3. DIFFERENTIATION POTENTIAL OF SMALL CELLS

3.1 Introduction

3.1.1 Differentiation potential of stem cells

One of definitions of stem cells is multidifferentiation potential. Also the degree of their stemness is determined by their differentiation potential. According to results of section 2, sphere forming cells expressed pluripotent cell markers. Therefore in this section, we aimed to confirm their differentiation potential *in vivo* and *in vitro*.

3.2 Experimental

3.2.1 *In Vitro* Differentiation Assays.

In vitro differentiation assays were examined following the published differentiation culture conditions for murin ES cells.

Mesoderm lineage differentiation assay. Dissociated muscle cells were stained with anti-αsmooth muscle actin antibody, anti-Myosin antibody and anti-Desmin antibody. Chondrocytes were stained with Safranin-O and Fast Green. Osteocytes

were stained with ALIZARIN RED S. After 21 days, adipocytes were stained with Oil Red O.

Ectoderm lineage (Neural lineage) differentiation assay. Cells were plated on ortinin-coated chamber slides and incubated with anti-βIII Tubuin mouse monoclonal, anti-O4 mouse monoclonal antibody and anti-GFAP mouse monoclonal antibody.

Endoderm lineage (Hepatic) differentiation assay. Differentiated cells were detected by immunohistochemistory using anti-α-fetoprotein mouse monoclonal antibody, anti-Albumin goat polyclonal antibody and anti-Cytokeratin 18 mouse monoclonal antibody. Results from immunohistochemistry were confirmed by RT-PCR.

3.2.2 *In Vivo* Differentiation.

Spheres were seeded onto biodegradable scaffolds and implanted into subcutaneous of NOD/SCID mice (Charles River laboratories). After 6 weeks, the implants were harvested and fixed with 10% formaldehyde, then examined by immunocytochemistry.

3.3Results

3.3.1 Differentiation potential of cells *in vitro*

When representative bone marrow derived spheres were dissociated into single cells and exposed to three different differentiation media, the cells differentiated to express specific genes of the three lineages, Map2 (ectoderm), MyoD (mesoderm) and alpha-fetoprotein (AFP, endoderm) (Fig. 10). The addition of a neural differentiation medium to the *in vitro* environment of cells from bone marrow spheres, resulted in expression of βIII tubulin (a marker for neuron) (Fig. 11). Alternatively, the addition of 20% fetal calf serum to the media resulted in the expression of markers representative of mesoderm; that is, α-smooth muscle actin (Fig. 11) as well as the mesenchymal cells, chondrocytes, osteocytes and adipocytes (Fig. 12). Thus, cells from spheres differentiated into all cell types of neural (neurons, oligodendrocytes and glias) and mesenchymal stem cell lineage (chondrocytes, osteocytes and adipocytes). When exposed to a hepatocyte differentiation media the expression of α-fetoprotein (Fig. 11), was seen, suggestive of differentiation into endodermal tissue.

3.3.2 Differentiation potential *in vivo.*

Bone marrow spheres and ES cells were transplanted subcutaneously into immune deficient mice to examine their tumor-initiating capacity. As a result, after 6 weeks ES cells formed a tumor. Spheres did not form tumor as big as ES cells did. We concluded that the proliferative potential of sphere cells was much weaker than that of ES cells (Fig. 13).

Next, we investigated if transplanted cells differentiated in vivo after transplantation. Transplanted cells were harvested after 6 weeks, and processed for

immunohistochemical analyses. According to results of immunohistochemical analyses, spheres differentiated into tissues derived from three germ layers *in vivo* (Fig. 14).

3.4 Summary of section 3

- Spheres differentiated into cells derived from all three germ layers *in vivo* and *in vitro*

3.5 Discussion

Spheres differentiated into cells derived from three germ layers in vitro. It is yet answered that it was either differentiation or trans-differentiation. Also it is hard to refer the difference from in vitro differentiation potential of mesenchymal stem cells. However, at least sphere forming cells enabled to generate various mature cells. In addition, in vivo differentiation assay proved that sphere forming cells were indeed stem cells, but distinct from ES cells in proriferative potential. The relationship between proliferative potential and differentiation potential has yet been understood. Spheres in this study showed differentiation potential which fulfill the critereia for both mesenchymal stem cells and neural stem cells. We believe that the spheres studied contain precursor cells to both mesenchymal and neural stem cells lineages.

It is important to note that cells described above, were propagated as non-adherent spheres, and are not known to exist *in vivo*. The *in vitro* behavior of cells contained in the spheres is likely to be very different from cells that reside *in vivo*. How these stem cells harbor in adult body and how they exert their potential

The spheres generated seemed to be composed of heterogenous populations of cells, with some markers expressed in some spheres, and other markers expressed in different spheres generated from cells isolated from the same tissue, at the same time. We believe that these differences also may be a function of the environment in which the cells were maintained.

