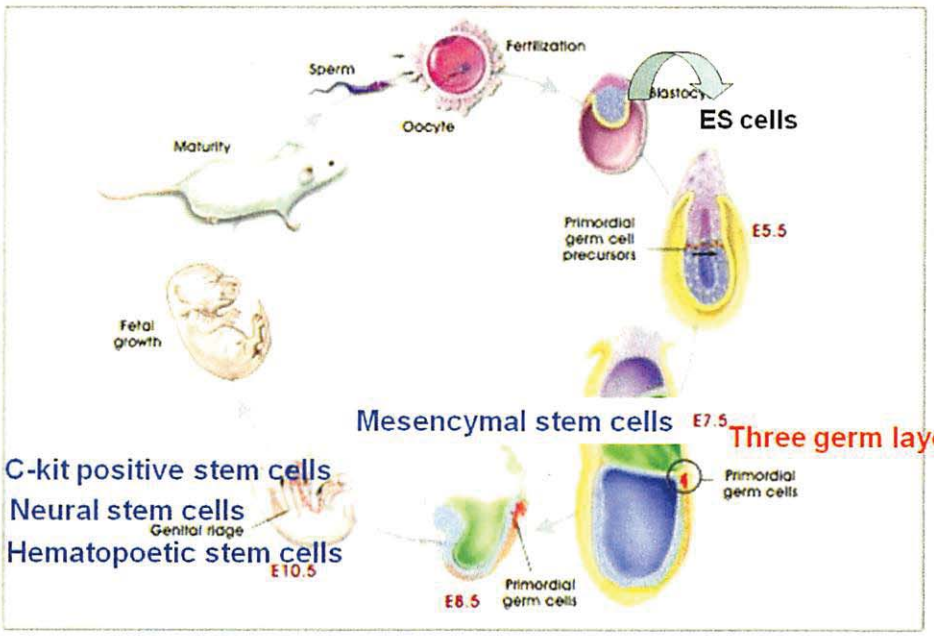


Figure 1 Development and adult stem cells

Adult stem cells are found after germ-layer differentiation in development. Therefore, adult stem cells cannot differentiate into cells crossing germ layers. Whereas, how do adult stem cells evolve during development and how are they maintained in the adult? Are they "leftover" embryonic stem cells, or do they arise in some other way? These questions are yet solved until today.

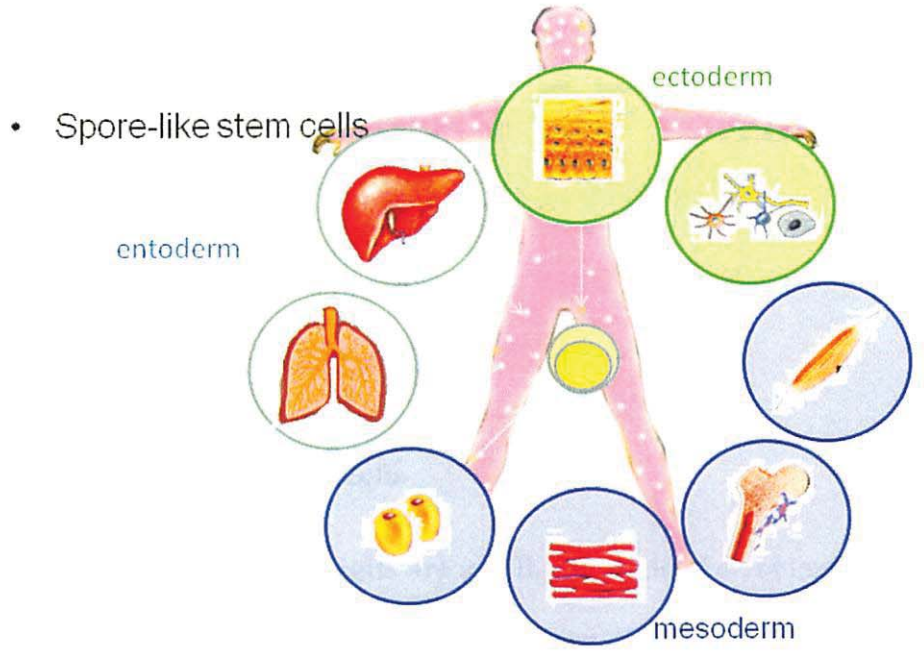
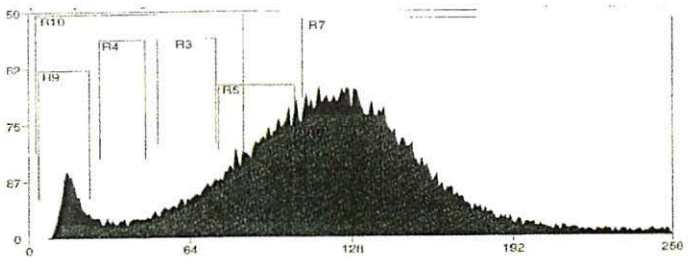


