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ヒト臍帯血由来間葉系幹細胞の培養液は、ヒトの皮膚の若返り機能を促進する。

Conditioned media from human umbilical cord blood-derived mesenchymal T stem cells stimulate rejuvenation function in human skin

HIghligts

USC-CM は、皮膚の若返りを促進する GDF-11 を含み、HDF - 繊維芽細胞由来、脂肪由来の間葉系幹細胞に比べて繊維芽細胞の移動やコラーゲン合成などを強力に促進します。

USC-CM は、ヒトの皮膚密度を増加させ、皮膚のしわを減少させました。

USC-CM は、フューチャーセルジャパン(株) が製造販売するベビーステム、セルコードの原料である
Kang Stem Bio Tech 社製の臍帯血由来培養上清液です。

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Conditioned media from human umbilical cord blood-derived mesenchymal stem cells stimulate rejuvenation function in human skin

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ヒト臍帯血由来間葉系幹細胞の培養液は、ヒトの皮膚の若返り機能を促進する。

Abstract（抄録）

皮膚の老化を抑制する治療法の開発は重要な研究テーマです。肌の老化防止に焦点を当てた若返りは、大きな課題のひとつです。最近の研究では、間葉系幹細胞（MSCs）が、創傷治癒に重要な多くのサイトカインを分泌することが示唆されています。この研究では、ヒト臍帯血由来間葉系幹細胞馴化培地（USC-CM）による皮膚の創傷治癒とコラーゲン合成の効果を調査しました。USC-CM には、上皮成長因子（EGF）、線維芽細胞成長因子（bFGF）、血小板由来成長因子（PDGF）、肝細胞成長因子（HGF）、コラーゲンタイプ 1 , さらに特に若返り因子の 1 つである成長分化因子 -11（GDF-11）など、皮膚の若返りに関連する多くの有用な成長因子があることがわかりました。in vitro の結果では、USC-CM が臍帯血由来以外の MSC 馴化培地（CM）のものと比較してヒト皮膚線維芽細胞（HDFs）の成長と細胞外マトリックス（ECM）産生を促進することを示しました。さらに、GDF-11 の役割を評価しました。結果は、GDF-11 が線維芽細胞の成長、移行、および ECM 生成を促進することが示されました。in vivo の結果では、USC-CM の局所治療が抗しわ効果を示し、女性の皮膚密度を有意に増加させたことを示した。結論として、USC-CM には、HDF の成長と ECM 生成を増加させることにより、肌の若返りを促進する GDF-11 を含むさまざまな成長因子を含むことが明らかとなりました。

1. はじめに

肌の若返りは、アンチエイジングのためにさまざまな医療や製品が使用され化粧品の焦点となっています。皮膚の老化は、生理学的および環境的要因により、年齢に依存する老化（内因性老化）と光老化（外因性老化）の 2 つの異なるタイプに分けることができます [1–3]。皮膚の健常性の障害は、皮膚の老化の最も顕著な兆候の 1 つです。年齢依存性の老化では、老化の主な兆候は皮膚のしわの出現ですが、光老化では、老化の兆候は、しわ、弾力性の喪失、変色、角質増殖、不規則な色素沈着や新生物などのさまざまな症状を伴います [4,5]。

グリコサミノグリカン、コラーゲン、エラスチンからなる皮膚の細胞外マトリックス（ECM）は、皮膚の形態や成長や弾力性などの機能に不可欠です [6]。皮膚の老化に伴って発生する ECM の劣化は、ヒアルロニダーゼ、エラスターゼ、コラゲナーゼなどの酵素の活性の増加に関連しています [7]。光老化は、ヒト皮膚線維芽細胞（HDFs）によるコラゲナーゼの産生を引き起こします。これは、コラーゲン産生を変性させ、しわとして皮膚に露出します。コラーゲンは真皮の重量の最大 70%を占めます。主要なコラーゲンはタイプ 1 と 3 で、真皮の引張強度としわの最小化に大きく関与しています。エラスチンは組織の主要なタンパク質成分であり、外圧に対し皮膚が元の位置に戻ることを補助しています。それは自然な弾力性と強さとなり、人体の組織修復において重要な役割を果たします [8]。皮膚の強度と弾力性が、真皮内のコラーゲン（タイプ 1 と 3 の両方）フィブリルとエラスチンの適切で均一な配置によって支配されていることは注目に値します [9]。

間葉系幹細胞（MSCs）は、骨髄、脂肪組織、臍帯血などのさまざまな組織に由来する多能性細胞です。原始的な臍帯血由来の間葉系幹細胞（UCB-MSCs）には、他の MSC と比較して生物学的な利点があります [10–12]。MSC は、表皮の継続的な再生の源であるため、皮膚で最も重要な細胞です [13]。最近の研究では、MSC がパラクリン効果を介して HDFs を刺激し、皮膚の創傷治癒を促進することが示唆されています [14,15]。
MSC は、上皮成長因子（EGF）、塩基性線維芽細胞成長因子（bFGF）、トランスフォーミング増殖因子ベータ（TGF-b）など、細胞増殖と皮膚組織の維持に重要な多くのサイトカインや成長因子を分泌します。
細胞増 [16,17]。しかし、皮膚の若返りのための成長因子におけるそれらの有益な役割が何であるかはまだ未知です [18]。
成長分化因子 -11（GDF-11）は、TGF-b スーパーファミリーのメンバーであり、脊椎動物の前後軸に沿った位置の同一性を特定するために広汎に作用する分泌シグナルです [19]。骨形成タンパク質 -11 としても知られる GDF-11 は、共生動物実験における若返り因子と見なされています [20]。若い動物と高齢動物が共通の血液循環を持っている場合、高齢の動物は多くの面で若くなる現象が起き、若い動物の血液からの可溶性因子が老齢の動物に影響を与える可能性があります。　Katsimpardi らは、若い動物の血清中の GDF-11 は、若返りのための極めて重要な可溶性因子であると主張しました [21]。GDF ファミリーと肌の若返りまたは肌の ECM との関係はこれまで証明されていません。唯一知られていることは、GDF ファミリーのメンバーである GDF-5 が、主にコラーゲンタイプ 1 で構成される複数の組織に影響を及ぼし、尾の腱や皮膚などの非体重負荷組織に一貫した生体力学的効果をもたらすことです [22]。本研究では、若い血液由来の hMSC が、さまざまな分泌された可溶性因子で人間の皮膚の老化を減衰することができる若返り因子を生成する可能性があると仮定しました。

2.Materials and Methods (実験方法)

2.1. Culture of AD-MSC, BM-MSC and UCB-MSC　脂肪、骨髄、臍帯血由来間葉系幹細胞の培養
UCB-MSC は、FORMIZ WOMEN's Hospital（IRB No. 219255-201305- BR-001、ソウル、韓国）によって承認されたヒト臍帯血から前述の方法で分離されました [23]。ヒト脂肪組織サンプルは、KODI MEDICAL（ソウル、韓国、IRB No. 219255-201407-BR-001-01）から入手しました。ヒト骨髄由来間葉系幹細胞は、SEVERANCEHOSPITAL（ソウル、韓国、IRB No. 4–2008–0643）から入手しました。AD-MSC、BM-MSC、および UCB-MSC を培養し、10％ウシ胎児血清（FBS）を含む KSB-3（Irvine Scientific）で 37° C および 5%CO2 で継代 5 まで増殖させました。）（Gibco）そして以前に報告されたようにそれを特徴づけた [24,25]。

2.2　HDF-CM、AD-MSC-CM、BM-MSC-CM および USC-CM の作製
HDF、AD-MSC、BM-MSC、および UCB-MSC（1.98×105 細胞 / フラスコ）を T-25 フラスコに播種し、10％ FBS を含む KSB-3（Irvine Scientific）で 48 時間培養しました。PBS で 2 回洗浄した後、培地を KSB-2 培地に交換しました。EGF（10 ng / ml）および bFGF（10 ng / ml）を含む DMEM（Gibco）、その後の 96 時間のインキュベーション期間。MSC と HDF の馴化培地（CM）を収集し、1500 rpm で 5 分間遠心分離し、最後に 0.22μm シリンジフィルターを使用してろ過しました。馴化培地は、GDF-11 ELISA キット（R＆D システム）を使用して、製造元のプロトコルに従って測定しました。

2.3. ヒト抗体アレイ
Human Antibody Array 1000（#AAH-BLM-1000-4、RayBiotech）を製造元の指示に従って使用し、ヒトタンパク質を分析しました。膜は検出バッファーを使用し、濃度計を使用して定量化されました。現像後、フィルムをスキャンし、Image J ソフトウェア（米国国立衛生研究所）を使用して画像を処理および定量化しました。シグナル強度は、比較のために内部のポジティブコントロールに対して正規化されました。

