manipulation. Genetic manipulation promotes the viability of stem cell engraftment by overexpression of cytoprotective genes. The common overexpressed genes in promoting the survival of mesenchymal stem cells (MSCs) include v-Akt Murine Thymoma Viral Oncogene (AKT) [10], B-cell lymphoma 2 (Bcl-2) [11], heat shock protein 20 (Hsp20) [12], nuclear factor related (erythroid-derived 2)-like 2 (Nrf2) [13], heme oxygenase-1 (HO) [14,15], endothelial nitric oxide synthase (eNOS) [16], connexin 43 (Cx43) [17], and hypoxia inducible factor-1α (HIF-1α) [18]. Other overexpressed genes, such as wild-type p53 inducible phosphatase-1 (WIP-1) [19] and lipocalin 2 (Lcn2) [20], could decrease MSC senescence during the process. However, genetic manipulation of MSCs has limited clinical benefit due to its inherent risks during genetic modification, such as random integration into the host genome inducing mutations [21].

Despite an initial focus on the suppression of inflammatory and immune responses and the promotion of cell survival rate as well as migration and homing of transplanted cells, preconditioning strategies now attract more attention for rejuvenation of regenerative and repair potentials of pre-engraftment cells [22,23]. As expansion is always needed to increase cell numbers for clinical application, it is critical to achieve expansion without compromising differentiation potential. Thanks to the discovery that crosstalk between MSCs and other cells in the native niche modulated MSCs' properties [24,25], the *in vitro* establishment of these communications has been demonstrated [26,27]. This review paper focuses on summarizing up-to-date environmental preconditioning strategies during *ex vivo* expansion and discussing their influence on adult stem cell proliferation and chondrogenic potential, which is important for cartilage tissue engineering and regeneration using autologous stem cells that become prematurely senescent due to donor age and suffer replicative senescence because of extensive expansion. We hypothesize that, from the clinical perspective, environmental preconditioning based rejuvenation is a simpler and safer strategy to program pre-engraftment stem cells for better survival and enhanced proliferation and differentiation capacity without the undesired effects of some treatments, such as genetic manipulation [21].

Hypoxic preconditioning

In native cartilage, cells are exposed to very low oxygen tension – about 7% (53 mmHg) in the superficial zone and 1% (5–8 mmHg) in the deep zone of articular cartilage [28]. There have been many studies investigating the effects of hypoxia on chondrogenic differentiation of MSCs in an attempt to determine the best point in the culture process to expose MSCs to hypoxic conditions. For example, should MSCs be expanded in hypoxia, differentiated in hypoxia, or should both expansion and differentiation take place in hypoxic conditions in order to attain the best results? Increasing evidence suggests that hypoxic pretreatment can not only promote cell survival and migration ability post-engraftment [29,30] but also can benefit cell rejuvenation, in terms of proliferation and differentiation capacity (Table 1) [31].

Evidence of cell proliferation capacity

Expansion of MSCs in hypoxic conditions has been shown to prevent stem cell senescence and yield higher proliferation rates, enhanced tissue forming capacity, and smaller, more densely organized, spindle-like cells than expansion in normoxic conditions [32,33]. Choi et al. found that hypoxic (2% oxygen) treatment significantly increased stemness markers, reduced expression 1 (REX1), SRY (sex determining region Y)-box 2 (SOX2), octamer-binding protein 4 (OCT4), and NANOG, along with HIF-1α in human adipose stem cells (ASCs) [34]. The proliferation rate of ASCs under hypoxic incubation was also significantly enhanced, evidenced by an increase in cell number but a decrease in the mean population doubling time (PDT) despite no alteration of surface markers, including CD73, CD90, and CD105. Xu et al. found that 2% oxygen treatment yielded a significantly higher cell number and more DNA synthesis as well as shorter PDT in mouse ASCs [35]. They also found that hypoxic treatment significantly reduced the matrix metalloproteinase (MMP) family genes, MMP2, MMP3, MMP8, and MMP13.

Krinner et al. found that hypoxic (5% oxygen) treatment promoted *in vitro* population growth of ovine bone marrow stromal cells (BMSCs) as demonstrated by significantly larger colonies compared to those under normoxic conditions [36]. Similarly, Zscharnack et al. found that hypoxic treatment (5% oxygen) of ovine BMSCs significantly increased colony numbers and sizes but diminished senescence, as shown by lower levels of granularity and senescence-associated (beta)-galactosidase positive cells [37]. Boyette et al. found that hypoxic treatment (5% oxygen) in human BMSCs enhanced colony formation and proliferation, evidenced by 5-ethynyl-2′-deoxyuridine (EdU) incorporation, but with no change in Ki67 staining [38]. They also found that metabolic activity was increased after 96 h of hypoxic treatment. However, hypoxia was not found to have any impact on cell death and apoptosis rates.

Martin-Rendon et al. found that exposure to hypoxia (1.5% oxygen) for 24 h demonstrated a moderate increase in total colony numbers of umbilical cord blood (UCB) CD133+ cells and a significant increase in viable cell numbers of human BMSCs [39]. In normoxia, there was low expression of endogenous HIF-1α in human BMSCs but not in UCB CD133+ cells; however, exposure to hypoxia for 24 h stabilized/upregulated HIF-1α in both cell populations. Hypoxia likely increased cell proliferation in a cell source-dependent manner. Kalpakci et al. found that hypoxic treatment (5% oxygen) resulted in a significant decrease

in number of dermis isolated adult stem cells at days 7, 9, and 11 of culture and a lower colony forming unit – fibroblast (CFU-F) compared with normoxic culture [40].

Evidence of chondrogenic potential

Accumulating evidence indicates that hypoxic preconditioning can promote MSC chondrogenic potential and concurrently inhibit unintended differentiation into the osteogenic lineage. Krinner et al. found that 5% oxygen preconditioning enhanced chondrogenic potential in ovine BMSCs [36]. Similarly, Müller et al. found that 4% oxygen preconditioning enhanced human BMSCs' chondrogenic differentiation in both micromass and gelatin hydrogel culture systems [41]. Adesida et al. found that hypoxia preconditioned human BMSCs yielded pellets with enhanced chondrogenic capacity in spite of oxygen tension during pellet culture [42]. Also of note, Xu et al. found that hypoxic preconditioning enhanced chondrogenic differentiation but decreased osteogenic differentiation in mouse ASCs [35]. These results indicate that hypoxia during expansion may prepare MSCs specifically for chondrogenesis.

However, conflicting reports exist regarding hypoxic preconditioning, possibly due to varied situations, such as hypoxic extent and donor cell type. Martin-Rendon et al. found that hypoxic preconditioning (1.5% oxygen) did not change the differentiation potential of UCB CD133+ clonogenic myeloid cells but promoted human BMSCs' chondrogenic potential despite having no effect on adipogenic and osteogenic differentiation [39]. Li and Pei found that hypoxic preconditioning did not promote chondrogenic potential in porcine synovium-derived stem cells (SDSCs) [43]. Pilgaard et al. found that, despite enrichment with CFU-Fs during expansion under hypoxia (5% and 1% oxygen), human ASCs did not exhibit an enhanced chondrogenic differentiation in subsequent chondrogenic induction [44]. Furthermore, Boyette et al. found that hypoxic preconditioning decreased chondrogenesis in human BMSCs in a pellet culture, which worsened if combined with hypoxic treatment during chondrogenic induction; however, human BMSCs preconditioned in 21% oxygen differentiated robustly in pellet culture under both 5% oxygen and 21% oxygen conditions [38].

