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厚生労働科学研究費補助金

難病・がん等の疾患分野の医療の実用化研究事業
（再生医療関係研究分野）

ヒト成体間葉系幹細胞の再生医療実現のための ゲノム科学に基づく品質管理と体内動態研究

平成２５年度　総括・分担研究報告書

研究代表者　落谷　孝広

平成２６（２０１４）年５月

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ヒト成体間葉系幹細胞の再生医療実現のためのゲノム科学に基づく品質管理と体内動態研究

研究代表者　落谷孝広　国立がん研究センター研究所分子細胞治療研究分野・分野長

研究要旨

平成 25 年度に計画していた研究内容は、①hAD-MSC の均質性評価系の構築の基盤研究、②hAD-MSC の肝硬変モデルマウスにおける有効性評価及び IVIS による体内動態の解析である。

均質性評価系構築の基盤研究の進捗としては、ストレス負荷で変化する分泌型 miRNA の同定を目的とし、継代ストレス（P4,P6,P11）による分泌型 miRNA の発現プロファイル解析を進めている。現在までに、培養上清中の分泌型 miRNA 量が継代ストレス負荷により増加するという結果が得られており、継代ストレスが分泌型 miRNA 発現に影響を及ぼすことが示唆された。現在は個々の分泌型 miRNA 発現量について Micro array 解析を実施中であり、平成 25 年度中には 20～30 種類のターゲット miRNA の同定を完了した。またデジタル PCR による高感度 miRNA 解析についても既に培養上清を用いた条件検討に着手しており、分泌型 miRNA による品質管理の実践については計画より早く実用化できると考えている。

肝硬変モデルマウスにおける有効性評価及び IVIS による体内動態解析については、四塩化炭素誘発肝硬変モデルに対して hAD-MSC を既に投与しており、AST 及び ALT 等の改善を図る事に成功した。一方で本試験モデルは最短でも 8 週間を要するため、別途四塩化炭素誘発急性肝障害モデルを用いた検討も並行して進めている。現在までに急性肝障害モデルを用いた hAD-MSC の有効性の確認、さらに hAD-MSC に最適な蛍光色素及び標識条件の確立は終了し、疾患モデルにおいて、hAD-MSC の肝臓への集積を示す IVIS の結果も得られている。

A．研究背景、目的

（背景）

本提案では、最新のゲノム解析技術を用い投与前の細胞の均質性、移植後の細胞の生着状況、体内循環状態、分化状況の定量的解析を行うことにより移植細胞の詳細な動態解析の実現を目的としている。これにより、ヒト脂肪組織由来間葉系幹細胞である hAD-MSCの安全かつ効果的な投与条件を定量データから予測することが可能となり、安全性の高い再生医療実現へ向け幅広い応用が期待できる。

このような背景のもと、本研究ではゲノム解析技術を応用し、種々ストレス下における分泌型 miRNAの発現プロファイルからバイオマーカーを同定し、さらにデジタルPCRによる高感度定量解析を行うことで、新しい細胞の均質化方法を開発することを目的としている。　また移植細胞の体内動態および生着細胞の機能解析についても、デジタル PCR及び並列型シーケンサーを用いたゲノム解析技術を応用した新しい定量解析法の開発を本研究の目的としている。

平成25年度の研究分担として、国立がん研究センター とロート製薬では、hAD-MSCのストレス負荷による培養上清中分泌型miRNAの発現プロファイル解析及びストレス応答性のバイオマーカーとなるmiRNAの同定、さらにhAD-MSCの肝障害モデルマウス及び肝線維化モデルマウスを用いた有効性評価、及び体内動態解析の従来法として蛍光標識hAD-MSCによる高感度in vivo imaging技術を用いた体内動態解析を行った。

B．研究方法

①ストレス応答性 miRNA の探索及び同定

Lonza 社から購入した hAD-MSC を培養フラスコで 4 継代培養した細胞（P4 細胞）および 11 継代培養した細胞（P11 細胞）を作製、それぞれの培養上清からエクソソームを精製した。精製したエクソソームを Agilent 社 Human miRNA microarray により P4 細胞及び P11 細胞から分泌された miRNA 発現プロファイルを比較した。

②hAD-MSCs の肝障害モデルマウス及び肝線維化モデルマウスによる有効性評価

●肝障害モデル

BALB/c マウスの腹腔内に四塩化炭素を投与し肝障害を誘発した。四塩化炭素投与 1 日後に $1x10^6$cells/body の用量で hAD-MSC を尾静脈から投与した。細胞投与 1 日後にそれぞれのマウスから採血を行い、各種生化学検査を行った。

●肝線維化モデル

BALB/c マウスの腹腔内に四塩化炭素を週 2 回、8 週間投与し肝線維化を誘発させた。8 回目の四塩化炭素投与後、$1x10^6$cells/body の用量で hAD-MSC を尾静脈から投与した。細胞投与 4 週後、マウスから

採血及び肝臓を摘出し、各種生化学検査及びMasson trichrome染色により線維化部分を染色し、画像解析により線維化部分の面積を算出した。

③蛍光標識hAD-MSC sによる体内動態解析

hAD-MSCにDiRを添加しDiRラベルhAD-MSCsを作製した。DiR-MSCsをマウス肝線維化モデルに1 x10^6cells/bodyの用量で尾静脈から投与、投与2時間、1日、2日、3日、1週間、2週間、3週間、4週間における蛍光シグナルの継時変化をIVISにより解析した。

（倫理面への配慮）

本研究課題で用いるヒト脂肪由来間葉系幹細胞はインフォームドコンセントを得て取得されたものであり、海外の市販品を購入するため倫理的な問題は起こらない。また本研究課題においてはヒトへの臨床応用は実行されない。

動物実験は、国立がん研究センターおよび委託試験先である三菱メディエンスの定める動物実験指針の基準に従うとともに、動物倫理委員会の承認を得たうえで、動物の苦痛軽減に努め、動物愛護の精神に基づく実験を行う。

C．研究結果

1. hAD-MSCの均質性評価系の構築の基盤研究：

均質性評価系構築の基盤研究の進捗としては、ストレス負荷で変化する分泌型miRNAの同定を目的とし、継代ストレス(P4,P6,P11)による分泌型miRNAの発現プロファイル解析を進めている。現在までに、培養上清中の分泌型miRNA量が継代ストレス負荷により増加するという結果が得られており、継代ストレスが分泌型miRNA発現に影響を及ぼすことが示唆された。現在は個々の分泌型miRNA発現量についてMicro array解析を実施中であり、平成25年度12月中には20～30種類のターゲットmiRNAを同定する予定である。またデジタルPCRによる高感度miRNA解析についても既に培養上清を用いた条件検討に着手しており、分泌型miRNAによるQCの実践については計画より早く実用化できると考えている。　（達成度:８０%/１００%）

2. hAD-MSCの肝硬変モデルマウスにおける有効性評価及びIVISによる体内動態の解析：

肝硬変モデルマウスにおける有効性評価及びIVISによる体内動態解析については、四塩化炭素誘発肝硬変モデルに対してhAD-MSCを既に投与しており、平成25年12月末には有効性の結果がでる予定である。一方で本試験モデルは最短でも8週間を要するため、別途四塩化炭素誘発急性肝障害モデルを用いた検討も並行して進めている。現在までに急性肝障害モデルを用いたhAD-MSCの有効性の確認、さらにhAD-MSCに最適な蛍光色素及び標識条件の確立は終了し、疾患モデルにおいて、hAD-MSCの肝臓への集積を示すIVISの結果も得られている。（達成度:80%/100%）

3. デジタルPCRによる生着細胞の絶対定量：

予定より早くマウス肝障害モデルにおけるhAD-MSCの有効性及びIVISによるイメージング解析の目途が立ったことから、平成26年度に実施予定であるデジタルPCRによる生着細胞の絶対定量について計画を前倒して着手している。現在デジタルPCRによる生着細胞数の絶対定量法の確立に向け、マウス肝臓組織にhAD-MSCを一定量混合し調整したDNAを用いた定量法のバリデーションを実施中である。　（達成度:20%/100%：平成26年度の前倒し）

D．考察

hAD-MSCsから分泌されるエクソソーム中の特定のmiRNAに着目し、その発現プロファイルの変化をhAD-MSCsの品質管理に応用する目的で、各種ストレス下で変化するmiRNAの同定を進めている。H25年度の研究では継代ストレスによるmiRNAの発現プロファイルの変化に着目し、P4細胞及びP11細胞における分泌型miRNA発現をマイクロアレイにより解析を行い、約40種類のmiRNAを同定することができた。今後、qRT-PCRによる検証を行うことでさらにmiRNAを絞り込み、東京医科歯科大学で進めているmiRNAコピー数のデジタルPCRによる測定を行う予定である。

hAD-MSCsの急性肝障害モデルマウスによる有効性評価においては、細胞投与により血清中AST及びALTの低下が認められた。本効果については再現性も確認されており、hAD-MSCの肝障害への有効性が示唆された。一方で、四塩化炭素の頻回投与による肝線維化モデルマウスによる有効性評価においても血清中AST及びALTが低下傾向を示すと共に、hAD-MSCs投与により肝線維化面積の縮小が認められた。しかしながら再現性の確認は必要であり、今後評価項目を追加して試験を行う予定である。

hAD-MSCsの肝線維化モデルマウスにおける体内動態を評価すべく、蛍光色素(DiR)で標識したhAD-MSCs投与後のマウス全身の蛍光強度変化をIn Vivo Imaging System(IVIS)を用いて観察した。投与直後は、肺付近に強い蛍光シグナルがみられ、その後、肝臓付近にも蛍光シグナルが観察された。

