フトウェアとして、アジレント社 Avadis NGS を用いた。

**（倫理面への配慮）**

本年度行われた RNA-seq 解析は、ヒト線維芽細胞株として汎用されている MRC5 細胞株をもとに作製された神経幹細胞を用いて行われた。

## C．研究結果

(1) RNA-seq 解析で得られた配列情報の概要：

NSC-iPS 細胞を用いて、CMV 感染前および感染後 3 タイムポイント（1 日、4 日、6 日）に回収した細胞より抽出した全 RNA を用いて cDNA ライブラリーを作製後、次世代シーケンサーによる網羅的発現解析を行った。感染前後の 4 サンプルについて、発現された配列情報を Gene type ごとに分類した結果を表 1 に示す。一定以上（raw counts > 20）の発現を示す配列は、4 サンプル全てにおいて 15,000 種類以上同定された。各 Gene type ごとの同定数は、protein-coding：約 14,000 種類と最も多く、全体の 9 割を占めた。その他に、long non-coding RNA (lincRNA)：550 種類、pseudogene：500 種類、antisense RNA：300 種類、などの配列が同定された（表 1）。また、少数とはいえ、miRNA：20 種類、non-coding RNA：15 種類、polymorphic pseudogene：10 種類、small nucleolar RNA (snoRNA)：8 種類なども同定された。なお、各 Gene type の同定数については、感染前後の各タイムポイント間で大きな変動は見られなかった。

(2) CMV 感染前後で発現変動する配列の解析：

CMV 感染前と比較して、感染後の各タイムポイントで発現レベルが変動した配列数を Gene type ごとに示す（表 2）。感染前に比べて、感染 1 日後に発現増強した配列数は 312、発現減弱した配列数は 228 であった。感染 4 日後に発現増強した配列数は 1,574 に増加し、発現減弱した配列数も 464 に増加した。感染 6 日後には、発現増強および発現減弱した配列数は、感染 4 日後よりさらに増加した。

次に、Gene type ごとの比較では、CMV 感染前後で発現変動を示した配列のうち、最多の Gene type は protein coding 配列であった（表 2）。CMV 感染 1 日後に発現増強した protein coding 配列は 250 種類、発現減弱した配列は 159 種類であった。CMV 感染 4 日後および 6 日後に発現変動した protein coding 配列数はさらに増加した。この結果から、CMV 感染前と比較して、感染後には時間経過とともに発現変動する protein coding 配列数が増加する傾向にあることが明らかとなった。CMV 感染後に増加傾向を示す Gene type には、antisense RNA、lincRNA、pseudogene 配列なども含まれていた。この結果より、複数の Gene type において、CMV 感染後に発現変動する配列数が時間経過とともに増加することが明らかとなった。

また、RNA-seq 解析の結果、個々の転写配列の発現動態に関する情報も得られた。そこで、転写配列が CMV 感染にともなって発現変動することを確認する目的で、一部の配列について RT-PCR 解析を行った（図 1）。CMV 感染 1 日後に発現ピークを示す転写配列（図 1、Gene A）、CMV 感染 2~3 日後に発現ピークを示す転写配列（図 1、Gene B, C, D）、CMV 感染 7 日後に発現ピークを示す転写配列（図 1、Gene E）など、発現ピークの異なる転写配列が同定された。この結果より、RNA-seq 解析で得られた発現動態に関する情報が RT-PCR 解析によって裏付けられた。

(3) Gene ontology (GO)解析：

CMV 感染前と比較して、感染後の各タイムポイントで発現増強あるいは減弱する配列群を用いて GO 解析を行い、p-value の低い方から上位 3 種類を抽出した。表 3 には、Molecular function に関する解析結果を示す。発現増強を示す配列群を用いた GO 解析の結果、感染 1 日後で double-strandede RNA binding や 2'-5'-oligoadenylate synthetase activity などが抽出され、感染 4 日後および感染 6 日後で sequence-specific DNA binding および transcription factor activity などが抽出された。一方、発現減弱を示す配列群を用いた GO 解析の結果、感染 4 日後および感染 6 日後で calcium

binding や extracellular matrix structural constituent などが抽出された。

(4) 転写産物 isoform の発現解析：

CMV 感染細胞において、未知 isoform の発現を示す遺伝子が 2,000 種類以上、既知 isoform の発現を示す遺伝子が 10,000 種類以上同定された（表４）。さらに、CMV 感染前後の発現を比較した結果 (Splicing Index 0.25 <)、CMV 感染前後で発現変動する未知 isoform をコードする遺伝子は、感染 1 日後には 23 種類、感染 4 日後には 49 種類、感染 6 日後には 74 種類存在し、時間経過とともに増加した。また、感染前後で発現変動する既知 isoform をコードする遺伝子についても、感染 1 日後には 607 種類、感染 4 日後には 695 種類、感染 6 日後には 813 種類同定され、時間経過とともに増加した。これらの結果より、NSC-iPS 細胞において、既知 isoform に加えて未知 isoform をコードする遺伝子が発現していること、それら遺伝子の一部は CMV 感染前後で発現変動する isoform をコードしていることが示唆された。

## D．考察

本年度は、先天性 CMV 感染症における神経病態の形成機構を明らかにする目的で、CMV の標的細胞の一つであるヒト神経幹細胞において、CMV 感染前後で発現変動する転写配列を同定した。その結果、同定された配列の Gene type のうち、protein coding 配列が全体の約 9 割を占めた。その一方で、lincRNA, miRNA, antisense RNA など多様な Gene type の配列も発現していることが明らかとなり、RNA-seq 解析が様々な Gene type の発現解析に有用であることが確認された。また、RNA-seq 解析で得られた転写配列の発現動態に関する情報が、RT-PCR 解析によって裏付けられたことから、各転写配列の発現動態を明らかにする上でも RNA-seq 解析は有用な手法であると考えられた。

GO 解析では、CMV 感染にともなう細胞変化の分子機構を明らかにする上で、着目すべき機能遺伝子群に関して新たな情報が得られた。今後は、GO 解析によって得られた情報をもとに、これまでにない視点からの病態解析に結びつけることが課題である。

転写産物 isoform の発現解析では、CMV 感染細胞において既知および未知 isoform をコードする遺伝子群が発現していることが明らかとなった。また、一部の遺伝子は、CMV 感染にともなって発現変動する isoform をコードしている可能性が示唆された。今後は、CMV 感染にともなう各 isoform の発現変動と病態形成との関連について明らかにする必要があると考えらえた。

## E．結論

先天性 CMV 感染症の病態形成メカニズムを明らかにするために、次世代シーケンサーを用いて網羅的発現解析を行った。その結果、CMV はヒト神経幹細胞に感染することで、転写配列のダイナミックな発現変動を引き起こすことが明らかになるとともに、当該疾患における神経病態を解明する上で基盤となる情報が得られた。

## F．研究発表

### １．論文発表

該当なし

### ２．学会発表

ヒト人工多能性幹細胞を用いた神経幹細胞への実験的 HCMV 感染系の確立. 中村浩幸、廖華南、南　佳ほり、阿久津英憲、梅澤明弘、井上直樹、藤原成悦. 第 60 回日本ウイルス学会学術集会（2012 年 11 月 14 日、大阪）

## H．知的財産権の出願・登録状況

### １．取得特許

該当なし

### ２．実用新案登録

該当なし

### ３．その他

該当なし

表 1

| | Expressed (raw counts > 20) | | | | |
|---|---|---|---|---|---|
| Gene Type | Day 0 | Day 1 | Day 4 | Day 6 | total |
| All entities | 15317 | 15534 | 16186 | 15904 | 52357 |
| 3prime_overlapping_ncrna | 1 | 1 | 1 | 1 | 12 |
| ambiguous_orf | 5 | 5 | 5 | 3 | 20 |
| antisense | 291 | 319 | 320 | 284 | 3523 |
| lincRNA | 534 | 544 | 594 | 533 | 5479 |
| miRNA | 20 | 21 | 17 | 15 | 1756 |
| miRNA_pseudogene | | | | | 15 |
| misc_RNA | 3 | 5 | 3 | 3 | 1187 |
| misc_RNA_pseudogene | | | | | 3 |
| ncrna_host | 3 | 4 | 3 | 3 | 14 |
| non_coding | 16 | 16 | 15 | 13 | 104 |
| polymorphic_pseudogene | 10 | 10 | 11 | 11 | 27 |
| processed_transcript | 210 | 220 | 246 | 217 | 1255 |
| protein_coding | 13675 | 13806 | 14367 | 14300 | 19975 |
| pseudogene | 481 | 509 | 519 | 443 | 12129 |
| retained_intron | 4 | 4 | 4 | 4 | 10 |
| rRNA | | | | | 530 |
| rRNA_pseudogene | 6 | 6 | 6 | 6 | 179 |
| scRNA_pseudogene | 3 | 4 | 2 | 2 | 787 |
| sense_intronic | 7 | 8 | 11 | 7 | 394 |
| sense_overlapping | 2 | 2 | 1 | 1 | 18 |
| snoRNA | 7 | 11 | 10 | 2 | 1521 |
| snoRNA_pseudogene | | | | | 73 |
| snRNA | 3 | 3 | 7 | 6 | 1944 |
| snRNA_pseudogene | | | | | 73 |
| NEWGENE | 34 | 34 | 41 | 47 | 59 |