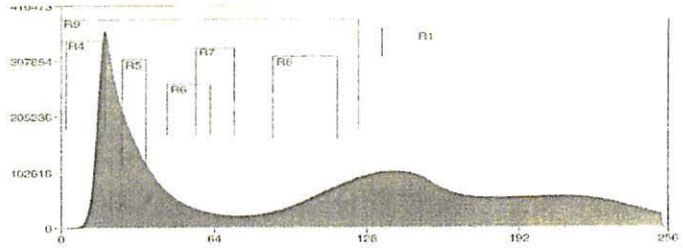
Figure 2 Hypothesis of this study.

Very small stem cells (spore-like stem cells) which possess the same potential exist in all tissues consisting adult body.

(A)



(B)



(C)

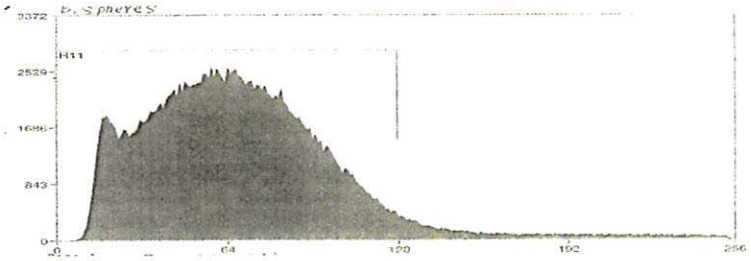


Figure 3 Effect of trituration

After trituration, the population of small cells increased. Also, after culture, over 80% of survived cells in spheres was less than 8 micro meters in diameter. (A) Cell size population range of native bone marrow. (B) Cell size population range of triturated bone marrow. (C) Cell size population range of cells in spheres. Vertical axis indicates cell number. Horizontal axis indicates Forward scatter.

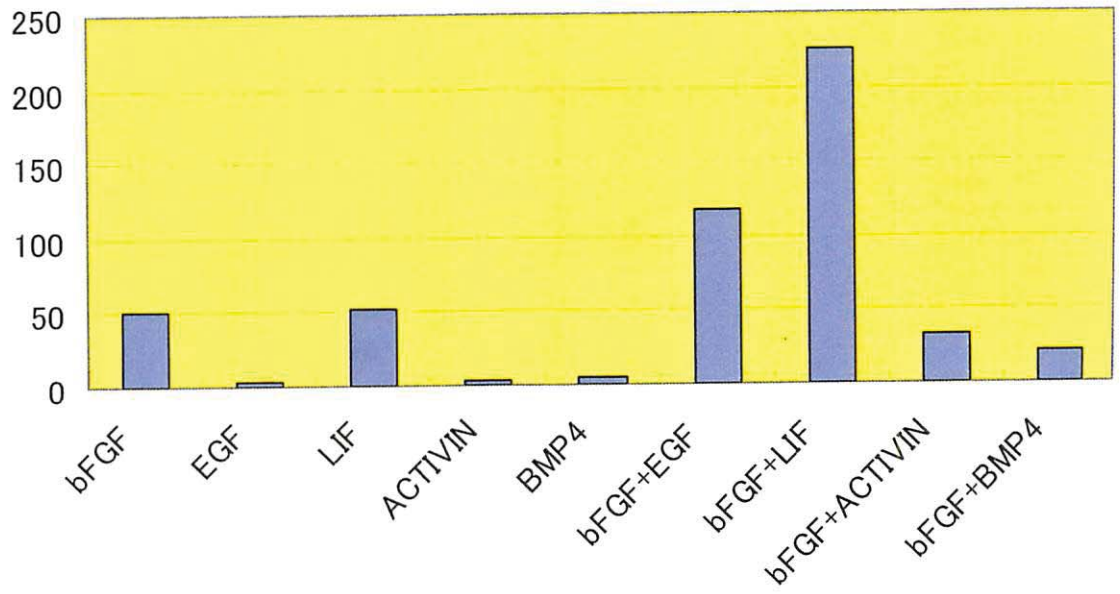


Figure 4 Culture conditions of small cells

Growth factors were chosen according to the culture conditions for various pluripotent stem cells and adult stem cells. Combination of bFGF and EGF is used for neural stem cels. Combination of bFGF and LIF is used for primitive neural stem cells. Combination of bFGF and ACTIVIN is used for epi stem cells. Combination of bFGF and BMP4 is used for human ES cells.

