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研究課題名（和文）Understanding the effects of substrate stiffness on rejuvenation of aging MSCs via single cell analysis.

研究課題名（英文）Understanding the effects of substrate stiffness on rejuvenation of aging MSCs via single cell analysis.

研究代表者

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研究成果の概要（和文）：本研究では、ゲル状ハイドロゲルが間葉系幹細胞（MSCs）の老化を遅延／逆転させる若返り能力を持つことを実証した。ハイドロゲルは、老化したMSCsの増殖と骨形成分化を増加させ、運動性を高めることができた。シングルセルリアルタイムPCR分析の結果、ゲル上のMSCにおいて、SIRT、FOXO、AMPK、NAMPTといった長寿と若返りに関連する経路のシグナル伝達分子が活性化されていることが示された。これらの結果から、ハイドロゲルは、酸化還元ホメオスタシスをよりよく維持し、細胞骨格の緊張を緩和し、アクチン動態の回復を促進し、MSCsの長寿と若返りに関連するシグナル伝達経路を活性化できることが示された。

研究成果の学術的意義や社会的意義
Increasing of aging population and aged-related diseases increase the health problems. Identification of key targets for preventing or ameliorating senescence is essential for precisely engineered mechanical substrate to slow or reverse the age-related reduction of MSC properties.

研究成果の概要（英文）：This study demonstrated the rejuvenation capacity of the gelatinous hydrogels to delay/reverse senescence of the mesenchymal stem cells (MSCs).The hydrogels could increase the proliferation and osteogenic differentiation and enhance the motility of the aged MSCs. Single cell real time PCR analysis results illustrated the activation of signaling molecules in longevity and rejuvenation related pathways such as SIRT, FOXO, AMPK and NAMPT in the MSCs on gels. These results demonstrated that the the hydrogels could better maintain redox homeostasis, reduce cytoskeletal tension, enhance the recovery of actin dynamic and activate the signaling pathways related to longevity and rejuvenation of the MSCs.

研究分野：Molecular Biology

キーワード：MSC aging　cellular senescence　rejuvenation　substrate elasticity　gelatinous hydrogel　redox homeostasis　DNA damage response　single cell analysis

様　式　C－１９、F－１９－１（共通）

１．研究開始当初の背景

Mesenchymal stem cells (MSCs) from bone marrow are widely used in clinical applications due to their therapeutic properties. However, *in vitro* expansion of MSCs on tissue culture dish (TC) induce aging (replicative senescence), which reduce their quantities and qualities with undefined mechanism.
This study hypothesizes that cultivation and passaging the MSCs on TC increase cytoskeletal tension, cellular stress and induce aging while the soft substrates could reduce cytoskeletal tension, increase their dynamics and the modulate the antioxidant system to maintain the redox and metabolic homeostasis and delay or reverse aging. The mechano-regulation of aging were elucidated from many angles including the redox modulation, cytoskeletal dynamic and the effect on therapeutic properties of MSCs on the compliance hydrogels. Single cell real time PCR analysis was performed to investigate the specific gene expression patterns encoded within RNA of the individual MSCs to gain a better understanding of the substrate stiffness induced MSC rejuvenation in single cell resolution.

２．研究の目的

The multiplicity and heterogeneity of the MSCs obscured the understanding in their biology including the mechanism of aging for both *in vivo* and *in vitro*. Not all of the cells are capable of differentiate to the specific lineages, not every cell stop dividing and only some subpopulation undergo senescence. Single cell analysis has emerged as a powerful tool to explore heterogeneity in single cell level. The aging-related-single cell analysis highlight new findings that difficult to demonstrate in bulk analysis. However, this application in the field of biomaterials still limited.
This study aims to understand the mechano-regulation of MSC aging and rejuvenation via the substrate stiffness by focusing on of the effect of substrate stiffness on CSK dynamics, redox balance and the implication on MSCs aging. Single cell expression profiling was performed to gain a better understanding of mechano-regulation of MSC aging in single cell level. Identification of key targets for preventing or ameliorating senescence is essential for intervention of aging and aged-related diseases and to precisely engineered mechanical substrate to slow or reverse the age-related reduction of MSC properties.