表 2

| **Gene Type** | **Up-regulated** (from day 0 to 1) | **Down-regulated** (from day 0 to 1) | **Up-regulated** (from day 0 to 4) | **Down-regulated** (from day 0 to 4) | **Up-regulated** (from day 0 to 6) | **Down-regulated** (from day 0 to 6) |
|---|---|---|---|---|---|---|
| ambiguous_orf | | | | 1 | 1 | 1 |
| antisense | 21 | 15 | 43 | 29 | 49 | 48 |
| lincRNA | 16 | 33 | 79 | 47 | 95 | 85 |
| miRNA | | 1 | | 3 | | 4 |
| ncrna_host | | | | | 2 | |
| non_coding | 1 | | 1 | 4 | 2 | 4 |
| polymorphic_pseudogene | | | 3 | 2 | 4 | 1 |
| processed_transcript | 8 | 9 | 27 | 12 | 21 | 22 |
| protein_coding | 250 | 159 | 1384 | 327 | 1980 | 928 |
| pseudogene | 15 | 11 | 35 | 35 | 43 | 63 |
| scRNA_pseudogene | 1 | | | | | |
| sense_intronic | | | 1 | 1 | 1 | 1 |
| sense_overlapping | | | | 1 | | 1 |
| snoRNA | | | | 1 | | 5 |
| snRNA | | | | | | 1 |
| TR_C_gene | | | 1 | 1 | | 1 |
| Total | 312 | 228 | 1574 | 464 | 2198 | 1165 |

図 1

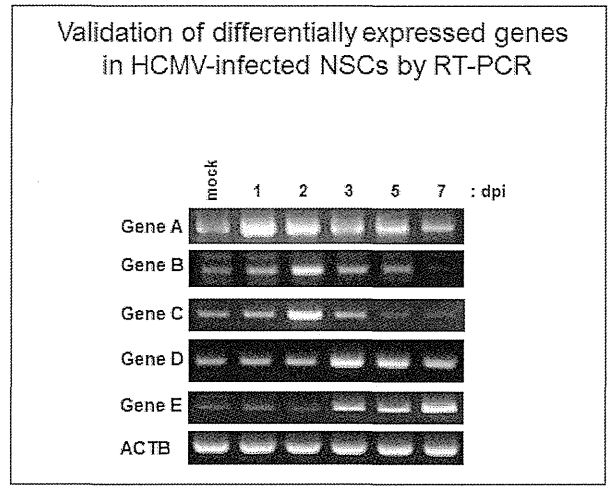


表 3

Molecular Function

| | Up-regulated | | Down-regulated | |
|---|---|---|---|---|
| | GO Term | corrected p-value | GO Term | corrected p-value |
| Day1 | double-stranded RNA binding | 0.03126192 | | |
| | 2'-5'-oligoadenylate synthetase activity | 0.042557 | | |
| | NAD+ ADP-ribosyltransferase activity | 0.042557 | | |
| Day4 | sequence-specific DNA binding | 1.95E-11 | calcium ion binding | 9.75E-08 |
| | transcription factor activity | 4.05E-10 | extracellular matrix structural constituent | 1.08E-05 |
| | transmembrane transporter activity | 1.11E-09 | metalloendopeptidase activity | 2.01E-04 |
| Day6 | sequence-specific DNA binding | 1.32E-18 | extracellular matrix structural constituent | 2.68E-14 |
| | transcription factor activity | 6.17E-16 | calcium ion binding | 1.12E-13 |
| | channel activity | 3.55E-12 | receptor binding | 1.74E-11 |

raw > 20 at each condition

表 4

| | Day 1 | Day 4 | Day 6 |
|---|---|---|---|
| Genes with Novel isoform | 2016 | 2383 | 2636 |
| Splicing Index 0.25 <: vs Day0 | 23 | 49 | 74 |
| Genes with Known isoform | 11332 | 11207 | 10767 |
| Splicing Index 0.25 <: vs Day0 | 607 | 695 | 813 |
| Genes with one isoform | 39009 | 38767 | 38954 |
| Splicing Index 0.25 <: vs Day0 | - | - | - |
| Total | 52357 | 52357 | 52357 |

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分担研究報告書

# 分担研究課題

研究分担者　　氏名　服部正平　所属　東京大学

研究要旨：本研究は、次世代シークエンサー等の大規模配列解析装置を駆使し、小児科・産科領域の難治性疾患や稀少疾患に関連するヒト常在菌叢ゲノム（マイクロバイオーム）を含めた遺伝因子を解明することを目的とする。

## A．研究目的

本研究は、種々の小児科・産科領域の難治性疾患や稀少疾患の患者の腸内細菌叢等のマイクロバイオームについて次世代シークエンサーを用いた解析技術の開発、並びにマイクロバイオーム（遺伝的要因）と疾患との関連性を解明する。本研究は、マイクロバイオームの制御による疾患の緩解という新しい治療法の開発につながる。

## B．研究方法

健常者及び疾患患者の腸内細菌叢等のヒト常在菌叢のゲノムDNA（マイクロバイオーム）を調製し、次世代シークエンサーを用いて、16Sリボソーム RNA 遺伝子（16S）解析及びメタゲノム解析を行い、疾患患者の菌種組成と細菌遺伝子の特徴を解明する。この中で、次世代シークエンサーを用いたゲノム情報取得法の確立と大量のゲノム情報の情報学的解析手法の開発等、高速で精度の高い解析パイプラインの構築も進める。

### （倫理面への配慮）

本研究ではヒト常在菌の分離とその DNA を抽出し、遺伝子情報等を解析する。この遺伝子情報は被験者本人のヒト遺伝子情報ではないが、個人に固有の情報であるため、法令等により保護される個人情報として取り扱う。よって、本研究内容について被験者に対して文書と面接による十分な説明（研究の目的、解析データから被験者が特定できない匿名化、個人名が特定されない形式での情報公開、同意後における撤回または継続の中止の容認とそれらのサンプル及び解析結果の廃棄等）を行い、インフォームドコンセントを取得する。以上について、被験者のサンプルを収集する国立成育医療研究センター等の研究者が所属する機関の「ヒトゲノム倫理審査委員会」の承認を得ていることを文書で確認後、本研究を開始した。

## C．研究結果

昨年度に引き続き、ロシュ社製 GS シークエンサーを用いて日本人健常者の腸内細菌叢の16S 及びメタゲノム解析を進めた。昨年度に解析した 24 名の被験者に、今年度はさらに 27 名の被験者からのサンプルを解析し、小児を含めた計 51 名の被験者から 16S 及びメタゲノムデータを収集した。これらには経時的に得たサンプルも含まれ、解析した総サンプル数は 118 サンプルとなった。まず、16S 解析による菌種組成解析では、定量性の評価を行い、従来よりも高い定量性をもった 16S 解析法を開発した（論文印刷中）。本改良法では 16S 遺伝子増幅の PCR プライマーを改良し、その高い定量性を人工の mock 細菌叢を用いることによって証明した。また、GS シークエンサーから得られる16S データの精度も評価し、クラスタリング等のバイオインフォマティクス条件の改良も行った。

118 サンプルのメタゲノム解析では、合計約 7.5Gb のユニーク配列データを取得し、約 330 万のユニークな遺伝子を同定した。得られた遺伝子については、KEGG 解析等により代謝経路を含めた機能解析を行った。これら遺伝子については 2010 年に発表された欧州人メタゲノムデータ（約 330 万遺伝子）との比較解析を行い、

日本人データに有意にその存在割合の高いまたは低い（日本人腸内細菌叢に特徴的な）遺伝子と代謝系（例えば、日本人では炭水化物代謝系や短鎖脂肪酸生成系が欧州人よりも多い等）を解明した。このほか、個人が有する遺伝子数（40～65 万）と日本人全体が有する遺伝子数（約 550 万）を推定した。

昨年度導入したイオン PGM シークエンサーに関しては、細菌ゲノム解析や細菌叢メタゲノム解析を進め、ルーチン使用としての技術を確立した。なお、イオン PGM の 10～100 倍の能力を有するイオン Proton を今年度導入し、その操作の練習／習得並びに試運転を行った。

## D．考察

昨年度に確立した次世代シークエンサーを用いた腸内細菌叢の菌種構成及び遺伝子組成の解析におけるウェットとバイオインフォマティクス解析手法を駆使して、日本人細菌叢に特徴的な機能・代謝系の同定や総遺伝子数等、これまでよりもより網羅的で広範囲な菌種及び遺伝子組成を定量性高く解析することができ、多くの新知見を得ることができた。

## E．結論

この 2 年間の研究開発により、ヒト腸内細菌叢の解析手法の実行性の証明とデータ品質の高度化を達成できたと考える。今後は、これらの基礎データ及び解析技術を元に、また、より解析能力の優れたイオン PGM 及び Proton を駆使して、健康と疾病の小児サンプルの解析を重点的に進める。本研究で開発した技術は健常者と疾患患者細菌叢との比較解析において、疾患に関連して有意に増減する菌種と遺伝子／機能の特定におおいに有用であると考えられる。また、得られる成果は常在細菌叢をターゲットとした新規な緩解法あるいは治療法の開発に繋がると期待できる。

## F．研究発表

### 1．論文発表

なし。

### 2．学会発表

・服部正平他：Cold Spring Harbor Meeting: The Biology of Genomes ‘Metagenomics of Japanese gut microbiomes’（ポスター発表）（平成 24 年 5 月）。
・服部正平：第 15 回プロバイオティクス学会‘Genomics of Human Microbiome Using Next-Generation Sequencers’（招待講演）（平成 24 年 6 月）。
・服部正平：第 16 回腸内細菌学会‘ヒト常在細菌叢研究の新しい道標’（招待講演）（平成 24 年 6 月）。
・服部正平：第 12 回日本抗加齢医学会‘日本人腸内マイクロバイオームの特徴’（招待講演）（平成 24 年 6 月）。
・服部正平：日本遺伝学会第 84 回大会公開市民講座‘人間の体内に共生する細菌の網羅的なゲノム解析から細菌とヒトの共生を読み解く’（招待講演）（平成 24 年 9 月）。
・服部正平：第 44 回小児感染症学会‘ヒトマイクロバイオーム研究の現状と展望’（招待講演）（平成 24 年 11 月）。
・服部正平：第 9 回日本消化管学会‘ヒトマイクロバイオームの全体像を読み解く’（招待講演）（平成 25 年 1 月）。
・服部正平：第 46 回日本無菌生物ノートバイオロジー学会‘ヒトマイクロバイオームのメタゲノム科学’（招待講演）（平成 25 年 1 月）。

