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Review Article (Invited)

Guideline for design of substrate stiffness for mesenchymal stem cell culture based on heterogeneity of YAP and RUNX2 responses

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Mesenchymal stem cells (MSCs) have the potential for self-renewal and multipotency to differentiate into various lineages. Thus, they are of great interest in regenerative medicine as a cell source for tissue engineering. Substrate stiffness is one of the most extensively studied exogenous physical factors; however, consistent results have not always been reported for controlling MSCs. Conventionally used stiff culture substrates, such as tissue-culture polystyrene and glass, enhance nuclear localization of a mechanotransducer YAP and a pre-osteogenic transcription factor RUNX2, and bias MSCs towards the osteogenic lineage, even without osteogenic-inducing soluble factors. The mechanosensitive nature and intrinsic heterogeneity present challenges for obtaining reproducible results. This review summarizes the heterogeneity in human MSC response, specifically, nuclear/cytoplasmic localization changes in the mechanotransducer yes-associated protein (YAP) and the osteogenic transcription factor RUNX2, in response to substrate stiffness. In addition, a perspective on the intracellular factors attributed to response heterogeneity is discussed. The optimal range of stiffness parameters, Young's modulus, for MSC expansion culture to suppress osteogenic differentiation bias through the suppression of YAP and RUNX2 nuclear localization, and cell cycle progression is likely to be surprisingly narrow for a cell population from an identical donor and vary among cell populations from different donors. We believe that characterization of the heterogeneity of MSCs and understanding their biological meaning is an exciting research direction to establish guidelines for the design of culture substrates for the sophisticated control of MSC properties.

Key words: osteogenic differentiation, regenerative medicine, mechanotransducer

◀ *Significance* ▶

Heterogeneity is an inevitable feature of biological system. This review summarizes heterogeneity in a human mesenchymal stem cell response, specifically, nuclear/cytoplasmic localization changes in a mechanotransducer, YAP and an osteogenic transcription factor, RUNX2, which are associated with osteogenic differentiation, in response to the substrate stiffness. We found that the optimal range of stiffness parameter for expansion culture to satisfy suppression of unintended osteogenic differentiation and cell cycle progression is likely to be surprisingly narrow and varies with cell population. The response heterogeneity will be a basis for the design of culture substrate for sophisticated control of cellular properties.

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Introduction

Technology for reconstituting functional cells, tissues, and organs *in vitro* is fundamental to regenerative medicine. Mesenchymal stem cells (MSCs) have the potential for self-renewal and multipotency to differentiate into various tissue lineages, including bone, cartilage, fat, and muscle [1]. Mesenchymal stem cells can differentiate into a specific cell type under specific conditions *in vitro* and limited microenvironments *in vivo*; thus, MSCs are of great interest in stem cell biology and regenerative medicine as a cell source for tissue engineering [2]. One critical unresolved issue is heterogeneity in MSCs closely correlated with therapeutic efficacy, such as differentiation potential, proliferation capacity, and ability to secrete cytokines [3].

Innate factors, such as donor and tissue source variations, are attributed to MSC heterogeneity [4]. Additionally, long-term culture is indicated to readily induce MSC heterogeneity [5]. Lineage commitment with the loss of self-renewal and multilineage potential at early stages of MSC differentiation *in vitro* is induced by physical factors, such as stiffness [6], topography [7,8], geometrical constraints [9,10], and the extracellular matrix proteins and the way of their binding [11,12] on the substrate, and cell density [13], as well as the soluble chemical factors. This review focuses on substrate stiffness, which is one of the most extensively studied exogenous physical factors but has not always reported consistent results for controlling human MSCs. Specifically, heterogeneity of MSC response to the substrate stiffness is considered in terms of the mechanical signaling of a mechanotransducer, yes-associated protein (YAP) [14-16], and a master osteoblast transcription factor, Runt-related transcription factor 2 (RUNX2) [17].