2.4. 培養

HDF（1×103 細胞 /well）を 96 ウェルプレートに播種し、KSB-3 培地で 24 時間培養しました。洗浄後、培地を対照培地（KSB-2 培地）またはさまざまな馴化培地（HDF-CM、AD-MSC-CM および USC-CM）に交換しました。GDF-11 で HDF の増殖を測定するために、培地を対照培地（DMEM）またはさまざまな濃度の GDF-11（0.1μg/ ml、0.2μg/ ml）に交換しました。72 時間後、CCK-8 キット（Dojindo、Gaithersburd、USA）を使用して HDF の増殖を測定しました。HDF を 10μl の CCK-8 溶液に加え、3 時間インキュベートした後、マイクロプレートリーダー（Tecan、Mannedorf、Switzerland）を使用して 450nm で吸光度を測定しました。各ウェルの光学密度値を使用して、検量線と比較することにより相対的な細胞数を計算しました。HDF のタンパク質含有量は、DCTM タンパク質アッセイキット（Bio-Rad）を使用して定量しました。

2.5. スクラッチアッセイ

ibidi Culture-Insert（No。81176、ibidi GmbH）を使用して、7×105 個の HDF を細胞培養システムに播種しました。このアプローチは、500μm の厚さの分離壁を備えた 2 つの細胞培養リザーバーを提供します。細胞遊走の測定のために、シリコンインサートは 24 時間後に取り外されました。作成されたギャップを洗浄し、各ウェルにコントロール培地（KSB-2 培地または DMEM）またはさまざまな馴化培地（HDF-CM、AD-MSC-CM、USC-CM）、およびヒト GDF-11（R&D システム）を充填しました。HDF は、位相差顕微鏡法によって創傷の 72 時間後に写真を撮り、Image J 分析を使用して手動で測定しました。データは、コントロールに対する移行の比率として報告されました。

2.6. HDF と HDF-CM、AD-MSC-CM、USC-CM の共培養

HDF（2×105 細胞 /well）を 6well plate に播種し、KSB-3 培地で 24 時間培養しました。洗浄後、各ウェルにコントロール培地（KSB-2 培地）またはさまざまな馴化培地（HDF-CM、AD-MSC-CM、USC-CM）を充填し、24 時間培養しました。

2.7. HDF と GDF-11 の共培養

HDF（2×105 細胞 /well）を 6well plate に播種し、KSB-3 培地で 24 時間培養しました。洗浄後、さまざまな濃度の GDF-11(0.01μg/ml, 0.1μg/ml) を添加した無血清 DMEM で 24 時間培養しました。

2.8. 逆転写ポリメラーゼ連鎖反応（RT-PCR）およびリアルタイム PCR

RNA ミニキット（Invitrogen）を使用して全細胞 RNA を抽出した後、cDNA 合成キット（Bioneer）を使用して逆転写を行いました。cDNA は、Accupower PCR プレミックス（Bioneer）を使用して増幅するか、SYBR Green PCR マスターミックス（Applied Biosystems）を各プライマーとともに使用して定量 PCR を行いました。各遺伝子発現レベルは、ハウスキーピングコントロールとして GAPDH または RPL13A で正規化されました。プライマー配列は補足表 1 に記載されています。

2.9. ウエスタンブロット分析

細胞を収集、洗浄し、PRO-PREPTM タンパク質抽出溶液（iNtRON Biotech）で抽出しました。タンパク質の 50μg を 8% ドデシル硫酸ナトリウム - ポリアクリルアミドゲル上で電気泳動により分離しました。タンパク質をニトロセルロースメンブレン（Bio-rad）に転写しました。メンブレンをコラーゲンタイプ 1、コラーゲンタイプ 4、フィブロネクチン、エラスチン、TGFb 受容体 1、SMAD2、p-SMAD2、SMAD3、p-SMAD3 および GAPDH（1：1000; abcam）の抗体とともにインキュベートした。次に、メンブレンを洗浄し、西洋ワサビペルオキシダーゼ (HRP) に結合した二次抗体（1：5000、abcam）とともにインキュベートしました。メンブレンは、ECL（GE Healthcare）を使用し定量化されました。平均ピクセル密度は、Image J Anarysis を使用して定量化されました。コラーゲンタイプ 1、コラーゲンタイプ 4、フィブロネクチン、エラスチン、TGFb 受容体 1、SMAD2、p-SMAD2、SMAD3、および p-SMAD3 の代表的なウエスタンブロットは、細胞溶解物抽出物がある場合にも総タンパク質または GAPDH のいずれかでタンパク質を補正します。

2.10. In vivo human test

ヒトでの in vivo 試験は supplementary materials and Methods を参照してください。

2.11. 統計分析

データは、3 つの独立した実験で統計分析は、SPSS バージョン 17.0（SPSS Inc.）を使用して実行されました。正規性は、シャピロ - ウィルク検定で検定されました。ベースラインでのグループ間の均一性は、ANOVA によってテストされました。グループ間の差異は、スチューデントの T 検定、ANOVA、およびダネット検定を使用した事後分析による ANOVA によって計算されました。P <0.05、0.01、および 0.001 の場合に、統計的有意性が考慮されました。

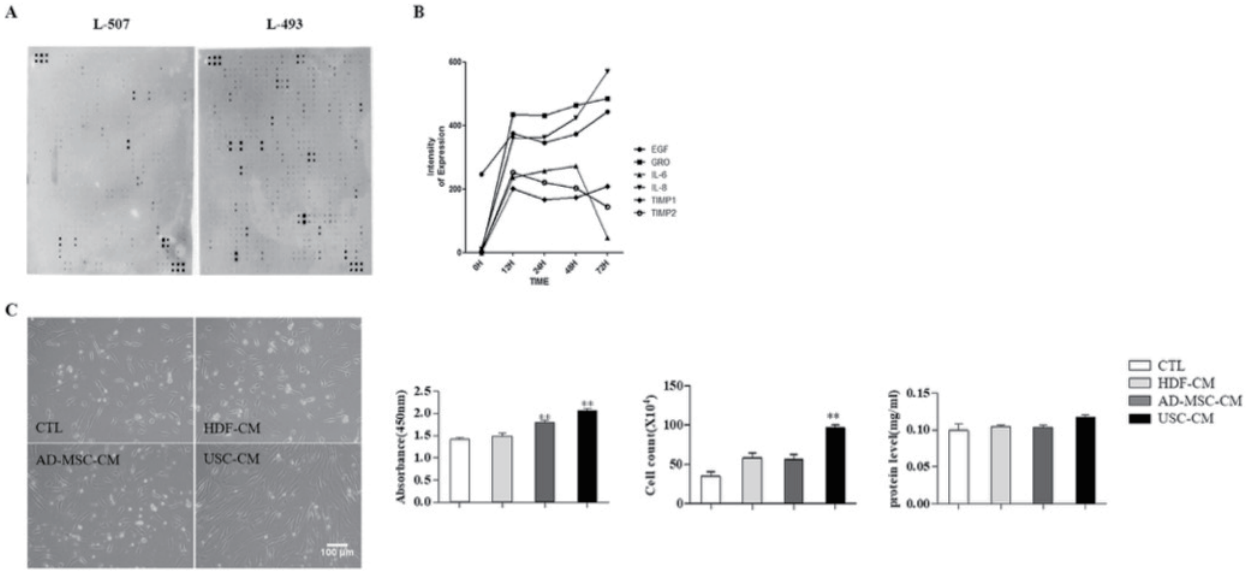


図 1. 馴化培地（CM）の選択。（A）USC-CM のヒト抗体アレイ分析。（B）USC-CM のサイトカイン発現のタイムコース。（C）さまざまな CM（培養上清）（CTL; KSB-2 培地、HDF-CM、AD-MSC-CM、および USC-CM）で培養後の HDF の成長と総タンパク質含有量（x100）。データは平均 ±SEM として表示。** P <0.01