## H．知的財産権の出願・登録状況

### 1．取得特許

・服部正平他：特願2012-050007 炎症性腸疾患の検出方法及びヒト唾液細菌叢の検査方法. 平成24年（出願）。
・服部正平他：U.S. Provisional Application No. 61/607,360. Human-derived bacteria that induce proliferation or accumulation of regulatory T cells. 平成24年（出願）。

### 2．実用新案登録

なし。

### 3．その他

なし。

# III. 研究成果の刊行に関する一覧表

# 研究成果の刊行に関する一覧表

雑誌

| 発表者氏名 | 論文タイトル名 | 発表誌名 | 巻号 | ページ | 出版年 |
|---|---|---|---|---|---|
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# IV. 研究成果の刊行物・別刷

CHEMICAL REVIEWS

Review

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# Biomimetic Cell Culture Proteins as Extracellular Matrices for Stem Cell Differentiation

Akon Higuchi,*[,†,‡,§] Qing-Dong Ling,[§,∥] Shih-Tien Hsu,[⊥] and Akihiro Umezawa[‡]

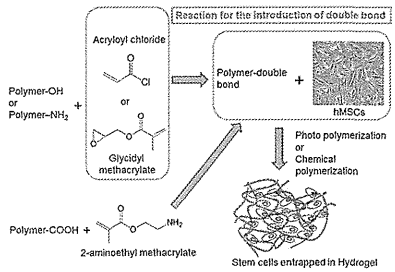
[†]Department of Chemical and Materials Engineering, National Central University, Jhongli, Taoyuan, 32001 Taiwan

[‡]Department of Reproductive Biology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan

[§]Cathay Medical Research Institute, Cathay General Hospital, No. 32, Ln 160, Jian-Cheng Road, Hsi-Chi City, Taipei, 221 Taiwan

[∥]Institute of Systems Biology and Bioinformatics, National Central University, No. 300, Jhongda RD., Jhongli, Taoyuan, 32001 Taiwan

[⊥]Taiwan Landseed Hospital, 77 Kuangtai Road, Pingjen City, Tao-Yuan County, 32405 Taiwan




## CONTENTS



## 1. INTRODUCTION

Each year, millions of people suffer loss or damage to organs and tissues due to accidents, birth defects, and disease. Stem cells are an attractive prospect for tissue engineering and regenerative medicine because of their unique biological properties. Embryonic stem cells (ESCs) derived from

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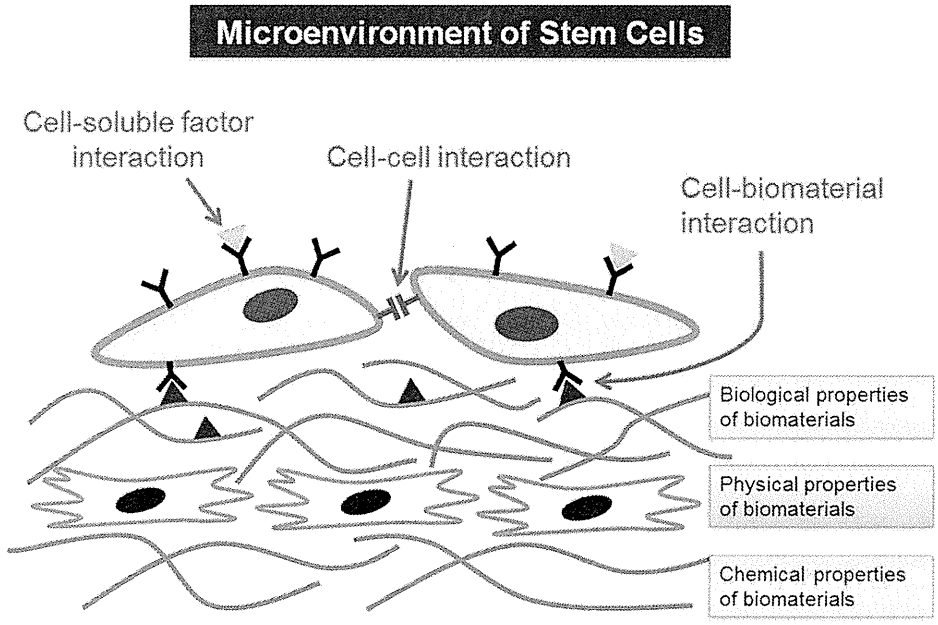


**Figure 1.** Schematic representation of the microenvironment and niches of stem cells and their regulation by the following factors: (a) soluble factors, such as growth factors or cytokines, nutrients, and bioactive molecules; (b) cell–cell interactions; (c) cell–biomaterial interactions. Biological, physical, and chemical properties of biomaterials also regulate stem cell fate.

preimplantation embryos have the potential to differentiate into any cell type derived from the three germ layers—the ectoderm (epidermal tissues and nerves), mesoderm (muscle, bone, and blood), and endoderm (liver, pancreas, gastrointestinal tract, and lungs).[1] The basis of pluripotency lies in conserved regulatory networks composed of numerous transcription factors and multiple signaling cascades. Together, these regulatory networks maintain human ESCs (hESCs) in a pluripotent and undifferentiated state, and alterations in the stoichiometry of these signals promote differentiation. hESCs have been shown to generate multipotent stem and progenitor cells in vitro and are capable of differentiating into a limited number of cell fates, and thus they have great potential for use in transplantation of cells and tissues into patients.[2]

Although hESCs are promising donor sources for cell transplantation therapies,[1] they face immune rejection after transplantation. Furthermore, ethical issues regarding human embryos hinder their widespread usage. These concerns can be circumvented if pluripotent stem cells can be derived directly from patients' own somatic cells.[3] Recently, pluripotent stem cells similar to ESCs, known as induced pluripotent stem cells (iPSC's), were derived from adult somatic cells by inducing a "forced" expression of certain pluripotent (stem cell) genes[4−6] such as Oct3/4, Sox2, (c-myc), and klf-4, or certain miRNAs[7] or proteins (piPS).[8] iPSC's are believed to be similar to ESCs in many respects, including the expression of certain stem cell genes and proteins, chromatin methylation patterns, doubling time, embryoid body formation, teratoma formation, viable chimera formation, pluripotency, and differentiability.

The pluripotent nature of iPSC's opens many avenues for potential stem cell-based regenerative therapies and for development of drug-discovery platforms.[9,10] The nearest-term therapeutic uses of iPSC's may exist in the transplantation of differentiated nerve cells or $\beta$-cells for treatment of Parkinson's Disease and diabetes, respectively, which arise from disorders of single cell types. However, there are several barriers to the clinical application of iPSC's, such as the use of viral vectors, cultivation using xeno-derived materials [e.g., mouse embryonic fibroblasts (MEFs)], and the extremely low efficiency of iPSC generation.[11]

Stem cells have also been isolated from a variety of somatic tissues, including hematopoietic stem cells (HSCs) derived from umbilical cord blood and mesenchymal stem cells (MSCs) derived from bone marrow, umbilical cord blood, umbilical cord, dental pulp, and tissues such as fat. There have been no reports to date of MSCs or fetal stem cells differentiating into tumors, unlike ESCs and iPSC's. Consequently, HSCs, MSCs, and fetal stem cells are the most promising sources of cells for tissue engineering and cell therapies. Currently, MSCs are thought to be the most widely available autologous source of stem cells for practical and clinical applications. Fetal stem cells derived from amniotic fluid are pluripotent cells capable of differentiating into multiple lineages, including cell types of the three embryonic germ layers. Bone marrow MSCs, adipose-derived stem cells (ADSCs), and amniotic fluid stem cells may be more suitable sources of stem cells in regenerative medicine and tissue engineering than ESCs and iPSC's because of ethical concerns regarding their use and concerns about xenogenic contamination arising from the use of mouse embryonic fibroblasts (MEFs) as a feeder layer for ESC and iPSC culture.[11]

Stem cell characteristics, such as proper differentiation and maintenance of pluripotency, are regulated not only by the stem cells themselves but also by the microenvironment. Therefore, mimicking stem cell microenvironments and niches using biopolymers will facilitate the production of large numbers of stem cells and specifically differentiated cells needed for in vitro regenerative medicine. Several factors in the microenvironment and niches of stem cells influence their fate: (i) soluble factors, such as growth factors or cytokines, nutrients, and bioactive molecules; (ii) cell–cell interactions; (iii) cell–biomacromolecule (or biomaterial) interactions; and (iv) physical factors, such as the rigidity of the environment (Figure 1). Some excellent review articles addressing the

engineering of stem cell microenvironments and niches using natural and synthetic biopolymers are listed in Table 1.[11−22]