In the signaling in response to the substrate stiffness, YAP translates information of the substrate stiffness into protein expression by translocation to the nucleus and regulate transcription [14,15]. YAP indirectly sense and respond to changes in the mechanical environment of the cell by monitoring the actin cytoskeleton that is highly responsive to mechanical stresses experienced by the cell [18]. YAP is inactivated when actin filaments are disrupted or when Rho GTPase is inhibited, whereas actin filament polymerization correlates with increased activity of YAP with its nuclear localization [19]. In the nucleus, YAP serves as a co-regulator for transcription factors that have crucial roles for bone homeostasis, including RUNX2 [20]. YAP binds to a short oligopeptide called the proline/tyrosine (PY) motif that presents in the transcription activation domains of RUNX2 [21]. The PY motif in combination with the functional nuclear matrix-targeting signal of RUNX2 is vital for targeting of the RUNX2-YAP complex to subnuclear sites where activation and repression take place, while the nuclear import of YAP is independent of RUNX2 [22]. The regulatory effects of YAP on RUNX2 vary, and sometimes positive and other times negative, depending on context: positive effects are promotion of *Runx2* expression [23] and RUNX2 activity, whereas negative one is repression of RUNX2-enhanced promoter activity of osteocalcin, which promotes osteogenesis and inhibits adipogenesis, as a cofactor to RUNX2 binding sites [22,24-26]

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Mesenchymal Stem Cell Lineage Commitment According to Substrate Stiffness

Human MSCs cultured on soft, collagen-coated polyacrylamide gels that mimic brain elasticity (Young's modulus, $E = 0.1–1$ kPa) can specify lineage towards neurons with no soluble induction factor [6]. Ten-fold stiffer gels that mimic striated muscle elasticity ($E = 8–17$ kPa) are myogenic, and further stiff gels that mimic collagenous bone ($E = 25–40$ kPa) or higher are osteogenic [6] (Fig. 1). Mesenchymal stem cell culture on tissue-culture polystyrene (TCPS; $E \sim 3$ GPa)
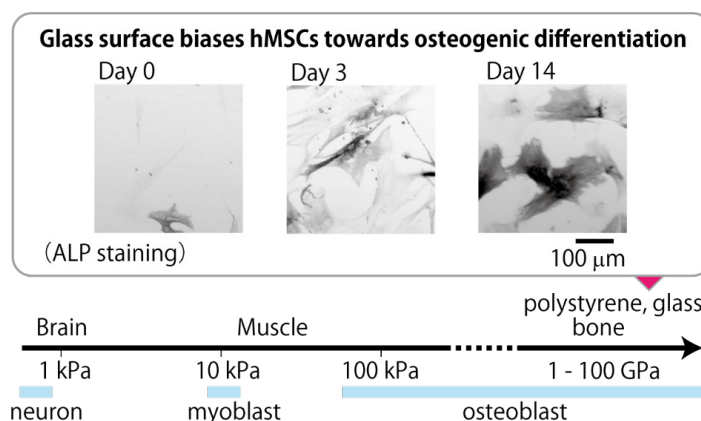

Figure 1 Mesenchymal stem cell lineage specification according to substrate stiffness. The micrographs are adapted from Yamazaki et al. 2021 [28].

activates the nuclear translocation of RUNX2, i.e., biases MSC towards the osteogenic lineage [23]. RUNX2 expression is also indicated in human myogenic progenitor cells [27], thus, myogenic differentiation bias could also be caused due to the MSC culture using the substrate with the stiffness to induce RUNX2 nuclear localization. RUNX2 nuclear localization is reversible for 1 day of culture on tissue-culture polystyrene after 3 days of culture on soft ($E \sim 2$ kPa) substrates, whereas it is irreversible for 7 days of TCPS culture [23]. The mechanosensitive nature together with intrinsic heterogeneity makes it challenging to obtain reproducible results by maintaining stemness in the growth of MSCs during all phases from basic research to clinical application.

Characteristics of YAP and RUNX2 Responses in Inter-Cell Populations from Different Donors and Intra-Cell Population from a Single Donor of Mesenchymal Stem Cell to Substrate Stiffness

YAP [14-16], and RUNX2 [17] are the key mediators of mechanical signaling to osteogenic lineage bias in response to mechanical cues from stiff substrates [23]. The intracellular localization of YAP and RUNX2 changes depending on the Young's modulus of the substrate and affects the transcriptional activities of genes that direct the lineage commitment of MSC. YAP and RUNX2 are localized in the nucleus of cells cultured on stiff substrates, whereas in extremely soft substrates, YAP and RUNX2 are localized in the cytoplasm of MSCs (Tables 1, 2). The threshold value of Young's modulus, E, is consistent for YAP among the studies (Table 1). Regarding RUNX2, the threshold values of E to switch their nuclear and cytoplasmic localization are different ranging from 1 to 10 kPa on order among studies (Table 2). One of the reasons for this inconsistency is attributed to the substrates. The materials used in stiffness studies may affect the surface chemistry, backbone flexibility, and binding properties of the adhesive ligands of the substrate, in addition to their bulk stiffness and porosity [29]. Another reason for the lack of consistency in the effects of substrate stiffness is the heterogeneity of MSC populations. This review specifically focuses on the heterogeneity of YAP and RUNX2 responses of MSC to substrate stiffness at the inter- and intra-cell population levels.