今後、投与後の各臓器における細胞数の定量化方法確立の検討として、東京医科歯科大学においてデジタルPCRの手法を用い、移植細胞の絶対定量評価の検討を進める予定である。

特記事項

1. 平成25年度計画の変更点（研究費補助金交付申請時の平成25年度研究計画からの変更点）：

・変更点（1）：　予定より早くマウス肝障害モデルにおけるhAD-MSCの有効性及びIVISによるイメージング解析の目途が立ったことから、平成26年度に実施予定であるデジタルPCRによる生着細

胞の絶対定量について計画を前倒して着手している。現在デジタル PCR による生着細胞数の絶対定量法の確立に向け、マウス肝臓組織に hAD-MSC を一定量混合し調整した DNA を用いた定量法のバリデーションを実施中である。

2. 評価委員会コメントへの対応状況

コメント１：品質管理はゲノム解析だけでは不足であり、再生医療経験者と組むべきである

再生医療経験者との連携については、すでにヒト幹指針での再生医療臨床研究事業の推進研究者と連携（情報交換）をはかっているところである。

コメント２：ゲノム解析に終始する事にならないか懸念

今回提案したゲノム解析以外に、品質管理の項目は、細胞の均質性、移植後の細胞の生着状況、体内循環状態、分化状況において、従来の細胞表面マーカーや、細胞生物学的な検討を併せて実施することが望ましい。

コメント３：細胞の供給源はどこか？

本研究期間においては、申請のとおりに市販のヒト間葉系幹細胞を使用して解析、最適化等の一連の作業をすすめ、平成 27 年度後半には、動物での有効性試験では実際に前臨床試験で使用するヒト間葉系幹細胞の候補ストックを使用する予定である。

コメント４：蛍光マーカーとの感度等の比較は？

蛍光による細胞標識法は、既知で標識可能な分子に対象が限定されること.強い紫外光照射による細胞障害，蛍光標識分子の退色，多重染色やそのスペクトル識別の困難さなどの欠点が有り、本研究で実行されるデジタル PCR の利便性、高感度には遥かに及ばない。さらにデジタル PCR 法は 1 細胞の解析も可能である。現在マウス肝臓組織に hAD-MSC を混合した評価系を用いて、PCR 条件の詳細についてバリデートを進めている。平成 25 年度中には、IVIS によるイメージング解析との感度の比較検討を実施する予定である。

コメント５：細胞機能の影響についての考慮は？

品質管理の項目は、細胞の均質性、移植後の細胞の生着状況、体内循環状態、分化状況において、従来の細胞表面マーカーや、細胞生物学的な検討を併せて実施することが望ましい。

E．結論

ストレス負荷による分泌型 miRNA をマイクロアレイ解析による比較検討により、バイオマーカー候補として約 40 種類の miRNA を同定することができた。今後 qRT-PCR などで検証することで候補となる miRNA を絞り込み、デジタル PCR による高感度発現解析を進める予定である。さらに、培養工程に係る他のストレスにより変化する分泌型 miRNA の同定を並行して行い、培養工程における QC ツールとしての実用化を目指した研究を進める予定である。

肝障害モデルマウスに hAD-MSCs を投与することにより、その有効性を確認することができた。さらに、蛍光標識した細胞の IVIS 解析を行うことで、移植後細胞の体内動態、特にターゲット部位への細胞の集積が確認できた。今後は、東京医科歯科大学で確立したデジタル PCR によるヒト由来細胞の絶対定量評価系と IVIS 解析の結果を比較検討を行い、新しい体内動態解析手法の開発を進める予定である。

F．健康危険情報

特に無し.

G．研究発表

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H. 知的財産権の出願・登録状況

なし

2. 実用新案登録

なし

1. その他

なし

厚生労働科学研究費補助金（難病・がん等の疾患分野の医療の実用化研究事業
（再生医療関係研究分野））
分担研究報告書

最新のゲノム科学による hAD-MSC の性状解析

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研究要旨

細胞を有効成分とした再生医療分野において、その品質管理（Quality Control）や移植細胞の体内動態解析、生着細胞の機能解析は安全性及び有効性を担保する上で大きな課題である。そこで本研究では分泌型 miRNA 発現プロファイルによる細胞品質管理法の検討、ゲノム解析手法を応用した移植細胞の体内動態及び生着細胞の機能解析法の検討を行った。H25 年度の研究において、細胞培養上清中の特定の分泌型 miRNA のコピー数解析を行い、培養上清中からの miRNA 発現量の絶対定量評価系を構築することができた。さらに、マウス肝臓に混入させたヒト由来細胞を用いてデジタル PCR 解析の予備検討を行い、マウス組織中のヒト由来細胞数を高感度で検出することができた。

A. 研究背景、目的

（背景）

再生医療分野において、①細胞の均質性担保（Quality Control）、②移植細胞の体内動態、生着状況、分化状況の解析は、再生医療製品の有効性及び安全性を予測する上で非常に重要である。これまでは細胞表面抗原の発現パターンによる移植細胞の Quality Control や、蛍光標識された移植細胞の免疫組織学的染色による動態解析などが行われてきたが、検出感度も低く定量評価とは程遠いのが現状である。

このような背景のもと、本研究ではゲノム解析技術を応用し、種々ストレス下における分泌型miRNAの発現プロファイルからバイオマーカーを同定し、さらにデジタルPCRによる高感度定量解析を行うことで、新しい細胞の均質化方法を開発することを目的としている。　また移植細胞の体内動態および生着細胞の機能解析についても、デジタルPCR及び並列型シーケンサーを用いたゲノム解析技術を応用した新しい定量解析法の開発を本研究の目的としている。

平成25年度の研究分担として、東京医科歯科大学では、hAD-MSC培養上清中の分泌型miRNAのデジタルPCRによる定量解析法の評価系構築、及びデジタルPCRによるマウス肝臓中のヒト由来細胞の定量解析法の評価系構築を行った。

B. 研究方法

①hAD-MSC 培養上清中の miRNA 定量法の構築

Lonza 社から購入した hAD-MSC をフラスコで 4 継代培養した細胞（P4 細胞）および 11 継代培養した細胞（P11 細胞）を作成、それぞれの培養上清からエクソソームを精製した。精製エクソソーム中の RNA を鋳型として miR-xxx 特異的逆転写プライマーを用いて逆転写を行った。得られた逆転写産物を鋳型として Digital Array™ IFC (Fluidigm)を用いデジタル PCR を行うことで miR-xxx のコピー数を測定した。

②マウス肝臓中のヒト由来細胞の定量法の構築

マウス組織中の異種（ヒト由来）細胞のデジタル PCR による絶対定量評価を行うにあたり、まずは予備検討として、採取したマウス肝臓 10mg あたり 1000 個のヒト脂肪由来間葉系幹細胞（hAD-MSCs）を添加し、異種細胞混合状態におけるヒト由来細胞をデジタル PCR で検出を行った。
ヒト由来細胞の検出には、ヒトRNaseP遺伝子及びマウス由来細胞の検出にはマウスTransferin遺伝子に対する特異的Taqmanプローブ(ヒト:FAMラベル、マウス:VICラベル)を用い、各ゲノムDNA を用いてDigital Array™ IFCによりデジタルPCRを行いマウス及びヒト由来細胞のコピー数を検出した。

（倫理面への配慮）

本研究課題で用いるヒト脂肪由来間葉系幹細胞はインフォームドコンセントを得て取得されたものであり、海外の市販品を購入するため倫理的な問題は起こらない。また本研究課題においてはヒトへの臨床応用は実行されない。

C.　研究結果

①hAD-MSC培養上清中のmiRNA定量法の構築
ヒトAD-MSCを4継代(P4)及び11継代(P11)を行った培養上清1mlより精製したエクソソームRNAを逆転写後、デジタルPCRによる定量を行った。その結果、P4は7コピー、P11は182コピー検出された。精製エクソソーム画分のRNA量が各々P4:7760pg、P11:12860pgであることから培養上清1mlあたりP4では1358コピー、P11では33436コピー存在することが推定された。

②マウス肝臓中のヒト由来細胞の定量法の構築
マウス肝臓10mgに1000個の細胞を混入した後、精製したゲノムDNAを鋳型としてデジタルPCR 11パネル(8415ウェル)を用いて検討を行った。その

結果、マウス由来ゲノムが7384コピー、ヒト由来ゲノムが15コピー検出された。
10mgのマウス肝臓中の細胞数が分からないので正確な理論値を算出することはできないが、固形組織1g中に約$1x10^9$個の細胞が含まれていることを参考に理論値を算出するとマウス肝臓10mg中にヒト由来細胞が約5000個含まれることとなり、本測定においてはヒト由来細胞を概ね正確に定量できていると考えられる。

D．考察

①hAD-MSC培養上清中のmiRNA絶対定量法の構築

hAD-MSC培養上清から精製したエクソソームを用いた、単位培養上清あたりのmiRNAコピー数はデジタルPCRで測定可能であることが分かった。

②マウス肝臓内のヒト由来細胞の絶対定量法の構築

今回の結果から、デジタルPCRを用いることによりマウス組織中に含まれるヒト細胞数の推定は可能であることが示された。今後、マウス組織に混入させる細胞数を振ることで検量線を書くことにより、ヒトAD-MSCを血中投与した際にマウスの肝臓内に移行したヒトAD-MSCの細胞数の算出が可能となると考えられる。

E．結論

hAD-MSCの培養上清中の分泌型miRNAの絶対定量解析法の構築については計画通り予備検討を進めることができた。今後はマイクロアレイ解析から同定した各種ストレス応答性のターゲットmiRNAを用いた絶対定量解析を進める予定である。