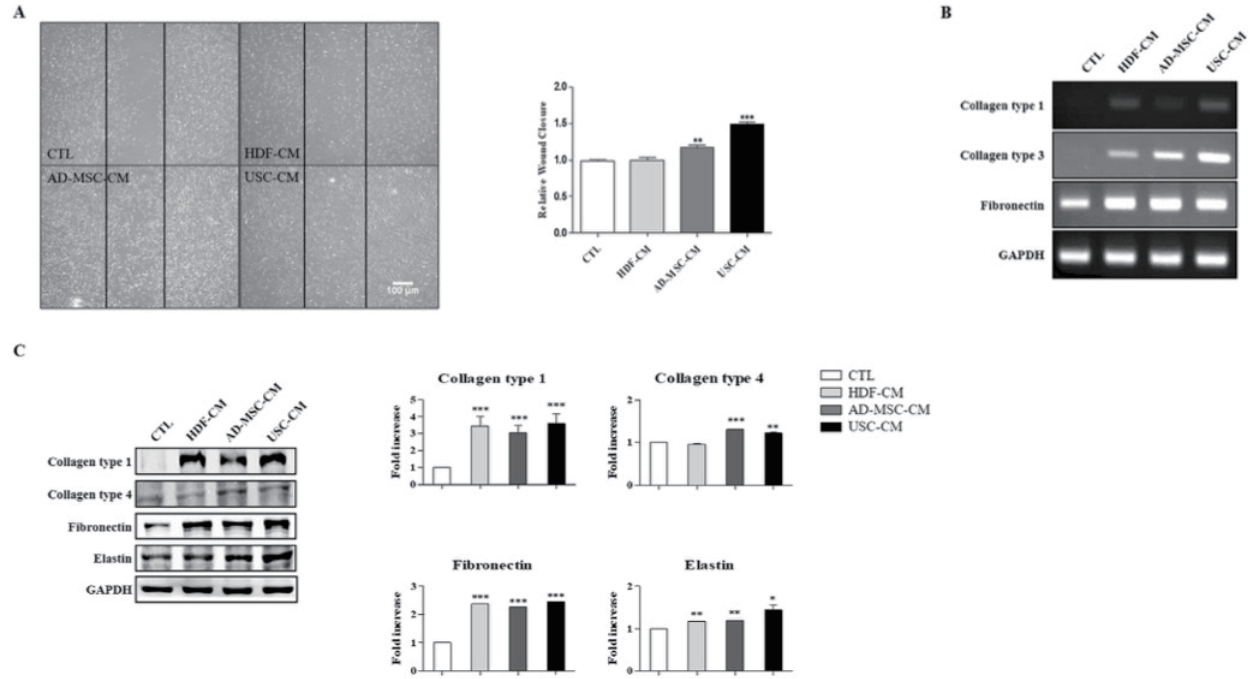


図 2. USC-CM は in vitro で HDF の移行と ECM の生成を促進しました。（A）CM 培養後の HDF の移行。USC-CM は、HDF の移行に最も効果があった（x40）（B）各培養上清で処理された HDF の ECM 遺伝子発現（CTL; KSB-2 培地、HDF-CM、AD-MSC-CM および USC-CM）。（C）各培養上清で処理された HDF の ECM 分泌。データは平均 ±SEM としてほ表示。* P <0.05、** P <0.01 および *** P <0.001。

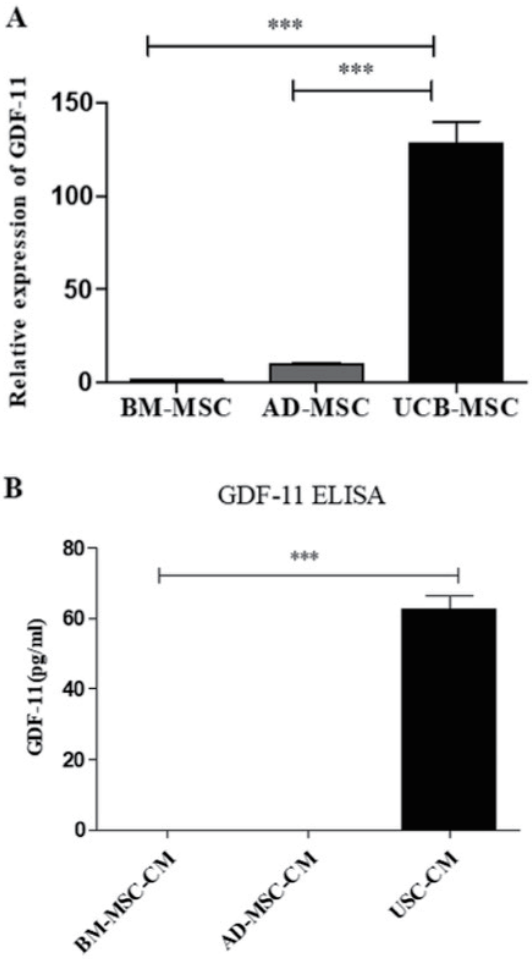
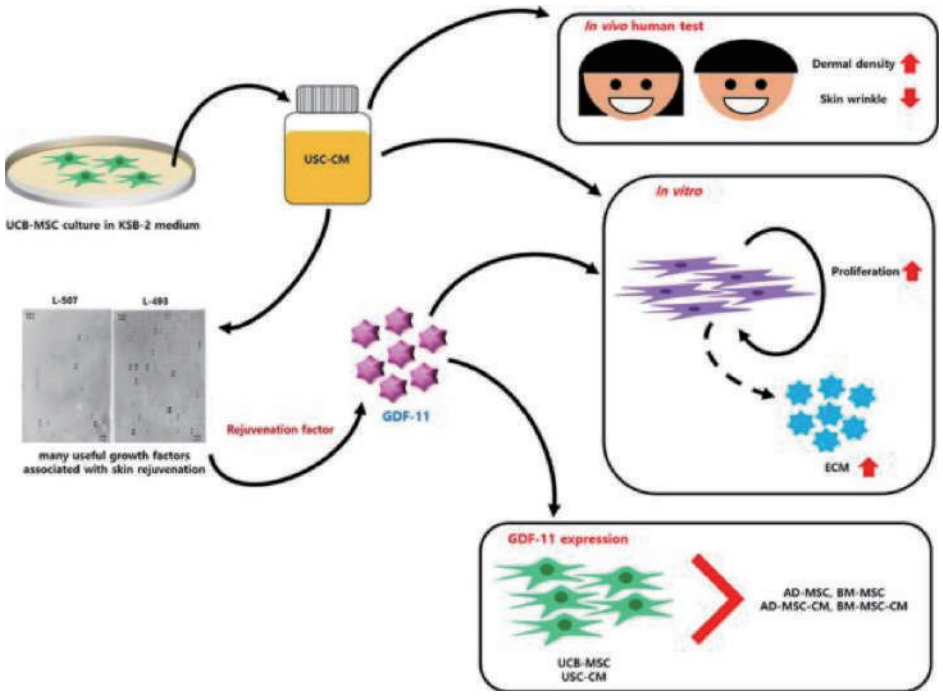


図 3.hMSC の GDF-11 発現。(A) 各培養条件での hMSC における GDF-11 の遺伝子発現。(B) ELISA キットを使用して GDF-11 の産生レベルを測定した。データは平均 ±SEM として表示。** P <0.01 および *** P <0.001。



Effect of USC-CM including GDF-11 in vivo and *in vitro*.