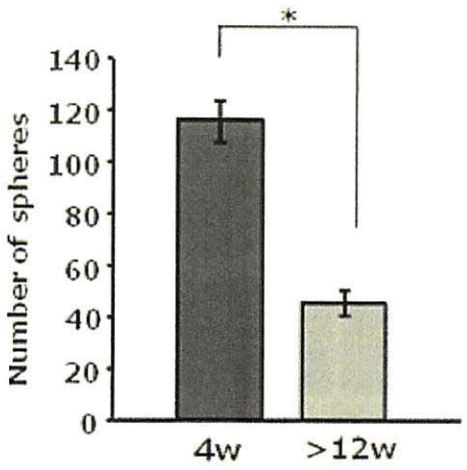


Figure 5 The ability of marrow derived cells to form primary spheres.

The number of spheres formed from 10^7 bone marrow cells derived from 4weeks old mice compared to mice >12 weeks old of age. 116 ± 5 spheres arose from 4weeks old mice and 45 ± 3 spheres arose from mice over 12 weeks of age (mean percentage ±SD, N=3, *$p<0.05$).

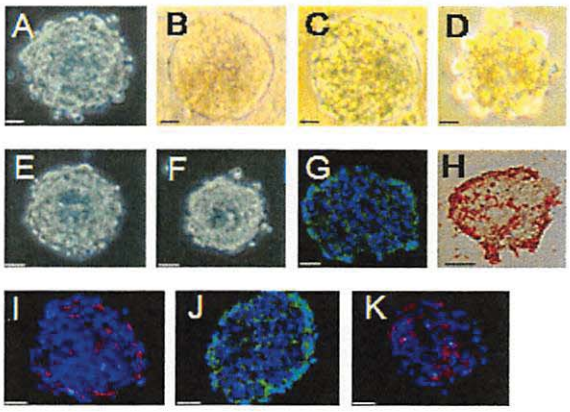


Figure 6 Spheres generation from Adult Bone marrow (Scale Bar A-H 20μm).

Primary spheres formed from non-adherent bone marrow cells at Day 5 (A). Sphere formation from cells representative of different adult tissue types. Muscle, lung and spinal cord were harvested with trituration and cultured in the maintain media. Spheres from all type of tissues arose within 5days. Spinalspheres (SS) (B), myospheres (MS) (C) and pneumospheres (PS) (D) at Day5. Secondary spheres formed from cells dissociated from primary marrowspheres (E) and tertiary spheres formed from cells dissociated from secondary marrowspheres (F). SSEA-1 expression is shown in green (G), Alkaline phosphatase expression is shown in red (H), C-kit expression is shown in red (I), Sca-1 expression is shown in green (J) and E-cadherin expression is shown in red (K) in primary bone marrow spheres.