マウス肝臓中のヒト由来細胞の絶対定量評価系については計画を前倒しで着手した。まずは摘出したマウス肝臓中に混合したヒト由来細胞を用いた予備検討の結果、マウス肝臓中のヒト由来細胞を高感度で定量することが可能であることが示された。今後、In vivo imagingによる解析との比較検討、疾患モデル動物でのhAD-MSCsの体内動態解析を進める予定である。

F．研究発表

1．論文発表

特になし。

2．学会発表

特になし。

G．知的財産権の出願・登録状況

（予定を含む。）

1.特許取得

特になし。

2.実用新案登録

特になし。

3.その他

特になし。

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（再生医療関係研究分野））
分担研究報告書

hAD-MSC の培養及び動物実験全般、デジタル PCR 及び並列型シーケンサ実務全般

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研究要旨

細胞を有効成分とした再生医療分野において、その品質管理（Quality Control）や移植細胞の体内動態解析、生着細胞の機能解析は安全性及び有効性を担保する上で大きな課題である。そこで本研究では分泌型 miRNA 発現プロファイルによる細胞品質管理法の検討、ゲノム解析手法を応用した移植細胞の体内動態及び生着細胞の機能解析法の検討を行った。H25 年度の研究において、継代ストレス負荷における分泌型 miRNA 発現プロファイルの比較検討を行い、ストレス負荷により発現量が著しく変化する miRNA を約 40 種類同定した。また、移植細胞の肝障害動物モデルを用いた有効性の検討、および蛍光標識細胞を疾患動物モデルに投与し、高感度 in vivo imaging system（IVIS）によりその体内動態を解析し、移植細胞の継時的な動態変化について確認することができた。

A．研究背景、目的

（背景）

再生医療分野において、①細胞の均質性担保（Quality Control）、②移植細胞の体内動態、生着状況、分化状況の解析は、再生医療製品の有効性及び安全性を予測する上で非常に重要である。これまでは細胞表面抗原の発現パターンによる移植細胞の Quality Control や、蛍光標識された移植細胞の免疫組織学的染色による動態解析などが行われてきたが、検出感度も低く定量評価とは程遠いのが現状である。

このような背景のもと、本研究ではゲノム解析技術を応用し、種々ストレス下における分泌型miRNAの発現プロファイルからバイオマーカーを同定し、さらにデジタルPCRによる高感度定量解析を行うことで、新しい細胞の均質化方法を開発することを目的としている。　また移植細胞の体内動態および生着細胞の機能解析についても、デジタルPCR及び並列型シーケンサーを用いたゲノム解析技術を応用した新しい定量解析法の開発を本研究の目的としている。

平成25年度の研究分担として、ロート製薬では、hAD-MSCのストレス負荷による培養上清中分泌型miRNAの発現プロファイル解析及びストレス応答性のバイオマーカーとなるmiRNAの同定、さらにhAD-MSCの肝障害モデルマウス及び肝線維化モデルマウスを用いた有効性評価、及び体内動態解析の従来法として蛍光標識hAD-MSCによる高感度in vivo imaging技術を用いた体内動態解析を行った。

B．研究方法

①ストレス応答性 miRNAの探索及び同定

Lonza社から購入したhAD-MSCを培養フラスコで4継代培養した細胞（P4細胞）および11継代培養した細胞（P11細胞）を作製、それぞれの培養上清からエクソソームを精製した。精製したエクソソームをAgilent社Human miRNA microarrayによりP4細胞及びP11細胞から分泌されたmiRNA発現プロファイルを比較した。

②hAD-MSCsの肝障害モデルマウス及び肝線維化モデルマウスによる有効性評価

●肝障害モデル

BALB/cマウスの腹腔内に四塩化炭素を投与し肝障害を誘発した。四塩化炭素投与1日後に1x10^{6}cells/bodyの用量でhAD-MSCを尾静脈から投与した。細胞投与1日後にそれぞれのマウスから採血を行い、各種生化学検査を行った。

●肝線維化モデル

BALB/cマウスの腹腔内に四塩化炭素を週2回、8週間投与し肝線維化を誘発させた。8回目の四塩化炭素投与後、1x10^{6}cells/bodyの用量でhAD-MSCを尾静脈から投与した。細胞投与4週後、マウスから採血及び肝臓を摘出し、各種生化学検査及びMasson trichrome染色により線維化部分を染色し、画像解析により線維化部分の面積を算出した。

③蛍光標識hAD-MSC s による体内動態解析

hAD-MSCにDiRを添加しDiRラベルhAD-MSCsを作製した。DiR-MSCsをマウス肝線維化モデルに1 x10^{6}cells/bodyの用量で尾静脈から投与、投与2時間、1日、2日、3日、1週間、2週間、3週間、4週間における蛍光シグナルの継時変化をIVISにより解析した。

（倫理面への配慮）

本研究課題で用いるヒト脂肪由来間葉系幹細胞はインフォームドコンセントを得て取得されたものであり、海外の市販品を購入するため倫理的な問題は起こらない。また本研究課題においてはヒトへの臨床応用は実行されない。

動物実験は、国立がん研究センターおよび委託試験先である三菱メディエンスの定める動物実験指針の

基準に従うとともに、動物倫理委員会の承認を得たうえで、動物の苦痛軽減に努め、動物愛護の精神に基づく実験を行う。

C．研究結果

①ストレス応答性 miRNAの探索及び同定

Agilent社Human miRNA microarrayを用い、P4細胞及びP11細胞におけるmiRNA発現プロファイルを比較した。P4細胞とP11細胞の発現量において、その発現量に5倍以上の差が認められたmiRNAを約40種類ターゲット候補miRNAとして同定した。

②hAD-MSCsの肝障害モデルマウス及び肝線維化モデルマウスによる有効性評価

●肝障害モデル

四塩化炭素で誘発した肝障害モデルマウスの血清中Aspartate aminotransferase (AST)、Alanine aminotransferase (ALT)を測定したところ、細胞非投与群において高値を示した。一方、hAD-MSCs投与群では血清中のAST及びALTが共に細胞非投与群に比べて低値を示しており、hAD-MSCs投与により肝障害レベルが改善する可能性が示唆された。

●肝線維化モデル

四塩化炭素の反復投与により肝線維化を誘発したモデルマウスにおいて、血清中AST、ALT及びLactate Dehydrogenase(LDH)を測定したところ、細胞非投与群において高値を示した。さらにMasson trichrome染色により肝組織中に四塩化炭素により誘発された線維化が確認できた。一方で、hAD-MSCs投与群では血清中のAST、ALT及びLDHが細胞非投与群に比べて低値を示し、さらに線維化部分の面積を比較したところ細胞投与群において線維化面積の低下が認められた。

③蛍光標識hAD-MSC s による体内動態解析

四塩化炭素誘発マウス肝線維化モデルにDiR-MSCs投与したところ、投与二時間後ではほとんどの細胞が肺に集積していることが示唆された。投与1日後から2日後にかけて肺から肝臓への蛍光シグナルの移行が認められ、以後継時的に肝臓における蛍光シグナルは減弱していた。

D．考察

hAD-MSCsから分泌されるエクソソーム中の特定のmiRNAに着目し、その発現プロファイルの変化をhAD-MSCsの品質管理に応用する目的で、各種ストレス下で変化するmiRNAの同定を進めている。H25年度の研究では継代ストレスによるmiRNAの発現プロファイルの変化に着目し、P4細胞及びP11細胞における分泌型miRNA発現をマイクロアレイにより解析を行い、約40種類のmiRNAを同定することができた。今後、qRT-PCRによる検証を行うことでさらにmiRNAを絞り込み、東京医科歯科大学で進めているmiRNAコピー数のデジタルPCRによる測定を行う予定である。

hAD-MSCsの急性肝障害モデルマウスによる有効性評価においては、細胞投与により血清中AST及びALTの低下が認められた。本効果については再現性も確認されており、hAD-MSCの肝障害への有効性が示唆された。一方で、四塩化炭素の頻回投与による肝線維化モデルマウスによる有効性評価においても血清中AST及びALTが低下傾向を示すと共に、hAD-MSCs投与により肝線維化面積の縮小が認められた。しかしながら再現性の確認は必要であり、今後評価項目を追加して試験を行う予定である。

hAD-MSCsの肝線維化モデルマウスにおける体内動態を評価すべく、蛍光色素(DiR)で標識したhAD-MSCs投与後のマウス全身の蛍光強度変化をIn Vivo Imaging System(IVIS)を用いて観察した。投与直後は、肺付近に強い蛍光シグナルがみられ、その後、肝臓付近にも蛍光シグナルが観察された。

今後、投与後の各臓器における細胞数の定量化方法確立の検討として、東京医科歯科大学においてデジタルPCRの手法を用い、移植細胞の絶対定量評価の検討を進める予定である。

E．結論

ストレス負荷による分泌型miRNAをマイクロアレイ解析による比較検討により、バイオマーカー候補として約40種類のmiRNAを同定することができた。今後qRT-PCRなどで検証することで候補となるmiRNAを絞り込み、デジタルPCRによる高感度発現解析を進める予定である。さらに、培養工程に係る他のストレスにより変化する分泌型miRNAの同定を並行して行い、培養工程におけるQCツールとしての実用化を目指した研究を進める予定である。

肝障害モデルマウスにhAD-MSCsを投与することにより、その有効性を確認することができた。さらに、蛍光標識した細胞のIVIS解析を行うことで、移植後細胞の体内動態、特にターゲット部位への細胞の集積が確認できた。今後は、東京医科歯科大学で確立したデジタルPCRによるヒト由来細胞の絶対定量評価系とIVIS解析の結果を比較検討を行い、新しい体内動態解析手法の開発を進める予定である。