Table 1 Substrate stiffness to switch the response of YAP

Material	surface	Response type, substrate stiffness, E	reference
poly(ethylene glycol) hydrogel	RGD peptide	intranuclear localization, ≥ 4 kPa, maximum at 6 kPa cytoplasmic localization, at 2 kPa	[23]
poly(ethylene glycol) hydrogel	RGD peptide	intranuclear localization, ≥ 11 kPa	[30]
poly(ethylene glycol) hydrogel	RGD peptide	intranuclear localization, at 32.7 kPa diffusing, at 5.5 kPa	[31]
polyacrylamide gel	fibronectin	intranuclear localization, at 38 kPa cytoplasmic localization, at 3 kPa	[32]
polyacrylamide gel	collagen I	intranuclear localization, at 20 kPa diffusing, at 1 kPa	[33]
polydimethylsiloxane	collagen I	intranuclear localization, at 32 kPa cytoplasmic localization, at 0.5 kPa	[34]

Table 2 Substrate stiffness to switch the response of RUNX2

Material	surface	Response type, substrate stiffness, E	reference
poly(ethylene glycol) hydrogel	RGD peptide	intranuclear localization, ≥ 4 kPa, maximum at 6 kPa cytoplasmic localization, at 2 kPa	[23]
Polyacrylamide gel	collagen I	intranuclear localization, at 20 kPa diffusing, at 1 kPa	[33]
Polyacrylamide gel	fibronectin	intranuclear localization, maximum at 30 kPa diffusing, at 20 kPa	[35]
Polyacrylamide gel	fibronectin	intranuclear localization, maximum at 40 kPa diffusing, at 0.5 kPa	[36]

Fig. 2 summarizes the characteristics of YAP and RUNX2 responses in three different populations, each derived from three different donors, with different differentiation potentials of human bone marrow-derived MSCs [37]. The three populations of human MSCs show different levels of competitive calcium and oil droplet deposits, i.e., competitively balanced different osteogenic and adipogenic differentiation potentials (Fig. 2A). Dependence of the percentage of cells with YAP in the nucleus on the substrate Young's modulus, E, in a MSC population from an identical donor is no discrete step-like transition, but a linear increase (Fig. 2B). This change means that the substrate Young's modulus to switch the localization of YAP is different even in MSCs isolated from a single donor. The percentage of cells with YAP in the nucleus increases linearly with an increase in E, reaching saturation with an elastic modulus of 10 kPa or higher. The response characteristics is the same among the different MSC populations from different donors with different differentiation potentials. RUNX2 localization changes in response ffto substrate stiffness are also heterogeneous among MSCs isolated from a single donor (Fig. 2C). The percentage of cells with RUNX2 in the nucleus increases linearly with increasing E, reaching saturation. In contrast to YAP, RUNX2 response characteristics are heterogeneous at the MSC population level, and each MSC population has a unique linear function with different slopes and intercepts. In addition, the Young's modulus, which saturates the percentage of cells with RUNX2 in the nucleus, is $E = 5$ kPa, which is lower than that for YAP, and 20–50% of the cells retained RUNX2 in the nucleus even at 0.8 kPa (leftmost data point of Lot 3 in Fig. 2C), indicating that RUNX2 is robustly retained in the nucleus compared with YAP.

The contradiction of previous reports on RUNX2 localization in $E = 1$–10 kPa substrates may result from differences among the MSC populations in RUNX2 nuclear retentivity and the rate of enhancement of nuclear RUNX2 with substrate stiffness. Specifically, in some MSC populations, the percentage of cells with RUNX2 in the nucleus is more than half for the $E = 4$ kPa substrate, as in lot 1 in Fig. 2C. This is consistent with a previous report that RUNX2 nuclear localization is significantly increased in MSCs cultured on substrates with $E = 4$ kPa [23]. In contrast, in some MSC populations, the percentage of cells with nuclear RUNX2 is about a half for $E = 4$ kPa substrate, as shown in lot 3 in Fig. 2C.