The effects on MSCs during expansion at normoxic levels and differentiation into chondrocytes at hypoxic levels have also been studied. MSCs under hypoxia for chondrogenic induction showed decreased proliferation, but elevated expression of sulfated glycosaminoglycan (GAG) and chondrogenic genes [45–48]. Interestingly, hypoxic conditions in pellet culture upregulated *HIF-2α* and downregulated *COL10A1* (type X collagen) despite normoxia or hypoxia during cell expansion [42]. Hypoxic treatment also produced a mechanically functional hyaline cartilage-like tissue compared to the cells differentiated in normoxia [49]. Recently, Leijten et al. found that normoxia in pellet culture promoted the expression of the hypertrophic cartilage-enriched gene transcripts of *COL10A1, MMP13*, and pannexin 3 (*PANX3*) levels while hypoxia enhanced the articular cartilage-enriched gene transcripts of gremlin 1 (*GREM1*), frizzled-related protein (*FRZB*), and Dickkopf Wnt signaling pathway inhibitor 1 (*DKK1*) which act as inhibitors of hypertrophic differentiation [50]. In addition, they also found that, in a nude mouse model, hypoxia-preconditioned implants retained cartilage; on the other hand, normoxia-

preconditioned implants readily underwent endochondral ossification [50]. These studies have shown that differentiation of MSCs *in vitro* in hypoxic conditions following normoxic expansion improves chondrogenesis when compared to cells differentiated in normoxic conditions.

Conflicting data also exist on the effect of hypoxia on chondrogenic induction. One study has shown that murine ASCs, when differentiated at 2% oxygen, exhibited increased proliferation and fewer chondrogenic markers compared to those differentiated in 21% oxygen [51]. Interestingly, the same investigators later showed that, when murine ASCs were expanded in 2% oxygen, then differentiated at normoxic levels, some chondrogenic markers [GAG content and *COL2A1* (type II collagen) expression, but not expression of *SOX9* or *ACAN* (*aggrecan*)] were increased compared to those expanded in 21% oxygen [52]. The differences between these two reports could have been a result of reactive oxygen species (ROS) accumulation during long-term exposure to hypoxic conditions in differentiation, which would have led to cell damage and decreased chondrogenic potential [35].

Potential mechanisms

The mechanism by which hypoxia exerts its effect on cells is mainly regulated by HIF-1, which is composed of two subunits, α and β [53]. Compared to the presence of the β subunit in the nucleus, the α subunit is constitutively expressed in the cytoplasm, where it is bound by von Hippel-Lindau (vHL) tumor suppressor protein, which is an E3 ubiquitin ligase that targets HIF-1α for degradation by the 26S proteasome. This vHL/HIF-1α interaction is oxygen dependent through a group of prolyl hydroxylases (PHDs), the most important of which is PHD domain-containing protein 2 (PHD2) [54,55]. These enzymes hydroxylate proline residues at Pro402 and Pro564 on HIF-1α [56] and at Pro405 and Pro531 on HIF-2α [57] and require Fe^{2+} and α-ketoglutarate as co-factors for their catalytic activity [58–60]. As a result of hydroxylation of these proline residues, vHL is able to bind HIF-1α and target it for destruction. However, as oxygen concentration decreases, the overall PHD2 function decreases as well, increasing the amount of HIF-1α that is able to translocate into the nucleus and bind to its counterpart, HIF-1β [59]. This complex binds to the hypoxia response element (HRE) on the genome, which induces expression of hypoxia-regulated genes.

Although oxygen concentration is directly associated with the HIF-1 pathway, there are still mechanisms by which oxygen indirectly regulates cells' response to hypoxia. For example, the AKT/phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways might be important in upstream regulation of HIF-1. When cultured in hypoxic conditions, levels of phosphorylated AKT and p38 MAPK are elevated and, when AKT and p38 MAPK are inhibited, HIF-1α is unable to translocate to the nucleus [47,61]. Recent evidence indicates that the increase of stem cell survival after hypoxic preconditioning is largely through the stabilization of HIF-1α *via* a hypoxia-induced increase of phosphorylated AKT and p38 MAPK [47], resulting in the upregulation of the glycose-6-phosphate transporter and promotion of MSC survival [62]. Hypoxic preconditioning also modulates the pro-survival and pro-angiogenic factors of MSCs by

upregulation of vascular endothelial growth factor (VEGF) and B-cell lymphoma 2 (BcL-2) [30], for example, *via* the AKT and Extracellular Signal-regulated Kinase (Erk) involved complex pathways [61,63]. Hypoxic preconditioning also plays a critical role in mobilization and homing of MSCs through its capacity to induce HIF-1 mediated expression of stromal cell-derived factor-1 (SDF-1) [64] and its receptor C-X-C chemokine receptor type 4 (CXCR4) [65]. Additionally, CREB-binding protein (CBP) and p300 co-activator encourage transcription of HRE genes by binding the HRE/HIF-1 complex in an oxygen-regulated manner that is independent of the HIF-1 pathway [66].

High oxygen tension has been shown to damage DNA, proteins, and lipids [67] by generation of abnormally high free-radical-derived ROS [68], causing senescence either by the p53-mediated pathway or by accelerating telomere loss [69]. In contrast, low oxygen tension activates HIF-1α, delaying senescence through the activation of macrophage inhibitory factor and inhibition of the p53-mediated pathway [70], regulating cell proliferation [71] and cell differentiation [72–74]. Mouse ASCs with the deletion of HIF-1α exhibited diminished chondrogenic capacity with no significant change in osteogenic differentiation and enhanced adipogenic differentiation [52]. Although HIF-1α has conventionally been thought responsible for the upregulation of chondrogenic gene expression, recent studies have shown that chondrogenesis may be more dependent on signaling through HIF-2 α. For example, MSCs expanded and differentiated within hypoxic conditions demonstrated no marked HIF-1α expression but showed upregulation of HIF-2α[42,45]. Interestingly, human articular chondrocytes with HIF-1α knockdown cultured in hypoxia (1% oxygen) had no effect on chondrogenic gene expression, while knockout of HIF-2α resulted in a marked decrease in expression of chondrogenic genes like *SOX9* and *COL2A1* [75]. These results indicate that the effects of hypoxia may be carried out by different mechanisms, depending on the level of hypoxia and the cell type.

FGF preconditioning

Growth factors are important in mediating the development and maintenance of hyaline cartilage [76]. As a result, the use of growth factors during MSC *ex vivo* expansion and chondrogenic induction has been well studied and yielded promising results. In the cytokines and growth factors, transforming growth factor alpha (TGFα) [77], interferon-gramma (IFN-γ) [78], SDF-1 [79,80], epidermal growth factor (EGF) [81], and insulin-like growth factor I (IGF-I) [82] have been extensively studied. Along with hypoxic preconditioning, these approaches are considered environmental manipulation, in which there is a fine-tuned balance between self-renewal and differentiation potential of MSCs [83,84], particularly for basic fibroblast growth factor (FGF-2) mediated preconditioning in MSCs' chondrogenic and osteogenic potential (Table 2) [85].

Evidence of cell proliferation and chondrogenic potential

FGF treatment could promote MSC proliferation, which is independent of species, such as mouse [86], human [87], and porcine [43], or of varied tissues, such as adipose [86], infrapatellar fat pad [88], bone marrow [89], and synovium [43]. Interestingly, FGF-2 administration during expansion is associated with downregulation of some important

surface markers (such as CD49a, CD90, and CD146) but upregulation of chondrogenic potential, indicating that a difference in surface marker distribution does not result in impaired differentiation [87,90,91]. Microarray analysis did not find a clear pattern as to the mitogenic effect of FGF-2 on human BMSCs [92].