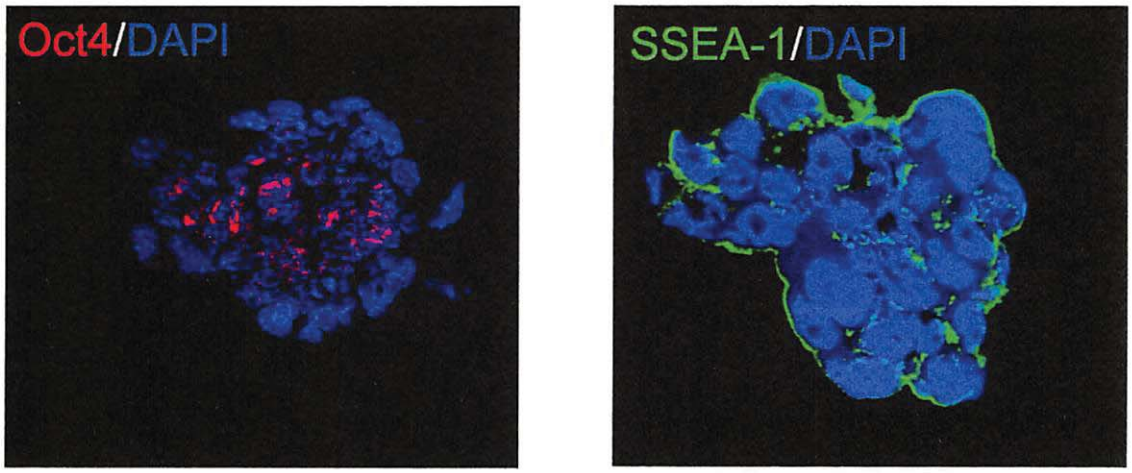


Figure 7 Pluripotent marker expressions

Spheres at day 5 expressed pluripotent cell markers Oct4 and SSEA-1.

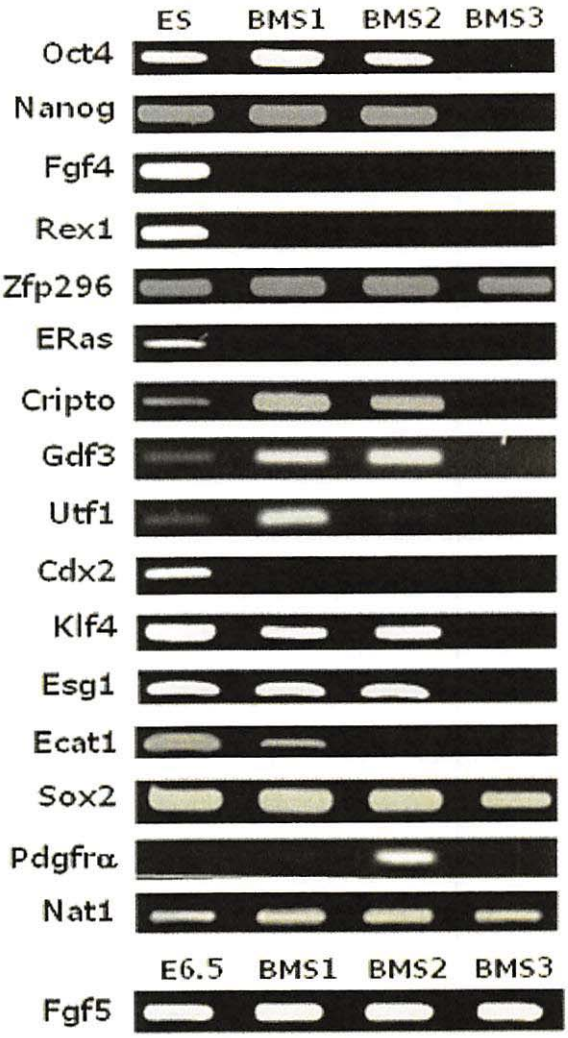
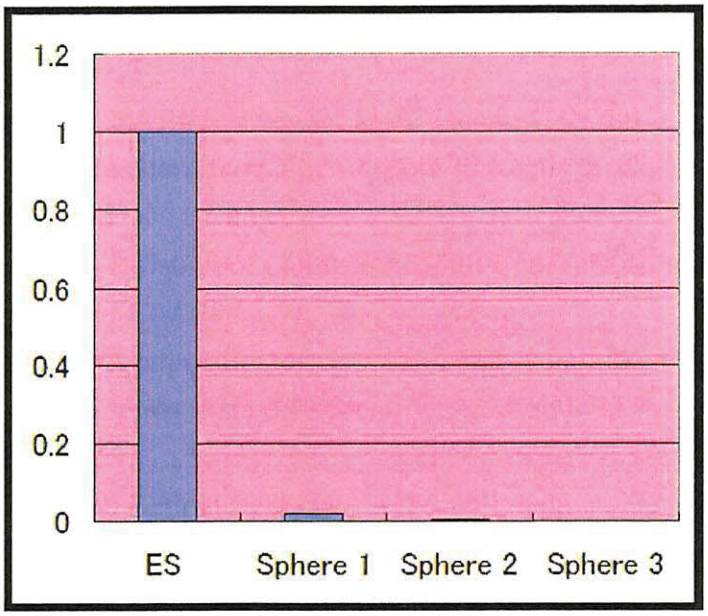


Figure 8 Gene expression profiles of cell contained in bone marrow spheres.
Gene expressions of each bone marrow sphere were analyzed by RT-PCR. Gene expressions of cells in each bone marrow sphere were compared to those of embryonic stem cells.