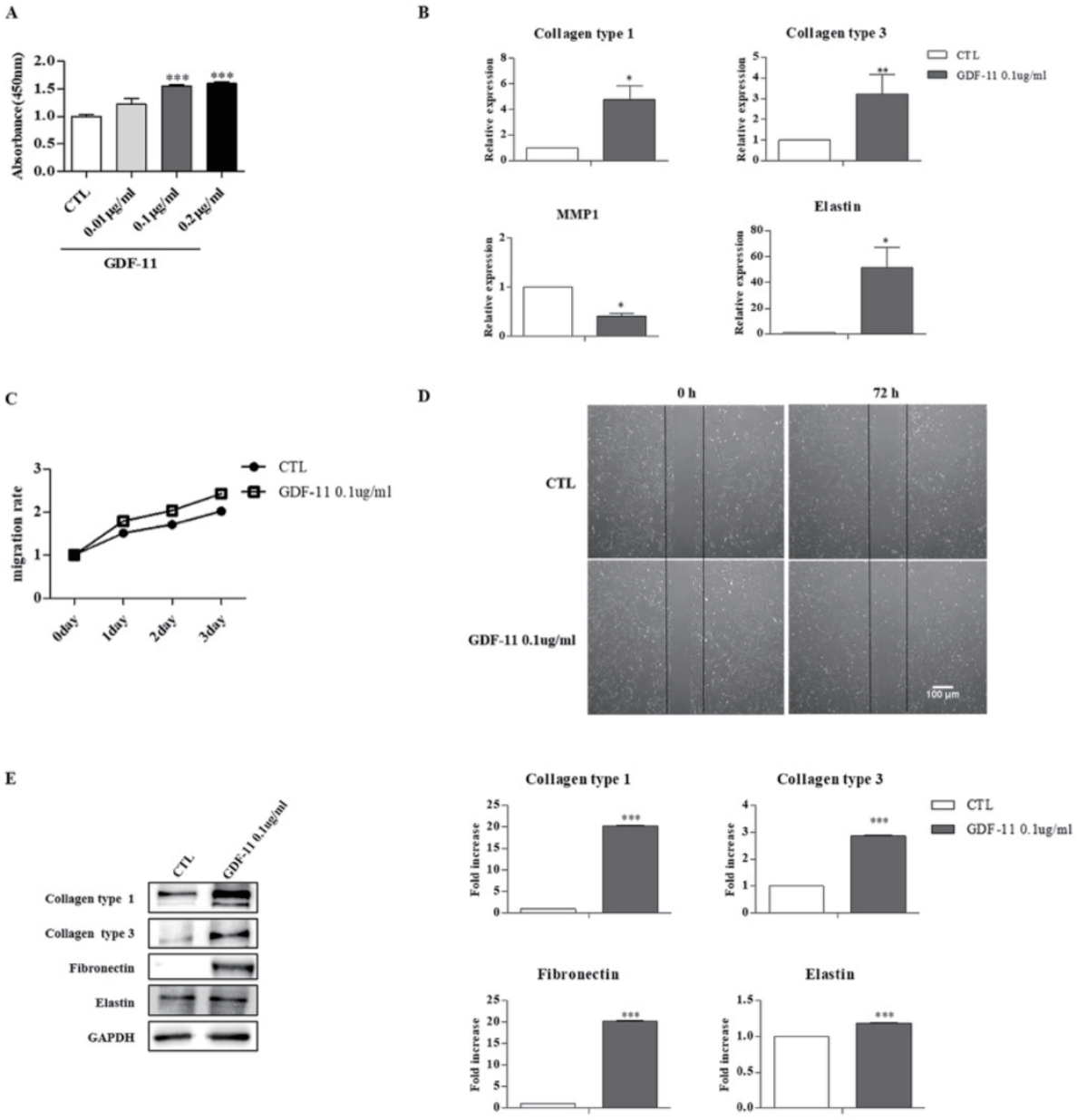


図 4.GDF-11 は in vitro で HDF の移行と ECM の生成を促進しました。(A) GDF-11 (0.01μg/ ml、0.1μgml) 処理で処理された HDF の増殖。(B) GDF-11 で脅威にさらされている ECM 発現 HDF の qRT-PCR 分析。(C) GDF-11 で処理した後の HDF の経時的成長率。(D) GDF-11 で 72 時間処理した後の HDF のスクラッチアッセイ。(E) GDF-11 で処理された HDF の ECM 分泌のウエスタンブロット分析。データは平均 ±SEM として表示。* P <0.05、** P <0.01 および *** P <0.001。

4. 考察

組織学的に、光老化した皮膚は、ECM 組成の顕著な変化を示しています。HDF は、細胞外マトリックスタンパク質と接着分子の供給源であるため、これらの変化において重要な役割を果たします [26]。幹細胞には自己複製と分化の可能性があります。これらの利点により、幹細胞治療は病気を防ぎ組織の修復を行います。ヒト MSC は、さまざまな成長因子、サイトカイン、およびその他のいくつかの ECM 調節物質を分泌します [27–29]。

本研究では、UCB-MSC の馴化培地が EGF、bFGF、TGF-b、PDGF、HGF、コラーゲンタイプ 1 などのさまざまな成長因子を含み、HDF の移動と増殖において最も顕著な効果を示したことを発見しました。hMSC-CM の他のソースに。USC-CM 処理は、HDF でのコラーゲンタイプ 1、コラーゲンタイプ 3、フィブロネクチン、エラスチンの産生を増加させ、コラーゲン産生に関与する重要な細胞になります。真皮の主要なコラーゲン成分はコラーゲン 1 型と 3 型であり [30]、コラーゲンのリモデリングは顔の若返りに重要な役割を果たします。

USC-CM には若返り因子の 1 つである GDF-11 が含まれていることがわかりました。GDF-11 の発現は若い動物の血液で見られました。それは加齢とともに減少し、中枢神経系を含む複数の組織の加齢を逆転させます [21,31,32]。ただし、HDF に対する GDF-11 の影響は、これまでまだ明らかとなっていませんでした。本報では、GDF-11 が HDF のコラーゲン 1 型および 3 型、エラスチン、フィブロネクチンなどの ECM タンパク質の成長と分泌を刺激することを初めて発見しました。これらの発見は、GDF-11 の若返り効果が以前に報告された特定の器官に加えて人間の皮膚にも適用される可能性があることを示唆しています。

USC-CM は、HDF からのコラーゲン、フィブロネクチン、エラスチンなどの ECM の生成を刺激することができ、USC-CM のこれらの機能の 1 つが GDF-11 の生成と分泌を担っています。UCB-MSC は、AD-MSC および BM-MSC と比較して最も多くの GDF-11 を生成しました。本論文では、GDF-11 が HDF と HDF の移行による ECM の生成のみを刺激できることを示しました。ただし、USC-CM には、HDF の ECM 生成の加速に寄与する可能性のある他の未定義の要因があります。これらの他の要因は、GDF-11 の唯一の効果を確認するために、近い将来に特定および調査する必要があります。

BM-MSC による皮膚創傷修復は、同種異系の新生児皮膚線維芽細胞を比較することにより、正常マウスと糖尿病マウスで示されました [33]。したがって、本研究では、USC-CM が線維芽細胞の遊走を刺激することで創傷治癒効果を加速できることを動物実験において調査した（データは示されていない）。さらに、私たちの USC-CM 治療は、AD-MSC-CM と同様に、メラノーマ B16 細胞におけるメラニンの合成とチロシナーゼの活性を阻害することが期待されています [34]。

私たちの以前の研究の研究は、さまざまな成長因子を含む大量の EGF がエクソソーム形態として USC-CM に存在することを発見しました [35]。エクソソームと細胞膜は一般的な脂質膜であるため、エクソソームで分泌される EGF などの主要なサイトカインは皮膚組織に容易に統合されます。これらは、USC-CM を含む化粧品が人間の肌に効果的である理由の 1 つです。当社の USC-CM は低温脱水粉末であり、使用前に化粧品ベースと混合されています。安定性試験の結果は、この混合物が冷蔵状態で 1 週間安定していることを示しました（データは示していません）。Lemestr らは GDF-11 が線維芽細胞の老化を減少させることを示した [36]。しかしながら、GDF-11 がヒトの皮膚の再生を促進できるかどうかはまだ不明です。この研究では、ヒトの皮膚における臍帯血由来の間葉系幹細胞の馴化培地の効果に焦点を当てました

In vivo 試験では、すべてのボランティアが申請前にこの冷蔵保管条件に署名しました。USC-CM と化粧品ベースの混合物は局所的に処理されますが、USC-CM の有効な要素は使用前に保存されたエクソソームでカプセル化されており、深部皮膚組織に簡単に統合できるため、深部皮膚に浸透できます。

まとめると、hUCB-MSC から分泌された GDF-11 は、HDF における細胞の成長と ECM タンパク質の発現を刺激する可能性があります。さらに、USC-CM の局所治療は、in vivo 研究で皮膚のしわを減らすことができます。これらの発見は、GDF-11 の若返り機能が中枢神経系に加えて皮膚にまで拡大できることを示唆しています。

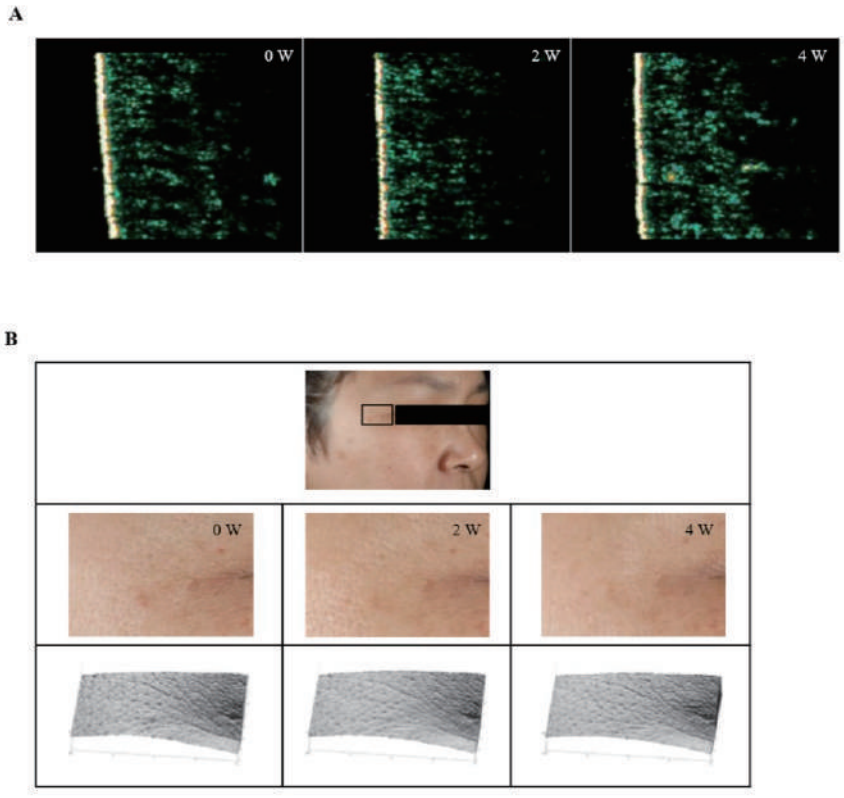
Supplementary Table 2. Concentration of secreted proteins of USC-CM

Secreted proteins	Concentration (pg/ml)
bFGF	2634
EGF	2305.72
Fibronectin	80
GDF-11	50
HGF	191.42
KGF	45
PDGF-AA	192.06
TGF-b1	90.25
Collagen type 1	4.15×10^8
VEGF	69.06

bFGF: basic fibroblast growth factor; EGF: epidermal growth factor; GDF-11: growth differentiation factor-11; HGF: hepatocyte growth factor; KGF: keratinocyte growth factor; PDGF-AA: platelet derived growth factor-AA; TGF-b1: transforming growth factor-beta 1; USC-CM: human umbilical cord blood-derived mesenchymal stem cell conditioned media; VEGF: vascular endothelial growth factor.