**Table 1. Key Review and Articles Dealing with Biopolymers for Culture and Differentiation of Stem and Progenitor Cells**

| author | contents | ref (year) |
|---|---|---|
| Lee and Mooney | hydrogels for tissue engineering | 12 (2001) |
| Little et al. | biomaterials for neural stem cell microenvironments | 13 (2008) |
| Higuchi et al. | polymeric materials for ex vivo expansion of HSCs | 16 (2009) |
| Mei et al. | combinatorial development of biomaterials for clonal growth of human pluripotent stem cells | 17 (2010) |
| Melkoumian et al. | synthetic peptide-acrylate surfaces for long-term self-renewal of hESCs | 18 (2010) |
| G. J. Delcroix et al. | adult cell therapy for brain neuronal damages and the role of tissue engineering | 22 (2010) |
| Higuchi et al. | biomaterials for the feeder-free culture of hESCs and human iPSC's | 11 (2011) |
| Balakrishnam and Banerjee | biopolymer-based hydrogels for cartilage tissue engineering | 14 (2011) |
| Kim et al. | design of artificial extracellular matrices for tissue engineering | 15 (2011) |
| Engler et al. | matrix elasticity directs stem cell lineage | 19 (2006) |
| Gilbert et al. | substrate elasticity regulates skeletal muscle stem cell self-renewal | 20 (2010) |
| Huebsch et al. | harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate | 21 (2010) |

These articles focus on biopolymers employed for maintenance of pluripotency of hESCs, iPSC's, or hematopoietic stem cells (HSCs),[16−18] and for specific differentiation lineages such as chondrocytes (cartilage), muscle cells, and neural cells.[13,14,20] There have been no review articles specifically describing extracellular matrix (ECM) scaffolds (ECM in 3D) or ECM-immobilized dish coatings (ECM in 2D) that guide stem cell fates and differentiation. Therefore, this review focuses on the chemical, physical, and biological characteristics of natural biopolymers, especially ECM proteins, which are the major functional biopolymers, and deals with the ability of these biopolymers to guide differentiation of MSCs into osteogenic, chondrogenic, adipogenic, cardiomyogenic, and neural cell lineages.

## 2. CELL SOURCES AND ANALYSIS OF DIFFERENTIATION LINEAGES OF MSCS

### 2.1. Cell Sources

Human MSCs (hMSCs), including fetal stem cells, are one of the most widely available autologous sources of stem cells for clinical applications. hMSCs can be obtained from bone marrow,[23,24] adipose tissue,[25,26] dental pulp,[27] and urine,[28] among other sources. Fetal stem cells can be obtained from amniotic fluid,[29−31] umbilical cord,[32−34] menstrual blood,[35,36] umbilical cord blood,[25,34,37] and placenta.[38,39] hMSCs derived from bone marrow and fat are primarily used for biomaterials research on stem cell culture and differentiation because bone marrow MSCs and ADSCs are easily accessible and can be obtained in large quantities. Bone marrow MSCs (BMSCs) are now commercially available from several companies. Stem cell research is facilitated with these stem cell sources because it is not necessary to obtain permission from ethics committees of the Institutional Review Board (IRB) for use of commercially available MSCs. Otherwise, informed consent from donors and permission from the IRB must be obtained.

### 2.2. Analysis of Differentiation Lineages

MSCs are multipotent stem cells that can be differentiated into various mesodermal lineages, including osteoblasts, chondrocytes (cartilages), adipocytes, myocytes, and cardiomyocytes.[19,40,41] MSCs are also reported to be able to differentiate into ectodermal lineages (e.g., neuron, oligodendrocyte, astrocyte, neural stem cells, and dopamine-secreting cells)[22,42−45] and endodermal lineages (hepatocytes and $\beta$-cells),[31,46−52] although with lower probability than mesoderm lineages. Table 2 summarizes methods for characterizing specific differentiated cells from MSCs.[11,34,46,48,51−87]

MSCs differentiate into an osteogenic phenotype in vitro when supplements such as ascorbic acid, $\beta$-glycerophosphate, dexamethasone, and/or bone morphogenic protein 2 (BMP-2) are added to the culture medium. Figure 2 shows the expression of several genes and proteins, as well as mineral deposition, by MSCs upon osteogenic differentiation. Runt-related transcription factor 2 (Runx2, also known as Cbfa1, Pebp2$\alpha$A, and AML3) is a master regulator of osteogenic gene expression and osteoblast differentiation, and it is an early marker of osteogenesis.[88−90] Runx2 activity is stimulated by mitogen-activated protein kinase (MAPK) signaling and is negatively regulated by thrombin-like enzyme 2 (TLE2). Alkaline phosphatase (ALP) activity is an early osteogenic marker, and osteopontin and osteocalcin are late osteogenic markers.[88] Mineral deposition is generated in the late stage of osteogenic differentiation and is detected by Alizarin Red staining (calcium deposition) and von Kossa staining (calcium phosphate deposition).[57,60,62]

MSCs commit to a chondrogenic phenotype when supplied with transforming growth factor-$\beta$1 (TGF-$\beta$1). Chondrogenic differentiation of MSCs is typically determined by immunostaining for specific proteins, such as collagen type II and Sox9, dye labeling of glycosamino glycans, and evaluation of expression of chondrogenic proteins or transcription factors (such as collagen type II and type X, cartilage oligomeric protein, aggrecan, and Sox9) (Table 2).[63,64,67,70,91] Sulfated glycosaminoglycans (sGAG's) are visualized by staining with Alcian blue.[91] Accumulation of sulfated proteoglycans are also visualized by Safranin O staining.[72]

Only a few groups have investigated adipogenic differentiation of MSCs cultured on natural and artificial biomaterials[53,62,70,74,75,92] because adipose tissue is in less demand in clinical usage than osteoblasts and cartilage cells. Adipogenic differentiation is also analyzed by immunostaining for specific proteins (vimentin), dye staining of oil droplets, and measuring expression of transcription factors or other marker proteins, such as peroxisome proliferator-activated receptor$\gamma$ [PPAR$\gamma$] and adipocyte Protein 2 (aP-2).[53,61,62,74,75,92] aP-2 is a carrier protein for fatty acids that is primarily expressed in adipocytes.[93] Preadipocytes and mature adipocytes contain multiple or single lipids in cell bodies, respectively. Therefore, Oil Red O or Nile red staining of preadipocytes and mature adipocytes is frequently used for the detection of lipids.

Neural differentiation of MSCs is primarily analyzed by observing characteristic morphologies of neurons, astrocytes, oligodendrocytes, and microglia. Neuronal progenitor cells and early-stage neurons are also identified by Sox1, Sox2, and CD133 gene expression and by nestin and $\beta$-tubulin-III


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**Table 2. Characterization of Differentiation of MSCs into Specific Lineages [Osteoblasts and Chondrocyte (Cartilages)]**

| differentiation lineage | characterization | specification | ref (example) |
|---|---|---|---|
| 1. Osteoblast | morphology | spread shape tends to differentiate into osteoblasts, bonelike nodule formation | 53−55 |
| | protein level (immunostaining) | **collagen I**, osteocalcin, osteonectin | 56, 57 |
| | surface marker analysis and immunostaining | osteopontin, bisphosphonate [2-(2-pyridinyl)ethylidene-BP] (PEBP), alkaline phosphatase (ALP) | 34, 58 |
| | enzyme activity | **alkaline phosphatase** | |
| | gene level | **runt-related transcription factor 2** [Runx2 or core binding protein A-1 (CBFA-1)], **osterix** (OSX), **osteocalcin** (OCN), **osteopontin** (OPN), **bone sialoprotein** (BSP), alkaline phosphatase, integrin-binding sialoprotein (IBSP), bone γ-carboxyglutamate protein (BGLAP) | 34, 58−61 |
| | dye staining | **Alizarin Red staining** (calcium) | 62 |
| | mineral deposition | **von Kossa staining** (calcium phosphate) | 57, 60 |
| 2. Chondrocytes | protein level (immunostaining) | **collagen type II** (Col II), **collagen type X** (Col X), **aggrecan** (AGN), Sox-9, chondroitin-4-sulfate, chondroitin-6-sulfate, sulphated glycosaminoglycans | 56, 57, 63−68 |
| | glycosaminoglycan assay | glycosaminoglycan content | |
| | dimethylmethylene blue (DMMB) assay | proteoglycan (PG) content | 69 |
| | hydroxyproline assay | collagen content | 65 |
| | gene level | **collagen II**, collagen IX (Col IX), **collagen X**, collagen XI (Col XI), **aggrecan**, Sox 5, Sox 6, **Sox 9**, **cartilage oligomeric protein** (COMP), xylosyltransferase I (XT-1), α-4-*N*-acetylhexosaminyltransferase (EXTL2), β-1,4-*N*-acetylgalactosaminyltransferase (GalNAcT), glucuronyl C5 epimerase (GlcAC5E) | 63, 64, 67, 70−73 |
| | dye staining | **Safanin O staining (proteoglycan)**, **Alcian blue staining (proteoglycan)**, EVG-staining, Masson's trichrome staining | 34, 62, 64, 67, 70, 72 |
| 3. Adipocytes | morphology | round shape cells tends to differentiated into adipocytes | 53, 54 |
| | protein level | vimentin, adipocyte lipid-binding protein (ALBP) | 53, 74 |
| | enzyme activity | glycerol-3-phosphate dehydrogenase activity | 75 |
| | gene level | PPARγ, aP-2 | 61 |
| | staining | Oil red O and Nile red staining for lipid droplet | 62 |
| 4. Neural cells | morphology | neuronal-like cells having long neurites | 76 |
| | protein level | nestin, neuron-specific class III β-tubulin (TuJ1), galactosylceramidase (GalC), glial fibrillary acidic protein (GFAP), β-tubulin-III, microtubule-associated protein 2 (MAP2), O4, tyrosine hydroxylase (TH), neurofibromatosis (NFM), neurone-specific enolase (NSE) | 76−81 |
| | gene level | nestin, Musashi 1, neuron-specific class III β-tubulin (TuJ1), glial fibrillary acidic protein, microtubule-associated protein 2, Sox1, Sox2, CD133, tyrosine hydroxylase, neurofibromatosis, Nurr1, dopamine transporter (DAT), dihydropyrimidinase-related protein 2 (DRP-2), purine-sensitive aminopeptidase (PSA) | 11, 61, 76, 81, 82 |
| 5. Cardiomyocytes | morphology | contractile cells | |
| | protein level | **cardiac troponin T (cTnT)**, **desmin**, myosin light chain (MLC), myosin heavy chain (MHC) | 81 |
| | gene level | Nkx2.5, GATA-4, MYH-6, TNNT2, TBX-5, myosin light chain (Mlc2a, MLC-2 V), tropomyosin, cTnI, ANP, **desmin**, **myosin heavy chain** (α-MHC, **β-MHC**), **cardiac troponin T**, Isl-1, and Mef2c | 11 |
| | electrocardiogram | electrocardiogram | |
| 6. Smooth muscle cells | protein level | α-smooth muscle actin (ASMA), h1-calponin (CALP), SM2 | 83 |
| | gene level | α-smooth muscle actin, h1-calponin, caldesmon, Smemb, SM22α, SM1, SM2 | 83 |
| 7. Epidermis | protein level | keratin 10 (early marker), filaggrin (intermediate marker), involucrin (late marker) | 84 |
| | gene level | keratin 10 (early marker), filaggrin (intermediate marker), involucrin (late marker) | 84 |
| 8. Hepatocyte | morphology | oval cell morphology, small round cell morphology | 46 |