The characteristics of nuclear/cytoplasmic YAP localization depending on substrate stiffness are similar in different MSC populations, regardless of differences in osteogenic and adipogenic differentiation potential [37]. In contrast to YAP,
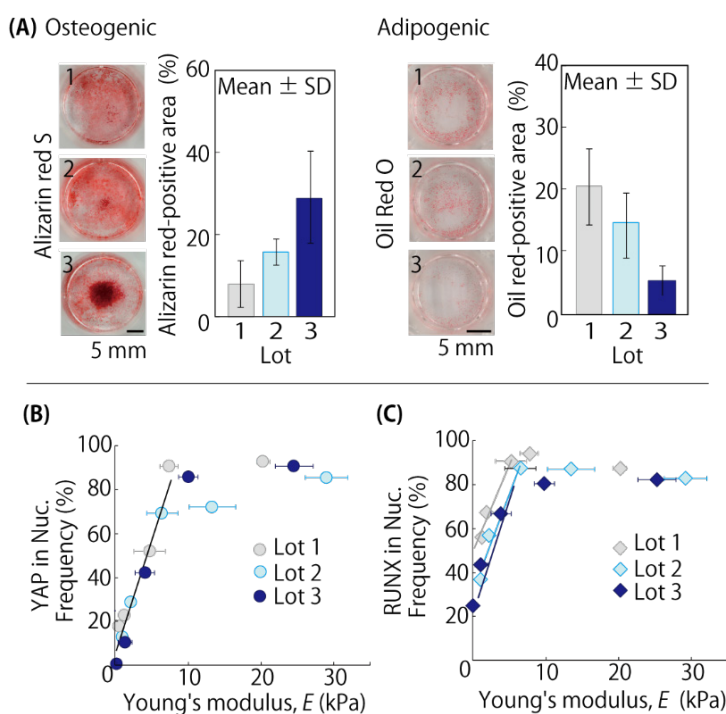

Figure 2 Characteristics of YAP and RUNX2 responses in inter- and intra-cell population of mesenchymal stem cell to styrenated gelatin substrate stiffness. (A) Differentiation potentials of human bone marrow-derived MSCs used for the analysis were evaluated by induction of osteogenic and adipogenic differentiations. The osteogenic differentiation was induced with an osteogenic induction medium (Lonza, Japan) on a tissue culture treated polystyrene plate (Young's modulus, E, is the on the order of GPa) coated with collagen I. The adipogenic differentiation was induced with adipogenic induction and maintenance media (Lonza, Japan) on a tissue culture treated polystyrene plate (Young's modulus, E, is the on the order of GPa). (B) YAP and (C) RUNX2 responses to substrate Young's modulus. The frequencies of cells with (B) YAP or (C) RUNX2 are plotted against Young's modulus. Modified from Yamazaki et al. 2021 [37].

the response characteristics of RUNX2 in terms of nuclear/cytoplasmic localization are different among MSC populations; in particular, the baseline degree of nuclear retentivity of RUNX2 [37], which will correspond to the extrapolated values of the percentage of cells with RUNX2 nuclear localization against 0 kPa in Fig. 2C. RUNX2 is known to be a transcription factor that localizes to the nucleus and regulates osteogenic differentiation-related genes in the early phase of osteogenic differentiation, whereas in the later phase, it needs to be suppressed to form mature bone [17,38,39]. The baseline degree of nuclear retentivity of RUNX2 may be correlated with the efficiency of RUNX2 release in the later phase of osteogenic differentiation in MSCs, for those with sufficient potential for RUNX2 nuclear localization in the early stage.

What Causes the Heterogeneity in RUNX2 Response to Substrate Stiffness Among Mesenchymal Stem Cells?

Runx2 expression is promoted by YAP [23]. The heterogeneity of RUNX2 nuclear localization in the inter-cell population from different donors of MSCs is attributed to downstream of *Runx2* expression because the characteristics of YAP localization change in response to substrate stiffness are the same among different MSC populations.

One presumed factor regulating RUNX2 localization is the actin cytoskeleton, but that seems not just the case but by an interplay of the actin cytoskeleton and other protein signaling that affect the chromatin structure. The development of the actin cytoskeleton has been reported to be positively correlated with substrate stiffness in numerous studies [40-42]. In MSCs cultured on stiff substrates, the actin cytoskeleton is well-developed, and YAP and RUNX2 are localized in the nuclei. Actin disruption by latrunculin B, an actin depolymerization reagent, or Y-27632, a Rho kinase inhibitor, in MSCs cultured on a glass substrate, which is an extremely stiff substrate, result in YAP diffusion (Fig. 3A) [43]. In contrast, RUNX2 is localized in the nucleus (Fig. 3B) [43].