F．研究発表

特になし。

1．論文発表

JSM Regenerative Medicine, in press

2．学会発表

特になし。

G．知的財産権の出願・登録状況

（予定を含む。）

1.特許取得

特になし。

2.実用新案登録

特になし。

3.その他

特になし。

別紙４

研究成果の刊行に関する一覧表レイアウト

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

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REVIEW

The therapeutic potential of mesenchymal stem cell-derived extracellular vesicles

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Extracellular vesicles (EVs), membrane vesicles that are secreted by a variety of mammalian cell types, have been shown to play an important role in intercellular communication. The contents of EVs, including proteins, microRNAs, and mRNAs, vary according to the cell type that secreted them. Accordingly, researchers have demonstrated that EVs derived from various cell types play different roles in biological phenomena. Considering the ubiquitous presence of mesenchymal stem cells (MSCs) in the body, MSC-derived EVs may take part in a wide range of events. In particular, MSCs have recently attracted much attention due to the therapeutic effects of their secretory factors. MSC-derived EVs may therefore provide novel therapeutic approaches. In this review, we first summarize the wide range of functions of EVs released from different cell types, emphasizing that EVs echo the phenotype of their parent cell. Then, we describe the various therapeutic effects of MSCs and pay particular attention to the significance of their paracrine effect. We then survey recent reports on MSC-derived EVs and consider the therapeutic potential of MSC-derived EVs. Finally, we discuss remaining issues that must be addressed before realizing the practical application of MSC-derived EVs, and we provide some suggestions for enhancing their therapeutic efficiency.

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Cell biology / Exosome / Extracellular vesicle / Mesenchymal stem cells / Microvesicle / Therapeutics

1 Introduction

The presence of membrane vesicles in the extracellular space was observed as early as in 1960s [1], but their significance remained obscure for a long time. These vesicles were later classified into two types according to their secretory processes, namely, exosomes and shedding vesicles [2, 3]. Exosomes were discovered around 30 years ago as small vesicles that were released when multivesicular endosomes fused with the plasma membrane [4, 5]. These vesicles, however, were long regarded as cellular garbage cans for discarding unwanted molecular components [2, 6–8]. Meanwhile, shedding vesicles were also found in many biological processes as vesicles that directly bud from the cell plasma membrane [9]. However, shedding vesicles were also considered for a long time to be inert cellular debris resulting from cell damage or dynamic plasma membrane turnover [3, 10]. It is only recently that major advances have been made in the identification of their biological significance as tools of intercellular communication.

Some confusion exists in the literature regarding the terms "exosomes" and "microvesicles." The difference between these two terms is generally based on size: exosomes are in the range of 10–100 nm, and microvesicles are in the range of 100–1000 nm. However, because this research area

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Abbreviations: **Aβ**, β-amyloid peptide; **AD**, Alzheimer's disease; **ADSC**, adipose tissue-derived mesenchymal stem cell; **AKI**, acute kidney injury; **BM**, bone marrow; **CM**, conditioned medium; **DC**, dendritic cell; **EBV**, Epstein-Barr virus; **EC**, endothelial cell; **EGFR**, epidermal growth factor receptor; **ESC**, embryonic stem cell; **EV**, extracellular vesicle; **HLSC**, human liver stem cell; **IFN**, interferon; **IL**, interleukin; **iPSCs**, induced pluripotent stem cells; **MCAo**, middle cerebral artery occlusion; **MIR**, myocardial ischemia/reperfusion injury; **miRNA**, microRNA; **MSCs**, mesenchymal stem cells

is still in its infancy, these definitions are flexible. Some researchers use these terms according to strict definitions, whereas others use the terms interchangeably. In particular, the term microvesicles have been often used more widely for membrane vesicles regardless of their intracellular origin, including exosomes and shedding vesicles. Recently, the International Society for Extraceluar Vesicles have recommended researchers to use the term "extracellular vesicles (EVs)" as an umbrella term for all types of vesicles present in the extracellular space, including exosomes, shedding vesicles, melanosomes, prostasomes, and apoptotic bodies. Following this recommendation, we use the term EVs throughout the paper.

EV research has dramatically changed because of two major breakthroughs, and it is now attracting much interest from various fields. The first breakthrough occurred in 1996 when Raposo's group found that EVs derived from immune cells function as activators of the immune system [11]. Many groups have since reported that EVs derived from certain cell types contain functional proteins that can activate biological events. These findings have established the novel concept that EVs serve as carriers for intercellular communication. The second breakthrough came with the findings that EVs shuttle functional mRNAs and microRNA (miRNA). In 2006, Ratajczak's group found that mRNA could be delivered by EVs to target cells and translated into the corresponding proteins [12]. In the next year, Lötvoll's group found that EVs contain not only mRNA but also miRNA [13]. Furthermore, in 2010, three groups independently demonstrated that the miRNAs contained in EVs also traveled between cells and suppressed the expression of target genes in recipient cells [14–16]. EV function, the mechanisms of EV biogenesis and secretion, and the molecular composition of EVs, including proteins, lipids, and nucleic acids, have been comprehensively studied [17]. For example, release of exosomes and shedding vesicles are regulated by calcium-dependent manner [3, 18]. On the other hand, Simon's group revealed that ceramide triggered budding of exosome vesicles into multivesicular endosomes, providing evidence for an alternative pathway to ESCRT (endosomal sorting complex required for transport) machinery-independent pathway for exosome biogenesis [19]. Consistent with this observation, Kosaka et al. demonstrated that decreased activity of neutral sphingomyelinase 2 (nSMase2), a rate-limiting enzyme in ceramide biosynthesis, resulted in the reduced secretion of miRNAs [16]. In addition, Théry's group demonstrated that Rab27 isoforms play essential roles in the exosome secretion pathway [20].

One major interest in this research area is the potential for various EV functions in therapeutic applications. While secreted EVs exhibit some common, shared contents, they also express molecules that reflect the originating cells [21]. Accordingly, there are many reports showing that functions of EVs reflect, at least in part, those of the originating cells despite the differences between the contents of EVs and those of the originating cells. Furthermore, it should be noted that EV-derived molecules can still help the parent cells play their roles even in a noncanonical manner [22]. From these evidences, the hypothesis can be drawn that cells utilize their EVs to fulfill their roles. This suggests that EVs released from cells that are capable of repairing damaged tissue may also have therapeutic ability. Some of the most promising candidate parent cells are mesenchymal stem cells (MSCs). A rapidly increasing number of reports have suggested that using MSC-derived EVs in treatment for several diseases is feasible. In this article, we first summarize the wide range of functions among EVs released from different cell types and highlight evidence showing that EVs reflect the phenotype of their parent cell. Then, we describe the various therapeutic effects of MSCs, with particular emphasis on the significance of the MSC paracrine effect. In subsequent sections, we survey the latest reports on MSC-derived EVs and discuss their therapeutic potential. Finally, we point to issues that require attention before realizing the practical application of EVs, and we provide some suggestions for enhancing therapeutic EV effects.

2 The functionality of EVs is origin-dependent

EVs have functions that depend on the phenotype of their parent cell. Because cells package cellular material into EVs, it is reasonable to speculate that EV content echoes that of its parent cell. Indeed, the molecular contents of EVs, including proteins, mRNAs, and miRNAs, are reported to imitate, at least in part, their parent cells. Selective enrichment of specific molecules in EVs, however, is also observed. The mechanisms underlying the EV packaging process require further study. Nonetheless, it should be noted that as described below, various types of cells produce EVs with functions that mirror those of their parent cells.

2.1 EVs in the immune system

The first evidence for EV functionality came from immunology studies. In 1996, Raposo et al. revealed that B-lymphocytes secreted antigen-presenting EVs [11]. They found that EVs released from human and murine B-lymphocytes induced an antigen-specific major histocompatibility complex class II-restricted T cell response. Further, they later demonstrated that dendritic cells (DCs) secreted antigen presenting EVs that expressed major histocompatibility complex class I and class II proteins and T-cell costimulatory molecules [23]. Importantly, the DC-derived EVs served as a novel cell-free vaccine. Tumor peptide-pulsed, DC-derived EVs primed specific cytotoxic T lymphocytes in vivo and eradicated murine tumors. Furthermore, mast cell-derived EVs were also reported to participate in immune reactions. Skokos et al. showed that mast cell-derived EVs activated B- and T-lymphocytes [24] and induced phenotypic and functional maturation of DCs [25].

Recent reports have revealed EV-mediated miRNA transfer between immune cells. Pegtel et al. demonstrated that

EV-mediated miRNAs secreted by Epstein-Barr virus (EBV) infected B cells were transferred to uninfected recipient cells such as DCs [14]. The internalized EBV miRNAs repressed target genes including CXCL11, an immunoregulatory gene. Of note, they found that in peripheral blood mononuclear cells from patients with increased EBV load, EBV miRNAs were present not only in B cells but also in uninfected non-B cells, suggesting in vivo miRNA transfer. Mittelbrunn et al. also reported EV-mediated unidirectional transfer of miRNAs from T cells to antigen-presenting cells [26]. Interestingly, Mittelbrunn et al. found that immune synapse between these two cell types significantly increased the efficiency of EV-mediated delivery of the miRNAs.

2.2 Tumor cell-derived EVs in cancer immunity

In addition to immune cells, tumor cells also release EVs that are related to cancer immunity. At the early stages of carcinogenesis, cell-intrinsic barriers to tumor development seem to be associated with stimulation of an active antitumor immune response, a process known as cancer immunosurveillance [27,28]. Cancer cells avoid immunosurveillance through the outgrowth of poorly immunogenic tumor-cell variants and through subversion of the immune system, which allows cancer cells to achieve immunotolerance [27, 28]. Tumor cell-derived EVs have been reported to play roles in both immunosurveillance and immunotolerance.