Supplementary Fig. 2. *In vivo* tests of USC-CM contained cosmetics.

(A) Dermal density measurement after daily USC-CM contained cosmetics treatment. Density was increased following time course. (B) Decreased skin wrinkle of eye end area after daily USC-CM contained cosmetics treatment.



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Conditioned media from human umbilical cord blood-derived mesenchymal stem cells stimulate rejuvenation function in human skin

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ABSTRACT

Developing treatments that inhibit skin aging is an important research project. Rejuvenation, which focuses on prevention of skin aging, is one of the major issues. Recent studies suggested that mesenchymal stem cells (MSCs) secrete many cytokines, which are important in wound healing. In this study, we investigated the effect of human umbilical cord blood-derived mesenchymal stem cells conditioned media (USC-CM) in cutaneous wound healing and collagen synthesis. We found that USC-CM has many useful growth factors associated with skin rejuvenation, such as Epithelial Growth Factor (EGF), basic Fibroblast Growth Factor (bFGF), Platelet Derived Growth Factor (PDGF), Hepatocyte Growth Factor (HGF), Collagen type 1, and especially, one of the rejuvenation factors, the growth differentiation factor-11 (GDF-11). Our in vitro results showed that USC-CM stimulate growth and extracellular matrix (ECM) production of Human Dermal Fibroblasts (HDFs) compared to those of other MSCs conditioned media (CM) from different origins. Moreover, we evaluated the roles of GDF-11. The results showed that GDF-11 accelerates growth, migration and ECM production of HDFs. Our in vivo results showed that topical treatment of USC-CM showed anti-wrinkle effect and significantly increased dermal density in women. In conclusion, USC-CM has various useful growth factors including GDF-11 that can stimulate skin rejuvenation by increasing growth and ECM production of HDFs.

1. Introduction

Skin rejuvenation has become the focus of cosmeceuticals, in which various medical treatments and products are used for anti-aging. Skin aging can be divided into two different types of age-dependent aging (intrinsic aging) and photo-aging (extrinsic aging) owing to the physiological and environmental factors [1–3]. Impairment of skin integrity is one of the most prominent indications of skin aging. In age-dependent aging the main indication of aging is the appearance of wrinkles on skin, while in photo-aging the indications of aging are accompanied with various symptoms such as wrinkles, loss of elasticity, discoloration, hyperkeratosis, irregular pigmentation, and other various neoplasms [4,5].

The skin extracellular matrix (ECM) that consists of glycosaminoglycans, collagen and elastin, is crucial for skin morphology and functions such as growth and elasticity[6]. The degradation of ECM that occurs along with skin aging is related to the increase of the activity of enzymes such as hyaluronidase, elastase and collagenase[7]. Photo-aging causes production of collagenase by Human Dermal Fibroblasts (HDFs), which degenerates collagen production and is exposed on skin as wrinkles. The collagen accounts for up to 70% of the weight of the dermis. The major collagens are type 1 and 3 that are largely responsible for the tensile strength of the dermis and minimization of the wrinkles. Elastin is a major protein component of tissues that helps skin to return to its original position when it is poked. It provides natural elasticity and strength and plays an important role in tissue reparation of the human body[8]. It is noteworthy that the strength and resiliency of skin are governed by proper and uniform arrangement of collagen (both type 1 and 3) fibrils and elastin in the dermis [9].

Mesenchymal stem cells (MSCs) are multipotent cells derived from a variety of tissues including bone marrow, adipose tissues and umbilical

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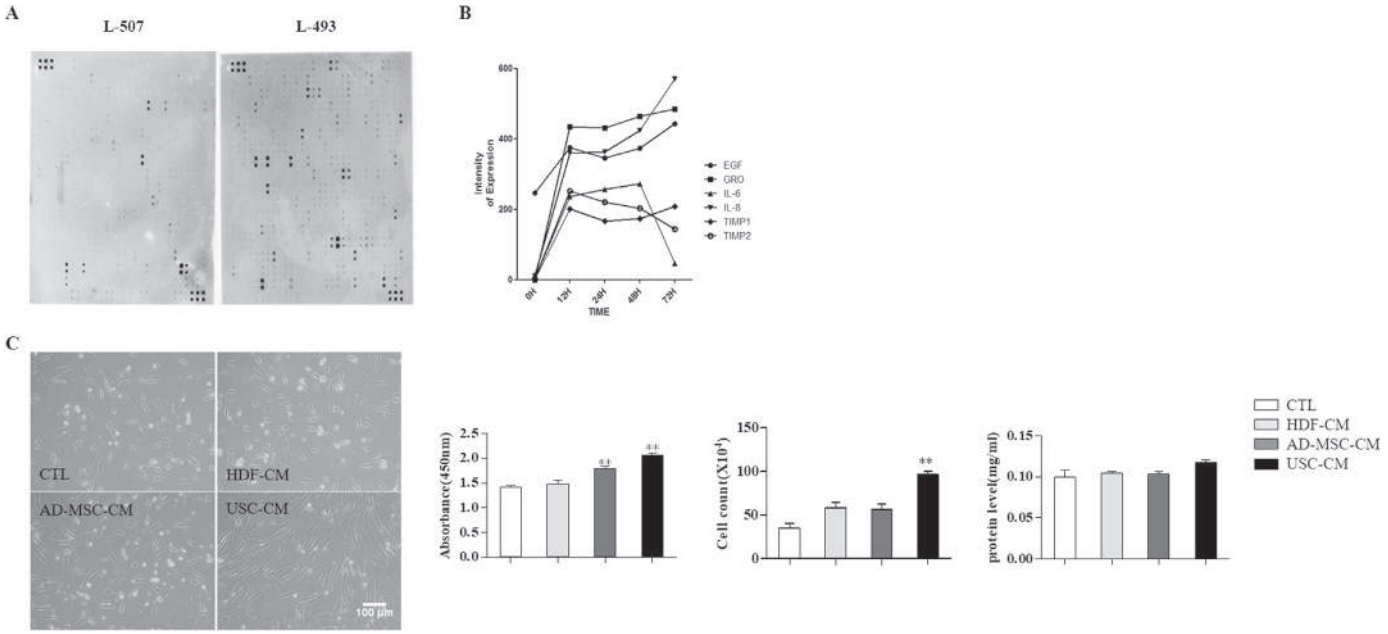

Fig. 1. Selection of conditioned media (CM). (A) Human antibody array analysis of USC-CM. (B) Time line of cytokine expression of USC-CM. (C) HDFs growth and total protein content after various CM (CTL; KSB-2 media, HDF-CM, AD-MSC-CM and USC-CM) (x100). Data are represented as the mean ± SEM.** $P < 0.01$.