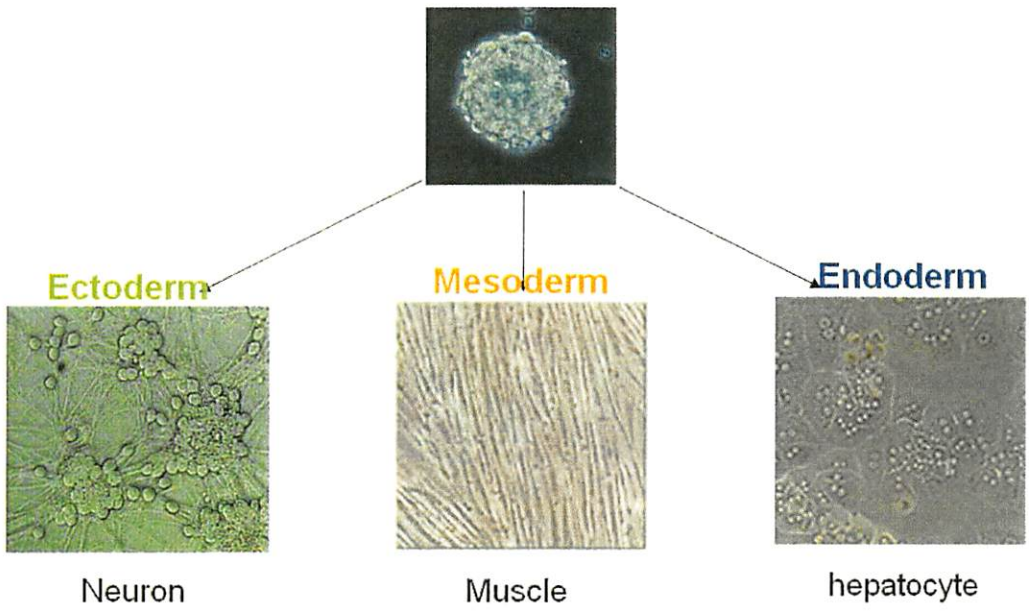

Figure 10 *in vitro* differentiation of bone marrow spheres

After 6 weeks of culture, cells change their figurations into those of cells representative of three germ layers.

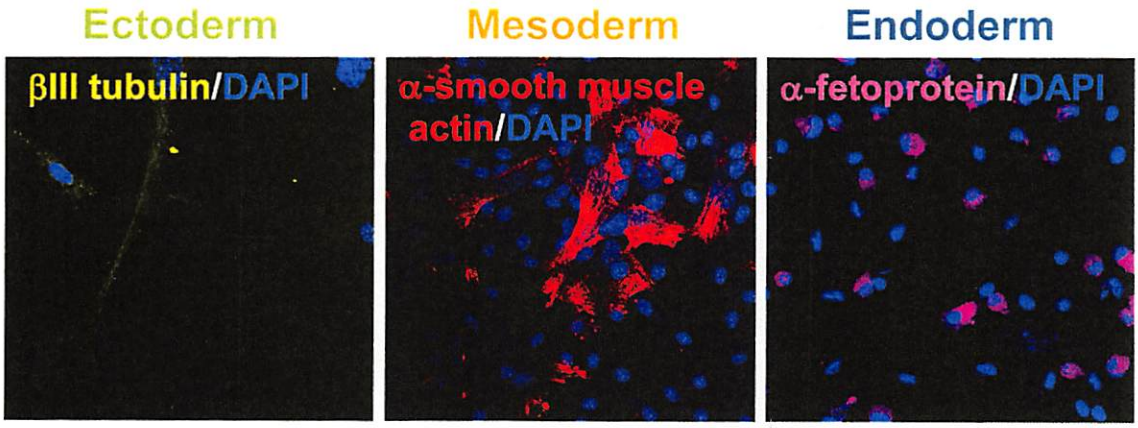

Figure 11 *In vitro* differentiation assay of cells from 3 germ layers.
Marrowspheres were dissociated and plated in each appropriate medium. Cells from spheres, differentiated into cells representative of the three germ layers. Neural cells (left), muscle cells (middle) cells, hepatocytes (right). Neurons stained with βIII tubuline (left),. Muscle cells stained with α-smooth muscle actin (middle). Hepatocytes were stained with α-fetoprotein (right).

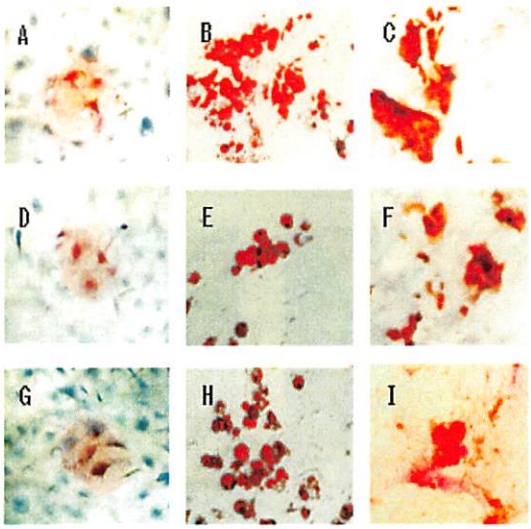

Figure 12 Mesenchymal lineage differentiation.

