Origin of mesenchymal stem cell

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Differentiation pathways of in vitro ES cell culture

- Adipocyte
- Osteocyte
- Chondrocyte
- Mesenchymal Stem Cell
- Paraxial Mesoderm
- Lateral Mesoderm
- Blood cell
- Endothelial cell
- Hepatocyte
- Gut cell
- Osteocyte

What is Mesenchymal stem cell (MSC) ?

Definition

- Fibroblastic morphology
- Self-renewal
- Adipocytes
- Chondrocytes
- Osteocytes

Marshak, D et al, Science, 1999
Problems of MSCs Research

Utilities
Multipotency for differentiation
Prevention from GVHD in transplantation
Support for regeneration of cardiac muscles

Problems
Developmental pathway is unclear
Specific surface markers are undefined
Transplantation methods are not established

ES cell study are useful for understanding what MSC is

Aims: To identify MSC progenitors and to define MSC differentiation pathway
Visualization of cell lineages by surface marker staining

Paraxial mesoderm marker

PDGFRα

Lateral mesoderm marker

VEGFR2

In vitro ES cell differentiation

Mesoderm-like cells

Paraxial mesoderm marker (PDGFRα)

Lateral mesoderm marker (VEGFR2)
**PDGFRα and VEGFR2 expressions during ES cell differentiation**

<table>
<thead>
<tr>
<th>Day 3</th>
<th>Day 3.5</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Paraxial mesoderm marker, PDGFRα

Lateral Mesoderm marker, VEGFR2

1 day culture

Double positive cells can give rise to single positive cells
PDGFRα single positive cells express paraxial mesoderm markers

Monolayer culture

Collagen IV coated mES cell

PDGFRα and VEGFR2 staining

FACS

PDGFRα+ VEGFR2+

Analyses

PDGFRα single positive cells express paraxial mesoderm markers

Relative value

qPCR

Paraxial mesoderm Markers

Lateral mesoderm Markers

DP: Double positive

PSP: PDGFRα single positive

VSP: VEGFR2 single positive

VEGFR2 PDGFRα Tbx6 Fst Mesp2 GATA2 VECD SCL
Two types of mesoderm-like cell in ES cell culture

PSP: PDGFR\(\alpha\) single positive

- Bone cells
  - Alizalin red staining for osteocytes

- Cartilage cells
  - Alcian Blue staining for Chondrocytes

- Bone cell markers
  - Bglap1, Bglap2

- Cartilage cell markers
  - Col2a1, Col10a1

VSP: VEGFR2 single positive

- Vascular endothelial cell colonies
  - (VE-Cadherin staining)

- Blood cells
  - Erythroblast, Erythrocyte

- Fetal globin expression
  - \(\beta\)H1 globin expression

- Cartilage cell markers (Q-PCR)
  - Col2a1, Col10a1

- Bone cell markers (Q-PCR)
  - Bglap1, Bglap2

- Vascular endothelial cell colonies (Q-PCR)
  - 

- Fetal globin expression (Q-PCR)
  - \(\beta\)H1 globin expression

- PDGFR\(\alpha\)+, VEGFR2- (PSP)
Retinoic acid (RA) treatment at early phase enhances adipogenesis

Mesoderm-like cell development

Day 0

Monolayer Culture

Day 5

ES cells

Day 9

+ Insulin, dexamethasone, IBMX and troglitazone

Day 18

Adipogenesis

Oil red O staining

Dani C. and Austin S. et al, Development, 1997

+ RA

On day 2 and 3
RA treatment induces PDGFR$\alpha^+$ mesenchymal cells

No RA treatment

<table>
<thead>
<tr>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>40.1%</td>
<td>20.4%</td>
<td>21.9%</td>
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</tbody>
</table>

Mesodermal cells

Mesoderm Markers
- Brachyury
- Mesp2
- Mesogenin

Mesenchymal Markers
- OB-CAD
- Vimentin
- PDGFR$\beta$
- $\beta$ Actin

RA treatment

<table>
<thead>
<tr>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.47%</td>
<td>15.5%</td>
<td>56.9%</td>
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</table>

Mesenchymal cells

Mesoderm and Mesenchymal Marker, PDGFR$\alpha$
**ES cell-derived PDGFRα⁺ Mesenchymal stem cells**

**Cell Growth**

- **PDGFRα⁺ mesenchymal cells**
- **PDGFRα⁺ mesoderm cells**

<table>
<thead>
<tr>
<th>Passage</th>
<th>Cell number</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>10⁴</td>
</tr>
<tr>
<td>10</td>
<td>10⁸</td>
</tr>
<tr>
<td>20</td>
<td>10¹⁴</td>
</tr>
<tr>
<td>30</td>
<td>10¹⁹</td>
</tr>
</tbody>
</table>

- **No RA**
- **RA**

**Cloning**

- **Fibroblastic morphology**
- **Adipocytes**
  - Oil red O staining
- **Osteocytes**
  - Alizarin red staining
- **Chondrocytes**
  - Alcian blue staining

**Markers**

- PPARγ
- Adiponectin
- Osteopontin
- Osteocalcin
- Col2a1
- Sox9
What kinds of cells are progenitors for MSCs?