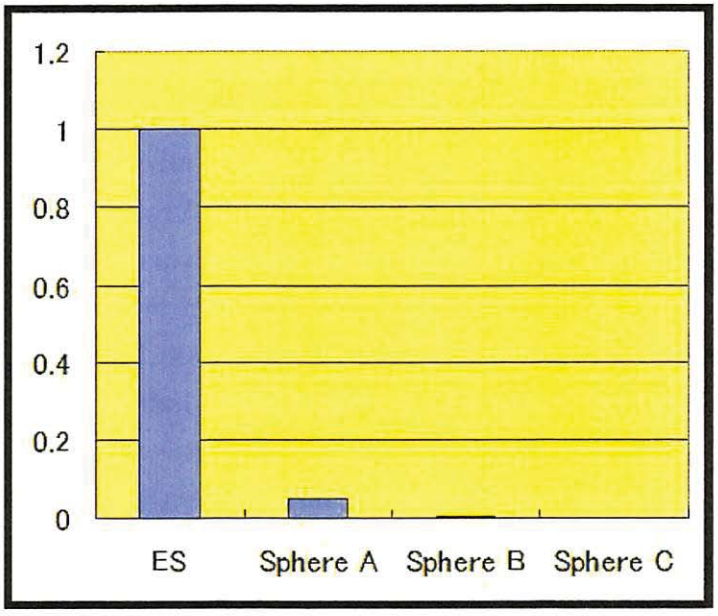


Figure 9 Quantitative PCR for Oct4 and Nanog

Spheres expressed 1/20-1/2000 of Oct4 gene of ES cells (upper). Also, spheres expressed 1/50-1/2000 of Nanog gene of ES cells (bottom).