Dissociated spheres were plated into serum-containing medium and cultured for 14-21 days. Plated cells differentiated into mesenchymal lineage cells even plated cells were from spheres derived from endoderm or ectoderm tissues.

Marrow spheres differentiated into condrocytes (A), adipocytes (B) and osteocytes (C). Pnemospheres differentiated into condrocytes (D), adipocytes (E) and osteocytes (F). Spinalspheres differentiated into condrocytes (G) and adipocytes (H).

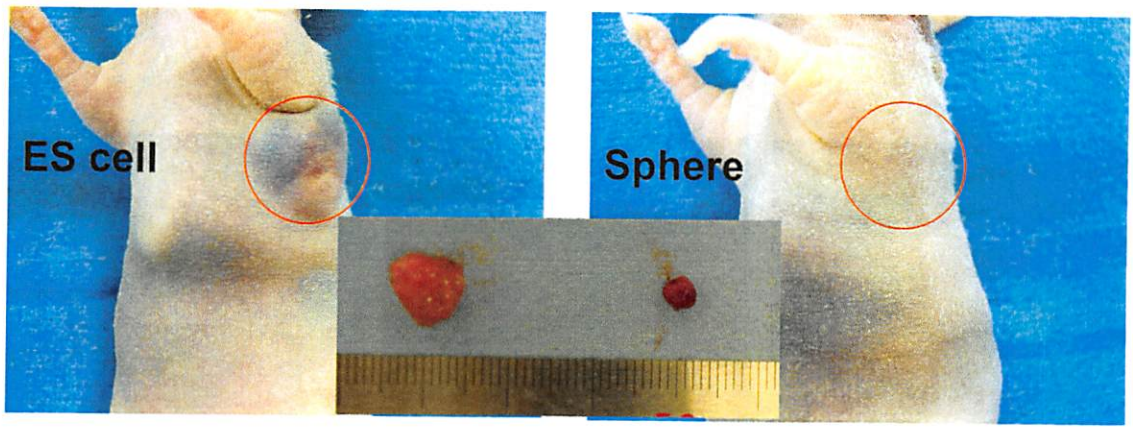

Figure 13 Teratoma forming assay

10^7 bone marrow cells and ES cells were injected subcutaneously into immunedificient mice. After 6 weeks of implantation, cell masses were harvested.

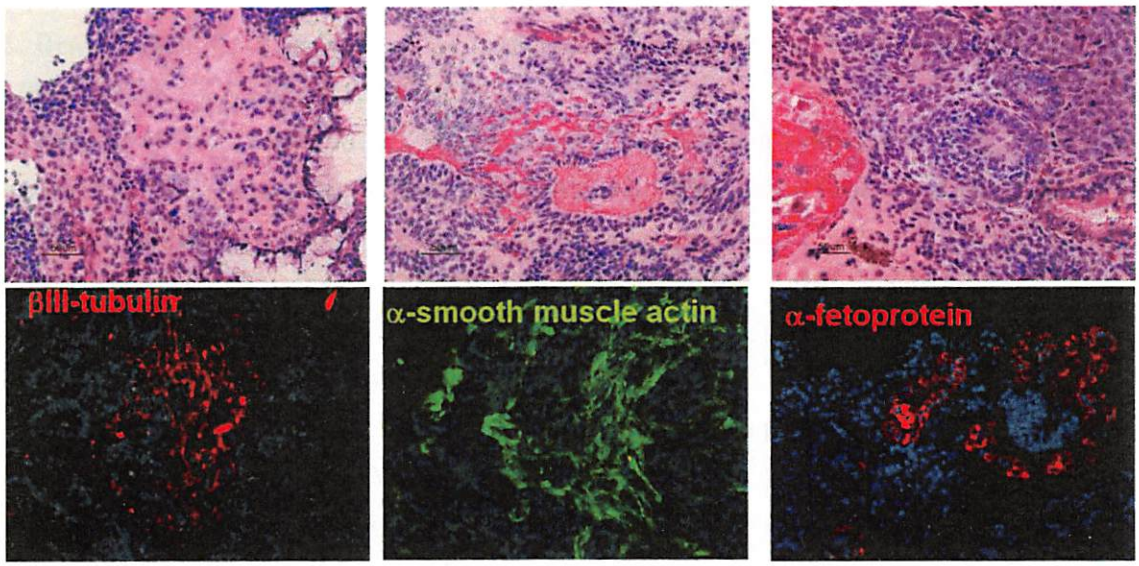

Figure 14 Teratoma like mass from bone marrow spheres contained nerve expressing betaIII-tubuline (left)(ectoderm), muscle expressing desmin (middle)(mesoderm) and duct like structure expressing AFP (right)(endoderm).

3.6 References

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