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Table 2. continued

| differentiation lineage | characterization | specification | ref (example) |
|---|---|---|---|
| | protein level | CXCR4 (endoderm), α-fetoprotein (AFP), albumin (ALB), asialoglycoprotein receptor (ASGPR), cytochrome P450 ($CYP_1A_1$), hepatocyte nuclear factor-1α (HNF-1α), hepatocyte nuclear factor-3β (HNF-3β), hepatocyte nuclear factor-4α (HNF-4α), CCAAT-enhancer binding protein α (C/EBPα), cytokeratin-18 (CK18), cytokeratin-19 (CK19), low-density lipoprotein (LDL), GATA4 | 46, 51, 52, 86, 87, 113 |
| | gene level | Sox17 (endoderm), Foxa2 (endoderm), Gata6 (endoderm), α-fetoprotein, albumin, hepatocyte nuclear factor-1α, hepatocyte nuclear factor-3β, hepatocyte nuclear factor-4α, cytokeratin 18, cytokeratin-19, asialoglycoprotein receptor, tryptophan oxygenase (TO), cytochrome P450 (CYP1A1, CYP2B6), CCAAT-enhancer binding protein α, glucose 6-phosphate (G6P), GATA4 | 46, 51, 52, 86, 87, 113 |
| | urea assay | urea production | 46, 51, 113 |
| | albumin assay | albumin production | 52, 86, 113 |
| | glycogen assay | glycogen production | 46, 52, 113 |
| | α-fetoprotein assay | α-fetoprotein production | 52, 86 |
| | pentoxyresorufin (PROD) assay | cytochrome P450 activity | 113 |
| | staining | periodic acid–Schiff (PAS) staining for glycogen storage | 46, 113 |

immunostaining. Mature neurons express neuron-specific class III β-tubulin (Tuj1), microtubule-associated protein 2 (MAP2), neuron-specific enolase (NSE), and purine-sensitive aminopeptidase (PSA). Oligodendrocytes express galactosylceramidase (GalC) and O4. Dopaminergic neurons express tyrosine hydroxylase (TH), neurofibromatosis (NFM), and dopamine transporter (DAT). Nerve cells are electrically excitable cells that transmit information by electrical and chemical signaling. Therefore, electrical and action potentials in nerve cells can be monitored using electrodes.

## 3. PREPARATION OF CULTURE MATRIX

Biomimetic stem cell cultures can be categorized as two-dimensional (2D) or three-dimensional (3D). 2D culture is useful for basic research to investigate the fundamental interactions between cells and immobilized nanosegments on dishes, but 3D culture of stem cells in biomaterials is essential for clinical applications. Figure 3 shows some examples of biomaterial designs for carrying stem cells, as well as direct injection of biomaterials without cells. The injection of hydrogels or scaffolds containing stem cells is categorized as 3D cultures. Cell sheets prepared on a surface-grafting polymer having low critical solution temperature (LCST), such as poly(*N*-isopropylacrylamide) (poly(NIPAM)), can be prepared on 2D dishes.[94,95] Recently, patch sheets of immobilized antibodies or ligands targeting specific stem cells, which recruit the stem cells from the patient's body, are reported to be effective in gathering autologous stem cells at sites of injury.[40] The following sections describe methods for (a) surface immobilization of ECM proteins and ECM-mimicking peptides on 2D culture dishes and (b) preparing hydrogels or scaffolds containing ECM proteins and ECM-mimicking peptides for 3D culture of stem cells.

### 3.1. ECM Immobilization on 2D Dishes

Typically, 2D cell culture dishes are coated with ECM proteins or ECM-mimicking peptides. Tables 3 and 4 show examples of the ECM proteins and ECM-mimicking peptides used to coat culture dishes and their binding sites on stem cells.[16,18,53,58,71, 83,91,96−118] Collagen types I, II, and IV, gelatin, laminin, laminin-1, laminin-5, vitronectin, and fibronectin are typically used as coating materials.[58,71,83,91, 96−98,100−102] ECM-mimicking peptides (e.g., RGD, DGEA, YIGSR, IKVAV, KRSR, P15, and GFOGER) are commonly used as coating or grafting materials.[16,18,53,97,103−118] Covalent binding is preferable for long-term effects in culture, but noncovalent coating is the simplest method for the preparation of dishes with immobilized ECM proteins or ECM-mimicking peptides. Figure 4 summarizes typical surface reactions for the covalent immobilization of ECM proteins and peptides on dishes. Proteins and ECM-mimicking peptides should be used in aqueous solution, as they are unstable biomolecules. Reactions between amino groups and between amino groups and carboxylic acids can be used to bind ECM proteins and ECM-mimicking peptides to plastic dishes. These plastic surfaces should therefore have amino groups, carboxylic acid groups, or hydroxyl groups to bind and immobilize ECM proteins or peptides. For dishes made of polyesters, such as poly(ε-caprolactone) (PCL), poly(glycolic acid) (PGA), poly(lactic acid) (PLA), or poly(lactic acid-*co*-glycolic acid) (PLGA), treatment with a diamine, such as hexamethylene diamine, generates amino groups on the surface by an aminolysis reaction. Then, ECM proteins and ECM-mimicking

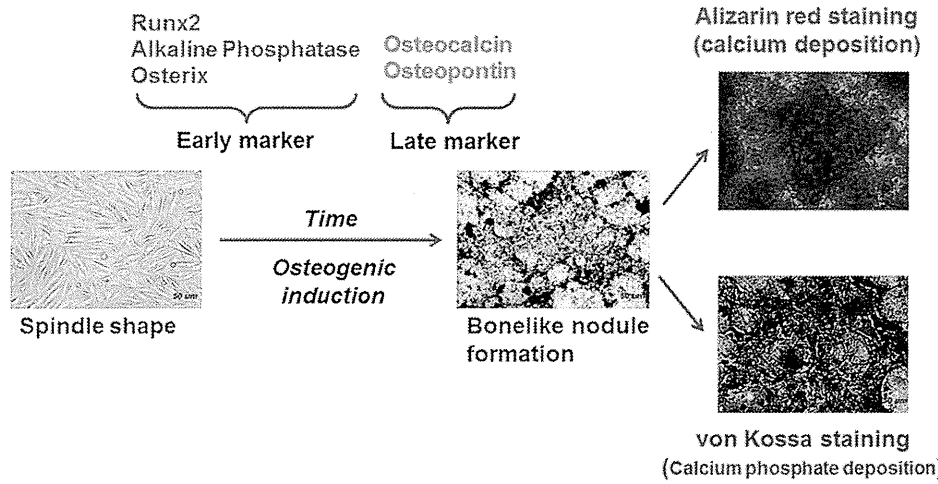


**Figure 2.** Osteogenic differentiation of MSCs, gene expression, and mineral deposition at early and late stages.

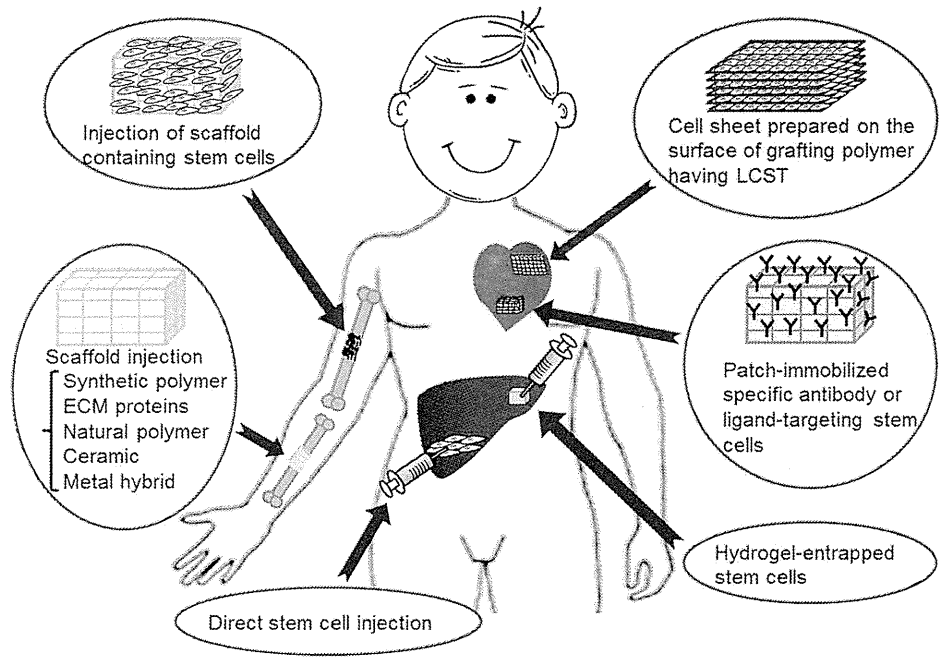


**Figure 3.** Some examples of biomaterial designs with and without stem cells for the injection of biomaterials in clinical applications: (a) injection of scaffold containing stem cells, (b) injection of scaffold without cells, (c) direct stem cell injection, (d) injection of cell sheets, (e) injection of patch-immobilized specific antibody or ligand-targeting stem cells, and (f) injection of hydrogel-entrapped stem cells.