Given that supplementation during *ex vivo* expansion supports a proliferative state, subsequent withdrawal of FGF-2 is proposed to contribute to leaving the cell cycle and coming into a differentiation-competent state. For example, compared to treatment during pellet culture inhibiting chondrogenic differentiation [93,94], FGF-2 pretreated stem cells were reported to promote and retain expanded MSCs' chondrogenic potential even after 30 population doublings, whereas the non-pretreated stem cells lost chondrogenic differentiation after around 20 population doublings, which is more than 1000-fold difference in the number of cells [95]. Furthermore, FGF-2 pretreated MSCs from porcine infrapatellar fat pad were found to generate the most mechanically functional cartilage tissue [96]. Preconditioning with a specific growth factor cocktail [1 ng/mL TGF-β1, 5 ng/mL FGF-2, and 10 ng/mL platelet-derived growth factor-BB (PDGF-ββ)] in monolayer culture led to remarkable improvement in biomechanical and biochemical properties of bovine SDSC-seeded tissue constructs [97].

Interestingly, during the study on limb development, ten Berge et al. found that FGF-8 and Wnt3a signals worked together to promote limb bud cell proliferation while retaining cells in an undifferentiated condition; once both types of stimulations were withdrawn, the cells switched to chondrogenic differentiation [98]. Inspired by this report, Narcisi et al. observed that combined pretreatment with Wnt3a greatly promoted the effect of FGF2 on human BMSC proliferation by increasing cell doublings from 20 to 30. They also found that co-preconditioned cells acquired enhanced chondrogenic potential; inhibition of Wnt3 signals during differentiation prevented calcification while preserving hyaline cartilage properties following transplantation in a mouse model [99]. In a three-dimensional (3D) pellet model, intriguingly, Centola et al. found that, contrary to their initial hypothesis, Wnt3a treatment induced human BMSCs to a five-fold increase in cell number despite a continuing decrease of total DNA content in the 3D construct; preconditioning with Wnt3a improved cells' chondrogenic potential, which was antagonized by treatment with FGF2 [100].

Different from hypoxic pretreatment, which inhibits differentiation toward osteogenesis [85], more evidence showed that FGF-2 preconditioning could promote not only type II collagen but also type X collagen [43,91,92], suggesting that FGF-2 expanded MSCs yielded pellets with an enhanced capacity toward endochondral bone formation [93]. This finding indicates that stem cells pretreated with FGF-2 alone would benefit bone tissue engineering rather than cartilage tissue engineering, which is worth noting for future clinical application.

Potential mechanisms

Two potential mechanisms have been proposed for FGF-2 mediated MSC rejuvenation toward chondrogenesis. The first one is that MSCs with inherent chondrogenic potential are preferentially selected by pretreatment with FGF-2 during monolayer culture, in terms of selection mechanism [89]. Investigations found that *ex vivo* enrichment of MSCs by FGF-2

had long telomeres and could maintain chondrogenic potential for greater numbers of population doublings, despite low or non-detectable expression levels of telomerase activity [89,101]. This finding indicates that long telomeres in FGF-2 pretreated stem cells may be useful genetic markers for chondrogenic progenitor cells. Low expression of telomerase activity might be explained by the findings that expression of ectopic telomerase expands the life expectancy of BMSCs without impacting the proliferation rate and BMSCs transduced with telomerase display a promoted bone formation capability [102,103]; however, telomerase-deficient mice exhibited impaired differentiation of MSCs, indicating that the low levels of telomerase activity may be necessary to maintain the growth of MSCs [104].

Another potential mechanism is that the chondrogenic potential of MSCs may be generally enhanced by pretreatment with FGF-2 [105], possibly by inducing FGF-receptor 2 and N-Cadherin, early mesenchymal condensation markers, and the key transcription factor Sox9 for chondrogenesis [86,106], in terms of priming mechanisms. FGF-2 treatment during expansion caused significant downregulation of chondrogenic genes [92], but showed robust upregulation of these genes in subsequent chondrogenic induction, resulting in greater matrix production per cell [45]. Proteomics analysis indicated that FGF-2 preconditioning mediated an incomplete dedifferentiation of chondrocytes and an incomplete differentiation of SDSCs as observed by the management of a round of extracellular matrix (ECM) related proteins, both of which enabled expansion of cells that have great chondrogenic potential once seeded in three-dimensional (3D) culture [107]. Another example is that, in extensive MSC monolayer cultures, the level of integrin α10, expressed by chondrocytes in cartilage, was downregulated while the level of integrin α11, expressed by subsets of the fibroblastic lineage, was reversed by FGF-2 treatment, thus keeping MSCs more multipotent and also inducing cell proliferation and *SOX9* upregulation [108].

Considering that Wnt and MAPK signals play critical roles in cartilage regeneration *via* crosstalk [109], FGF-2 pretreated stem cells exhibited an upregulation of secreted frizzled-related protein 1 (*SFRP1*) and downregulation of pregnancy-specific beta-1-glycoprotein 1 (*PSG1*), two typical Wnt signals, and upregulation of angiopoietin 1 (*ANGPT1*) and midkine (*MDK*), two upstream regulators of MAPK signaling [95], indicating that these two signals are also closely associated with the enhancement of chondrogenic potential in FGF-2 expanded cells.

Decellularized ECM (dECM) preconditioning

Increasing evidence indicates that the culture medium of MSCs, which is called secretome or conditioned medium, contains the biological factors secreted by MSCs that could be used in regenerative medicine [110,111]. Furthermore, dECM deposited by stem cells becomes another promising approach to rejuvenate either stem cells or primary cells for chondrogenesis (Table 3) [112].

Evidence of cell proliferation and chondrogenic potential

In 2009, we found that dECM deposited by SDSCs dramatically enhanced porcine SDSC expansion (Fig. 1A/B) and subsequent chondrogenic differentiation in a pellet culture system (Fig. 1C/D/E) [113]. Later we reported that, compared to the negligible rejuvenation

effect of hypoxic preconditioning alone, FGF-2 and dECM preconditioning greatly enhanced porcine SDSC proliferation and chondrogenic potential (Fig. 2A/B) [43]. Compared to the culture substrate provided by a plastic flask, after six-day expansion, FGF-2 treatment resulted in a 2.65-fold increase in cell number while dECM treatment generated a 13.23-fold increase in cell number. The combined treatment of hypoxia, FGF-2, and dECM produced around a 35.81-fold increase in cell number (Fig. 2C). FGF-2 preconditioning yielded SDSC pellets with higher chondrogenic and hypertrophic differentiation while dECM expanded cells yielded pellets with much lower hypertrophic marker expression (Fig. 2D/E/F). Enhanced chondrogenic potential of SDSCs rejuvenated by dECM preconditioning has also been demonstrated in an *in vivo* study in which dECM-expanded SDSCs had better performance than plastic flask-expanded SDSCs in resurfacing partial-thickness cartilage defects of a minipig model *via* boosting type II collagen and sulfated GAG expression [114].