Tumor cell-derived EVs can contribute to tumor rejection by the host immune system. The first functional study on tumor-derived EVs by Zitvogel's group reported that tumor cell-derived EVs transferred tumor antigens to DCs, which resulted in $CD8^+$ T cell-dependent anti-tumor effects [29]. Accordingly, Théry's group highlighted the importance of EVs for an efficient anti-tumor immune response [30]. They showed that tumor cells secreting an antigen in an EV-associated form induced antitumor immune responses more efficiently than those secreting the same antigen as a soluble protein. Furthermore, tumor cell-derived EVs also played an important role in activation of the innate immune system by stimulating migratory and cytolytic activity of natural killer cells [31].

In contrast to the above reports, tumor cell-derived EVs have also been reported to promote tumor immune resistance. Andreola et al. reported that melanoma cell-derived EVs induced T-cell apoptosis [32]. They showed that this effect could be ascribed to EV-bound Fas ligand, a transmembrane protein belonging to the tumor necrosis factor family that plays a pivotal role in the induction of Fas receptor-mediated apoptosis. A similar effect was confirmed for prostate cancer cell-derived EVs [33]. Clayton et al. showed that mesothelioma cell-derived EVs selectively impaired lymphocyte response to IL-2 by suppressing cytotoxic T lymphocytes and natural killer cells [34]. Intriguingly, these EVs did not impair regulatory T-cell response to IL-2. Instead, their inhibitory function was enhanced by tumor EVs. These effects worked in concert to contribute to the tumor immune resistance. Consistently, Chalmin et al. found that tumor cell-derived EVs activated the immunosuppressive activity of myeloid-derived suppressor cells, which are thought to contribute to tumor progression [35].

2.3 Tumor cell-derived EVs can modulate the surrounding environment

In addition to cancer immunity, tumor cell-derived EVs seem to have various functions that affect tumor invasiveness, tumor cell proliferation, and the formation of pro-metastatic and pre-metastatic niches. Many studies have reported that EV-mediated effects occur through EV cargo proteins including growth factors/cytokines and membrane-bound receptors such as epidermal growth factor receptor (EGFR). In addition, recent reports have suggested a pathological significance for EV-mediated transfer of tumor cell-derived miRNAs.

Tumor cell-derived EVs can interact with surrounding cells and promote malignancy in an autocrine/paracrine-dependent manner. Gutwin et al. reported that ovarian carcinoma cell-derived EVs mediated the secretion of the L1 adhesion molecule (CD171) that is overexpressed in human ovarian and endometrial carcinomas and is associated with a poor prognosis [36]. The authors found the presence of L1 on the EV surface, and the membrane-bound L1 was subsequently cleaved into a soluble form that triggered cell migration and phosphorylation of ERK. Higginbotham et al. revealed a novel, EV-mediated pathway for EGFR ligands [37]. Higginbotham et al. found that human breast and colorectal cancer cells released EVs containing full-length, signaling competent EGFR ligands. Interestingly, these EVs increased the invasiveness of recipient breast cancer cells over an equivalent amount of the soluble form of EGFR ligands. In addition, an oncogenic form of EGFR, EGFRvIII, can be transferred via this EV-mediated pathway to recipient cells. In aggressive human brain tumors, gliomas, only a small percentage of cells possess the EGFRvIII gene; however, most of these cells exhibit a transformed, carcinogenic phenotype [38]. Al-Nedawi et al. showed that EVs containing EGFRvIII were released from glioma cells, merged with the plasma membranes of cancer cells lacking EGFRvIII, and led to the transfer of oncogenic activity, including the activation of transforming signaling pathways, changes in expression of EGFRvIII-regulated genes, morphological transformation, and an increase in anchorage-independent growth capacity [39]. Furthermore, Skog et al. demonstrated the presence of EGFRvIII mRNA in EVs extracted from primary glioblastoma cells [40]. Their observation that recipient cells translated messages delivered by EVs supports the idea that EGFRvIII delivery among glioblastoma cells involves EV-mediated transfer of both its message and the translated product.

Tumor cell-derived EVs are delivered not only to tumor cells but also to neighboring and/or distant normal cells. These EVs provide a supportive environment for

tumor progression. Several groups have reported that tumor cell-derived EVs can be transferred to vascular endothelial cells (ECs) where they promote angiogenesis. Zöller's group found that tetraspanin 8 (Tspan8)-expressing tumor cells enhanced tumor growth by inducing angiogenesis and that Tspan8-bound EVs also promoted in vitro angiogenesis [41]. They also showed that Tspan8-CD49d association contributed to EV binding to ECs and that internalized EVs modulated the fate of ECs and EC progenitors [42]. In the same manner as described above [39], EV-mediated EGFR transfer also occurred between tumor cells and recipient ECs, which resulted in the onset of vascular endothelial growth factor expression in ECs, and autocrine activation of its key signaling receptors, i.e. vascular endothelial growth factor receptor2 [43]. Sheldon et al. found that a Notch ligand delta-like 4 was expressed on tumor cell EVs, and transferred to ECs in vitro and in vivo [22]. Intriguingly, transferred delta-like 4 did not activate Notch signaling in the ECs, but instead inhibited the pathway. As a consequence, tumor-derived EVs switched the recipient ECs to tip cell phenotype, and induced angiogenesis. This finding highlights the notion that EV-derived molecules can help the parent cells play their roles even in a noncanonical manner. In addition to neighboring cells, tumor cell-derived EVs can reach distant cells. In 2005, Kaplan et al. demonstrated that vascular endothelial growth factor receptor1+ bone marrow (BM) progenitor cells migrated to and conditioned a premetastatic lung niche in response to systemically administered melanoma-derived conditioned medium (CM) [44]. This group recently reported that this effect was, at least in part, due to secreted EVs. Metastatic melanoma-derived EVs altered BM progenitor cells toward a prometastatic phenotype [45]. Moreover, Jung et al. reported that CD44v6 expressing tumor cell-derived CM is essential for premetastatic niche formation in lymph nodes and lung tissue [46]. Their data suggested that tumor cell-derived EVs, when assisted by the soluble fraction of CM, promoted premetastatic niche formation.

2.4 EVs in other biological phenomena

EV-mediated intercellular communication is not limited to the immune system and cancer pathology. Rather, it seems that most cell types produce EVs, and their functions vary accordingly. Ratajczak et al. demonstrated that embryonic stem cell (ESC)-derived EVs may contribute to cell-fate determination and may be a critical component in the self-renewal and expansion of stem cells [12]. Deregibus et al. reported that endothelial progenitor cell-derived EVs activated an angiogenic program in ECs via transfer of mRNA [47]. Recently, our group reported that in contrast to tumor cells, noncancerous cells secreted EVs containing tumor-suppressive miRNAs and inhibited the proliferation of tumor cells [48]. This finding suggests that EV-derived tumor-suppressive miRNAs act as an inhibitory signal for cancer cells in a cell-competitive process.

3 MSCs and their therapeutic potential

The possibility of cell therapy using pluripotent stem cells has attracted much attention from researchers and the general public alike, but such technology is not yet within reach. Although pluripotent ESCs have long been predicted as a cell source for regenerative medicine, use of ESCs has been hampered by the possibility of immune rejection and ethical issues. Induced pluripotent stem cells (iPSCs) have recently gained increasing attention as a cell source that can circumvent the problems associated with ESCs. However, despite their promising potential, many hurdles must be overcome before human iPSC-based therapy will appear in clinics (reviewed in [49]). For example, similar to the case with ESCs, therapeutic application of iPSCs involves the risk of teratoma formation. Therapy with iPSC also involves genetic modification, which could possibly give rise to various obstacles [50, 51]. Thus, there still exists a need for an alternative to iPSCs in order to make cell therapy a viable option.

MSCs, a type of adult stem cell, have emerged as a very attractive candidate for cell therapy applications [52–55]. MSCs can be isolated from adult connective tissue such as BM and adipose tissue and can differentiate into mesodermal cell lineages. MSCs have already been clinically applied during breast reconstruction following conservative surgery for breast cancer, and research is now underway on clinical applications for MSCs in disorders such as chronic heart disorders, acute myocardial infarction, and stroke [56]. Moreover, in the last decade, it has been demonstrated that MSCs have many other functional properties. They can differentiate into cells from unrelated germline lineages, resist immunosurveillance, home to injured tissue, and secrete factors with immunosuppressive, anti-apoptotic, and trophic effects [52–55]. These clinically useful features of MSCs have provoked enthusiasm for their application in a wide range of clinical situations.

3.1 What are MSCs?

MSCs are multipotent stem cells present in mesodermal tissue [52–55]. In the 1970s, Friedenstein et al., using BM, first isolated spindle-shaped, clonogenic cells in monolayer cultures that could differentiate into colonies resembling small deposits of bone or cartilage [57, 58]. Other groups extended the observations of Friedenstein et al. throughout the 1980s [52] and established that these cells were multipotent and could differentiate into osteoblasts, chondrocytes, adipocytes, and even myoblasts. To date, similar cells have also been isolated from a variety of other connective tissues such as adipose tissue, periosteum, perichondrium, cartilage, umbilical cord blood and tissue, amniotic membrane, and synovial tissue [52–55]. These cells are currently referred to as MSCs because of their ability to differentiate into mesenchymal-type cells (Fig. 1).