peptides can be covalently immobilized using hexamethylene diisocyanate (HMDIC), 1,6-dimethyl suberimidate dihydrochloride (DMS),[119] or NHS/EDC reagent,[18] where NHS is *N*-hydroxysuccinimide and EDC is *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (Figure 4). EDC is a water-soluble carbodiimide that is generally used in the 4.0–6.0 pH range. Therefore, it is possible to immobilize ECM proteins and ECM-mimicking peptides in aqueous solution using NHS/EDC reagents. The covalent bonding between amino groups can be reacted with aqueous DMS.[119]

Genipin is generally used to cross-link proteins, such as collagen and gelatin, and chitosan via amino groups.[120,121] Genipin can also be used for the immobilization of ECM proteins and peptides on the surface of culture dishes with amino groups (Figure 4). NHS/EDC, DMS, and genipin are the recommended reagents to covalently immobilize ECM proteins and ECM-mimicking peptides on culture dishes.

### 3.2. 3D Culture in Hydrogels

Hydrogels are physically or chemically cross-linked polymer networks that are able to absorb large amounts of water. Injectable hydrogels containing stem cells can be delivered to sites of damage in patients with minimal invasiveness, and the hydrogels ensure that stem cells remain localized to the damaged sites more effectively than injected cells alone. Physical cross-linking is performed on ECM proteins with thermosensitive properties of lower critical solution temperature (LCST) or upper critical solution temperature (UCST), such as collagen and gelatin. Collagen can be dissolved in aqueous solutions at low temperature and forms gels at ~37 °C because of its LCST characteristics, and gelatin can be dissolved in aqueous solution at high temperatures and forms gels at room temperature because of its UCST. Therefore, stem cells can be dissolved in ECM protein solutions and efficiently entrapped in ECM gels at 20–37 °C. However, most ECM

**Table 3. ECM Immobilized on Dishes for Adhesion, Differentiation, And Proliferation of Stem Cells and Some Examples of the Literature**

| ECM | binding site of cells | ref |
|---|---|---|
| collagen I | integrin ($\alpha V\beta 3$, $\alpha 2\beta 1$) | 58, 96 |
| collagen I | integrin ($\alpha 1\beta 1$) | 97 |
| collagen I | integrin ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$) | 71 |
| collagen II | integrin ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$) | 71, 91 |
| collagen IV | integrin ($\alpha 2\beta 1$, CD44) | 98 |
| gelatin | | 99 |
| fibronectin | integrin ($\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha V\beta 3$, $\alpha IIb\beta 3$, $\alpha V\beta 6$, $\alpha V\beta 5$) | 58, 96 |
| laminin | integrin ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$) | 100 |
| laminin-1 (laminin 111) | integrin ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$, $\alpha 9\beta 1$), $\alpha$-dystroglycan, suifade, and heparan sulfate proteoglycan | 83, 101 |
| laminin-5 (laminin 332) | integrin ($\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$) | 102 |
| laminin-10/11 | integrin ($\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$) | 100 |
| vitronectin | integrin ($\alpha V\beta 3$, $\alpha V\beta 5$) | 58, 96 |

proteins and ECM-derived oligopeptides (ECM peptides) need other forms of cross-linking to trap stem cells and generate hydrogels. Typically, photocross-linking and chemical cross-linking of ECM proteins and ECM peptides are used. There are several excellent reviews that discuss hydrogel preparation and reaction in detail.[12,14] Therefore, this section deals briefly with the preparation of ECM hydrogels using photocross-linking and chemical cross-linking with cross-linking agents. The application of ECM hydrogels containing stem cells is discussed in section 5 for specific ECM proteins and ECM peptides.

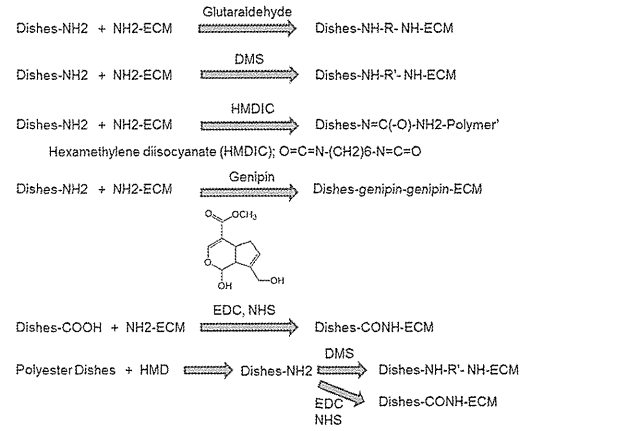


**Figure 4.** Surface reactions of covalent immobilization of ECM proteins and ECM-mimicking peptides on dishes.

**3.2.1. Photocross-Linking of ECM Proteins and ECM Peptides.** Hydrogels containing stem cells can be easily prepared by UV irradiation of ECM proteins and ECM–peptide solutions. These preparations can be used as injectable hydrogels via photocross-linking. However, it is first necessary to introduce double bonds into ECM proteins and ECM peptides for photocross-linking. ECM proteins and ECM peptides have $-OH$, $-NH_2$, and $-COOH$ functional groups. Double bonds can be introduced into ECM proteins and ECM

**Table 4. ECM-Mimicking Peptides Immobilized on Dishes for Adhesion, Differentiation, And Proliferation of Stem Cells**

| ECM-mimicking peptide | ECM proteins for mimicking | binding site of cells | ref |
|---|---|---|---|
| **DGEA** | collagen I | integrin ($\alpha 2\beta 1$) | 103–105 |
| GTPGPQGIAGQRGVV (P15) | collagen I | integrin ($\alpha 2\beta 1$) | 103, 106 |
| $(RADA)_4$GG**DGEA** | collagen I | integrin ($\alpha 2\beta 1$) | 116 |
| $(RADA)_4$GGFPGERGVEGPGP | collagen I | | 116 |
| GFOGER | collagen | integrin ($\alpha 2\beta 1$) | 103, 107, 108 |
| MNYYSNS | collagen IV | | 109 |
| RGD | collagen I | integrin ($\alpha V\beta 3$) | 97, 110 |
| **ELIDVPST** (CS-1) | fibronectin | integrin ($\alpha 4\beta 1$); VLA-4 | 16, 111 |
| FN-40 | fibronectin | integrin ($\alpha 4\beta 1$, VLA-4) | 16, 112 |
| FN-120 | fibronectin | integrin ($\alpha 5\beta 1$); VLA-5 | 16, 112 |
| FN–CH296 | fibronectin | integrin ($\alpha 4\beta 1$, $\alpha 5\beta 1$) | 16, 112 |
| KGGAVTG**RGD**SPASS | fibronectin | integrin ($\alpha 5\beta 1$); VLA-5 | 18, 113 |
| **GRGDS**PK | fibronectin | integrin ($\alpha 5\beta 1$); VLA-5 | 18, 113 |
| KNNQKSEPLIGRKKT | fibronectin | heparin-binding domain | 53 |
| **RGDS** | fibronectin | | 109 |
| PHSRN | fibronectin | | 109 |
| KYGAAS**IKVAV**SADR | laminin | | 18, 114 |
| **YIGSR** | laminin | | 109 |
| **IKVAV** | laminin | | 115 |
| PPFLMLLKGSTR | laminin-5 (laminin332) | integrin ($\alpha 3\beta 1$) | |
| (RADA)4-GG**PDSGR** | laminin | | 116 |
| (RADA)4-GGSDPG**YIGSR** | laminin | | 116 |
| (RADA)4-GGIKVAV | laminin | | 116 |
| KGGPQV**TRGD**VFTMP | vitronectin | integrin ($\alpha V\beta 5$) | 18, 117 |
| KGGNGEP**RGD**TYRAY | bone sialoprotein (BSP) | | 18, 118 |
| PEO4-NGEP**RGD**TYRAY | BSP-linker | | 18, 118 |
| **RGD** | osteopontin | integrin ($\alpha V\beta 3$) | 97 |


dx.doi.org/10.1021/cr3000169 | Chem. Rev. 2012, 112, 4507–4540

peptides by the reactions of acryloyl chloride,[122] glycidyl methacrylate,[12,123] and 2-aminoethylmethacrylate[12,124] (Figure 5). Figure 5 also shows a schematic for preparation method of

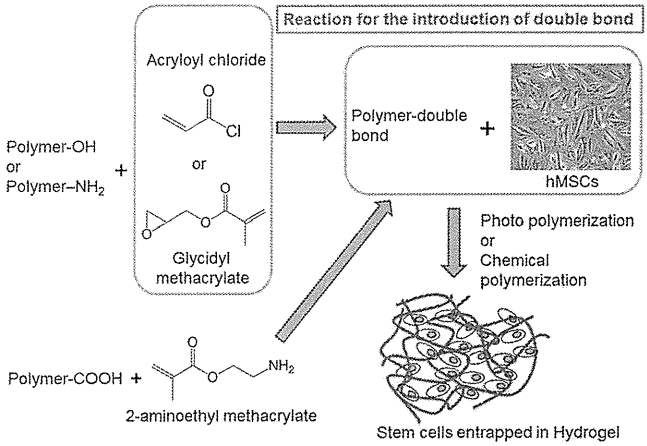


**Figure 5.** Schematic of the preparation method of hydrogels with entrapped stem cells by photopolymerization.

hydrogels with entrapped stem cells by photopolymerization. Aqueous solutions containing stem cells and macromers of ECM proteins and ECM peptides are irradiated with UV light to generate hydrogels with entrapped stem cells.