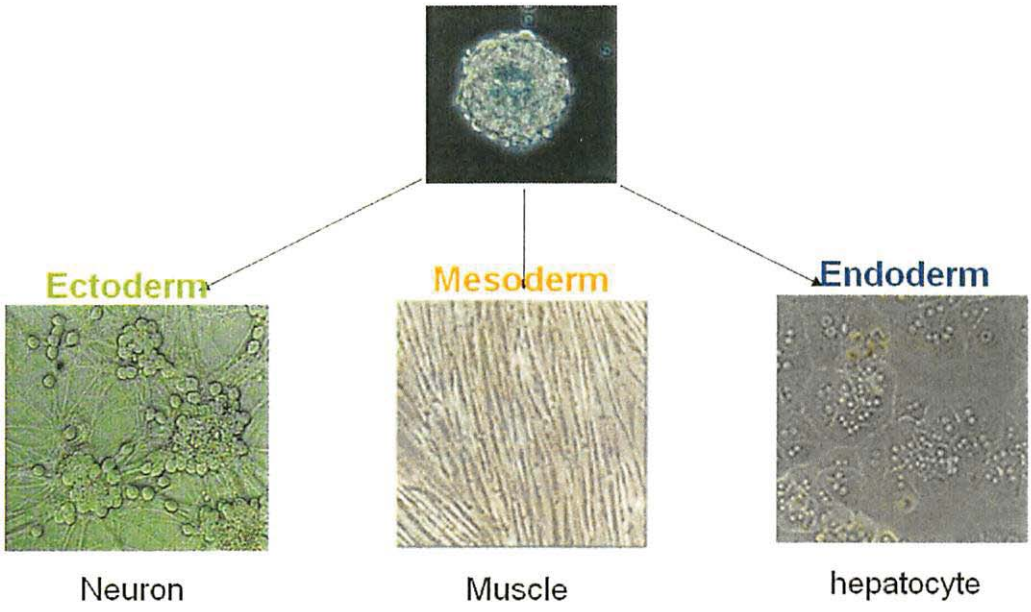


Figure 10 *in vitro* differentiation of bone marrow spheres

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

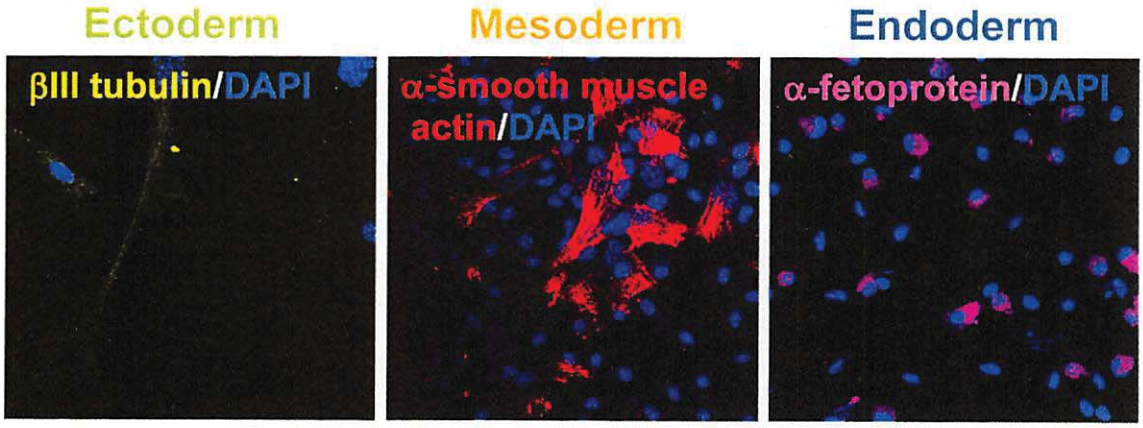


Figure 11 *In vitro* differentiation assay of cells from 3 germ layers.
Marrowspheres were dissociated and plated in each appropriate medium. Cells from spheres, differentiated into cells representative of the three germ layers. Neural cells (left), muscle cells (middle) cells, hepatocytes (right). Neurons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

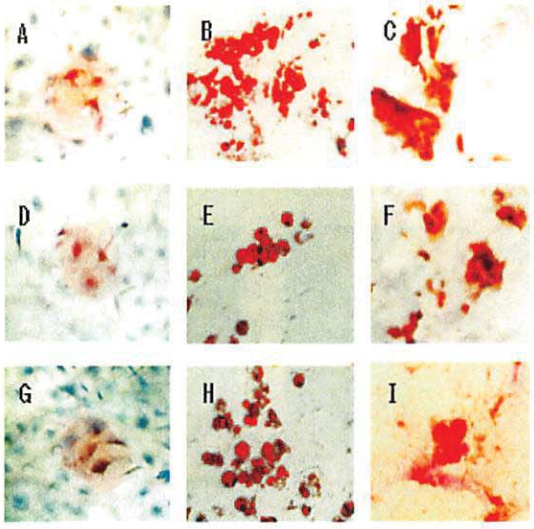


Figure 12 Mesenchymal lineage differentiation.

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).