ES cells → ?? → PDGFRα⁺ → adipocytes, chondrocytes, osteocytes

Mesenchymal stem cells
## RA treatment induces neuro-epithelial cells

<table>
<thead>
<tr>
<th>Condition A</th>
<th>Condition B</th>
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</thead>
<tbody>
<tr>
<td>-RA</td>
<td>+RA</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Day</th>
<th>Condition A</th>
<th>Condition B</th>
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</thead>
<tbody>
<tr>
<td>3</td>
<td>-RA</td>
<td>+RA</td>
</tr>
<tr>
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<td></td>
<td></td>
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<tr>
<td>5</td>
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</table>

### Mesoderm
- **Brachyury**
- **Mesogenin**

### Endoderm
- **Fxda2**
- **GATA4**

### Ectoderm
- **Otx2**
- **Sox1**
- **Neurogenin1**
- **β-actin**

RT-PCR
Visualization of neuroepithelium as Sox1-GFP+ cells

Sox1 Expression


Sox1-GFP Knock-in ES cells

J. Aubert et al. PNAS, 2003
Neuro-epithelium is a origin of ES cell-derived MSC

With RA
Day 4
Sox1-GFP

30 4.5
6 days after purification

17

Adipogenesis

Sox1+ Purification
PDGFRα

Sox1-GFP
Mesenchyme marker

Neuroepithelium marker

PDGFRα
Differentiation pathway from ES cell to MSC

ES cells → Sox1+ PDGFRα- → Sox1- PDGFRα+ → adipocytes

+RA → Neuro-epithelial cells → Mesenchymal stem cells → chondrocytes osteocytes
Tetraploid chimera

Only ES cells can contribute to embryo.

4N tetraploid embryo

2N ES cell carrying GFP marker

Sox1-GFP
Tetraploid chimera

E9.5

Sox1 Nestin TO-PRO

Review by Tam, PP. and Rossant, J. Development, 2003
Visualization of Sox1+ cells in mouse embryos

Neuro-epithelium is the earliest origin of MSCs.
Tracing of Sox1\(^+\) neuro-epithelial and P0\(^+\) neural crest cells

**Sox1-Cre Knock-in mouse**

Cre gene is specifically expressed in Sox1\(^+\) neuro-epithelium cells

**P0-Cre transgenic mouse**

Cre gene is specifically expressed in P0\(^+\) neural crest cells

**Rosa26-STOP-YFP mouse**

YFP marker are constitutively expressed during mouse development
YFP expression in Sox1-Cre and P0-Cre YFP mice

Sox1-Cre

P0-Cre
YFP expression in Sox1-Cre and P0-Cre embryos

Sox1-Cre
(Neuroepithelium)

P0-Cre
(Neural crest)

E14.5 embryos

FACS Analysis

PDGFRα

YFP

21 1

30

13 9

5

21 1
Neuroepithelium-derived MSCs in mouse embryos

Colonies of MSCs, Colony Forming Unit-Fibroblast (CFU-F)

Sox1-Cre (neuroepithelium-derived)

<table>
<thead>
<tr>
<th>Sox1/YFP</th>
<th>PDGFRα</th>
<th>Colony Formation</th>
</tr>
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<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
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P0-Cre (Neural crest-derived)

<table>
<thead>
<tr>
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<tr>
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Neuroepithelium- and other tissue-derived PDGFRα+ MSCs are present in E14.5 mouse embryos
E14.5 Sox1⁺-derived PDGFRα⁺ cells shows a multipotency and a sustained growth in vitro
New differentiation pathway of MSCs

Sox1+ PDGFRα- → Neuro-epithelium

Sox1- PDGFRα+ → Neural crest → P0+ → MSCs

→ Neural and pigment cells

Takashima, Era and Nishikawa, Cell, 2007
MSCs activity of neonate Sox1\(^+\)-derived PDGFR\(\alpha^+\) cells

FACS pattern of Femur bone (from P0 to P7)

<table>
<thead>
<tr>
<th>CFU-F assay</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>YFP(^+) PDGFR(\alpha^+)</td>
<td>YFP(^-) PDGFR(\alpha^+)</td>
<td>YFP(^-) PDGFR(\alpha^-)</td>
<td>YFP(^+) PDGFR(\alpha^-)</td>
<td></td>
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Differentiation activity of clonal cell lines

Fibroblastic Morphology

 Passage 18

Adipogenesis

Osteogenesis

Chondrogenesis
NE-derived MSC are reduced after birth

Proportion of PDGFRα⁺ population

%  0.15

4-week old
Neonates

P<0.01

YFP⁺ PDGFRα⁺
YFP⁺ PDGFRα⁻
Two developmental waves of MSCs

Neuro-epithelium supplies the earliest and transient wave of MSCs

MSC activity

Embryo

After Birth

Development

Neuroepithelium origin

Unknown origin
Mesoderm?
Acknowledgements

Department of Cell Modulation
Institute of Molecular Embryology and Genetics (IMEG)
Kumamoto University

Genta Tsuzuki

RIKEN CDB
Stem Cell Biology Group
Yasuhiro Takashima
Shin-Ichi Nishikawa

Animal Resources and Genetic Engineering
Kazuki Nakao

Cambridge University
Austin Smith