Poly(ethylene glycol)diacrylate (PEODA) is typically added to the reaction solution to generate optimal hydrogels.[65,125−129] Yang et al. prepared PEODA hydrogels incorporating RGD adhesive peptides and goat BMSCs by photopolymerization. They found that RGD-conjugated PEODA hydrogels promoted the osteogenic differentiation of BMSCs, and RGD enhanced differentiation in a dosage-dependent manner, with the highest concentration (2.5 mM) in the reaction solution being optimal in their study.[125]

**3.2.2. Chemical Cross-Linking of Hydrogels.** Hydrogels of ECM proteins can also be prepared by chemical cross-linking. Similar to ECM protein immobilization on 2D dishes, as discussed in section 3.1, NHS/EDC, DMS, HMDIC, and genipin are typically used as cross-linking agents. Glutaraldehyde is not commonly used for the preparation of hydrogels in tissue engineering because it is relatively toxic to stem cells. DMS, HMDIC, and genipin allow cross-linking between amino groups, whereas NHS/EDC leads to cross-linking between carboxylic acids and amino groups in ECM proteins.

Chang et al. compared gelatin hydrogels cross-linked with genipin and gelatin hydrogels cross-linked with glutaraldehyde.[120] They found that the degree of inflammatory reaction in wounds treated with the genipin-cross-linked gelatin was significantly less severe than those covered with the glutaraldehyde-cross-linked gelatin in vivo.[120] In addition, the healing rates of wounds treated with the genipin-cross-linked gelatin were notably faster than those with glutaraldehyde-cross-linked hydrogels.[120]

### 3.3. 3D Culture in Scaffolds

Scaffolds seeded with stem cells can support 3D tissue formation artificially. It is optimal for scaffolds (a) to allow cell attachment and migration, (b) to allow diffusion of nutrients, growth factors, and waste secreted by cells, and (c) to have mechanical properties similar to the natural tissue. Most of the scaffolds have high porosity (>80%) and large pore size (200−800 $\mu$m), which allow diffusion of nutrients, growth factors, and waste, but these properties also lead to weak mechanical properties. Biodegradability of scaffolds is often required because scaffolds should be absorbed by the surrounding tissues without the necessity of surgical removal. It is preferable that the degradation rate of scaffolds should be matched to the speed of tissue formation. The degradation speed of scaffolds can be regulated by the degree of cross-linking. Scaffolds prepared from ECM proteins and ECM peptides are desirable because of their biodegradable characteristics. ECM proteins used for the preparation of scaffolds are typically collagen type I, collagen type II, gelatin, fibronectin, laminin, and vitronectin. ECM proteins can be used as (a) coating materials, (b) blending materials, and (c) main materials of scaffolds.

**3.3.1. Preparation of Scaffolds.** There are several methods used to prepare scaffolds for tissue engineering and 3D culture of stem cells, including (a) freeze-drying, (b) salt leaching, (c) porogen leaching, (d) use of nonwoven fabric or mesh, (e) nanotopography, and (f) electrospinning. In the freeze-drying method, ECM proteins are dissolved in a buffer solution. The ECM solution is frozen at −20 or −80 °C and then lyophilized in a freeze-dryer before being washed and stored (Figure 6). If necessary, the scaffolds are also cross-linked.

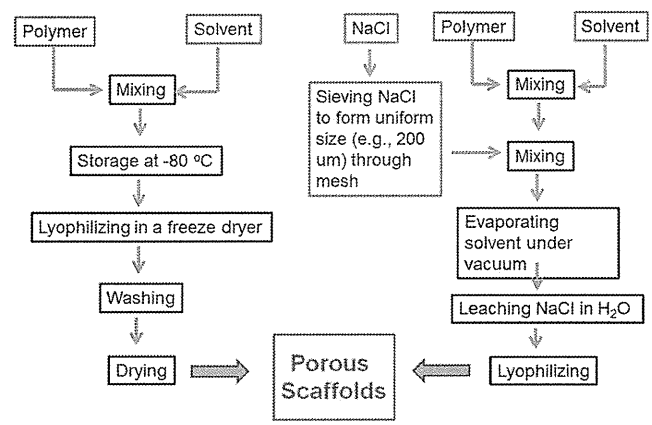


**Figure 6.** Typical preparation method of porous scaffolds by freeze-drying (a) and salt leaching (b).

The salt-leaching method is as follows. Biopolymers and/or ECM proteins are dissolved in a solvent. Salt, typically NaCl, is sieved to generate a uniform distribution of size using filtration through mesh and added into the solution. The solvent of the solution is vaporized under vacuum to generate dry scaffolds. Salt is then leached from the scaffolds by immersion in water after drying the scaffolds (Figure 6). The porogen-leaching method is a similar method to the salt-leaching method, but other uniformly sized particles, such as polymeric particles, are used instead of salt.

### 3.4. 3D Culture in Nanofibers

Peptide amphiphiles (PAs), which have a hydrophilic domain and a hydrophobic domain, are known to spontaneously generate self-assembled nanofibers above critical micelle concentrations.[109,116,130] MSC differentiation on self-assembled nanofibers using ECM peptides is discussed in section 5.8.1.

A typical method to create nanofibers is electrospinning. Electrospun scaffolds can support cell adhesion and growth and

promote differentiation of stem cells.[131] Nanofibers can be generated from a spinning nozzle when high voltage is applied between the spinning nozzle and a flat metal collector. Typical electrospinning products are flat and highly interconnected scaffolds with a nonwoven fabric sheetlike morphology. These characteristics hinder cell infiltration and growth throughout the scaffolds. Blakeney et al. have developed a three-dimensional cotton ball-like electrospun scaffold that consists of low-density, uncompressed nanofibers.[131] A grounded spherical dish and an array of needle-like probes were used instead of a traditional flat-plate collector to create a cotton ball-like scaffold. Scanning electron microscopy showed that the cotton ball-like scaffold consisted of electrospun nanofibers with a similar diameter, but with larger pores and less dense structures than traditional electrospun scaffolds.[131] The cotton-ball like scaffolds prepared from ECM proteins by electrospinning will be interesting for use as scaffolds for guiding specific lineages of stem cell differentiation.

## 4. PHYSICAL PROPERTIES OF BIOPOLYMERS (BIOMATERIALS) GUIDE STEM CELL DIFFERENTIATION FATE (LINEAGE)

The interactions between MSCs and ECM proteins are classified as physical, chemical, and biological. It has recently been recognized that stem cell differentiation is directed by physical properties of culture materials as well as by biochemical responses to growth factors and ECM proteins.[19,20,132] Cells in bone, muscle, liver, and brain tissues reside in different environments that have diverse physical properties.[133] The matrix stiffness for differentiated cells is known to influence focal-adhesion structure and the cytoskeleton.[134−139] Engler et al. reported that soft materials, with similar stiffness to the brain, tend to differentiate MSCs into neurogenic cells, whereas stiffer materials that mimic muscle guide MSCs into myogenic cells and rigid materials similar to collagenous bone induce osteogenic differentiation (Figure 7).[19] However, this work was performed on a 2D surface of hydrogels coated with collagen. The effect of stiffness in 3D culture may produce different results than in 2D culture.

Gilbert et al. also reported that the elasticity of culture materials regulates self-renewal of skeletal muscle stem cells.[20]

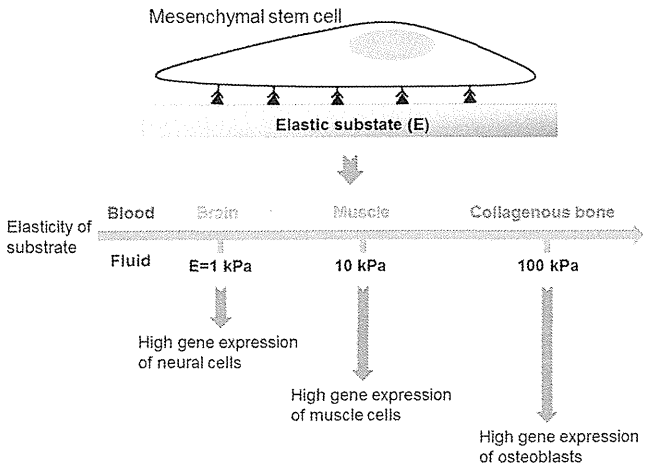


**Figure 7.** Physical properties decide the fate of stem cell cultured on biomaterials with different elasticity. Modified with permission from ref 19. Copyright 2006 Elsevier Inc.

Muscle stem cells (MuSC's) exhibit robust regenerative capacity in vivo, but this capacity is rapidly lost in culture. They showed that the elasticity of culture materials was a potent regulator of MuSC fate. MuSC's cultured on soft hydrogel substrates that mimicked the elasticity of muscle (12 kPa) self-renew in vitro and contributed extensively to muscle regeneration when transplanted into mice, unlike MuSC's grown on rigid plastic dishes (~106 kPa), as shown by histology and bioluminescence imaging. These studies provide evidence that propagation of adult muscle stem cells is possible by recapitulating physiological tissue rigidity.[20] This finding may contribute to future cell-based therapies for muscle-wasting diseases.

