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学位論文題目	Differentiation of Adipose Derived Stem Cells by Stimulating with Extracellular Vesicles Secreted from Dermal Papilla Cells Activated with Osteogenic and Adipogenic Inducers (骨芽細胞誘導因子と脂肪細胞誘導因子で活性化された毛乳頭細胞から分泌される細胞外小胞で刺激することによる細胞組織由来幹細胞の分化)
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論文内容の要旨

Dermal papilla ceils (DPCs) exist as spheroidal or globular shape m In-vivo condition and play crucial roles in hair regeneration, but they readily lose their hair forming ability during in vitro culture. Although the formation of spheroids partially restores the ability, shrinkage of the spheroids makes it difficult to maintain cellular viability. To address this problem, we stimulated DPCs with factors known to induce adipogenic and/or osteogenic differentiation, because DPCs share unique gene expression profiles with adipocytes and osteocytes. We isolated DPCs from versican (vcan)-GFP mice, in which GFP is expressed under the control of a vcan promoter, which is strongly active in DPCs of anagen hair follicles. In shortly, versican is a proteoglycan sulfate and serve the purpose as ECM (extracellular matrix molecule to cell-cell attachment. Isolated intact DPCs from anagen hair follicle showed intense GFP-fluorescence intensity, but it lost the fluorescence while these spheroids attached in monolayer culture surface. Even though in spheroid culture system, fluorescence getting declined and spheroid shaped became shrink which indicated the lost of hair inductive properties in DPCs. However, we analyze GFP fluorescence was most intense when the spheroids were made from DPCs cultured in a half-diluted combination of adipogenic and osteogenic media (CAO1/2), a Dulbecco's modified Eagle's medium-based medium (DMEM) that contains 10% FBS, 275 nM dexamethasone, 2.5 mM β-glycerol phosphate, 12.5 μg/mL ascorbic acid, 0.125 μM Isobutylmethylxanthine and 2.5 ng/mL insulin. The dose of each additive used was less than the optimal dose for adipogenic or osteogenic differentiation. We observed that, the media named CAOl/2 able to maintain GFP-fluorescence compare with the control DPCs which cultured with only 10% FBS contain DMEM, but the DPCs spheroid

shrinkage was unavoidable in both groups.

The shrinkage of the spheroids was avoided through the addition of fibroblast growth factor 2 (FGF-2) and platelet-derived growth factor-AA (PDGF-AA) to CAOl/2 and named CAO1/2-FP. Addition of FGF-2 and PDGF-AA was administrated in CAOl/2 medium as dose dependent manner. The concentrations of two growth factors FGF-2 and PDGF-AA were 20, 40 and 100 ng/mL, respectively. We noticed that these concentrations elevated the GFP-fluorescence of DPC spheroid which was significant during the culture days but unable to prevent spheroidal shrinkage. Interestingly the GFP-fluorescence and spheroid size were maintained during the 4day spheroid culture while the FGF-2 and PDGF-AA applied jointly in culture condition at higher concentration. In addition, the gene and protein expression of vcan, osteopontin, alkaline phosphatase and α-smooth muscle actin in the spheroids were augmented to levels similar to those of the intact dermal papillae, which exhibited restored hair-forming activity.

However, propagation and maintenance of DPCs became futile due loss the hair inductive properties over after several passage whereas isolation of DPCs itself laborious. To resolve this problem differentiation of DPCs properties in stem cells via extracellular vesicle (EV) could be an alternative way. Adipose derived stem cells (ASCs) are multipotent stem cells which are differentiated in several types of cells, if they are feed with appropriate condition media, for instance, chondrocyte medium differentiated ASCs into a chondrocyte cell. Recently, extracellular vesicle (EV)-mediated cell differentiation has gained attention in developmental biology due to genetic exchange between donor performed western-blot to scrutinize by specific and non-specific marker antibodies with DPC lysate and DPC-EVs lysate, respectively. Several antibodies are being used to detect EVs such as CD9, CD63, CD81, TSG101 and so on for positive marker, and Tubulin, CYC1 and HSP90 for negative marker. In our study, anti-CD63 antibody and anti-TSG101 antibody were applied to detect DPC-EVs. On the other hand, cytosolic fraction markers (HSP90 and CYC1) were detected only in cell lysate not in EVs lysate, which indicate that isolated EVs pure and were not contaminated by cell debris. In addition, Transmission electron microscope analyze confirmed that the average size of DPC derived EVs were between 80-170 nm.

To understand the effect of DPC-EVs on cell differentiation, DPC-EVs were characterized and incubated with ASCs, of monolayer and spheroid cell cultures in combination with the CAO1/2-FP medium specialized for DPCs described above. DPC-like properties in ASCs were initially evaluated by comparing several genes and proteins with those of DPCs via real-time PCR analysis and immunostaining, respectively. We also valuated the presence of hair growth-related microRNAs (miRNAs), specifically mir214-5P, mir218-5p, and mir195-5P. Here, we found that miRNA expression patterns varied in DPC-EVs between passage 4 (P4) and P5. We hypothesized that miRNA may enhance the expression of hair-inductive genes in ASCs, as well as increasing cell proliferation. However, it has been reported that miRNA expression changes in vitro-cultured DPCs in a passage-dependent manner, and a high expression of one such miRNA, mir195-5p, inhibited DPC proliferation when it was transfected. In particular, mir214 was significantly higher in P5 DPC-EVs than in P4 DPC-EVs. However in vivo, mir214 overexpression has been reported to be responsible for the reduction in HFs in developing skin, and for a delay in hair cycle progression during postnatal development. As we showed that the lower expression of mir214 in P4 DPC-EVs implies its potential role in providing DP-like properties to ASCs and DPC-EVs derived from the lower passage cells might have increased hair growth activities.