However, the rejuvenation effect of dECM on human adult stem cells, such as BMSCs [115] and SDSCs [116], is not as powerful as shown above for young porcine SDSCs [113], in terms of cell number increase of 2.51-fold (adult BMSCs) versus 2.35-fold (adult SDSCs) versus 17.5-fold (young SDSCs), respectively. Recent evidence indicates that the “aging” of dECM might influence the rejuvenation effect on adult stem cells [117]. A recent report demonstrated that dECM deposited by fetal SDSCs could better rejuvenate human adult SDSCs in both proliferation and chondrogenic potential [118]. Intriguingly, both fetal and adult dECM expanded SDSCs exhibited a decrease of MSC surface markers CD29, CD90, and CD105 in percentage but heavily at the median; an increase of stage-specific embryonic antigen-4 (SSEA4) both in percentage and at the median; and integrin β5 only in percentage. Consistent with the finding by Li et al. [118], Ng et al. found that adult human BMSCs were more proliferative (~1.6×) on fetal dECM than adult dECM and plastic flasks. However, the average Alcian blue staining for sulfated GAG on both fetal and adult dECMs were similar and higher than the plastic flask group but were not significantly different [119]. The authors stated that the lack of difference between the adult dECM and plastic flask groups in cell proliferation was likely due to more conducive plastic flasks used in this study compared to those used by others in previous literature [120,121]. Similarly, both adult and fetal dECM expanded BMSCs showed a decrease of surface markers CD90, CD105, and CD146 in percentage (not shown at the median) [119]. The correlation of known surface marker downregulation and MSC stemness needs to be further elucidated.

Evidence of anti-dedifferentiation and pro-redifferentiation potential

dECM expansion could rejuvenate not only adult stem cells but also primary chondrocytes. Pei and He found that dECM deposited by porcine SDSCs not only greatly enhanced porcine chondrocyte expansion but also delayed dedifferentiation and enhanced redifferentiation capacity up to passage 6 of expanded chondrocytes compared to expansion on plastic flasks where redifferentiation was retained only in the early passages [122]. Dedifferentiated or aged chondrocytes (from passage 4) [123,124] were found to regain their redifferentiation capacity with the aid of dECM expansion [122]. Similarly, Cha et al. found that the proliferation of rat primary chondrocytes grown on dECM was better than those grown on a plastic coverslip (control) or gelatin [125]. Passaged chondrocytes (passage 4)

cultivated on dECM acquired more synthesis of sulfated GAG, which is also reflected in the gene expression level; the dedifferentiating marker, *COL1A1*, was downregulated whereas the ratio between *COL2A1* and *COL1A1* and between *ACAN* and *COL1A1*, as an indicator of redifferentiation, was greatly boosted. This *ex vivo* expansion system also works for the rejuvenation of nucleus pulposus (NP) cells, another chondrocyte-like cell. There is increasing evidence to support that porcine NP cells expanded on dECM grew faster with a tiny size compared with those grown on plastic flasks; dECM pretreated NP cells also acquired a robust redifferentiation capacity [126,127].

Evidence of anti-oxidative and anti-inflammatory potential

Cartilage defects usually accompanied with posttraumatic inflammation present a challenge in cartilage repair and the biological constructs for implantation need to be able to survive this harsh environment [128]. Pei et al. found that expansion on dECM promoted the antioxidative and chondrogenic capacities of human adult SDSCs, slowing down the decrease of cell proliferation and the increase in apoptosis, and contributed SDSCs' resistance to cell-cycle G1 arrest resulting from hydrogen peroxide [116]. Furthermore, dECM preconditioning protected chondrogenically induced human adult SDSCs from interleukin-1 beta (IL-1β) induced inflammatory stress; sb203580 (a p38 MAPK inhibitor) preconditioning promoted dECM rejuvenated human adult SDSCs' ability against inflammation during chondrogenic induction [129].

Potential mechanisms

The mechanisms underlying dECM rejuvenation have not been elucidated. There is increasing evidence indicating that both chemical and physical stimulators in dECM play a critical role in the rejuvenation of expanded cells. He et al. found that the dECM deposited by SDSCs exhibited a nanosized 3D fibrillary structure as shown by scanning electron microscope (SEM) (Fig. 3A/C) with type I collagen as one of the essential structural proteins [113], which might contribute to a physical architecture that relays varied mechanical cues to their resident cells [130]. Furthermore, atomic force microscope (AFM) data suggested that SDSCs grown on rough dECM substrate exhibited greater height and more cell volume but lower Young's moduli compared to those plated on smooth plastic flasks (Fig. 3B/D/E) [131,132]. This finding was in line with another report [118], in which dECM deposited by fetal SDSCs exhibited lower stiffness compared to that from adult SDSCs, which might be associated with the lower expression of elastin in fetal dECM. Interestingly, the stiffness of expanded SDSCs was in line with that of the culture substrate, indicating that lower elasticity in fetal dECM might be associated with enhancement of adult SDSCs' proliferation and chondrogenic potential. This finding and others [133,134] support that matrix elasticity might contribute to lineage-specific differentiation.

Li et al. found that not only biomechanical impact but also chemical composition of dECM might play a role in promoting cell proliferation and differentiation potential [118]. Proteomics data (Table 4) showed that fetal dECM had advantageous expression of fibrilin-2, tenascin C, and clusterin over adult dECM. Both fibrillin-2 and tenascin C are actively involved in tissue regeneration [135–138] while a fair amount of clusterin found in fetal dECM might be responsible for less apoptosis detected in fetal dECM expanded adult

SDSCs since clusterin could inhibit apoptosis by interfacing with activated Bax [139]. On the other hand, adult dECM had more biglycan, decorin, dermatopontin, elastin, periotin, thrombospondin-1, and TGF-β1 than fetal dECM. Biglycan, decorin, and thrombospondin-1 were reported to inhibit cell proliferation [140,141] while dermatopontin, periotin, and TGF-β1 promoted cell differentiation [142–144], indicating that, compared to the action of fetal dECM on cell expansion, adult dECM contained extra matrix components preferring cell differentiation.

As critical pathways for chondrogenesis, non-typical changes of both MAPK and Wnt signals were reported in dECM mediated stem cell chondrogenesis [118]. The data showed that dECM preconditioning resulted in p-Erk downregulation in the cell expansion phase, upregulation in the condensation phase, and downregulation after 10-day chondrogenic induction. This trend was evident in those cells expanded using fetal dECM. The early downregulation of p-Erk expression was associated with a decline in cell senescence [145] but the later downregulation facilitated better chondrogenic differentiation because p-Erk signals during induction promoted chondrogenic differentiation at the early stage but inhibited it at the later stage [146]. The adaptation of p-Erk expression in expanded stem cells following dECM preconditioning and subsequent removal might be responsible for the rejuvenation in cell proliferation and chondrogenic differentiation. dECM pretreatment was also reported to downregulate Wnt3a, a typical canonical Wnt signal, while upregulating Wnt5a and Wnt11, two typical noncanonical signals, in expanded SDSCs [118]. This finding is contradictory to previous reports, in which Wnt3a stimulated MSC proliferation [147] while Wnt5a and Wnt11 mainly promoted cell migration and differentiation [148,149]. The contribution of dECM on stem cell proliferation and chondrogenic potential needs to be further elucidated.

Other factors for preconditioning

Two dimensional culture conditions for MSC expansion, including plating density and culture media and term, play a critical role in governing the chondrogenic potential of MSCs [150–152]. Low seeding density or formulation of the base medium also could promote progenitor cells' chondrogenic potential [85,89,153]. For instance, Li et al. found that expansion at a low seeding density (30 cells/cm^2) yielded human adult SDSCs with enhanced proliferation and chondrogenic differentiation capacity compared to those grown at a high seeding density (3000 cells/cm^2); downregulation of Erk1/2 and c-Jun N-terminal kinases (Jnk) expression and upregulation of p38 MAPK level might be associated with the retained stemness in the cells expanded at low density [151]. However, there also exists a conflicting report. Neuhuber et al. found that the initial seeding density was not critical for retaining a well-defined, multipotent MSC population despite the fact that a plating density of 200 cells/cm^2 favored rat BMSC growth compared to either 20 or 2000 cells/cm2 [154]. They also found that cell expansion from all seeding densities developed an increased proportion of flat cells over passaging.