The effect of physical interactions between MSCs and culture materials on stem cell fate is discussed in several articles.[19,20,61,133,140−154] Some landmark findings are summarized in Table 5, and some examples of physical effects on differentiation of MSCs cultured on ECM proteins are reviewed here.

**Table 5. Some Articles Discussing Physical Effect of Substrates on Differentiation of MSCs Cultured on the Substrates**

| authors | contents | ref (year) |
|---|---|---|
| J. R. Mauney et al. | **mechanical stimulation** promotes osteogenic differentiation of hBMSCs | 140 (2004) |
| J. S. Park et al. | differential effects of **equiaxial and uniaxial strain** on MSCs | 141 (2004) |
| V. E. Meyers et al. | **microgravity** disrupts collagen I/integrin signaling during osteogenic differentiation of hMSCs | 142 (2004) |
| V. I. Sikavitsas et al. | **flow perfusion** enhances the calcified matrix deposition of marrow stromal cells in scaffolds | 143 (2005) |
| H. Hosseinkhani et al. | **perfusion culture** enhances osteogenic differentiation of MSCs | 144 (2005) |
| A. J. Engler et al. | **matrix elasticity** directs stem cell lineage specification | 19 (2006) |
| R. D. Sumanasinghe et al. | osteogenic differentiation of hMSCs in collagen matrices: effect of **uniaxial cyclic tensile strain** | 145 (2006) |
| D. F. Ward et al. | **mechanical strain** promotes osteogenic differentiation of hMSCs | 61 (2007) |
| E. K. F. Yim et al. | **nanostructures** inducing differentiation of hMSCs into neurinal lihneage | 154 (2007) |
| B. Lanfer et al. | growth and differentiation of MSCs on **aligned collagen** matrices | 146 (2009) |
| Q. Li et al. | ECM with the **rigidity** of adipose tissue helps adipocytes maintain insulin responsiveness | 147 (2009) |
| M. Zscharnack et al. | **low $O_2$** expansion improves subsequent chondrogenesis of BMSCs in hydrogel | 148 (2009) |
| C. H. Huang et al. | interactive effects of **mechanical stretching** and ECM proteins on initiating osteogenic differentiation of hMSCs | 149 (2009) |
| P. M. Gilbert et al. | substrate **elasticity** regulates skeletal muscle stem cell self-renewal in culture | 20 (2010) |
| G. C. Reilly and A. J. Engler | intrinsic ECM properties regulate stem cell differentiation (**mechanobiology**) | 150 (2010) |
| J. M. Kemppainen and S. J. Hollister | differential effects of **designed scaffold** permeability on chondrogenesis by BMSCs | 151 (2010) |
| E. K. F. Yim et al. | **nanotopography**-induced changes in focal adhesions, cytoskeletal organization, and mechanical properties of hMSCs | 152 (2010) |
| J. Tang et al. | regulation of stem cell differentiation by cell−cell contact on **micropatterned** material surfaces | 153 (2010) |
| P. A. Janmey and R. T. Miller | mechanisms of **mechanical** signaling in development and disease | 133 (2011) |


dx.doi.org/10.1021/cr3000169 | *Chem. Rev.* 2012, 112, 4507−4540

### 4.1. Mechanical Stretching Effect of Culture Surface-Coated with ECM Proteins

Mechanical strain and ECM proteins play important roles in the osteogenic differentiation of hMSCs.[61,140,145,149] Several studies have shown that mechanical strain can promote osteogenic or other lineage differentiation in cells cultured on ECM proteins even in the absence of osteogenic supplements in the culture medium.[61,145,149]

Park et al. reported that mechanical strain regulated the expression of vascular smooth muscle cell (SMC) markers in MSCs (Figure 8).[141] Cyclic equiaxial strain downregulated smooth muscle (SM) $\alpha$-actin and SM-22$\alpha$ in MSCs on collagen- or elastin-coated membranes after one day and decreased the level of $\alpha$-actin in stress fibers. In contrast, cyclic uniaxial strain transiently increased the expression of SM $\alpha$-actin and SM-22$\alpha$ after one day, which subsequently returned to basal levels after the cells aligned in the direction perpendicular to the strain.[141] In addition, uniaxial but not equiaxial strain induced a transient increase in collagen type I expression. DNA microarray experiments showed that uniaxial strain increased SMC markers and regulated the expression of matrix molecules without significantly changing the expression of differentiation markers (e.g., ALP and collagen type II) in other cell types.[141] Their results suggest that uniaxial strain, which better mimics the type of mechanical strain experienced by SMCs, could promote MSC differentiation into SMCs if cell orientation is controlled.[141]

Ward et al. showed that application of a 3−5% tensile strain to a collagen type I substrate stimulated osteogenesis in attached hMSCs through gene focusing via a MAPK signaling pathway.[61] They found that mechanical strain led to an increase in the expression of osteogenic marker genes while simultaneously reducing expression of marker genes from three alternate lineages (chondrogenic, adipogenic, and neurogenic).[61] Mechanical strain also increased matrix mineralization (a hallmark of osteogenic differentiation) and activation of extracellular signal-related kinase 1/2 (ERK).[61] These results demonstrated that mechanical strain enhanced collagen type I-induced gene focusing and osteogenic differentiation in hMSCs through the ERK/MAPK signaling pathway.[61]

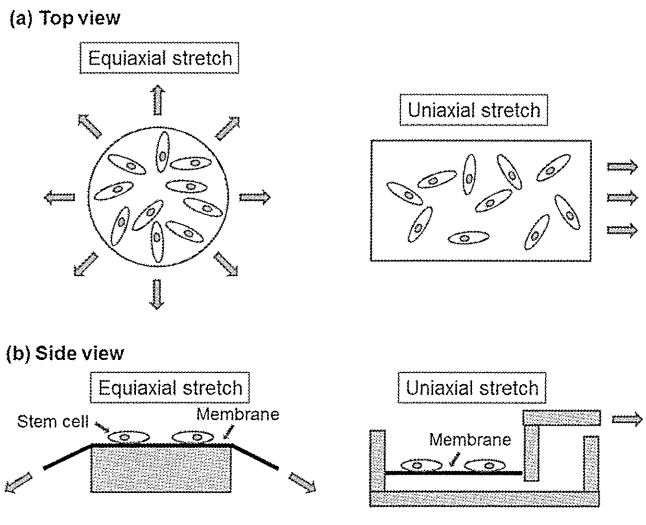


**Figure 8.** Schematic model of the apparatus that can apply equiaxial (a) and uniaxial (b) strain to MSCs. Modified with permission from ref 141. Copyright 2004 Wiley Periodicals.

Huang et al. investigated the combined effects of ECM proteins and mechanical factors (cyclic stretching) in driving hMSCs toward osteogenic differentiation.[149] hMSCs cultured in regular medium were grown on substrates coated with various ECM proteins (collagen type I, vitronectin, fibronectin, and laminin) and subjected to cyclic mechanical stretching.[149] All of the ECM proteins tested supported hMSC differentiation into osteogenic phenotypes in the absence of osteogenic supplements.[149] Cyclic mechanical stretching activated the phosphorylation of focal adhesion kinase (FAK), induced upregulation of the transcription and phosphorylation of Runx2, and subsequently increased ALP activity and mineralized matrix deposition.[149] Fibronectin and laminin exhibited greater effects of supporting stretching-induced osteogenic differentiation than did collagen type I and vitronectin.[149] It was suggested that the ability of ECM proteins and mechanical stretching to regulate osteogenesis in hMSCs may be exploited in bone tissue engineering by appropriate matrix design and by mechanical stimulation.[149]

### 4.2. Low Oxygen Expansion Promotes Differentiation of MSCs

Several groups have reported the effects of low oxygen tension on the differentiation of MSCs, especially in chondrogenic differentiation of MSCs cultured on ECM substrates.[148,155] Zscharnack et al. investigated the effect of low oxygen tension (5%) during the expansion of ovine MSCs on colony-forming unit-fibroblast (CFU-F) formation and chondrogenesis in pellet culture and in collagen type I hydrogels.[148] MSCs expanded in 5% $O_2$ showed a 2-fold higher CFU-F potential, and chondrogenic differentiation was enhanced in both pellet culture and collagen type I hydrogels. It was demonstrated that physiologically low oxygen tension during monolayer expansion of ovine MSCs was advantageous to improving cartilage tissue engineering in a sheep model.[148]

### 4.3. Other Physical Effect Affecting Differentiation of MSCs

There are several other physical effects that promote differentiation of MSCs on ECM protein surfaces. (i) Perfusion culture promotes osteogenic differentiation of MSCs cultured on ECM protein surface.[143,144] (ii) Microgravity disrupts collagen type I/integrin signaling during osteoblastic differentiation of hMSCs.[142] (iii) The mechanical properties of ECM proteins guide specific lineage differentiation of MSCs.[147,150,156,157] (iv) The topography of ECM proteins promotes differentiation of MSCs cultured on aligned or patterned substrates.[74,146,151−154,158]

## 5. MSC CULTURE ON ECM PROTEINS AND NATURAL BIOPOLYMERS

The ECM is the extracellular component of animal tissues that provides structural support for the cells, in addition to stimulating various important biological functions. ECM proteins are able to dictate whether cells will proliferate or undergo growth arrest, migrate or remain stationary, and thrive or undergo apoptotic death.[159] Therefore, the ECM proteins are an important factor in reproducing the biological niches of cells in vitro, which guides MSCs to differentiate into different lineages such as osteoblasts, chondrocytes, adipocytes, cardiomyocytes, neural cells, hepatocytes, and $\beta$-cells. The differentiation of MSCs in culture systems depends on the components, structure (morphology), origin, and quantity of ECM proteins that are used. Because ECM proteins are used as scaffolds for the organization of cells in tissues, ECM proteins