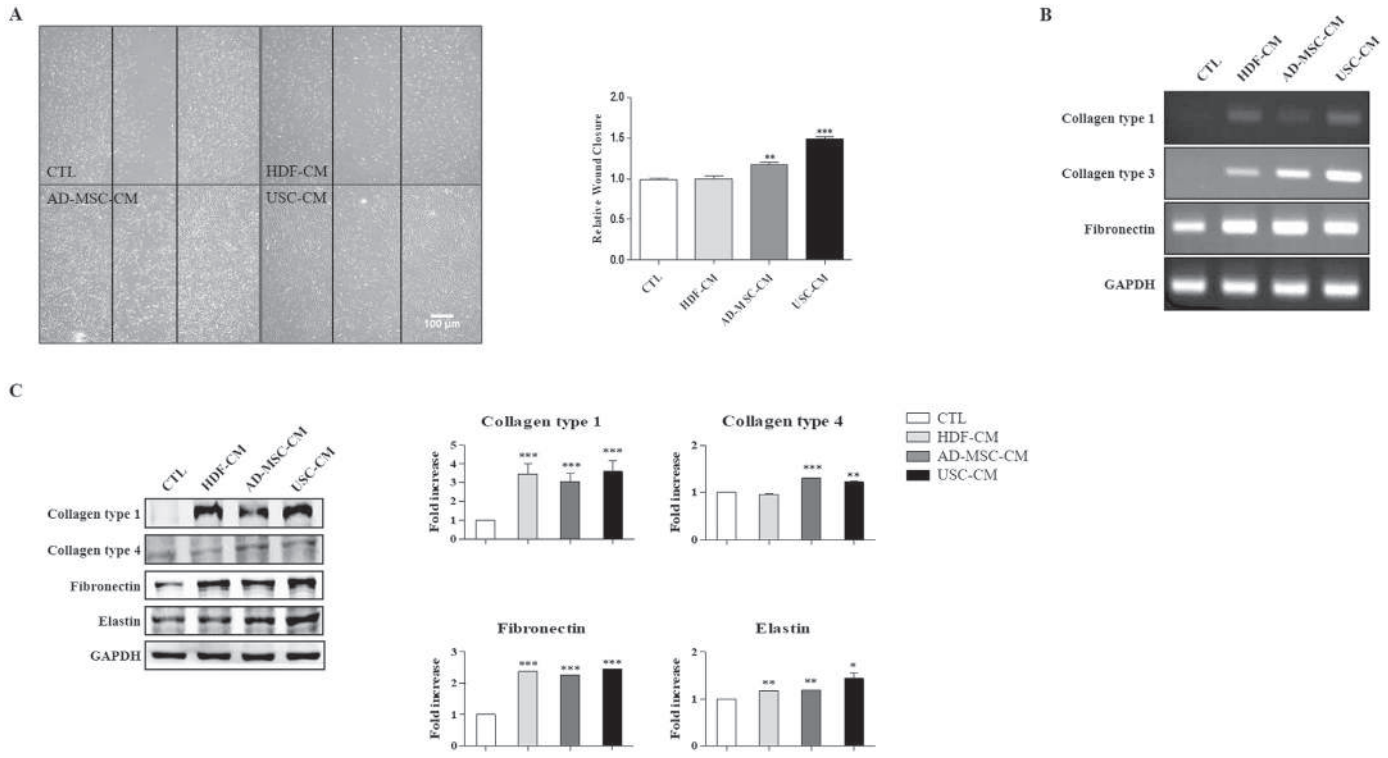

Fig. 2. USC-CM promoted HDFs migration and ECM production in vitro. (A) Migration of HDFs after CM culture. USC-CM is most effective in HDFs migration. (x40) (B) ECM gene expression of HDFs treated with each CM (CTL; KSB-2 media, HDF-CM, AD-MSC-CM and USC-CM). (C) ECM secretion of HDFs treated with each CM. Data are represented as the mean ± SEM.* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

cord blood. Primitive umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) have biological advantages, compared to other MSCs [10–12]. MSCs are the most important cells in the skin, as they are the source for continuous regeneration of the epidermis[13]. Recent studies suggested that MSCs stimulated HDFs via paracrine effects and enhanced cutaneous wound healing[14,15].

MSCs secrete many cytokines and growth factors such as Epidermal growth factor (EGF), basic Fibroblast growth factor (bFGF), Transforming growth factor-beta (TGF-b), which are important in cell growth and maintaining skin tissues[16,17]. However, it is still unclear what their beneficial roles in growth factors for skin rejuvenation[18].

Growth differentiation factor-11 (GDF-11) is a member of TGF-b superfamily and a secreted signal that acts globally to specify positional identity along the anterior/posterior axis of vertebrates[19]. GDF-11, also known as bone morphogenetic protein-11, is considered as a rejuvenation factor in symbiotic animal experiment[20]. When young

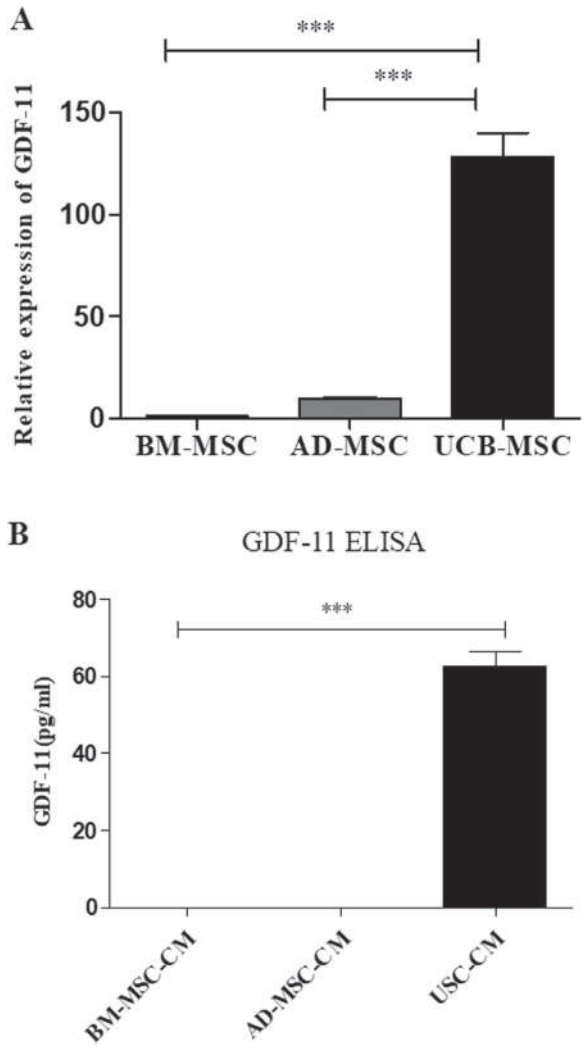

Fig. 3. GDF-11 expression of hMSCs. (A) Gene expression of GDF-11 in hMSCs with each culture condition. (B) The level of production GDF-11 was measured using ELISA kit. Data are represented as the mean ± SEM.** $P < 0.01$ and *** $P < 0.001$.

and old animals have a common blood circulation, the elder animal becomes younger in many aspects since soluble factors from blood of young animals can affect the elder animals. Katsimpardi et al. claimed GDF-11 in the serum from young animal is a pivotal soluble factor for rejuvenation [21] . The relationship between GDF family and skin rejuvenation or skin ECM has not been proven before. The only known thing is that GDF-5, a member of GDF family, influences multiple tissues composed primarily of Collagen type 1, with consistent biomechanical effects on non-weight-bearing tissues such as tail tendon and skin [22] . In this study, we hypothesized that young blood-originated hMSCs could produce rejuvenating factors that can attenuate the aging of human skins with various secreted soluble factors.

2. Materials and methods

2.1. Culture of AD-MSC, BM-MSC and UCB-MSC

UCB-MSCs were isolated from Human umbilical cord bloods approved by the FORMIZ WOMEN's Hospital (IRB No. 219255-201305-BR-001, Seoul, Korea) with previously described method [23] . Human adipose tissue samples were acquired from the KODI MEDICAL (Seoul, Korea, IRB No. 219255-201407-BR-001-01). Human bone marrow-derived mesenchymal stem cells were acquired from the SEVERANCE HOSPITAL (Seoul, Korea, IRB No. 4 –2008–0643). AD-MSC, BM-MSC and UCB-MSC were cultured and expanded up to passage 5 at 37℃ and 5% CO_2 in KSB-3 (Irvine scientific, Santa Ana, CA) with 10% fetal bovine serum (FBS) (Gibco) and characterized it as previously reported [24,25] .

2.2. Preparation of HDF-CM, AD-MSC-CM, BM-MSC-CM and USC-CM

HDF, AD-MSC, BM-MSC and UCB-MSCs (1.98×10^5 cells/Flask) were seeded in T-25 flask and cultured for 48 h in KSB-3 (Irvine Scientific, California) with 10% FBS. After PBS washing twice, the culture medium was changed to KSB-2 media; DMEM (Gibco) containing EGF (10 ng/ml) and bFGF (10 ng/ml), followed by incubation period of 96 h. Conditioned media (CM) of MSCs and HDFs were collected, centrifuged at 1500 rpm for 5 min, and finally filtered using a 0.22 μm syringe filter. The conditioned media were measured with GDF-11 ELISA kit (R&D systems, Minneapolis, MN) according to the manufacturer's protocol.

2.3. Human antibody array

Human proteins analyzed by using a Human Antibody Array 1000 (Cat. No. AAH-BLM-1000-4, RayBiotech) according to the manufacturer's instructions. Membranes were developed using detection buffer and quantified using a densitometer. After developing, films were scanned and the images processed and quantified using Image J software (National Institutes of Health). Signal intensity was normalized to internal positive controls for comparison.

2.4. Proliferation assay

HDFs (1×10^3 cells/well) were seeded in 96-well plates and cultured for 24 h in KSB-3 medium. After washing, the medium was replaced by control medium (KSB-2 media) or varying conditioned medium (HDF-CM, AD-MSC-CM and USC-CM). To measurement of HDFs proliferation with GDF-11, the medium was replaced by control medium (DMEM) or various concentrations of GDF-11 (0.1 μg/ml, 0.2 μg/ml). After 72 h, HDFs proliferation was measured using a CCK-8 kit (Dojindo, Gaithersburd, USA). HDFs were added to 10 μl of the CCK-8 solution, and incubated for 3 h, and then the absorbance was measured at 450 nm using a microplate reader (Tecan, Mannedorf, Switzerland). Optical density values from each well were used to calculate the relative cell numbers by comparing to the standard curves. The protein content of HDFs was determined by using a DC™ protein assay kit (Bio-Rad, Philadelphia, USA).