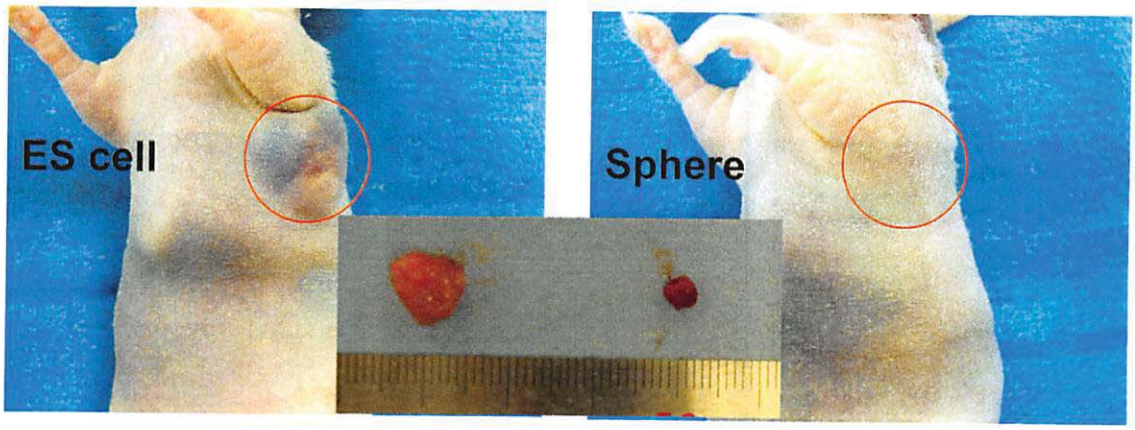


Figure 13 Teratoma forming assay

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

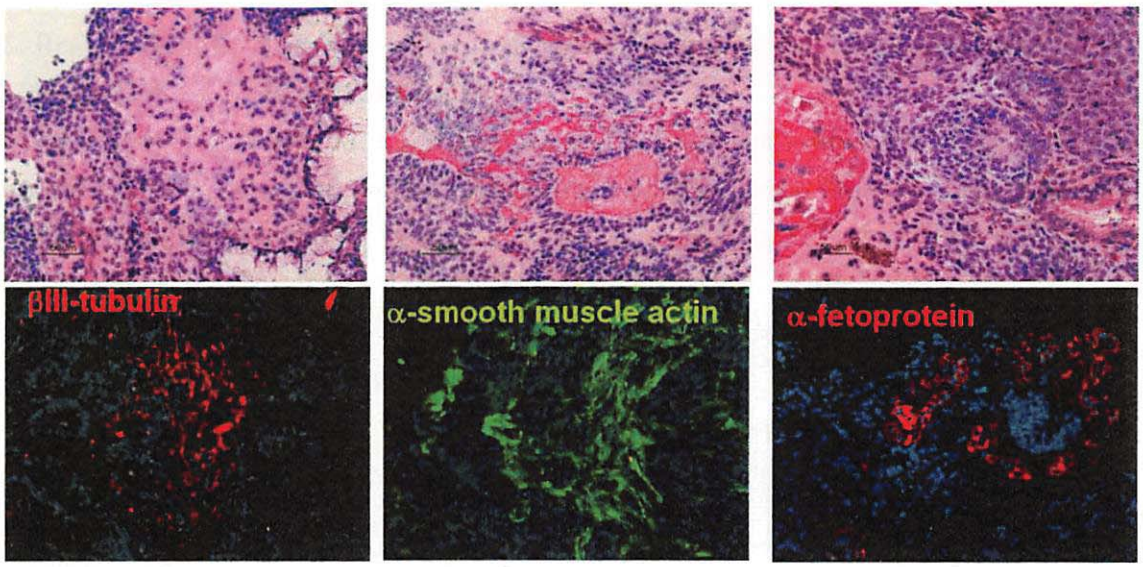


Figure 14 Teratoma like mass from bone marrow spheres contained nerve expressing betaIII-tubuline (left)(ectoderm), muscle expressing desmin (middle)(mesoderm) and duct like structure expressing AFP (right)(endoderm).

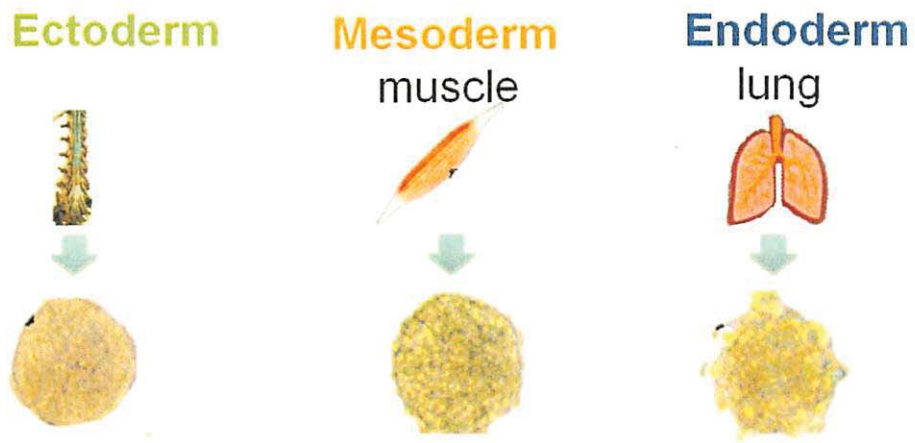


Figure 15 Sphere formation from cells representative of different adult tissue types.

Muscle, lung and spinal cord were harvested with trituration and cultured in the maintain media. Spheres from all type of tissues arose within 5days. Spinalspheres (SS) (B), myospheres (MS) (C) and pneumospheres (PS) (D) at Day5.