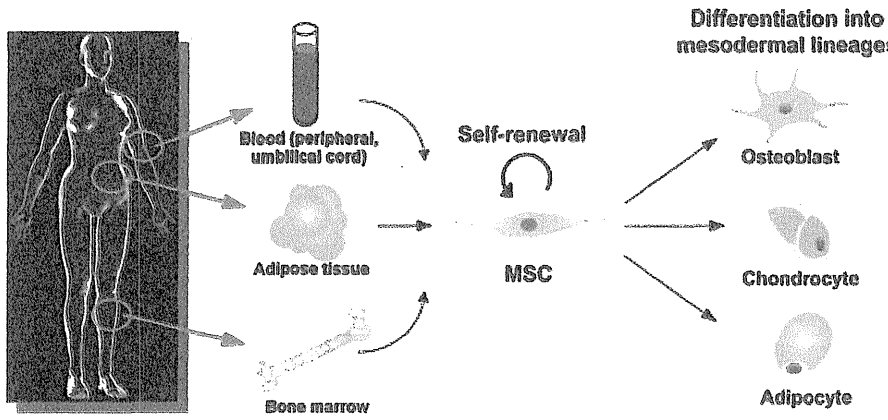

Figure 1. Origin and characteristics of mesenchymal stem cells. MSCs can be isolated from various tissues, including bone marrow, adipose tissue, and blood including peripheral and umbilical cord blood. MSCs have the capacity to self-renew and differentiate into multiple cell lineages including three major mesodermal lineages: osteoblasts, chondrocytes, and adipocytes.

The surface marker profiles for MSCs differ between species and are dependent on methods of isolation and culture, making a common standard and a precise definition for an MSC difficult. The current and widely accepted definition for MSCs was proposed in 2006 by the International Society for Cellular Therapy [59]. First, an MSC must be plastic adherent when maintained in standard culture conditions. Second, an MSC must express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14, or CD11b, CD79a, or CD19 and HLA-DR surface molecules. Third, an MSC must differentiate to osteoblasts, adipocytes, and chondroblasts in vitro.

Although MSCs initially attracted interest for their ability to differentiate into cells of mesodermal lineage in vitro and in vivo, the beneficial effects of MSCs appear to be due to other properties. MSCs can transdifferentiate into other lineages besides mesoderm, and they migrate to injured tissue beds, interact with injured host cells, and secrete paracrine-soluble and growth factors that modulate immune responses and alter the responses of the endothelium or the epithelium to injury (Fig. 2).

3.2 Multipotency of MSCs and their potential application in cell replacement therapy

A remarkable transition in MSC study occurred in the last decade when these cells were found to possess greater plasticity than that dictated by the established paradigms of embryonic development. MSCs have been shown to transdifferentiate into ectoderm including neuroectoderm cells [60], retinal pigment epithelial cells [61], and skin epithelial cells [62], and endoderm including hepatocytes [63], kidney tubular epithelial cells [64], and lung epithelial cells [65, 66]. In particular, a large number of papers have been published on the generation of neuroectoderm cells and hepatocytes.

Neuroectoderm differentiation was the first recognized transdifferentiation event in MSCs and has been extensively studied [60]. The brain has long been regarded as incapable of regeneration. Thus, the discovery of neural stem cells, which are capable of undergoing expansion and differentiation into neurons, astrocytes, and oligodendrocytes, has generated intense interest [67]. However, the location of neural stem cell sources severely limits clinical utility because they are deep in the brain. Kopen et al. first demonstrated that MSCs that were injected into the CNS of newborn mice migrated throughout the brain and adopted the morphological and the phenotypic characteristics of astrocytes and neurons [68]. These results were supported by similar observations from several other groups [69–71]. In parallel, MSCs were also shown to transdifferentiate in vitro into neural ectoderm cells [72, 73]. Many researchers have since investigated methods for in vitro induction of MSCs to neurons using various soluble factors [60, 74]. Results from these studies have raised expectations for the development of a novel therapeutic approach for neurodegenerative diseases using MSCs.

Another cell type that researchers have attempted to generate using MSCs is hepatocytes, which are liver parenchymal cells. The only treatment for end-stage liver diseases is liver transplantation, but transplantation is limited due to a shortage of donors. Many alternative therapies for liver failure are currently being developed and studied, including hepatocyte transplantation [75], extracorporeal bioartificial liver support devices [76], and heterotopic transplantation of engineered liver tissue [77]. In these studies, mature hepatocytes have been the main cell source. However, the use of a large amount of mature hepatocytes is constrained by availability in a clinical setting. Many researchers have attempted to obtain functional hepatocytes from progenitor cells including ESCs [78–80], iPSCs [81–83], fetal liver cells [84–86], and adult liver stem cells [87–89]. However, the use of these cell types has been hampered by ethical concerns as well as poor availability and accessibility [90]. The possibility of hepatic differentiation of MSCs has opened up a new avenue for improving the availability of functional hepatocytes [63]. Chen's group and Lee's group were the first to each independently report that BM-MSCs and umbilical cord blood-derived MSCs can differentiate into hepatocyte-like cells expressing liver-specific markers in vitro [91, 92]. These results were further confirmed by other groups [93–95]. In

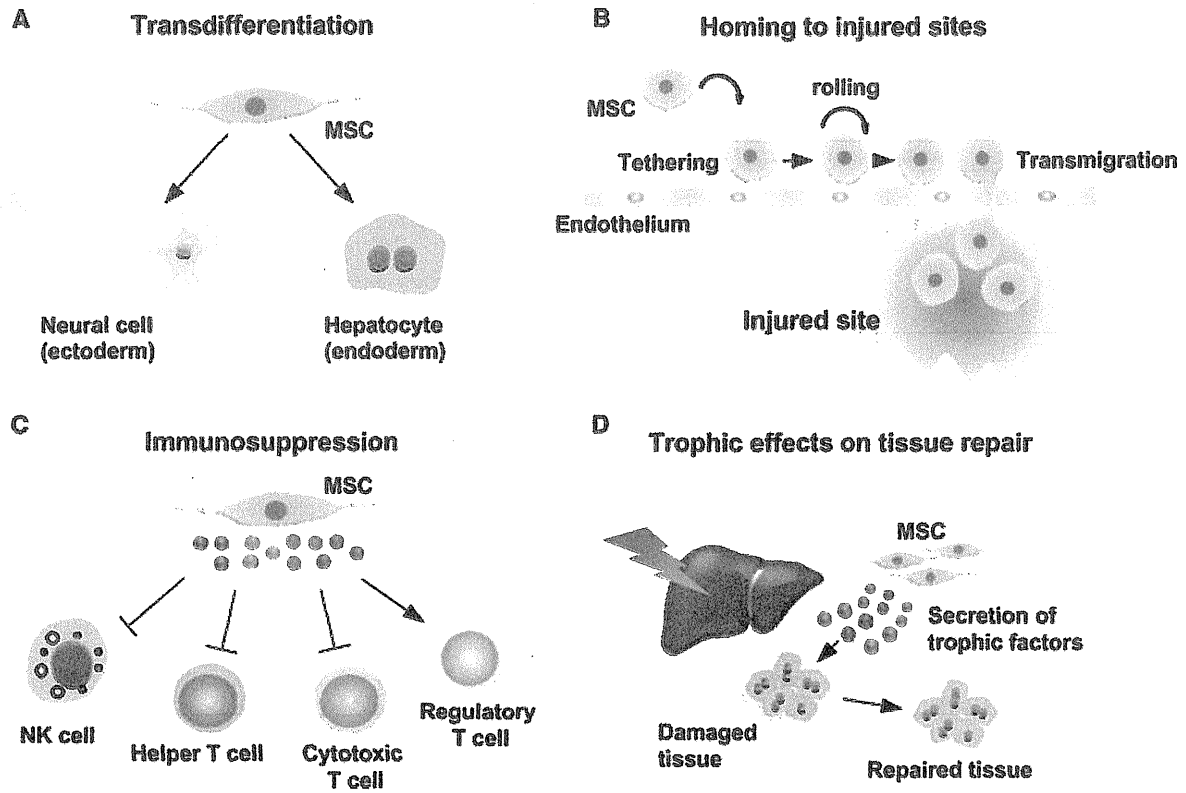

Figure 2. Various therapeutic effects of MSCs. (A) MSCs can transdifferentiate into cells of nonmesodermal origin including neurons (ectoderm) and hepatocytes (endoderm). (B) MSCs are capable of homing to injured tissues. The mechanism by which MSCs home to tissues and migrate across endothelium still remains unclear, but it is likely that injured tissue expresses specific receptors or ligands that facilitate trafficking, adhesion, and infiltration of MSCs to the site of injury in a manner similar to that in which leukocytes are recruited to sites of inflammation. (C) MSCs exhibit immunosuppressive activity through secretion of several cytokines that inhibit the activity of natural killer cells, helper T cells, and cytotoxic T cells while activating the generation of regulatory T cells. (D) MSCs produce trophic factors that promote repair of damaged tissue.

addition, hepatocyte-like cells were also generated from adipose tissue-derived MSCs (ADSCs) [96–99]. Our group reported that MACS-sorted CD105$^+$ fraction of ADSCs exhibited high hepatic differentiation ability in an adherent monoculture condition [98]. We further investigated the mechanism underlying ADSC plasticity by comparative analysis of the transcriptome and signal pathways and found that mesenchymal-to-epithelial transitions brought about the transdifferentiation of ADSCs into hepatocytes [100]. Furthermore, a clustering analysis revealed a striking similarity in gene clusters between ADSC-derived hepatocyte-like cells (ADSC-Hepa) and the whole liver, indicating that ADSC-Hepa were similar to mature hepatocytes.