2.5. Scratch assay

7×10^5 HDFs were seeded into the cell culture system by using the ibidi Culture-Insert (No. 81176, ibidi GmbH, Munich, Germany,). This approach provides two cell culture reservoirs with a separation wall of 500 μm thick. For the measurement of cell migration, the silicon inserts were removed after 24 h. The gaps created were washed and each well was filled with control medium (KSB-2 media or DMEM) or varying conditioned medium (HDF-CM, AD-MSC-CM and USC-CM) as well as Human GDF-11 (R&D systems, Minneapolis, USA). HDFs were photographed 72 h after wounding by phase-contrast microscopy and measured manually with Image J analysis. The data were reported as the ratio of migration relative to the control.

2.6. Co-culture of HDFs with HDF-CM, AD-MSC-CM and USC-CM

HDFs (2×10^5 cells/well) were seeded in 6-well plates and cultured for 24 h in KSB-3 medium. After washing, each well was filled with control medium (KSB-2 media) or varying conditioned medium (HDF-CM, AD-MSC-CM and USC-CM), followed by incubation period of 24 h.

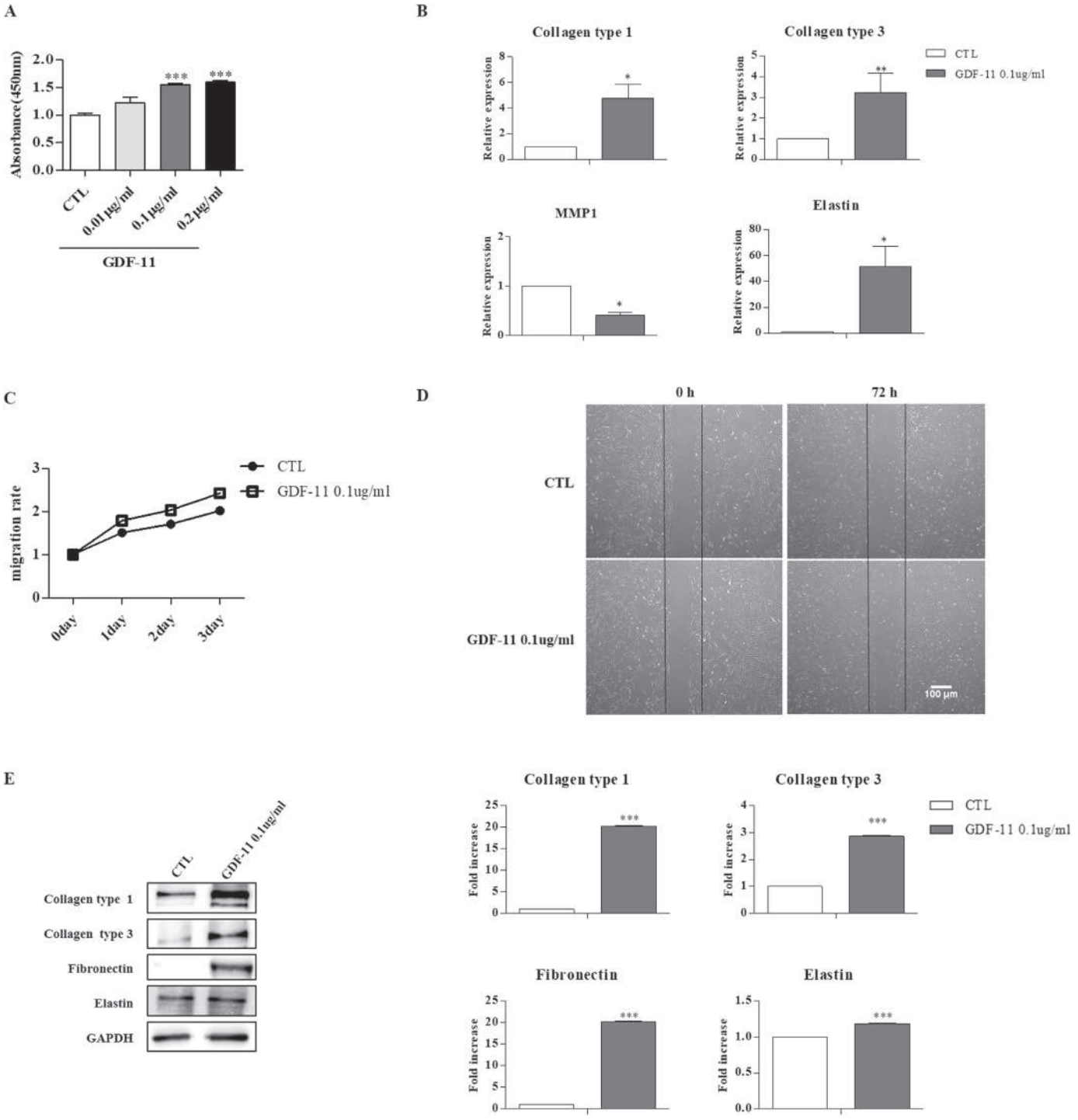

Fig. 4. GDF-11 promoted HDFs migration and ECM productionin vitro (A) The proliferation of HDFs treated GDF-11 (0.01 μg/ml, 0.1 μg ml) treatment. (B) qRT-PCR analysis for ECM expression HDFs threated with GDF-11. (C) Time-course growth rate of HDFs following treated with GDF-11. (D) Scratch assay of HDFs following treated with GDF-11 for 72 h. (E) Western blot analysis for ECM secretion of HDFs threated with GDF-11. Data are represented as the mean ± SEM. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

2.7. Co-culture of HDFs with GDF-11

HDFs (2×10^5 cells/well) were seeded in 6-well plates and cultured for 24 h in KSB-3 medium. After washing, different concentrations of GDF-11 (0.01 μg/ml, 0.1 μg/ml) with serum-free culture medium (DMEM) were changed for additional 24 h culture.

2.8. Reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR

Total cellular RNA was extracted using an RNA mini kit (Invitrogen, Waltham, USA), followed by a reverse transcription using a cDNA synthesis kit (Bioneer, Daejeon, Korea). cDNA was amplified using the Accupower PCR premix (Bioneer) or followed by quantitative PCR using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with each primer. Each gene expression level was normalized with

GAPDH or RPL13A as housekeeping controls. The primer sequences were listed in Supplementary Table 1.

2.9. Western blot analysis

Cells were collected, washed, and extracted with PRO-PREP™ protein extraction solution (iNtRON Biotech, Sungnam, Korea). Fifty micrograms of proteins were separated on an 8% sodium dodecyl sulfate-polyacryamide gel by electrophoresis. The proteins were transferred to Nitrocellulose membranes (Bio-rad, Hercules, CA). The membranes were incubated with antibodies of Collagen type 1, Collagen type 4, Fibronectin, Elastin, TGFb Receptor1, SMAD2, p-SMAD2, SMAD3, p-SMAD3 and GAPDH (1:1000; abcam, Cambridge, USA). Then, the membranes were washed and incubated with a secondary antibody conjugated to horseradish peroxidase (1:5000, abcam). Membranes were developed using ECL (GE Healthcare, Pittsburgh, USA) and quantified using a densitometer. Mean pixel density was quantified using Image J analysis Representative western blot of Collagen type 1, Collagen type 4, Fibronectin, Elastin, TGFb Receptor1, SMAD2, p-SMAD2, SMAD3 and p-SMAD3 those of either total protein or GAPDH to correct for protein loading in the case of cellular lysate extracts.

2.10. In vivo human test

In vivo tests on human were performed as described in the Supplementary Materials and Methods Section.

2.11. Statistical analysis

Data are representatives of three independent experiments. Statistical analysis was performed with SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Normality was tested with Shapiro-Wilk test. Homogeneity between groups at baseline was tested by ANOVA. Differences intergroup were calculated by student's T test, ANOVA, and ANOVA with a post hoc analysis using the Dunnett's test. Statistical significance was considered when $P < 0.05$, 0.01 and 0.001.

3. Results

3.1. UCB-MSC secretes cytokines that stimulate HDFs growth

For the anti-aging effect in human skin, HDFs growth and its production of ECM are the most essential factors. Therefore, we focused on the HDFs growth and the ECM production. UCB-MSC secrete various useful cytokines (Fig. 1A). They secrete several different cytokines, which were detected with cytokine dot-blot (Fig. 1A). In addition, we compared UCB-MSC conditioned media (USC-CM) with two different MSC-CM (AD-MSC-CM, BM-MSC-CM) using the human growth factor antibody array C1 (Cat. No. AAH-GF-1–8, RayBiotech) (Supplementary Fig. 1). USC-CM strongly contained skin-related proteins compared with AD- and BM-MSC-CM. Most of the major cytokines were secreted from UCB-MSC within 48 h (Fig. 1B). Four days of culture with KSB-2 media was evaluated as a proper culture method for making conditioned media. In this USC-CM, major growth factors were evaluated with ELISA (Supplementary Table 2). EGF, bFGF, HGF, PDGF and Collagen type 1 were highly increased in USC-CM. Additionally, HDFs and AD-MSC were cultured with KSB-2 media with the same culture method used for UCB-MSC. Four days later, each type of CM from UCB-MSC, AD-MSC and HDFs was collected and added to HDFs instead of the regular HDFs culture medium. After 72 h of culture in these conditioned media, we found that HDFs showed greater proliferation in USC-CM than in AD-MSC-CM and HDF-CM. There were no significant effects of total protein contents on HDFs (Fig. 1C). Therefore, we selected USC-CM, which was cultured with UCB-MSC for 4 days in KSB-2 media for the following experiments.

3.2. USC-CM promoted HDFs migration and ECM production in vitro

In order to determine HDFs proliferation with various conditioned media, scratch assay was performed (Fig. 2A). USC-CM showed the most distinguished proliferation and recovery/migration property compared to those of HDF-CM and AD-MSC-CM. Therefore, we evaluated the ECM gene expression of HDFs treated with each type of CM. The results of Collagen type 1 and 3 gene expression were significantly increased (Fig. 2B). These results were confirmed in protein levels with western blot analysis (Fig. 2C). Especially, from USC-CM culture Collagen type 1 and Elastin were most abundantly secreted from HDFs compared to HDF-CM and AD-MSC-CM. All data demonstrate that USC-CM strongly promoted HDFs migration, collagen synthesisin vitro compared with HDF- and AD-MSC-CM.

3.3. UCB-MSC expressed and secreted high level of GDF-11

Among secreted cytokines from UCB-MSC, one of the known rejuvenation factors, GDF-11, was found (Supplementary Table 2). The GDF-11 expression was compared among the three types of conventionally used human mesenchymal stem cells (hMSCs), BM-MSC, AD-MSC and UCB-MSC. Among these three types of hMSCs, UCB-MSC showed the most abundant RNA expression of GDF-11 as expected since UCB-MSC was originated from the youngest tissues (Fig. 3A). The RNA expression level of GDF-11 was more than 100 folds greater in UCB-MSC than BM-MSC and AD-MSC. Each of the hMSCs was cultured in KSB-2 media for 4 days to compare the GDF-11 protein expression levels among the three clones of hMSCs (Fig. 3B). GDF-11 protein secretion from UCB-MSC was also the highest among the 3 different hMSCs clones. This analysis demonstrated that GDF-11 expression/secretion was the highest in UCB-MSC compared to those of other hMSCs.

3.4. GDF-11 promoted HDFs migration and ECM production in vitro

UCB-MSC secretes various cytokines and can stimulate ECM production. Among these cytokines, GDF-11 was proven to be an essential factor for rejuvenation. To verify the role of GDF-11 in HDFs, we evaluated the cell growth and ECM gene expression of HDFs treated with GDF-11. We investigated HDFs growth ability of GDF-11 (0.01 μg/ml, 0.1 μg/ml). GDF-11 showed accelerated proliferation of HDFs and showed dose-dependent manner (Fig. 4A). Therefore, the optimal concentration of GDF-11 was decided as 0.1μg/ml. In addition, the results of Collagen type 1, 3 and Elastin gene expression were significantly increased. Furthermore, one of collagenase, MMP-1 expression was significantly decreased in GDF-11 (0.1 μg/ml) compared to control group (Fig. 4B). Consecutively, the proliferation of HDFs were significantly increased in GDF-11 (0.1 μg/ml) group compared to control group for 72 h (Fig. 4C). To determine the effect of GDF-11 (0.1 μg/ml) on migration of HDFs, scratch assay was performed. Images of the closing area at 0 and 72 h is shown in Fig. 4D. These expressions and secretions were confirmed in protein expression levels, which were detected in western blot analysis. The protein levels of Collagen type 1, 3, Fibronectin and Elastin were significantly increased in GDF-11 group compared to control group (Fig. 4E). All data demonstrate that GDF-11 promoted HDFs migration, collagen synthesisin vitro.

3.5. USC-CM contained cosmetics increased dermal density and decreased skin wrinkle in human

For in vivo test, 22 volunteers (18–55 years-old women) were selected. After IRB and signature of consent, 10% cryo-preserved USC-CM in cream base were treated daily to their skin. The subjects were observed every 2 weeks and the final observation was performed at 4 weeks from the starting point (Supplementary Fig. 2). In this in vivotest, there was no irritation, stinging or any adverse reaction observed. Dermal density was measured with ultrasound and it was increased as

following treatment times (Supplementary Fig. 2A). The skin density at 4 weeks after the treatment was significantly increased by 2.46% when compared to that of before treatment (Supplementary Table 3). The skin wrinkles of eye-end area were also decreased after the treatment (Supplementary Fig. 2B). These results were measured and counted with digital micro mirror devices. Four weeks after the treatment, skin wrinkles of eye-end area were significantly decreased (Supplementary Table 4). Especially, maximum of all peak-to-valley value (Rmax) and maxium profile peak-height (Rp) were significantly decreased.

4. Discussion

Histologically, photo-aged skin shows marked alterations in ECM composition. HDFs play key roles in these changes because they are the source of extracellular matrix proteins and adhesive molecules[26]. Stem cells have self-renewing and differentiation potentials. According to their benefits, stem cell therapy has prevent a disease and reparation of tissue. Human MSCs secrete various growth factors, cytokines and several other ECM regulating materials [27–29].

In the present study, we found that conditioned medium of UCB-MSCs contained a variety of growth factors such as EGF, bFGF, TGF-b, PDGF, HGF, Collagen type 1 and exhibited the most prominent effect in migration and proliferation of HDFs compared to other sources of hMSC-CM. USC-CM treatment increased the production of Collagen type 1, Collagen type 3, Fibronectin and Elastin in the HDFs, which makes it the key cell responsible for collagen production. The major collagenous components of dermis are Collagen type 1 and 3[30] and collagen remodeling plays an important role in facial rejuvenation.

We found USC-CM contained one of the rejuvenation factors, GDF-11. The expression of GDF-11 was found in the blood of young animals. It declines with aging, and reverses aging in multiple tissues including central nervous system[21,31,32]. However, the effects of GDF-11 on HDFs have not yet been addressed previously. In this paper, we discovered for the first time that GDF-11 stimulates growth and secretion of ECM proteins including Collagen type 1 and 3, Elastin and Fibronectin in HDFs. These findings suggest that rejuvenating effect of GDF-11 could be expended to human skin in addition to the specific organs previously reported.

USC-CM can stimulate the productions of ECM such as Collagen, Fibronectin and Elastin, from HDFs and one of these functions of USC-CM is responsible for GDF-11 production and secretion. UCB-MSC produced the highest amount of GDF-11 compared to AD-MSC and BM-MSC. In this paper, we showed that GDF-11 could solely stimulate ECM production from HDFs and HDFs migration. However, there are other undefined factors in USC-CM that can contribute in accelerating the ECM production of HDFs. These other factors must be identified and studied in the near future to confirm the sole effect of GDF-11.

Skin wound repair by BM-MSC was shown in normal and diabetic mice by comparing their allogeneic neonatal dermal fibroblasts [33]. Thus we investigated that USC-CM can accelerate wound healing effect in animal studies by stimulating fibroblast migration (data not shown). Furthermore, our USC-CM treatments are expected to inhibit the synthesis of melanin and the activity of tyrosinase in melanoma B16 cells as AD-MSC-CM do [34].

Research of our previous study found that high amount of EGF including various growth factors exists in USC-CM as exosome forms[35]. Major cytokines, such as EGF, secreted in exosomes are easily integrated to skin tissues since exosomes and cell membranes are common lipid membranes. These are one of the explanations why USC-CM contained cosmetics are effective to human skin. Our USC-CM is cryo-dehydrated powder and mixed with cosmetic base before use. Stability test results showed this mixture is stable for a week in refrigerated condition (data not shown). Lemestr et al. showed that GDF-11 decreased fibroblast senescence[36]. However, it is still unclear if GDF-11 can enhance skin regeneration via human applied. In this study, we focused on the effect of conditioned media from human umbilical cord blood-derived mesenchymal stem cells in human skin. In vivo tests, every volunteer signed this refrigerated storage condition before application. Even though USC-CM and cosmetic base mixture are treated topically, they can penetrate to deep skin since effective factors of USC-CM are encapsulated with exosomes preserved before usage and they can easily integrate to deep skin tissues.

Taken together, GDF-11 secreted from hUCB-MSCs could stimulate cellular growth and expression of ECM proteins in HDFs. Moreover, topical treatment of USC-CM could decrease skin wrinkles in vivo study. These findings suggest that the rejuvenating functions of GDF-11 can be expanded to the skin in addition to the central nervous system.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.10.007.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.10.007.

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