３．研究の方法

To investigate the effect of substrate stiffness on the MSC rejuvenation, the stiffness tunable gelatinous gels were fabricated using photocurable styrenated gelatin via LED illumination system. The surface elasticity of the gels was measured by microindentation analysis using atomic force microscope. The 3-5 kPa soft gels, 7-10 kPa moderate stiffness and 20-30 kPa stiff gels were used for cell cultivation. To obtain the information of how the young and aged cells interact with the mechanical substrates, the early (P5-6), middle (P7-10) and late passages (P11-25) of the MSCs were cultured on TC and gels and the cell/nuclear morphological changes, migration and differentiation properties were investigated. The mitochondrial ROS level of the MSCs was evaluated using specific dyes and analyzed by flow cytometry. The degree of senescence was evaluated by staining with SA-β-gal and fluorogenic probe SPiDER-β-gal. Then, the MSC expansion on various substrate stiffness was performed by serially passage the cells on the hydrogels and

TC. The expression of senescence markers including galactosidase β1 (GLB1), cell cycle regulators (p21, p53), and redox/anti-oxidant related genes (NRF2, SOD2) were evaluated using quantitative real time polymerase chain reaction (qPCR). The expression and organization of CSK related protein such as actin, vinculin and focal adhesions and DNA damage markers was investigated by immunofluorescence staining. Single cell qPCR was performed to delineate the expression of genes including CSK regulation, proliferation, epigenetic modification, differentiation, antioxidants, cytoprotective, longevity, apoptosis and senescence.

４．研究成果

This study demonstrated the rejuvenation capacity of the surface elasticity tunable gelatinous hydorgels to delay/reverse senescence phenotypes of the MSCs. The MSCs on hydrogels exhibited lower production of mitochondrial reactive oxygen species (ROS), decreased the expression of cell cycle inhibitors and senescence markers. In contrast, the rigid TC induced higher ROS, persistently activated redox-antioxidant system, induced cell cycle arrest and enhanced the MSC senescence. The hydrogels could attenuate the effects of hydrogen peroxide induced oxidative stress and suppressed senescence. Knocking down the master of redox regulator enhanced the senescence, suggesting the interconnection between mechanical signaling, redox homeostasis and cellular senescence. Treating the MSCs on TC with CSK tension inhibitors downregulated the senescence markers. The actin remodeling of the MSCs on hydrogels was resotred, as demonstrated by treatment with specific actin stabilizing agent. These results highlight the importance of the CSK tension and dynamics on cellular senescence.

The gelatinous hydrogels could rescue the therapeutic properties of the late passages MSCs, as suggested by the reducing the numbers of DNA damage positive cells, increasing the proliferation and osteogenic differentiation potencies, decreasing the nuclear and cell size and increased their motility. Single cell real time PCR analysis was performed to investigate the specific gene expression patterns encoded within RNA of the individual MSCs to gain a better understanding of the substrate stiffness induced MSC rejuvenation in single cell resolution. With the high throughput approach, senescence, antioxidant, cytoprotective, longevity, and rejuvenation related genes could be simultaneously quantified. The results illustrated the activation of several regulators and signaling molecules in longevity and rejuvenation related pathways such as SIRT, FOXO, AMPK and NAMPT in the MSCs maintained on gels.

In summary, the results from this study demonstrated that the MSCs cultured on the hydrogels possessed higher radical scavenging capacity and could better maintain redox homeostasis, reduce CSK tension, enhance the recovery of actin dynamic and activate the signaling pathways related to longevity and rejuvenation.

5．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計3件（うち招待講演　1件／うち国際学会　2件）

1．発表者名 Kuboki Thasaneeya
2．発表標題 Understanding the mecahno-regulation of substrate stiffness on rejuvenation mechanism of aging stem cell
3．学会等名 world congress of biomechanics（国際学会）
4．発表年 2022年

1．発表者名 Kuboki Thasaneeya
2．発表標題 Understanding the mecahno-regulation of substrate stiffness on rejuvenation mechanism of aging stem cell
3．学会等名 The Japan Society of Mechanical Engineers（招待講演）（国際学会）
4．発表年 2022年

1．発表者名 Kuboki Thasaneeya
2．発表標題 Understanding the mecahno-regulation of substrate stiffness on rejuvenation mechanism of aging stem cell
3．学会等名 Molecular Biology Society of Japan
4．発表年 2022年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

7．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関