3.3 The therapeutic significance of the secretory capacity of MSCs

The therapeutic potential of MSCs has been largely dependent on their secretory capacity rather than their differentiation capacity. The multipotency of MSCs led researchers to examine whether these cells would contribute to the repair of injured tissue by replacing damaged cells through differentiation into functional cells. Our group examined whether MSCs would reveal therapeutic abilities for repairing injured liver by transplanting ADSC-Hepa into nude mice with acute liver failure. As expected, markers of liver injury, including alanine aminotransferase, aspartate aminotransferase, and ammonia, decreased after ADSC-Hepa transplantation [99]. To our surprise, however, transplantation of undifferentiated ADSCs resulted in higher levels of serological recovery and suppression of histopathological damage compared to those achieved by ADSC-Hepa (unpublished data). Further analyses suggested that the therapeutic capacity for liver disorders from undifferentiated ADSCs was a paracrine effect resulting from factors secreted by these cells, including interleukin (IL)-1RA, IL-6, IL-8, granulocyte-colony stimulating factor, granulocyte macrophage, monocyte chemotactic protein-1, nerve growth factor, and hepatocyte growth factor [101]. These factors are known to be responsible for immunosuppression,

hepatocyte growth, and hematopoiesis. This finding is consistent with observations reported by other groups [102–104]. Similar observations have been made in the case of CNS injury. For example, researchers have reported the efficacy of the systemic administration of MSCs as treatment for experimental autoimmune encephalomyelitis, which is the animal model of human multiple sclerosis. Clinical efficacy of MSC treatment was sustained because of a significant reduction of demyelination and cellular infiltrates within the inflamed CNS and because of an impaired peripheral immune response against myelin antigens [105–108]. In addition to these beneficial effects, it was also observed that MSCs could engraft inside the CNS leading to reduced axonal loss [106, 107]. These observations show the significant anti-apoptotic effect of MSCs on neurons and lymphocytes, and they also show an antioxidant effect and the capacity to induce oligodendrogenesis and endogenous neurogenesis [109]. Collectively, these findings strongly suggest that given patients with a certain amount of potential for recovery, the secretory capacity of MSCs plays a more prominent role than MSC transdifferentiation in effecting tissue repair. However, in more serious cases where patients' organs neither maintain their own function nor have recovery potential, e.g. in the case of end-stage liver diseases, heterotopic transplantation of a tissue engineering-based auxiliary organ will be the better therapeutic option. In the latter case, the transdifferentiation capacity of MSCs has therapeutic potential. Thus, MSCs may provide options for both moderately and more serious patients.

Data from various studies suggest that MSCs can produce the immunomodulatory cytokines and associated trophic factors that correspond to a wide range of pathological conditions. MSCs can modulate both the innate and the adaptive immune systems [110]. MSC-mediated immunosuppression requires the previous activation of MSCs by immune cells, particularly secretion of the proinflammatory cytokine interferon (IFN)-γ with tumor necrosis factor, IL-1-α or IL-1-β. Upon stimulation of these proinflammatory cytokines, MSCs secrete immunosuppressive cytokines including prostaglandin E2, indoleamine 2,3-dioxygenase, transforming growth factor-β1, and IL-10 [110]. This activation pathway illustrates an important concept in MSC function. In addition to immunomodulatory effects, tissue repair by MSCs is not solely dependent on the rich mixture of soluble factors produced by MSCs in isolated cultures. Instead, MSCs are activated by cross-talk within the microenvironment that is generated by injured tissues. Activation results in the expression of factors that are most likely specific to the immediate needs of the tissue [111]. This idea is supported by various studies showing that MSCs exhibit therapeutic efficacy for not only specific disorders but also various types of disorders, including lung injury [112, 113], kidney disease [114], diabetes [115], myocardial infarction [116], and various neurological disorders [117].

In summary, it is widely argued that MSCs enable tissue repair in a wide range of disorders despite their low and/or transient levels of engraftment in vivo. Therefore, it is currently well accepted that MSCs contribute to tissue repair through secretion of immunomodulatory cytokines and trophic factors in response to pathological conditions without replacing the damaged cells.

4 Therapeutic potential of MSC-derived EVs

Collectively, the studies summarized above imply therapeutic potential for MSC-derived EVs. Research thus far has mostly focused on the secretion of cytokines and growth factors by MSCs. However, the fact that EVs can mirror the phenotype of their parent cell suggests that the therapeutic effects of MSC-derived factors are partly due to secreted EVs. In addition, the diversity of MSC-based therapeutic intervention implies the applicability of MSC-derived EVs to the treatment of various disorders. Furthermore, EVs may provide advantage over MSCs in that EVs can avoid the lung barrier, one of the major obstacles for systemic administration of MSCs [118].

In the last few years, several groups have investigated the therapeutic potential of MSC-derived EVs (Table 1). Their therapeutic effects have been observed in several different types of diseases, including kidney injury, cardiac injury, and brain injury. Although the number of reports are still limited, these findings strongly support the idea that MSC-derived EVs imitate the phenotype of parent MSCs and hold therapeutic potential for a wide range of diseases.

4.1 Kidney injury

The first study of the therapeutic effect of MSC-derived EVs focused on kidney injury. It is well recognized that MSCs contribute to repopulation of injured nephrons [64]. Repopulation is attributed to the transient recruitment of MSCs to the renal vasculature without direct incorporation of MSCs into regenerating tubules, suggesting that MSCs provide paracrine support to intrinsic repair mechanisms employed by the epithelial cells that survive injury. Indeed, MSCs were shown to protect the kidney from toxic injury by producing factors that limited apoptosis and enhanced proliferation of endogenous tubular cells. As a part of the paracrine effect, Camussi's group demonstrated that BM-MSC-derived EVs protected acute kidney injury (AKI), using a model mouse induced with glycerol [119]. The effect of EVs on the recovery of AKI was similar to that of MSCs. EVs induced proliferation and resistance to apoptosis in tubular epithelial cells. This effect was also confirmed using a lethal version of cisplatin-induced AKI [120]. Furthermore, the same group showed that a single administration of MSC-derived EVs immediately after ischemia-reperfusion injury protected against the development of both acute and chronic kidney injury [121].

Table 1. Reports on therapeutic potential of MSC-derived EVs

Species/MSC origin	Disease model	Mechanisms for the therapeutic effect	Molecules responsible for the therapeutic effect	References
Human/BM	Mouse model of acute kidney injury induced by glycerol	Induction of proliferation of surviving intrinsic epithelial cells	mRNAs (not specifically identified)	[119]
Human/BM	Mouse model of acute kidney injury induced by cisplatin	Induction of survival of tubular epithelial cells via anti-apoptotic effects	Not identified	[120]
Human/BM	Rat model of acute kidney injury induced by ischemia–reperfusion injury	Proliferative and anti-apoptotic effects on surviving intrinsic epithelial cells	Not identified	[121]
Mouse/BM	Rat model of 5/6 subtotal nephrectomy	Prevention of fibrosis, interstitial lymphocyte infiltrates, and absent tubular atrophy	Not identified	[122]
Human/ESC	Mouse model of myocardial ischemia/reperfusion injury	Not determined	Not identified	[128]
Human/fetus	Mouse model of myocardial ischemia/reperfusion injury	Not determined	Not identified	[129]
Rat/BM	Rat model of middle cerebral artery occlusion	Induction of neurite outgrowth of neural cells	miR-133b	[130]

They observed that EVs shuttle a specific subset of cellular mRNAs, such as those associated with the mesenchymal phenotype and with transcription control, proliferation, and immunoregulation [10, 119, 120]. In addition to these results, He et al. also described the potential renoprotective effect of MSC-derived EVs in the remnant kidney using a 5/6-nephrectomy mouse model [122].

4.2 Cardiac injury

Recent studies from Lim's group suggest that the therapeutic effect of MSC-derived paracrine factors on cardiovascular disease is, in large part, due to EV fractions. MSC transplantation in animal models of acute myocardial injury has been reported to reduce infarct size, improve the left ventricular ejection fraction, and increase capillary density and myocardial perfusion [123]. As is the case for other organs, transplantation of MSCs to treat cardiac disease was predicated on the hypothesis that these cells would engraft, differentiate, and replace damaged cardiac tissues [124]. However, it was observed that most transplanted MSCs remain in the lungs rather than the heart, which led to the hypothesis that their therapeutic effect is a result of paracrine effects [125, 126]. Consistent with this hypothesis, Lim's group demonstrated, using a porcine model of myocardial ischemia/reperfusion injury (MIR), that treatment with MSC-CM results in a reduction of myocardial infarct size [127]. Interestingly, in this report, they also showed that only the fraction of the CM containing products greater than 1000 kDa provided cardioprotection in a mouse model of MIR. They further demonstrated that the active component was enriched with 50–200 nm particles, which were identified as EVs. Further, the other fractions did not have protective effects on MIR [128, 129]. Currently, the specific molecules responsible for the protective effects remain unknown. EV-mediated injury protection may be dependent on cytokines and growth factors, similar to the case in MSC transplantation. Alternatively, miRNAs and/or mRNAs transferred from MSCs to damaged cardiac cells may provide therapeutic effects. More detailed

investigations are needed, but it is important to note that MSC-derived EVs mimic the phenotype of their parent cells and exhibit a protective effect on MIR.

4.3 Brain injury

MSC-derived EVs have been reported to contribute to tissue repair using a model of stroke in rats [130]. It has been widely shown that following intravenous administration, MSCs exhibit a therapeutic effect on stroke injury through secretion of neurotrophins and angiogenic growth factors [131]. In addition to these trophic proteins, Xin et al. recently found that EV-mediated secretion of miRNA contributes to the protective effect of MSCs on stroke [132]. It was previously demonstrated that miR-133b is specifically expressed in midbrain dopaminergic neurons and that it regulates the production of tyrosine hydroxylase and the dopamine transporter [132]. Xin et al. found that MSC treatment in rats that were subjected to middle cerebral artery occlusion (MCAo) had an increased miR-133b level in the ipsilateral hemisphere. The increase of miR-133b and subsequent induction of neurite outgrowth were shown to depend on EV-mediated miR-133b transfer from MSCs to neurons and astrocytes. Furthermore, Xin et al. found that exposure of MSCs to MCAo brain tissue extracts increased the miR-133b level in secreted EVs, suggesting that MSCs can increase the secretion level of therapeutic EVs in response to injury stimulus.

Recently, we have found a new therapeutic possibility for using MSCs-derived EVs against Alzheimer's disease (AD) [133]. One of the neuropathological hallmarks of AD is the accumulation of β-amyloid peptide (Aβ) in the brain because of an imbalance between Aβ production and clearance. Recently, we have found that ADSCs, but not BM-MSCs, potentially contribute to Aβ clearance. Moreover, we have found ADSC-derived EVs reflect their parent cells, and also hold potential as a therapeutic tool for AD. Therefore ADSC-derived EVs warrant further investigation as a promising novel therapy for AD patients.

5 Comprehensive characterization of MSC-derived EVs toward their potential therapeutic applications

Comprehensive characterization of MSC-derived EVs will provide further insight into their potential benefits for clinical applications. In the preceding section, we summarized research that found that MSC-derived EVs were therapeutically effective for several disease types. The wide range of known MSC therapeutic effects suggests that the potential for their EVs in therapeutic applications is broader than those currently identified. To further explore the therapeutic potential of MSC-derived EVs, proteome and mRNA/miRNA microarray analyses may provide useful information.

5.1 Proteomic feature of MSC-derived EVs

Despite its potential utility, proteomic data for MSC-derived EVs are still lacking and require better characterization. Presently, only one report on a proteomic analysis of MSC-derived EVs is available [134]. Kim et al. performed LC–MS/MS analysis of BM-MSC-derived EVs and identified 730 EV proteins. These EV proteins exhibit characteristics of MSCs. The EVs contained positive MSC markers but no negative markers, and 122 of 730 EV proteins were shared by proteomes from BM-MSCs and UBC-MSCs. Furthermore, a functional enrichment analysis identified candidate EV proteins that are thought to be involved in the therapeutic effects of MSCs, including surface receptors, signaling molecules, cell adhesion, and MSC-associated antigens. Indeed, their data successfully predicted the therapeutic roles that were previously reported for some of the identified proteins, such as adhesion molecules including fibronectin-1 [135] and galectin-1 [136]. Regarding other identified adhesion molecules, such as EZR and IQGAP1, no functional association with the therapeutic effects of MSCs has been reported. However, EZR and IQGAP1 were shown to regulate EC proliferation and angiogenesis, implying that they have potential roles in MSC-derived EV-based tissue repair [137, 138]. Comprehensive exploration of the proteome from MSC-derived EVs and subsequent database-based functional analyses may predict new potential therapeutic roles for MSC EVs.

5.2 mRNA/miRNA profiling of MSC-derived EVs

mRNA/miRNA microarray analyses of MSC-derived EVs have also provided information for exploring their therapeutic potential. Lims' group was the first to perform a microarray analysis, and they found that MSC-CM contained RNAs of less than 300 nt encapsulated in EVs [139]. They observed that 45 of the 60 miRNAs identified in EVs were also present in MSCs, suggesting that released EVs echo the contents of their parent cell. In addition, they also defined miRNAs that were found only in EVs, suggesting that miRNA secretion is, at least in part, a selective and nonrandom process. Although the authors did not refer to the possible involvement of the EV-miRNAs with therapeutics, their data may provide insight into therapeutic applications of MSC-derived EV-miRNAs. For example, miR-124, one of the miRNAs present only in EVs, is a well-characterized brain-specific miRNA, which is involved in neurogenesis [140] and was recently reported to be involved in CNS disease pathogenesis [141]. Camussi's group performed microarray analysis of the miRNAs contained in EVs released from BM-MSCs and the liver resident stem cells that are termed as human liver stem cells (HLSCs) [142]. This group had previously reported that HLSCs shared phenotypes in part with MSCs [143] and that their EVs accelerated hepatic regeneration in hepatectomized rats [144]. Collino et al. compared miRNA profiles in EVs to matched parent

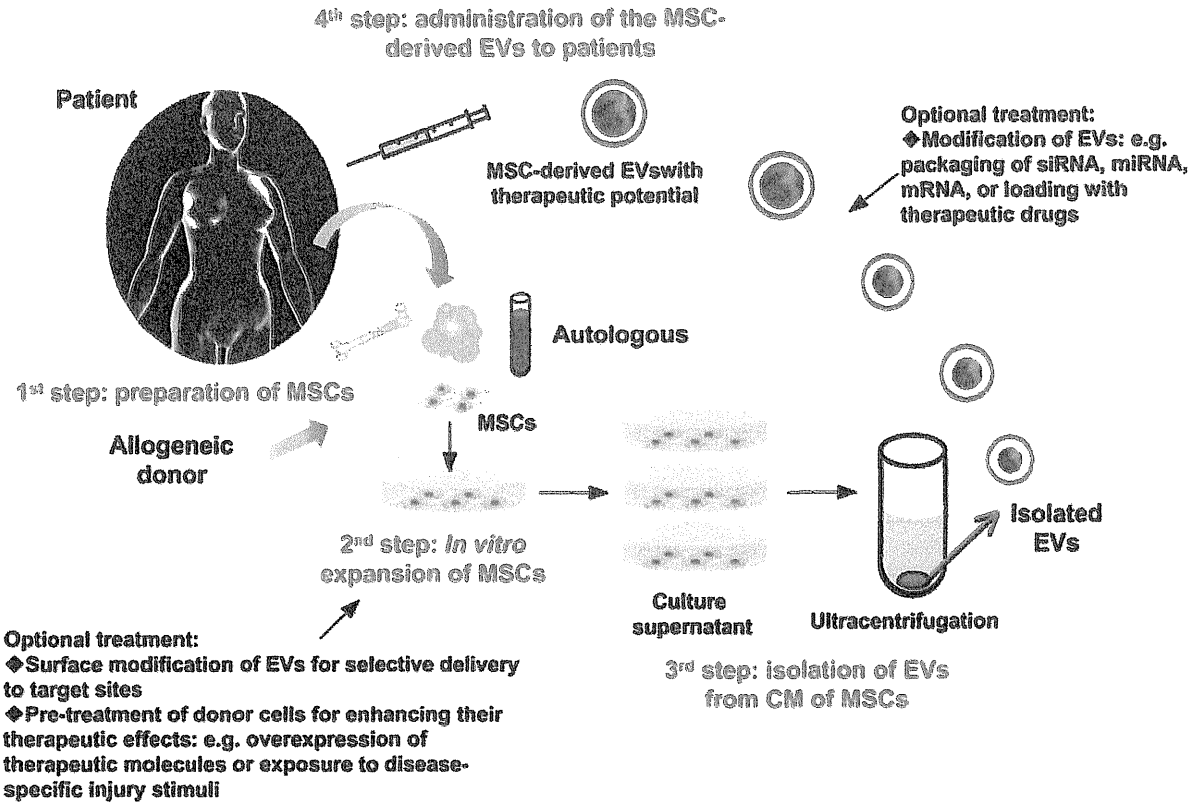

Figure 3. Schematic representation of the proposed strategy for clinical application of MSC-derived EVs. The first step is to prepare MSCs for production of EVs with therapeutic efficacy. MSCs can be obtained from a patient's own connective tissue such as BM, adipose tissue, and blood including peripheral and umbilical cord blood. In addition, the use of allogeneic MSCs is thought to be feasible because these cells evade and actively suppress the host immune response. The second step is to expand the prepared MSCs to obtain the required amount of EVs. At this step, optional treatments or gene engineering of the cultured MSCs may allow modification of EVs for selective delivery and/or enhancement of the therapeutic potential of the produced EVs. The third step is to harvest the EVs from the CM of MSCs. At this step, it will be possible to modify the harvested EVs by packaging siRNA, miRNA, and mRNA into the EVs, or loading the EVs with therapeutic drugs. The final 4th step is to administer MSC-derived EVs with therapeutic efficacy to the patient. For efficient delivery of the MSC-derived EVs, researchers are required to explore the best route of administration according to the target disease.

cells and identified miRNAs that were selectively enriched in EVs [142]. Gene ontogeny analysis implied that the selected miRNAs that were shuttled by EVs were associated with immune system regulation. This group also performed mRNA microarray analyses of BM-MSCs and HLSCs as well as their EVs and confirmed that mRNA profiles in EVs reflect their parent cell phenotypes [119, 144]. For example, BM-MSC-derived EVs shuttled a specific subset of cellular mRNAs, including those associated with differentiation into the mesenchymal phenotype and those associated with several cell functions involved in the control of transcription, proliferation, and cell immune regulation [119]. HLSC-derived EVs also shuttled a specific subset rather than a random sample of cellular mRNAs. They shuttled mRNAs that were related to several cell functions involved in the control of transcription, metabolism, and proliferation [144]. Out of the detected mRNAs, the CDK2 gene has been shown to be involved in liver regeneration, highlighting the selective transport of this mRNA from HLSCs to recipient hepatocytes [145].

6 Discussion

In this review, we have summarized the functionality of EVs in a variety of biological events and highlighted the therapeutic potential of MSC-derived EVs. Despite promising results in animal studies, research on MSC-derived EVs is still in its infancy, and strategies for EV therapeutics need to be refined before their clinical application. Here, we will point to three issues that need to be addressed to realize clinical applications of MSC-derived EVs. We also provide some suggestions for future research. Finally, we will emphasize the importance of careful investigation of safety issues regarding the clinical application of MSC-derived EVs.