Another candidate for regulating RUNX2 localization is the chromatin structure influenced by histone acetylation through histone deacetylase (HDAC). Mechano-signals from soft substrates have been suggested to affect the chromatin condensation state HDAC, although specific contributions have contradicted each other [31,44]. Human umbilical cord MSCs cultured on soft ($E = 1.5$ and 15 kPa) PDMS substrates for 7 days showed less developed actin cytoskeleton, more relaxed nuclei, more euchromatic, and less heterochromatic nuclear DNA regions, compared with the culture condition using TCPS and glass substrate, where the involvement of decrease in HDAC is indicated [44]. Another group has reported that 3 days of culture of human bone marrow-derived MSCs on $E = 5$ kPa polyethylene glycol (PEG)-based hydrogel decreases histone acetylation with an increase in HDAC activity and changes chromatin to more heterochromatic nuclear
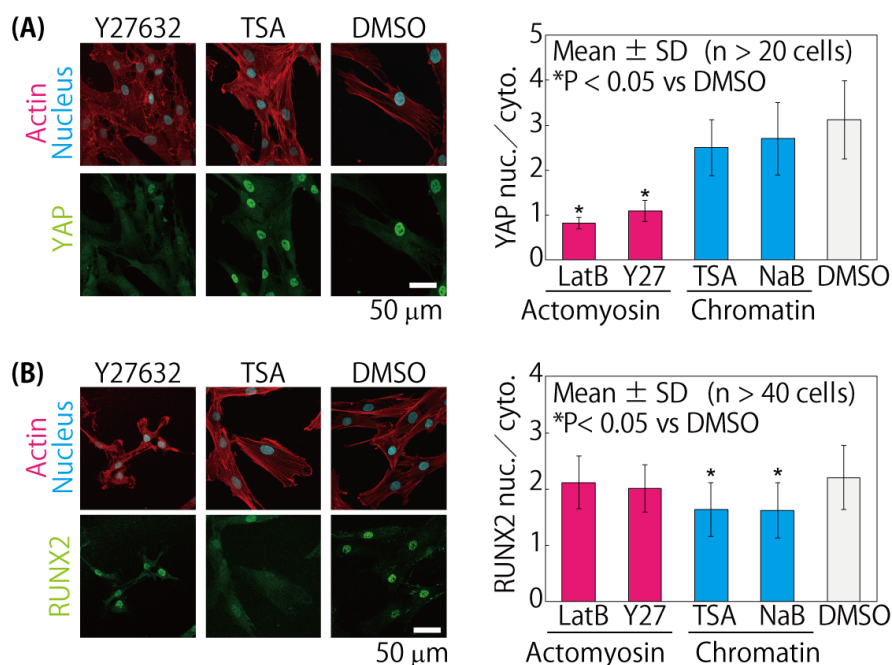

Figure 3 Cellular factors affecting (A) YAP and (B) RUNX2 nuclear/cytoplasmic localization. The effect of disturbance of actomyosin are evaluated by actin polymerization inhibitor, latrunculin B (Lat B), and by Rho-associated coiled-coil containing protein kinase (ROCK) inhibitor, Y27632 (Y27). The effect of chromatin decondensation is evaluated by histone deacetylase inhibitors, torichostatin A (TSA), and sodium borate (NaB). Modified from Yamazaki et al. 2020 [43].

DNA regions compared with the nuclei on stiffer $E = 32.7$ kPa substrate [31]. A study using histone deacetylase inhibitors, trichostatin A, and sodium borate, which induce chromatin decondensation, demonstrated that chromatin decondensation is associated with RUNX2 diffusion from the nucleus (Fig. 3) [43]. Collectively, the effect of a soft substrate on chromatin structure is sometimes chromatin decondensation and sometimes the opposite; however, the culture condition with a soft styrenated gelatin substrate that leads to RUNX2 diffusion is assumed to induce chromatin decondensation, probably due to increased histone acetylation with a decrease in HDAC.

Given that RUNX2 has a sequence of nuclear matrix targeting signals [45] and DNA motifs, structural changes in chromatin associated with histone acetylation state regulated by HDAC, nuclear matrix, or the combinations of them are assumed to be a factor that determine the retention of RUNX2 in the nucleus. Heterogeneity of the response of RUNX2 to substrate stiffness in inter-cell populations from different donors and intra-populations from a single donor of MSCs and related differentiation potentials could reflect their heterogeneity in chromatin and nuclear matrix structures, and their signaling through HDAC. In mouse fibroblast, mechanical cues from a microgrooved surface and an aligned nanofibrous scaffold are reported to induce decreased HDAC activity and improved reprogramming efficiency, which requires an intact actin cytoskeleton with proper amount and structure [46]. Whether the same is the case or not for the MSC response to the substrate stiffness remains to be clarified though, if the actin cytoskeletal structure is involved in the regulation of HDAC activity, the pathway should be highly independent from that for YAP.

Guideline for the Design of Substrate Stiffness for Human Mesenchymal Stem Cell Culture Without Loss of Stemness

A collagen I-coated soft ($E = 5$ kPa) polyacrylamide gel substrate is suggested to be useful for retaining the proliferative and differentiating potential of umbilical cord-derived and bone marrow-derived human MSCs during long-term expansion [47]. The underlying mechanism is most likely the suppression of YAP and RUNX2 nuclear localization, which decreases the quality of MSCs as stem cells through biased osteogenic differentiation [23]. Additionally, suppression of RUNX2 nuclear localization might also suppress the biased myogenic differentiation, based on the report that myogenic progenitor cells express osteoblast-specific proteins, bone-specific alkaline phosphatase and RUNX2 spontaneously with a potential of terminal muscle differentiation [27]. YAP and RUNX2 responses are heterogeneous in an MSC population from the bone marrow of a donor [37]. Substrate stiffness of $E = 5$ kPa is a condition that suppresses YAP nuclear localization, whereas, for RUNX2, $E = 5$ kPa is the point to reach the maximum frequency of cells with RUNX2 in the nucleus for some MSC populations [37].

In estimating the substrate stiffness for MSC expansion culture without the loss of stemness, it can be assumed that an extremely soft substrate can effectively suppress osteogenic bias; however, an extremely soft ($E = 0.25$ kPa) substrate coated with a mixture of collagen I and fibronectin is reported to halt the cell cycle of bone marrow-derived human MSCs [48]. It should also be noted that the extremely soft substrate favors neuronal differentiation, although relationship with YAP nuclear localization remains a matter of research [49-51]. In human pluripotent stem cells, two weeks culture with soft substrates, e.g., a polyethylene glycol gel ($E = 1$ kPa) and a polyacrylamide hydrogel ($E = 0.7$ kPa), reported to produce neurons more efficiently (>75%) than conventional differentiation methods [50]. Likewise, neuronal differentiation bias could be induced by an extremely soft substrate to suppress YAP nuclear localization. In contrast, for culture of MSC at a liquid-liquid interface, which can be regarded as Young's modulus is nearly equal 0 kPa substrate, neuronal differentiation is associated with YAP nuclear localization [51]. In any case, excessively soft substrate could be useless for their expansion culture maintaining proliferation activity without loss of stemness. The optimal range of Young's modulus for MSC expansion culture to satisfy the suppression of osteogenic differentiation bias and cell cycle progression is likely to be surprisingly narrow for each cell population and vary among cell populations. Knowledge of YAP and RUNX2 response heterogeneity in inter- and intra-populations of MSCs is key to building guidelines for the design of culture substrates for sophisticated control of MSC properties, such as differentiation potential, proliferation capacity, and ability to secrete cytokines, which underlie therapeutic efficacy.

Conclusion and Outlook

In this review, a design strategy for culture substrate, specifically its stiffness, for human MSCs is described based on heterogeneity in nuclear/cytoplasmic localization changes in mechanotransducer, YAP, and an osteogenic transcription factor, RUNX2, in response to substrate stiffness. Heterogeneity is an inevitable feature of biological systems, including MSCs. Controlling the biological properties of MSC populations will require a different approach than controlling artificial systems to avoid variability and ensure accurate signal transduction. We believe that further characterization of the heterogeneity of MSCs and understanding their biological meaning will be an exciting research direction to establish guidelines for the design of culture substrates for the sophisticated control of MSC properties.

Conflict of Interest

Author Contributions

H.M., M.Y., H.F., and S.K. wrote the manuscript and prepared figures. All the authors have agreed to the final version of this manuscript for publication.

Data Availability

The evidence data generated during the current study are available from the corresponding author on reasonable request.

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