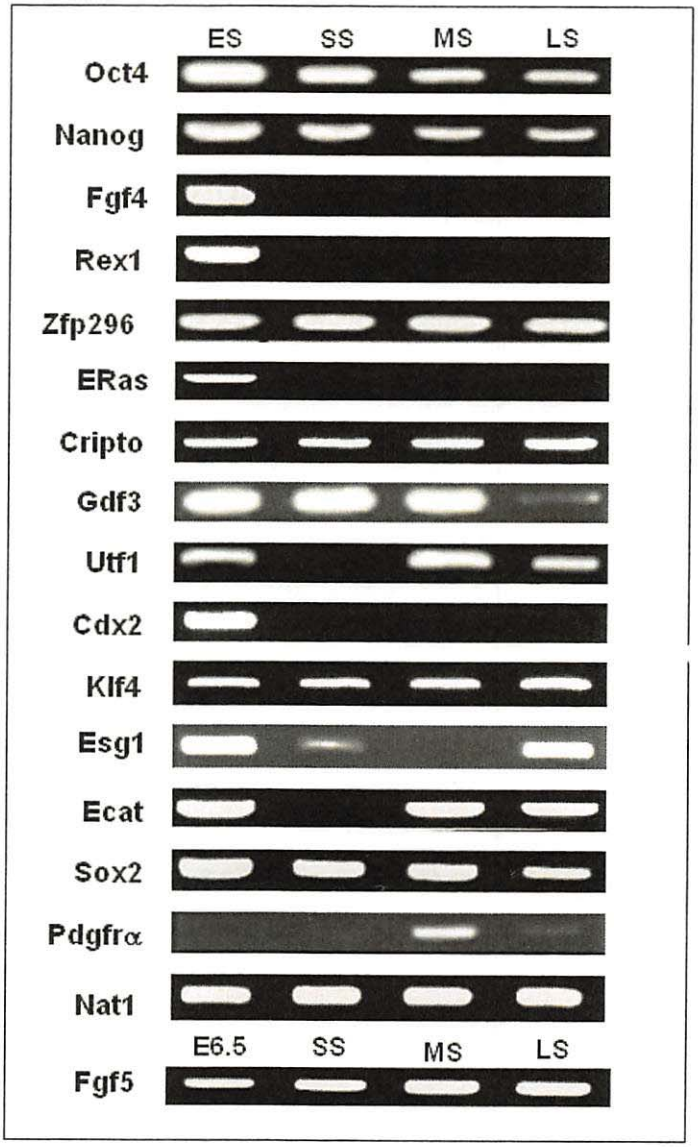


Figure 16 Gene expression profiles of oct4-expressing cells in spheres from each tissue. Spinalspheres (SS), myospheres (MS) and pneumospheres (PS) at Day5 were compared to those of embryonic stem cells.

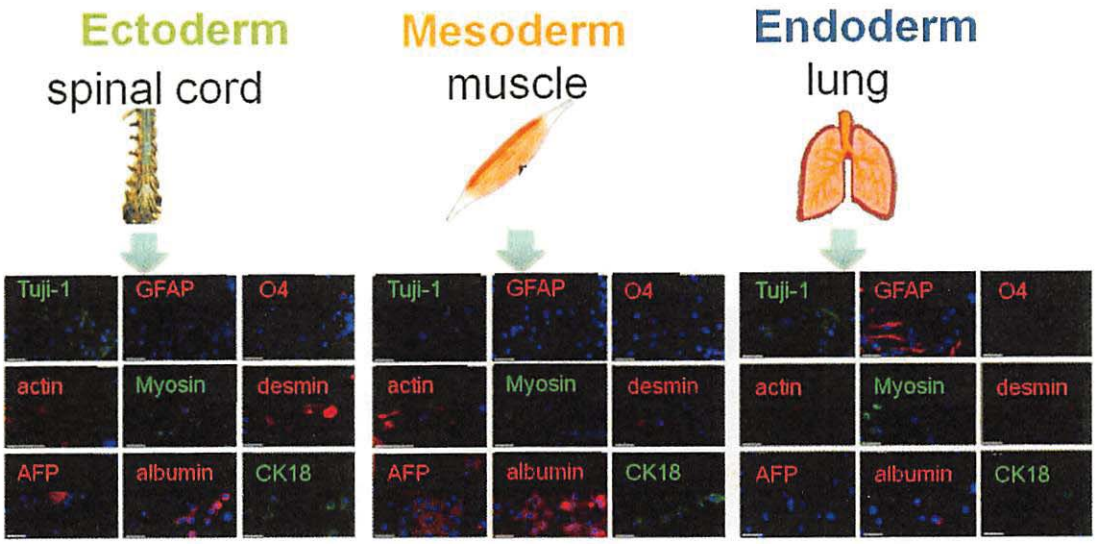


Figure 17 *In vitro* differentiation assay of cells from 3 germ layers.
Differentiation into cells representative of the 3 germ layers were confirmed by each specific gene expression of various lineages; Map2 (ectoderm), MyoD (mesoderm) and AFP (endoderm), however non-differentiated spheres did not express any of Map2, MyoD and AFP
Marrowspheres were dissociated and plated in each appropriate medium. Cells from spheres, differentiated into cells representative of the three germ layers. