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Cytoskeletal and Focal Adhesion Influences on Mesenchymal Stem Cell Shape, Mechanical Properties, and Differentiation Down Osteogenic, Adipogenic, and Chondrogenic Pathways

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Mesenchymal stem cells (MSCs) hold great potential for regenerative medicine and tissue-engineering applications. They have multipotent differentiation capabilities and have been shown to differentiate down various lineages, including osteoblasts, adipocytes, chondrocytes, myocytes, and possibly neurons. The majority of approaches to control the MSC fate have been via the use of chemical factors in the form of growth factors within the culture medium. More recently, it has been understood that mechanical forces play a significant role in regulating MSC fate. We and others have shown that mechanical stimuli can control MSC lineage specification. The cytoskeleton is known to play a large role in mechanotransduction, and a growing number of studies are showing that it can also contribute to MSC differentiation. This review analyzes the significant contribution of actin and integrin distribution, and the smaller role of microtubules, in regulating MSC fate. Osteogenic differentiation is more prevalent in MSCs with a stiff, spread actin cytoskeleton and greater numbers of focal adhesions. Both adipogenic differentiation and chondrogenic differentiation are encouraged when MSCs have a spherical morphology associated with a dispersed actin cytoskeleton with few focal adhesions. Different mechanical stimuli can be implemented to alter these cytoskeletal patterns and encourage MSC differentiation to the desired lineage.

Introduction

Mesenchymal stem cells (MSCs) show great promise for use in tissue-engineering applications because of their potential to regenerate many types of tissue, including bone, cartilage, adipose, muscle, and possibly nerve tissues.[1–6] Since MSCs can be isolated from adult patients, this allows for the possibility of using MSCs for patient-specific repair of bone and cartilage defects with tissue that will not provoke an immune reaction. Tissues engineered using MSCs are known to be sensitive to mechanical stimuli.[7–9] Mechanical forces can be used to induce or aid MSC differentiation into various mature cells. Cyclic tensile strain[9,10] and oscillatory fluid flow[11–13] have both been reported to increase osteogenic differentiation and decrease adipogenic differentiation, whereas uniaxial, unconfined compression[14] and cyclic hydrostatic pressure[15] increase chondrogenesis.[16,17] How such mechanical forces transmit signals to the cells and thus affect differentiation is currently a topic of great interest and study.

The cytoskeleton is known to play a role in mechanosensing and mechanotransduction.[18–21] The interactions between cytoskeletal proteins, integrins, and mechanical forces can influence cells to change shape, proliferate, and even differentiate.[18] Because of this, there has been increasing interest in the interaction between the cytoskeleton and the differentiation of MSCs. There appear to be important links between the mechanosensing role of the cytoskeleton and MSC fate. However, this is a burgeoning field of research, and much knowledge is yet to be acquired. This review provides information on what has been published in this relatively new field with specific emphasis on cytoskeletal and focal adhesion influence on the MSC shape, mechanical properties, and differentiation.

Cytoskeletal Properties of MSCs

As MSCs differentiate, their mechanical properties change according to their lineage specification. Young's modulus is a measurement of how much a material will deform in

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response to a stress placed on it. Materials with a higher Young's modulus are stiffer and do not deform as easily. Before differentiation, human MSCs (hMSC) have a Young's modulus of ~3.2 kPa.[22,23] However, the sample temperature can change the cell stiffness. hMSC measured at 20°C have been reported to have lower viscosity and higher stiffness than hMSC measured at 37°C.[24] Cytoskeletal structure has a large impact on hMSC' mechanical properties. Disrupting the actin cytoskeleton with cytochalasin significantly decreases the stiffness and increases the viscosity of hMSC.[23,24] However, disrupting the microtubule structure with nocodazole does not significantly change the stiffness.[23] This implies that much of the stiffness of hMSC is dependent on the actin cytoskeleton as opposed to the microtubules.

Two nonmechanical factors have been reported to influence the structure of the actin cytoskeleton in hMSC. If the perinuclear actin cap is inhibited, the formation of actin stress fibers in hMSC is prevented.[25] Disruption of actin configuration can also be influenced by population doubling of the cells. hMSC at passage 6 are more susceptible to actin cytoskeleton disruption by jasplakinolide than cells at passage 2.[26] Therefore, hMSC should be used at a low passage to ensure cytoskeletal integrity.

Mechanical Properties

As MSCs differentiate, they exhibit changes in their mechanical properties.[23,27] These changes in mechanical properties can be indicative of underlying cytoskeletal changes, especially in the actin cytoskeleton.[23,24] Actin stress fibers are one of the stiffest structures within hMSC,[28] and disrupting the actin cytoskeleton decreases cell stiffness.[23,24] The mechanical properties of MSCs depend on the actin fiber structure, which varies based on the differentiation pathway.

Studies have differed in their findings of the value of the elastic modulus of hMSC as shown in Table 1. Yu *et al.* reported that hMSC have an instantaneous Young's modulus of about 0.5 kPa and an equilibrium Young's modulus of about 0.1 kPa.[27] The viscoelastic properties of the cells were measured using micropipette aspiration of suspended cells. The instantaneous Young's modulus was measured over the initial aspiration of the cell, and the equilibrium Young's modulus was measured after an adequate amount of time to measure the cell equilibrium response.[27] On the other hand, Titushkin *et al.* reported a much larger Young's modulus of about 3.2 kPa.[23] They measured the Young's modulus by atomic force microscopy (AFM) indentation at a velocity of 2 μm/s.[23] This value was corroborated by Darling *et al.* who reported a Young's modulus of ~3.2 kPa for hMSC attached to a surface, and 2.5 kPa for spherical cells using AFM at an indentation rate of 6.25 μm/s.[22] However, the differing protocols for cell loading make it difficult to compare Young's moduli between studies.

Osteogenesis

Using AFM, Titushkin *et al.* reported that osteoblasts have a Young's modulus of ~1.7 kPa.[23] That study also found that hMSC exposed to an osteogenic medium for 10 days exhibited a significant decrease in the elastic modulus to ~2 kPa from 3.2 kPa.[23] Yu *et al.*, using the same procedure used for hMSC, showed an increase in both instantaneous and equilibrium Young's moduli when hMSC were cultured in an osteogenic differentiation medium for 21 days.[27] The instantaneous Young's modulus increased from 0.5 kPa to 0.9 kPa, while the equilibrium Young's modulus increased from 0.1 kPa to about 0.2 kPa.[27] As MSCs differentiate into osteoblasts, their mechanical properties become similar to those of osteoblasts; however, whether or not this equates to a significant increase or decrease in their stiffness is debatable.

Darling *et al.* reported that the elastic modulus of osteoblasts was dependent upon cell spreading and morphology. They reported that spherical osteoblasts had an elastic modulus of 2.0 kPa, whereas spread osteoblasts had an elastic modulus of 5.8 kPa.[22] These findings contradict the findings of Yu *et al.* whose measurements showed that osteoblasts were less stiff than hMSC, whereas Darling *et al.* determined that osteoblasts were stiffer than hMSC. However, differing methods used for measuring the stiffness could be causing the disparate responses. Because the cell shape affects mechanical properties, we would also expect differing cytoskeletal arrangements between cell shapes.

Table 1. Young's Modulus Measurements of hMSC Using Various Measurement and Culture Techniques

	Paper	*Measurement technique*	*Young's modulus*
hMSC	Yu *et al.*[27]	Micropipette aspiration	Instantaneous: 0.5 kPa Equilibrium: 0.1 kPa
	Titushkin *et al.*[23]	AFM indentation	3.2 kPa
	Darling *et al.*[22]	AFM indentation	Spread: 3.2 kPa Spherical: 2.5 kPa
Osteoblast	Titushkin *et al.*[23]	AFM indentation	1.75 kPa
	Darling *et al.*[22]	AFM indentation	Spread: 5.8 kPa Spherical: 2.0 kPa
Osteogenic Differentiation	Yu *et al.*[27]	Micropipette aspiration after 21 days	Instantaneous: 0.9 kPa Equilibrium: 0.2 kPa
	Titushkin *et al.*[23]	AFM indentation after 10 days	2 kPa
Adipocyte	Darling *et al.*[27]	AFM indentation	Spread and Spherical 0.61 kPa
Adipogenic Differentiation	Yu *et al.*[27]	Micropipette aspiration after 21 days	Instantaneous: 0.42 kPa Equilibrium: 0.09 kPa
Chondrocyte	Darling *et al.*[22]	AFM indentation	Spreadand Spherical: 1.2 kPa

hMSC, human mesenchymal stem cells; AFM, atomic force microscopy.

Adipogenesis

Adipocytes have been shown to have a much lower stiffness than hMSC and osteoblasts. Yu *et al.* showed a decrease in the Young's modulus of hMSC after 21 days of exposure to an adipogenic differentiation medium.[27] The instantaneous Young's modulus was measured to be 0.42 kPa, and the equilibrium Young's modulus was measured to be 0.09 kPa.[27] Darling *et al.* reported the elastic modulus of adipocytes to be ~0.61 kPa for both spread and spherical cells.[22] These relatively low moduli, which do not change with the cell shape, imply that adipocytes do not have a very dense cytoskeleton.[29]

Chondrogenesis

Darling *et al.* reported that chondrocytes have a stiffness that is midway between MSCs and adipocytes at ~1.2 kPa.[22] As with adipocytes, the modulus did not change significantly relative to cell spreading.[22] This might indicate that the actin cytoskeleton is not as important for mechanotransduction in chondrocytes as it is in osteoblasts.

Cell Shape

Osteogenesis

Cell shape and the cytoskeletal changes related to cell shape highlight how important the cytoskeleton may be in regulating MSC differentiation. As MSCs differentiate into osteoblasts, they become more elongated and spread.[30] When MSCs are plated at a low density, where they have the ability to spread, they have a greater osteogenic potential than cells plated at a high density.[29] Even if these cells are replated at a high density after 48 h, they still exhibit increased osteogenic potential.[29] Confining cells on micropatterns designed to prevent them from spreading inhibits osteogenic differentiation.[29] Therefore, cell shape plays an important role in early differentiation, and this effect is not due to cell–cell interactions.[29] Cell shape has been shown to be related to actin cytoskeleton regulation. MSCs plated on micropatterns that allowed them to spread expressed more RhoA, which is responsible for actin organization, than cells that were not allowed to spread.[29] hMSC that are more spread have actin and focal adhesion arrangements more similar to osteoblasts than hMSC that are less spread.[30] This exemplifies that the shape of MSCs, their osteogenic potential, and the actin cytoskeleton are likely related.

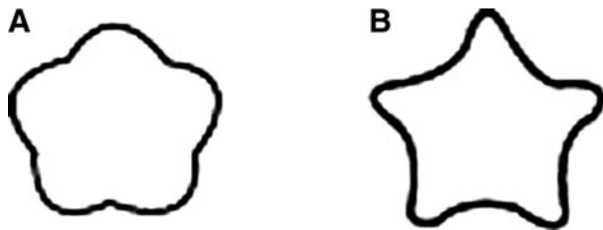

FIG. 1. **(A)** Flower shape used in Kilian *et al.*[31] **(B)** Star shape used in Kilian *et al.*[31] Mesenchymal stem cells (MSC) confined to the flower shape showed increased adipogenesis. MSC confined to the star shape showed increased osteogenesis. Adapted from Kilian *et al.*[31]

Cell spreading is not the only component of cell shape that influences cytoskeletal configuration and osteogenic differentiation. When MSCs are confined to micropatterned rectangles, rectangles with increased aspect ratios increase MSC osteogenic differentiation, indicating that more elongated cells are more likely to undergo osteogenesis.[31] When MSC are constrained to micropatterned flowers with convex edges (Fig. 1), osteogenesis decreases.[31] Constraining the cells to micropatterned stars, with concave edges (Fig. 1), increases osteogenesis.[31] The authors of that study analyzed F-actin in the flower patterns and found that it was mostly dispersed within the cells, whereas star-patterned cells had more prominent stress fibers along the outer edges with more disperse fibers in the interior.[31] Focal adhesion distribution was also found to change between the two shapes. In flower-patterned cells, vinculin was dispersed throughout the cell, whereas in star-patterned cells, vinculin was concentrated in the points of the stars.[31] Disrupting microtubules with nocodazole caused both patterns to become equally osteogenic, whereas disrupting the actin cytoskeleton with cytochalasin-D and Rho-kinase with Y-27632 caused both patterns to become equally nonosteogenic.[31] This implies that the ability of cell shape to determine MSC differentiation is dependent on cytoskeletal cues from both the actin cytoskeleton and the microtubule skeleton.

Adipogenesis

Cytoskeleton configuration can also affect the ability of MSCs to undergo adipogenesis. Like osteogenesis, adipogenesis has been shown to be dependent on cell shape and, by extension, the underlying cytoskeleton. MSCs are more adipogenic when the cells are rounded. When MSCs are geometrically constrained by micropatterns so that they are unable to spread, they become more adipogenic.[29] Similarly, when cells are plated at a high density, such that they cannot spread, they show a greater adipogenic potential.[29] Even when cells are later replated at a low density, high-density plating in the first 48 h of differentiation encourages adipogenesis.[29] As with osteogenic differentiation, the cell shape within the first 48 h appears to be essential to regulating the lineage specification to which MSCs commit.

Adipogenic differentiation is affected not only by cell spreading but also by cell geometry. When MSCs are confined to micropatterned squares, they are more adipogenic than when confined to rectangles.[31] This again exemplifies that when MSCs are maintained in a more rounded morphology, it increases the likelihood that they will undergo adipogenesis as opposed to osteogenesis. Furthermore, in direct contrast to osteogenic differentiation, when MSCs are constrained to micropatterned flowers, adipogenesis increases, whereas constraint to micropatterned stars decreases adipogenesis.[31] As noted previously in this review, the differences between these two patterns are dependent both on the actin and microtubule cytoskeleton; the trend found for osteogenic differentiation is opposite to adipogenesis.[31]

Actin Cytoskeleton and RhoA/ROCK Signaling

Osteogenesis

The actin cytoskeleton plays an important role in osteogenic differentiation of MSCs. Changes in the actin

cytoskeleton occur as MSCs differentiate. As suggested by the changing stiffness of MSCs, the arrangement of the cytoskeleton significantly changes as MSCs differentiate into osteoblasts. hMSC have thicker actin stress fibers, whereas osteoblasts exhibit a much more disperse actin cytoskeleton.[23] As MSCs undergo osteogenic differentiation, the actin cytoskeleton becomes more disperse and begins looking more similar to that of osteoblasts.[32] When the actin cytoskeleton is disrupted with Cytochalasin-D, MSCs become rounded.[32] Disrupting the actin cytoskeletal structure within the first 48 h of exposure to an osteogenic medium prevents hMSC from reaching the same alkaline phosphatase activity as cells with no cytoskeletal disruption.[32] This again exemplifies that the structure of MSCs in the first 48 h of differentiation is extremely important for osteogenesis. Disrupting the actin cytoskeleton decreases osteogenesis in favor of adipogenesis.[29] This result has been shown to be dependent on ROCK and RhoA. Inhibiting ROCK with Y-27632 causes decreased osteogenesis.[29,33] Expressing dominant-negative RhoA decreases osteogenesis, whereas constitutively active RhoA increases osteogenesis.[29] These findings indicate that actin cytoskeletal tension is necessary for MSC osteogenic differentiation. Because actin configuration is important in osteogenesis, the actin cytoskeleton can be used to predict if a cell is differentiating down an osteogenic pathway within 48 h of culture in an osteogenic differentiation medium.[34] By analyzing the organizational characteristics of the actin cytoskeleton, Treiser *et al.* have developed an algorithm that could accurately determine the osteogenic potential of various surfaces in only 48 h, a test that normally takes 2 weeks.[34] A follow-up to that study determined that genetic changes could be detected within the first 24 h.[35] These findings exemplify how interrelated the actin cytoskeleton is with osteogenic differentiation and again demonstrates that cytoskeletal activity in the first 48 h of differentiation determines the osteogenic potential of MSCs.

Adipogenesis

In the one article published to date investigating a link between the actin cytoskeleton and adipogenesis, disrupting the actin cytoskeleton with cytochalasin increased the adipogenic potential of MSCs.[29] This suggests that having a stable actin cytoskeleton inhibits adipogenesis. Inhibiting both ROCK and RhoA also increases the adipogenic potential of MSCs.[29] Therefore, it appears that increased actin polymerization in MSCs decreases adipogenic differentiation. Overall, cells with a less-organized and less-stiff actin cytoskeleton are more likely to differentiate into adipocytes.

Chondrogenesis

Actin also plays a role in chondrogenesis. In chick wing-bud MSCs, disrupting the actin cytoskeleton with cytochalasin-D encouraged chondrogenesis.[36] Various factors known to regulate the actin cytoskeleton play an important role in regulating chondrogenesis. As MSCs undergo chondrogenesis, they exhibit a decrease in RhoA activity.[37] This decrease in RhoA is at least partially responsible for chondrogenic differentiation of MSCs, as MSCs made to overexpress RhoA exhibited decreased chondrogenesis.[37] This shows that the cell causes the actin cytoskeleton to become more diffused through decreased RhoA activity to undergo chondrogenesis.

Manipulating the RhoA/ROCK pathway can also affect the chondrogenic potential of MSCs. In mouse limb-bud MSCs, treatment with Y27632 to inhibit ROCK increased GAG production of the cells and caused cortical actin organization.[38] This treatment also reduced the number of actin fibers and caused cell rounding.[38] Conversely, RhoA overexpression inhibited GAG synthesis and Sox9.[38] Therefore, the RhoA/ROCK pathway plays a role in regulating the markers of chondrogenic differentiation. Cytochalasin also has been shown to increase Sox9.[11] This strongly indicates that the mechanism for this regulation is likely related to the actin cytoskeleton. Therefore, decreased actin cytoskeletal organization appears to increase chondrogenesis in MSCs.

Microtubules

Despite the fact that they seem to play a negligible role in the mechanical properties of hMSC, there is evidence that the microtubule structure plays at least some role in MSC differentiation. To evaluate the effects of microtubules in MSCs, various treatments can be used to disrupt the microtubule structure. Normally, MSCs show a spindle-like microtubule morphology.[39] Taxol, which targets and stabilizes β-tubulin, and nocodazole, which prevents microtubule polymerization, cause significant changes to the microtubule structure of MSCs.[39] When exposed to taxol, hMSC exhibit a stabilized microtubule structure, and when exposed to nocodazole, hMSC have a depolymerized microtubule structure.[39] Of the two treatments, only taxol changes overall tubulin production within cells, increasing the amount of tubulin ninefold.[39] Since these chemicals can disrupt the microtubule structure, one could use this to assess the role microtubules play in the differentiation of hMSC. However, the limited effects of microtubule disruption mean that very few studies have been done to investigate their role in differentiation. Those that have been published to date are reviewed here.

Osteogenesis

Microtubules have been shown to play a minor role in osteogenic differentiation. As MSCs differentiate, the structure of microtubules does not change significantly.[32] However, cells in which the cytoskeleton has been disrupted show a quicker change in the actin cytoskeleton from the undifferentiated to the differentiated morphology.[32] Even if the actin cytoskeleton plays the primary cytoskeletal role in differentiation, microtubules might play a small role in the ability of cells to differentiate quickly.

Focal Adhesions

Osteogenesis

The relationship between focal adhesions and the cytoskeleton also changes as MSCs undergo osteogenic differentiation. Osteoblasts have more ERM (ezrin, radixin, moesin family) proteins than MSCs, indicating that there are more focal adhesions in osteoblasts.[23] When these ERM proteins are knocked down with siRNA, it decreases alkaline phosphatase production and mRNA expression of osteogenic markers.[40] Therefore, focal adhesions appear to play an important role in the ability of MSCs to undergo

osteogenic differentiation. It has been shown that the cytoskeletal changes necessary for osteogenesis are integrin dependent.[33,34,41] When cells are cultured on glass with no extracellular matrix (ECM) and then exposed to an osteogenic differentiation medium, cytoskeletal changes observable in the first 24 h in MSCs plated on fibronectin do not occur until 72 h after plating.[34] Since by 72 h the cells are capable of depositing their own ECM, integrins appear to require a place to bind before differentiation can occur. Osteogenic differentiation of hMSC has been shown to be focal adhesion kinase (FAK) dependent when cells are plated on collagen-1, but not when they are plated on fibronectin or vitronectin.[41] FAK knockdown decreased alkaline phosphate activity in hMSC plated on collagen-1 and vitronectin, but not fibronectin.[41] Another study that plated hMSC on polyacrylamide gels showed that FAK inhibition and alpha-2 integrin inhibition decrease osteogenesis.[33] Focal adhesions appear to be vital for osteogenic differentiation when cells are binding to collagen-1.

Adipogenesis

There has been only one study reported on the role of focal adhesions in MSC adipogenic differentiation. That study found that heparin promotes adipogenic differentiation by disrupting focal adhesions in immortalized and normal mouse MSCs by upregulating adipogenic genes.[42] This increase in adipogenesis was consistent for multiple types of adipogenic induction.[42] They concluded that having fewer focal adhesions increases the adipogenic potential of MSCs.[42]

Chondrogenesis

Cytoskeletal configuration and regulation have also been shown to play an important role in chondrogenesis. Like adipogenesis, chondrogenesis is encouraged by having a rounded cell shape. MSCs plated on surfaces modified with RGD (arginine, glycine, aspartic acid) peptides spread out, whereas MSCs on RGE (arginine, glycine, glutamic acid) peptide-modified surfaces, which prevent focal adhesion attachment, remain rounded.[43] MSCs on the RGD surfaces showed decreased chondrogenesis as evidenced by lower levels of mRNA for collagen II and aggrecan.[43] These surfaces also exemplified the importance of interactions between integrins and the actin cytoskeleton in chondrogenesis. While cells seeded on RGD surfaces had high levels of localized vinculin expression, MSCs on RGE surfaces expressed only low levels of vinculin that were not localized.[43] This implies that strong focal adhesion attachments are not necessary or beneficial to chondrogenesis.

Mechanical Interventions

Once we understand the cytoskeletal arrangements that encourage various types of MSC differentiation, we next need to understand how we can encourage MSCs to configure their cytoskeletons in a manner conducive to the type of differentiation that is desired. Micro- or nanoscale patterns can be used to control the cytoskeletal configuration of MSCs. Nanoscale ridges made of polydimethylsiloxane (PDMS) or polystyrene have been used to cause hMSC to align in the direction of the ridges.[44,45] Not only is this alignment translated into an alignment of the actin cytoskeleton but it also causes a deformation of the nucleus.[44] It has been shown that FAK is decreased in MSCs by nanopatterned ridges.[46] On polystyrene, but not PDMS, hMSC produce higher levels of vinculin than nanopatterned cells.[46] Nanopatterns also increase both microtubule expression and the stiffness of MSCs.[46]

Active mechanical interventions can also cause actin–cytoskeletal alignment in MSCs. In 3D culture, MSCs show actin–cytoskeletal alignment parallel to the compression direction both in cyclic and static unconfined compression.[47] Because the pellets are unconfined, there is likely an element of tensile strain in the direction perpendicular to applied compression. Therefore, it is not surprising that cyclic uniaxial stretch can also be used to cause actin cytoskeletal alignment in MSCs.[48] We have shown that actin fiber alignment in the direction of applied strain occurs in hMSC exposed to uniaxial cyclic tensile strain.[48] However, the alignment is more consistent in 10% strain versus 12% strain, which is likely due to cellular damage caused by the higher 12% strain.[48] Shear stress can also cause cytoskeletal changes consistent with osteogenic differentiation. Oscillatory fluid flow at 1 Hz increases RhoA and ROCK II and creates a denser actin cytoskeleton.[11] All of these methods could be used to increase cytoskeletal organization, and thus osteogenic differentiation.

Other mechanical interventions can be used to promote a more disperse actin cytoskeleton. Microgravity causes the actin cytoskeleton to rearrange in as little as 30 min.[49] The low-gravity environment causes MSCs to change from having prominent stress fibers to having a more amorphous actin distribution with actin redistributed from the edges to the center of the cells.[49,50] However, by 120 h in microgravity, the cells regain the actin structure of cells not subjected to a microgravity environment.[49] Cells cultured in microgravity and then returned to regular gravity also show a return to normal actin organization.[49] As expected from a treatment that reduces actin cytoskeleton organization, microgravity can also reduce RhoA activity.[50] In response to microgravity, it has been shown that vinculin redistributes in a manner colocalized with actin.[49] These changes were not noticeable until 6 h, much longer than the 30 min it took to notice changes in actin.[49] Microtubule organization was not affected by microgravity conditions.[49] Given the quick return to normal actin organization, microgravity might not be an effective technique for differentiation.

Electric fields can also disrupt the actin cytoskeleton of MSCs. Exposure to a direct current (DC) electric field of 2V/cm for 1 h has been shown to decrease stiffness and F-actin content of MSCs when cells were cultured in Hanks' balanced salt solution, but not in a complete growth medium.[51] This treatment also depolymerized the actin cytoskeletal structure.[51] However, the structure could be recovered after an hour in a complete growth medium with no electric field.[51] These treatments that decrease the actin cytoskeletal structure would be useful in encouraging MSC adipogenic or chondrogenic differentiation.

Integrin configuration can also be affected by mechanical interventions. Stiffer substrates cause increased alpha-2 integrin expression.[33] Therefore, stiffer substrates should be more suitable for MSC osteogenic differentiation, and this has been shown to be the case.[33,52] It has also been shown that focal adhesions adapt differently to various forces.

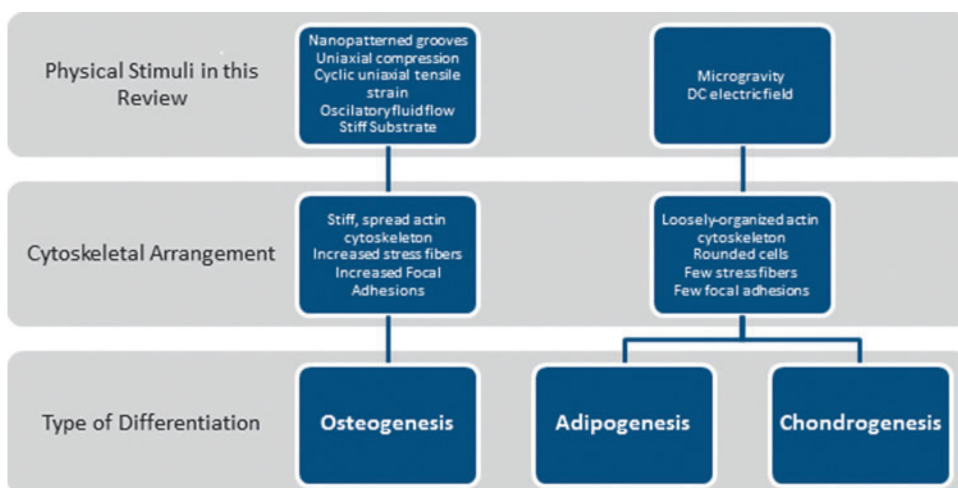

FIG. 2. Mechanical interventions have been shown to alter the cytoskeletal arrangement of MSC. This knowledge can be used to determine which mechanical interventions to use to encourage various types of differentiation. Color images available online at www.liebertpub.com/teb

Using ligand-coated magnetic microbeads, focal adhesions were shown to adapt within 15 s to multiple exposures to 3 s of 130 pN of force.[53] This reaction could be suppressed using Y27632, 2,3-butanedione 2-monoxime, or cell cooling to 4°C.[53] A stronger adaptation occurred after 15 s of a sustained 130 pN force; however, this reaction could not be inhibited by inhibiting myosin, stretch-activated channels, or Src tyrosine kinases.[53] Only cooling the cells to 4°C prevented this cellular reaction.[53] When cells were exposed to more than 60 s of strain on the beads, the cells began actively reacting to the strain by retracting against the movement of the beads.[53]

Conclusion

An important cytoskeletal factor in osteogenic, adipogenic, and chondrogenic differentiation of MSCs appears to be the actin cytoskeleton. Osteogenic differentiation appears to require a stiff, spread actin cytoskeleton. This is not surprising, because *in vivo*, osteoblasts are generally found as flat cells on the bone surface. Both disrupting actin and limiting the ability of MSCs to spread decrease the osteogenic potential of MSCs. These conditions are especially important within the first 48 h of differentiation. Disrupting the actin cytoskeleton or not allowing cells to spread within those first two vital days appears to prevent MSCs from ever reaching their full differentiation potential. Adipogenesis requires a cytoskeleton that does not have much organization or tension. Since adipocytes are normally spherical cells with little structural function, they do not need an organized cytoskeleton. Cells that are less spread have a greater potential to undergo adipogenesis. Disrupting the actin cytoskeleton will increase adipogenesis, especially if this is done in the first 48 h of exposure to an adipogenic environment. Chondrogenesis seems to share the same cytoskeletal configuration as adipogenesis. However, chondrocytes show a larger range of shapes *in vivo* than adipocytes. In articular cartilage, chondrocytes exhibit a spherical morphology in the intermediate zone, an elongated shape in the superficial tangential zone, and an oblong shape in the deep zone. However, these shape differences are not apparent in cytoskeletal configuration, leading to chondrogenic differentiation. The factors that can be modified to distinguish chondrogenic differentiation of MSCs from adipogenic differentiation of MSCs may be chemical and not cytoskeletal. Both adipogenesis and chondrogenesis can be induced by addition of chemical factors to the cell growth media. Focal adhesions are also important in differentiation. Osteogenesis requires larger numbers of focal adhesions, whereas adipogenesis and chondrogenesis are encouraged by preventing focal adhesion attachment.

The usefulness of understanding cytoskeletal properties of stem cells comes in the ability to use this knowledge to influence stem cell differentiation. Figure 2 shows our proposed routes for manipulating differentiation based on studies of cytoskeletal changes under various mechanical manipulations. Nanopatterned ridges, uniaxial compression, cyclic uniaxial tensile strain, oscillatory fluid flow, and stiff substrates should all encourage osteogenesis based on cytoskeletal responses to these treatments. Microgravity and DC electric fields cause MSCs to assume a rounded shape with little cytoskeletal structure, conducive to both adipogenesis and chondrogenesis. However, physical interventions that have not been tested for cytoskeletal configuration, such as unconfined uniaxial compression and hydrostatic pressure, have also been shown to be conducive to chondrogenic differentiation in MSCs and should be a subject of future cytoskeletal studies.[14–17] These cytoskeletal changes have been previously shown to translate into the predicted differentiation pathway. Increased stiffness in scaffold matrices has been shown to increase the expression of osteogenic proteins.[33,52] Microgravity has been shown to increase intracellular lipid accumulation, thus showing that microgravity does increase adipogenic differentiation.[50] However, cytoskeletal changes might not always predict differentiation. Oscillatory fluid flow, which has been shown to increase cytoskeletal organization, has also been reported to increase Sox9 production in MSCs when delivered at 1 Hz with a peak force of 1 Pa.[11] In this instance, it appears that a force that causes cytoskeletal alignment also increased MSC propensity for chondrogenesis.

The results for MSCs can also be used to predict behavior in other stem cell types. Many studies have shown that adipose-derived stem cells (ASC) have similar differentiation capabilities as MSCs.[54–56] Although they do differentiate

similarly, MSCs and ASC are not exactly alike. MSCs have been shown to be more osteogenic under certain conditions[57,58] and more chondrogenic under others.[57,59,60] Like MSCs, ASC are known to have increased osteogenesis when they are exposed to uniaxial cyclic tensile strain.[61–64] Therefore, we would expect that cyclic tensile strain causes ASC to develop a stiff, spread actin cytoskeleton like that found in MSCs exposed to cyclic tensile strain.

In conclusion, actin cytoskeleton and focal adhesion configuration can likely be used to determine how MSCs are going to differentiate based on external cues. Cytoskeletal structure is very important to understanding MSC differentiation and how we can regulate it for functional tissue engineering and regenerative medicine applications.

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Disclosure Statement

No competing financial interests exist.

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