Conclusion and Perspectives

Finding straightforward and efficient strategies for promoting *in vivo* survival and benefiting differentiation of transplanted stem cells is important for the success of stem cell based tissue regeneration. Adult stem cells are promising sources for tissue regeneration but present challenges by becoming senescent during *ex vivo* expansion as well as having a harsh environment for transplantation. An increasing number of studies on preconditioning strategies to refine *ex vivo* expansion microenvironment for promoting engraftment [155], homing [156], and viability [157–159] after stem cell transplantation, particularly for cardiac tissue regeneration [155,159], have been published. Different from the above-mentioned strategies for cell preconditioning, this paper, for the first time, summarizes and assesses current efforts at altering the *ex vivo* microenvironment *via* hypoxia, soluble factors, and/or dECM to improve stem cell survival and chondrogenic potential post-transplantation.

Besides the potential mechanisms discussed in each section, epigenetic changes are also proposed to play a critical role in this microenvironment mediated cell rejuvenation rather than genetic manipulation (Fig. 4) [160]. For instance, hypoxic stress often results in changes of gene expression that are affiliated with adaptations in chromatin structure by histone modifying and chromatin remodeling complexes [161,162]. Hypoxia also triggers microRNAs in the regulation of vascular endothelial growth factor (VEGF) for angiogenesis, some of which are downstream effectors of HIFs [163]; induction of HIF-1α in hypoxic preconditioned stem cells caused upregulation of miR-210 and this cytoprotective effect of hypoxic preconditioning could be negated by inhibition of HIF-1α or miR-210 [164]. This evidence indicates that hypoxic stress can cause epigenetic adaptation that, in adult stem cells, is dedicated not only to maintain cell stemness but also to drive cell differentiation [160]. Unfortunately, there are few reports to elucidate the rejuvenation effect of environmental preconditioning on adult stem cell proliferation and chondrogenic potential. Still in its infancy, the study of epigenetic effect on stem cells' rejuvenation deserves further in-depth investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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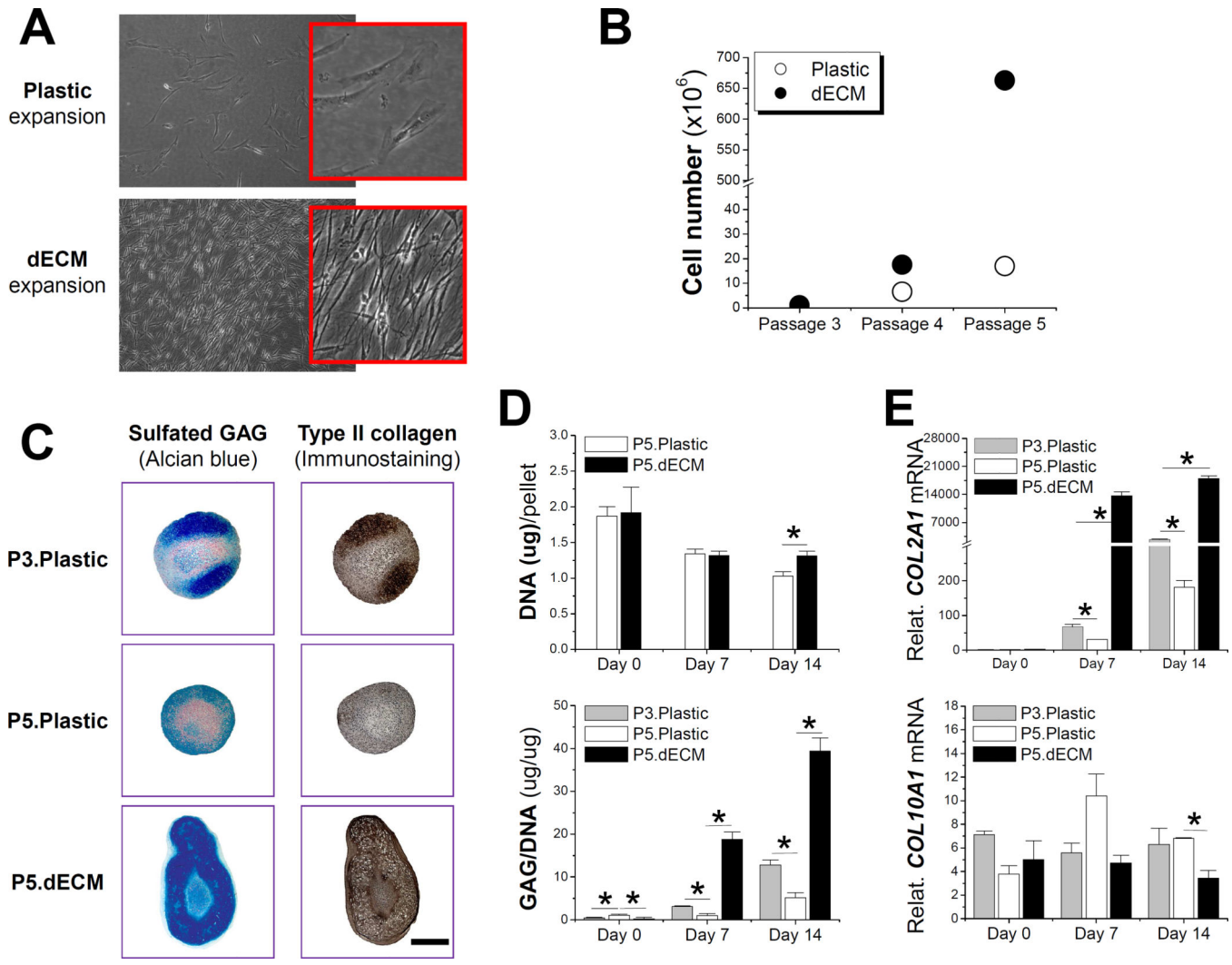

Fig. 1.

Effect of decellularized extracellular matrix (dECM) deposited by synovium-derived stem cells (SDSCs) on porcine SDSCs' proliferation and chondrogenic differentiation. (A) Cell morphology five days after expansion on dECM and Plastic flasks; (B) Cell proliferation from passage 3 (P3) SDSCs grown on either dECM or Plastic flasks for two consecutive passages; (C) Alcian blue staining for sulfated GAG and immunostaining for type II collagen (scale bar: 800 mm) of 14-day chondrogenically induced SDSCs in a pellet culture system after two passages on dECM (P5.dECM) or Plastic flasks (P5.Plastic) with pre-expansion SDSCs (P3.Plastic) as a control; (D) Biochemical analyses were used to detect DNA content per pellet and ratio of GAG to DNA; (E) TaqMan real-time polymerase chain reaction (PCR) was used to quantitatively assess chondrogenic markers - *COL2A1* (type II collagen) and *COL10A1* (type X collagen). * indicates a statistical difference (p<0.05). Data are shown as average ± SD for n=6 in biochemical analyses and n=5 in real-time PCR. Reprint with permission from He, F.; Chen, X.; Pei, M. *Tissue Eng. Part A* **2009**, 15, 3809. Copyright (2009) Mary Ann Liebert, Inc. Publications.

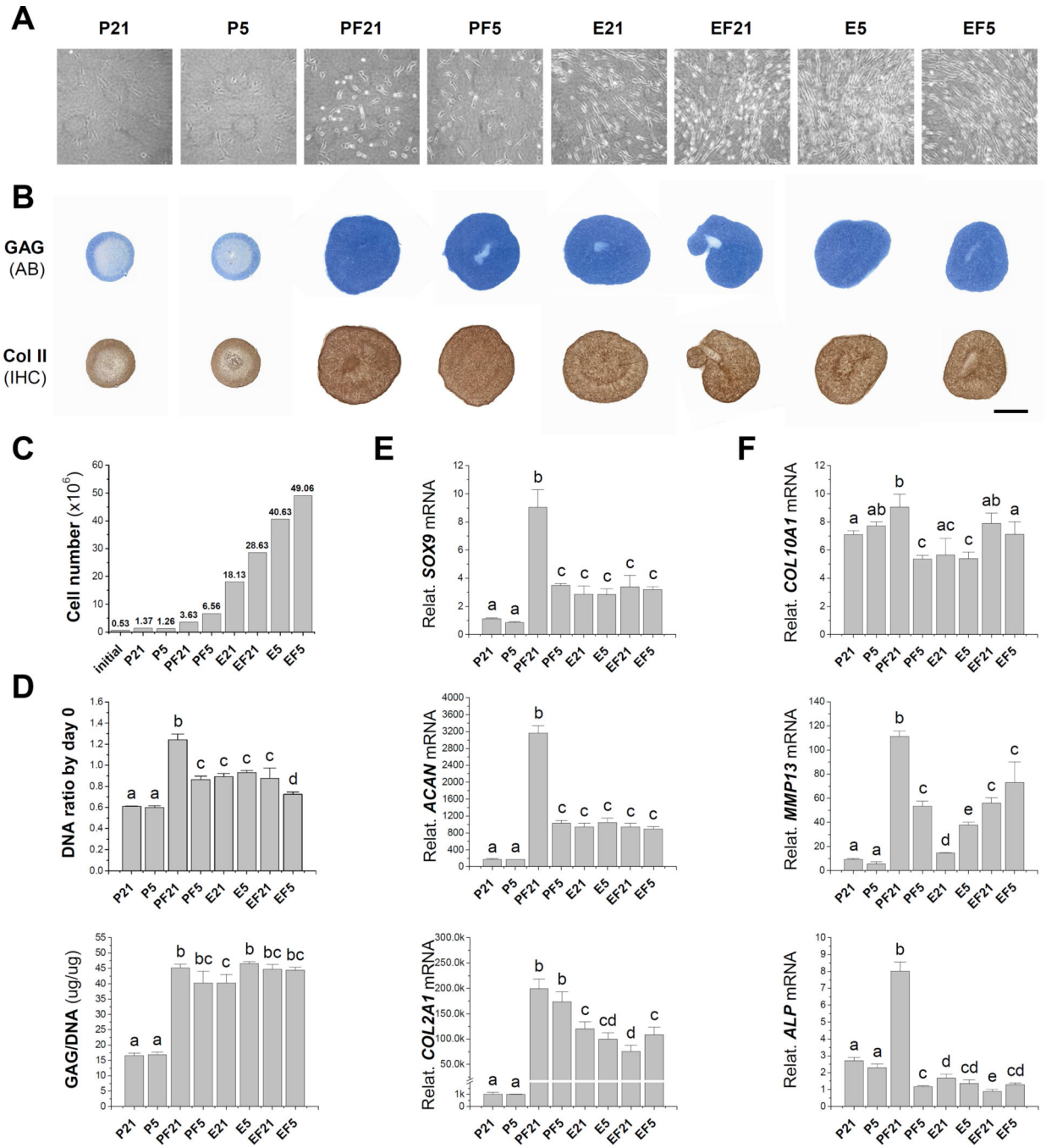

Fig. 2.

Optimization of preconditioning strategies to rejuvenate stem cells' chondrogenesis. (A) Synovium-derived stem cell (SDSC) morphology after five-day expansion on dECM ("E") or Plastic flasks ("P") in hypoxia (5% O_2, "5") or normoxia (21% O_2, "21") with or without 10 ng/mL of fibroblast growth factor-2 ("F"). (B) Alcian blue (AB) staining for sulfated GAG and immunohistochemistry (IHC) staining for type II collagen (scale bar: 800 mm) of 14-day chondrogenically induced SDSCs in a pellet culture system after one passaging culture with varied pretreatments. (C) Cell number increase after a six-day expansion with

initial cell number as 0.53×10^6 in one 175 cm^2 flask. (D) Biochemical analyses after a 14-day chondrogenic induction in a pellet culture system including DNA content per pellet (adjusted by day 0) and ratio of GAG to DNA. TaqMan real-time PCR analyses of chondrogenically induced pellets including chondrogenic markers [*SOX9* (SRY (sex determining region Y)-box 9), *ACAN* (aggrecan), and *COL2A1* (type II collagen)] (E) and hypertrophic markers [*COL10A1* (type X collagen), *MMP13* (matrix metalloproteinase 13), and *ALP* (alkaline phosphatase)] (F). *18S RNA* was used as an internal control. Groups not connected by the same letter are significantly different ($p < 0.05$). Data are shown as average ± SD for n=4. Reprint with permission from Li, J.; Pei, M. *Tissue Eng. Part A* **2011**, 17, 703. Copyright (2011) Mary Ann Liebert, Inc. Publications.

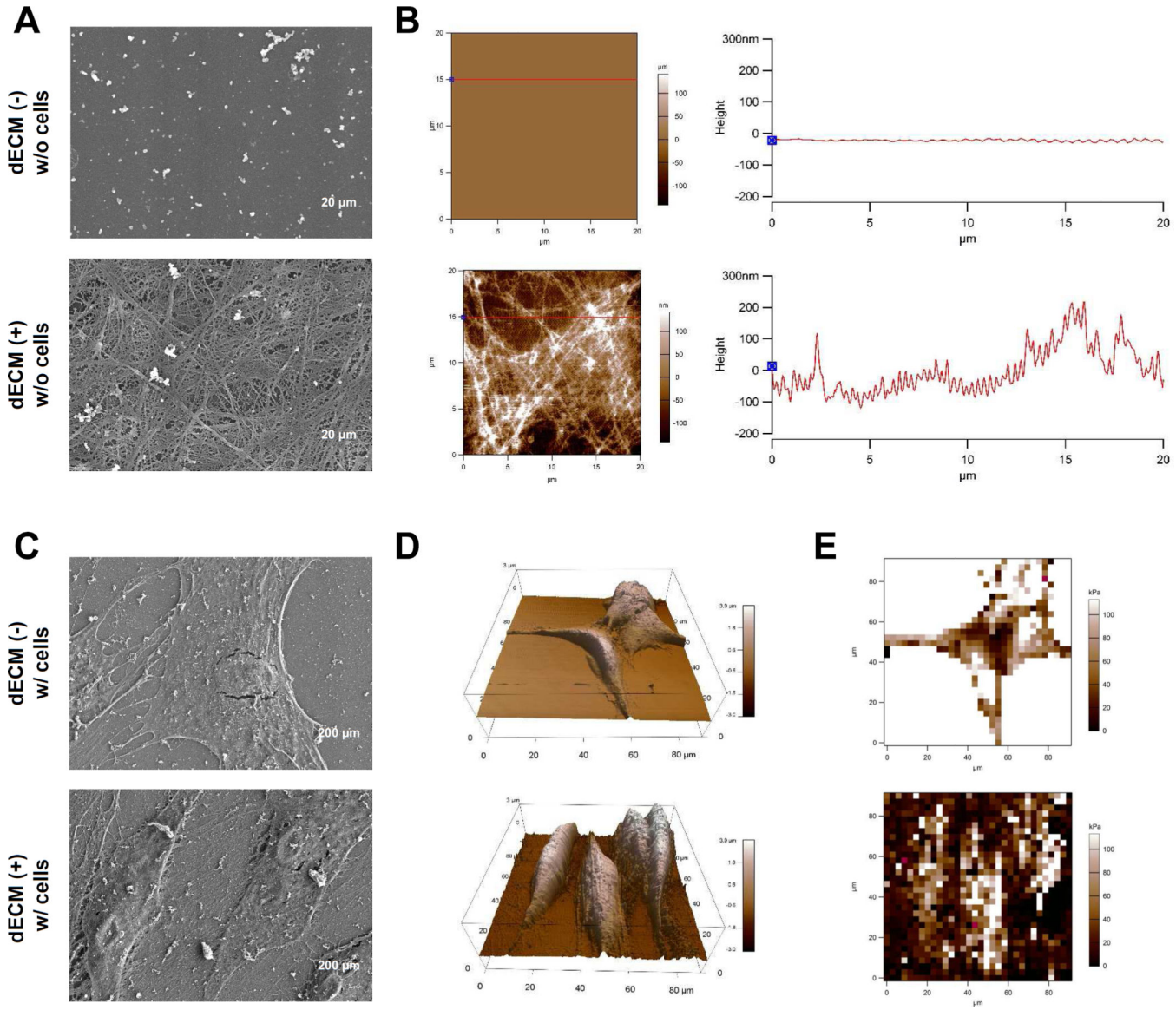

Fig. 3.

Morphological characterization of culture substrates and expanded cells. Surface topography of the two substrates, Plastic (flasks) and dECM, was characterized using scanning electron microscope (SEM) (A) and atomic force microscope (AFM) (B). Scale bar for SEM (A): 20 μm. Expanded SDSCs on either Plastic or dECM after fixation in glutaraldehyde were characterized using SEM (C) and AFM (D) for morphology and using AFM (E) for elasticity. Scale bar for SEM (C): 200 μm. Reprint with permission from Zhang, Y.; Li, J., Davis, M.E., et al. *Acta Biomater* **2015**, 20, 39. Copyright (2015) Elsevier Publications.

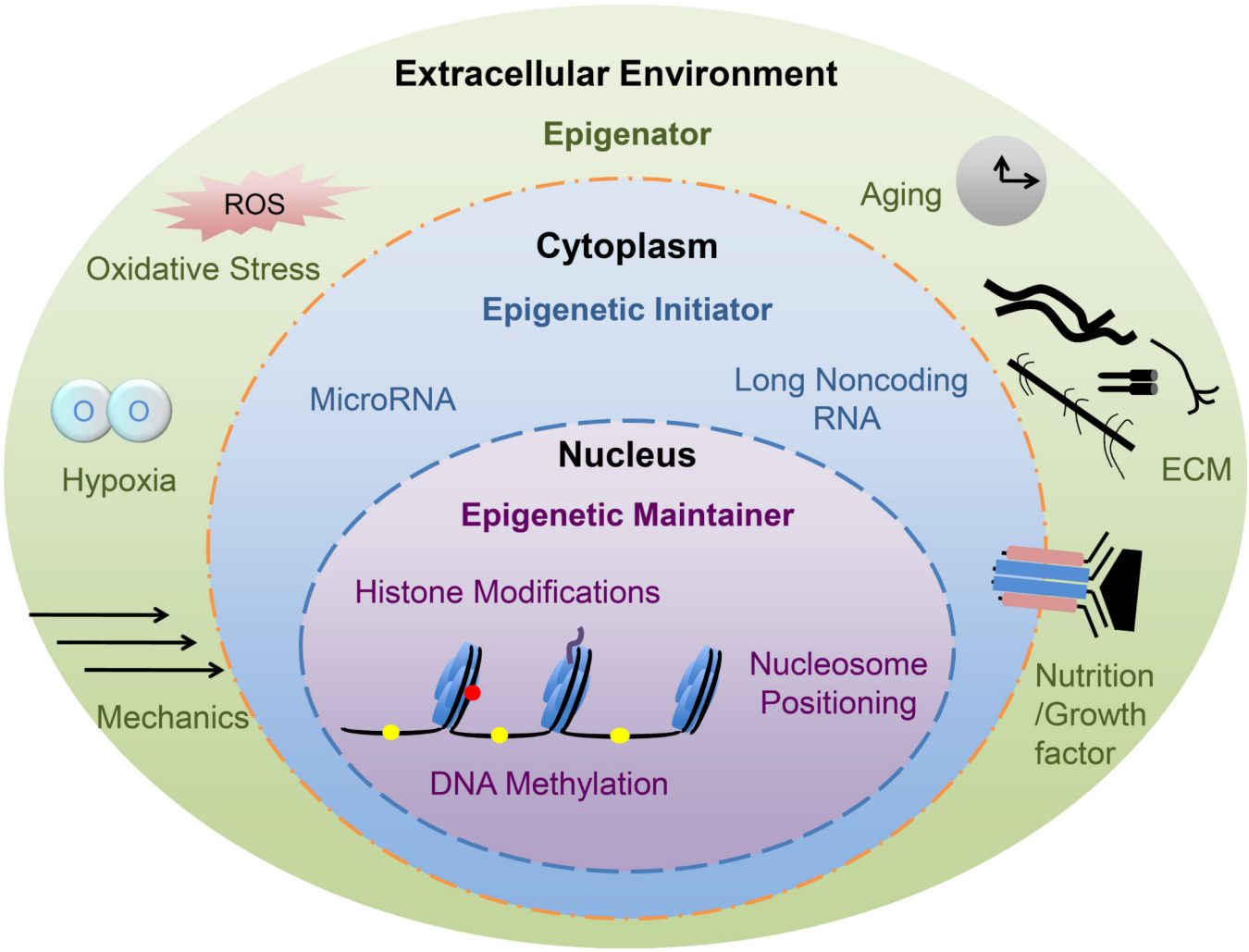

Fig. 4.

The landscape view of epigenetic events during cartilage regeneration. Reprint with permission from Li, J., Ohliger, J., Pei, M. *Stem Cell Dev.* **2014**, 23, 1178. Copyright (2014) Mary Ann Liebert, Inc. Publications.

Table 1

Hypoxia primed adult stem cells for chondrogenesis.

MSC	Passage	O_2 %	Proliferation	Chondro-	others	References
hASC	1	1,5,10,15	↑ CFU-F	↓ (pellet)	-	44
hASC	3	2 vs 21	↑ stemness/cell number; ↓ PDT	↑ (pellet)	↓ osteo- /adipo-	34
mASC	2	2 vs 21	↑ cell number/BrdU; ↓ PDT	↑ (pellet)	↓ osteo-	35
hBMSC	2	3 vs 21	↑ number of cell colonies	↑ (pellet)	-	42
hBMSC	-	4 vs 20	-	↑ (pellet/gelatin/gelatin+HA)	-	41
hBMSC	2	5 vs 21	↑ CFU-F/cell number	↑ (pellet) (ns)	osteo- (nc); ↓ adipo- (ns)	38
hBMSC	4	1.5 vs 21	↑ cell number	↑ (pellet)	nc	39
oBMSC	2	5 vs 20	↑ CFU-F; ↓β-gal/cell size	↑ (pellet/collagen I hydrogel)	-	37
oBMSC	2	5 vs 20	↑ number and size of cell colonies	↑ (pellet)	-	36
hIFPSC	2	5 vs 20	-	↑ (pellet)	-	45
gDSC	-	5 vs 20	↓ cell number/CFU-F	↑ (pellet)	-	40
pSDSC	3	5 vs 21	↑ cell number; ↓ cell size	nc	-	43
hUCB CD133(+)	1	1.5 vs 21	↑ clonogenic myeloid capacity	-	-	39

Abbreviation: CFU-F: colony forming unit-fibroblast; DSCs: dermis isolated stem cells; g: goat; h: human; HA: hyaluronan; nc: no change; ns: no significant difference; o: ovine/sheep; p: porcine; PDT: population doubling time; stemness markers: REX1, SOX2, OCT4, and NANOG; UCB: umbilical cord blood

Table 2

Growth factor primed stem cells for chondrogenesis.

MSC	Passage	Dose (ng/mL)	Proliferation	Chondro-	Adipo- /osteo-	Reference
mASC	1	5, 10, 50, 100 (F)	↑ cell number	↑ (pellet)	-	86
hBMSC	2–4	10 (F)	↑ growth index	↑ (pellet)	No difference	87
hBMSC	1–7	10 (F)	↑ PD	↑ (pellet)	-	95
hBMSC	2–4	1, 10 (F)	n/a	↑ (pellet)	-	165
hBMSC	-	1, 5, 10 (F)	↓ cell size & PDT	↑ (pellet)	-	92
hBMSC	2	5 (F)	-	↑ (pellet)	-	106
hBMSC	-	1 (F)	↑ TRF length; ↓ PDT	↑ (pellet)	-	89
hBMSC	1–2	10 (F)	-	↑ (pellet)		108
hBMSC	1	1 (F)	↑ colony size; ↓ colony number	↑ (pellet)	↑ osteo-	85
hBMSC	-	1 (F)	↑ PD	↑ (pellet)	-	101
hIFPSC	2	10 (F)	↑ cell number	↑ (pellet)	-	88
pIFPSC	1–2	5 (F)	↓ PDT	↑ (agarose hydrogel)	-	96
bSDSC	4	1 (T) + 5 (F) + 10 (P)	-	↑ (agarose disc)	-	97
dSDSC	2	1 (T) + 5 (F) + 10 (P)	↓ PDT	↑ (pellet)	-	107
hSDSC	1–2	0.1, 1, 10, 100 (F)	↓ cell size; ↑ cell number	↑ (pellet)	-	91
pSDSC	3	10 (T) + 50 (F) + 500 (I)	-	↑ (pellet)	-	166
pSDSC	3	10 (F)	↑ cell number; ↓ cell size	↑ (pellet); hypertrophy	-	43

Abbreviation: ASCs: adipose stem cells; BMSCs: bone marrow stromal cells; b: bovine; d: dog; F: basic fibroblast growth factor; h: human; I: insulin-like growth factor I; IFPSCs: infrapatellar fat pad derived stem cells; m: mouse; p: porcine; P: platelet-derived growth factor-BB; PD: population doubling; PDT: population doubling time; SDSCs: synovium-derived stem cells. TRF: telomere restriction fragment; T: transforming growth factor beta 1

Table 3

dECM primed stem cells for chondrogenesis.

MSC	Passage	dECM	Proliferation	Chondro-	Adipo- /osteo-	Reference
hBMSC	2, 5	Basement membrane-like ECM by PYS-2 cells, endothelial cells	↑cell number	↑(pellet)	↑adipo- osteo-	167
hBMSC	4	Chondrocyte-collagen microsphere	-	↑(pellet)	-	168
hBMSC	2, 5, 10	Mouse laminin 1, mouse laminin 5, or mouse collagen IV	↑cell number ↑CFU-F colony number and size	↑ (pellet) except laminin 5	↑adipo- /osteo-	169
hBMSC	7, 9	Human fetal BMSCs	↑cell number	↑	↑adipo- /osteo-	119
hBMSC		MC3T3 preosteoblasts			↑osteo-	34
hBMSC		NIH3T3 fibroblasts		↑(pellet)		34
hSDSCs	3	Human SDSCs	↑cell number and PI	↑ (pellet)	-	116,129,132
hBMSC	8	Human USCs		↑(pellet)	-	170
hSDSC	3	Human fetal and adult SDSCs	↑cell number and PI	↑(pellet)	↑adipo-,	118
pSDSC	2	Porcine SDSCs	↑p-cyclin D1	↑(pellet) and *in vivo* resurfacing	-	114
pSDSC	3	Porcine SDSCs, NPCs, and SDSCs/NPCs (1:1)	↑cell number	↑(pellet)	-	127
pIPFSC	3	Porcine SDSCs and IPFSCs	↑cell number	↑(pellet)	↑adipo-	171
hfSDSC	7	Human fetal SDSCs	↓β-gal	↑(pellet)	-	172
hBMSC	5	Human BMSCs	↑cell number	↑(pellet)	↑osteo-, ↓adipo-	115
pSDSC	3	Porcine SDSCs	↑cell number; ↓ cell size	↑(pellet)	-	43
pSDSC	3	Porcine SDSCs	↑cell number	↑(pellet)	↑adipo-, ↓osteo-	113

Abbreviation: BMSCs: bone marrow stromal cells; CFU-F: colony-forming unit-fibroblast; dECM: decellularized extracellular matrix; f: fetal; h: human; IFPSCs: infrapatellar fat pad derived stem cells; NPC: nucleus pulposus cell; p: porcine; PI: proliferation index; SDSCs: synovium-derived stem cells

Table 4

Select ECM and ECM interacting proteins identified in fetal and adult ECMs.

Swiss-Prot Accession	Select ECM Proteins	Assigned Spectra		% of protein in the insoluble pellet	
		FE	AE	FE	AE
P98160	Perlecan	91	95	22%	51%
P21810	Biglycan	37	95	14%	53%
P07585	Decorin	40	95	65%	54%
Q07507	Dermatopontin	16	35	100%	66%
P15502	Elastin	2	18	100%	89%
Q9Y6C2	EMILIN-1	131	113	1%	25%
Q9BXX0	EMILIN-2	35	46	46%	43%
P35555	Fibrillin-1	394	529	39%	53%
P35556	Fibrillin-2	76	2	0%	0%
P02751	Fibronectin	767	873	29%	43%
P23142	Fibulin-1	5	4	0%	50%
P98095	Fibulin-2	149	150	68%	63%
Q96RW7	Fibulin-6 (hemicentin-1)	1	12	0%	0%
P09382	Galectin-1	22	12	0%	0%
P55001	Microfibrillar-assoc. prot. 2	39	47	85%	81%
Q13361	Microfibrillar-assoc. prot. 5	28	37	86%	68%
P20774	Mimecan	16	6	0%	17%
Q14112	Nidogen-2	7	6	0%	0%
Q15063	Periostin	125	400	15%	57%
P24821	Tenascin	259	120	3%	36%
P22105	Tenascin-X	59	49	10%	0%
P07996	Thrombospondin-1	6	79	33%	68%
Q15582	TGFBI	164	258	45%	44%
P13611	Versican core protein	58	22	74%	50%

Swiss-Prot Accession		Assigned Spectra		% of protein in the insoluble pellet	
	Collagens	**FE**	**AE**	**FE**	**AE**
P02452	Collagen alpha-1(I) chain	250	494	11%	86%
P08123	Collagen alpha-2(I) chain	206	339	6%	59%
P02461	Collagen alpha-1(III) chain	41	31	0%	81%
P20908	Collagen alpha-1(V) chain	18	36	28%	47%
P05997	Collagen alpha-2(V) chain	7	12	0%	75%
P12109	Collagen alpha-1(VI) chain	602	799	79%	68%
P12110	Collagen alpha-2(VI) chain	634	633	87%	73%
P12111	Collagen alpha-3(VI) chain	3019	3542	67%	56%
Q99715	Collagen alpha-1(XII) chain	798	1563	30%	64%
Q05707	Collagen alpha-1(XIV) chain	163	47	17%	34%
	Additional Proteins				
Q6UY14	ADAMTS-like protein 4	8	18	0%	0%
P10909	Clusterin	5	0	0%	
Q08397	Lysyl oxidase homolog 1	22	23	36%	35%
P21980	Transglutaminase 2	18	44	28%	30%