DPC-EVs in combination with CAO1/2-FP accelerated ASC proliferation at low concentrations. We have observed that DPC-EVs, at concentrations as low as 2 pg/mL in combination with CAO1/-2FP, enhanced the proliferation of ASCs. This strongly suggests that DPC-EVs are biologically active and affect the transformation of ASC properties into DP-like properties and propagated hair inductive gene expression for versican (vcan), alpha-smooth muscle actin (α-sma), osteopontin (opn), and N-Cam (ncam). BVs were

endocytosed by recipient cells, and the RNA cargo in the EVs was transferred, which in turn altered the recipient cell gene expression profile and function. Comparison between the expression of hair inductive genes (vcan, a-sma, ctnb, and others), the protein VCAN, α-SMA and β-Catenin (CTNB), and hair inductive miRNAs (mir214-5P, mir218-5p and mir195-5p) of DPC-EVs revealed similarities between P4 DPC-EVs-treated ASCs and DPCs.

In conclusion, a combination of certain adipogenic and osteogenic inducers, together with fibroblast growth factor 2 and platelet-derived growth factor-AA, can promote differentiation toward the DPC lineage and early passage DPC-EVs, in combination with CAO1/2-FP, enabled ASCs to differentiate into DPC-like cells.

論文審査結果の要旨

毛髪の新生や再生に重要な役割を果たす毛乳頭細胞(DPC)は、脱毛症の治療や再生医療に有用であるものの、体外に取り出して培養すると毛髪形成能力を容易に失ってしまう。毛髪形成能力は３次元培養によってスフェロイド化すると部分的に回復するものの、この状態を長く維持することは困難であることから、治療や病態解明への利活用が難しい。これらの問題に対処するために、申請者の Taheruzzaman Kazi 氏は、安定して毛髪形成能力を発揮する DPC を入手する方法を見つけることに注力した。氏は DPC が脂肪細胞および骨細胞と固有の遺伝子発現プロファイルを共有することに着目し、脂肪細胞分化を誘導する因子と骨形成分化を誘導する因子を、それぞれの細胞種の分化に適した濃度の 1/4 ずつ組み合わせた培地(CAO1/2)で DPC を刺激すると、毛髪形成能力と相関するバーシカン(vcan)遺伝子の発現が増加することを発見した。しかし培養中にスフェロイドが収縮して細胞が維持できなくなる問題は解消されなかったことから、線維芽細胞増殖因子 2 (FGF-2)および血小板由来増殖因子 AA (PDGF-AA)を CAO1/2 に添加することでスフェロイドの収縮が回避できることを見いだし、この培地を CAO1/2-FP と名付けた。この培地で培養された DPC スフェロイドでは、vcan と同様に DPC で特異的に発現するオステオポンチン(opn)、アルカリホスファターゼ(alp)、およびα-平滑筋アクチン(asma)の遺伝子およびタンパク質の発現が、生体から取り出したばかりの毛乳頭細胞と同様のレベルまで増強されることを明らかにした。

Kazi 氏は次に、生体からの単離が難しく、継代培養中に毛髪形成能力を失ってしまう DPC を安定的に入手する方法を開発するため、DPC の前駆細胞と考えられ容易に入手可能な多能性幹細胞である脂肪由来幹細胞(ASC)を刺激して、毛髪形成能力を有する DPC に分化させることを試みた。毛髪形成能力増強に効果的であった CAO1/2-FP で培養した DPC から、細胞外小胞(EV)を単離生成した後、CAO1/2-FP とともに ASC に添加したところ、DPC で特異的に発現する vcan、opn、asma, ncam の発現が上昇した。DPC-EV が ASC を DPC 様の細胞へと分化させる理由を探るため、氏は EV に含まれるマイクロ RNA(miRNA)を解析した。発毛に関連するといわれている mir214-5P、mir218-5p、および mir195-5P の含有量を計測したところ、継代数が４代(P4)の DPC から調整した EV と５代(P5)の DPC から調整した EV では発毛関連 miRNA の発現パターンが異なることを明らかにした。P4 の DPC は毛髪形成能力を保っているが、P5 の DPC では失われてしまうことから、P4 DPC-EV で P5 DPC-EV より高発現していた mir195-5p が ASC に対する毛髪形成能力

の付与に関わっている可能性を指摘した。また、逆に P4DPC-EV で発現が低かった mir214 は、皮膚で過剰発現させたマウスで毛包数の減少および出生後の毛周期進行の遅延が報告されていることから、P5DPC-EV が ASC に毛髪形成能力を付与できない一因となっていることを示した。また、P4 DPC-EV で刺激された ASC では、Wnt 経路の重要な因子であるβ-カテニンが活性化していることも明らかにした。

Kazi 氏は、DPC が脂肪細胞と骨芽細胞の特性を併せ持つことに着目して、脂肪細胞分化誘導因子と骨形成分化誘導因子を含む培養液 CAO1/2-FP を用いた miRNA 発現の適切な調節が Wnt/β-カテニン経路を活性化することで、ASC を DPC へと分化させる方法の有用性を示した。この着眼点はユニークなもので、比較的安価な因子を組み合わせることで容易に入手可能な幹細胞を分化させる可能性を示したことは高く評価でき、申請者は博士の学位を授与するに適するものと判断した。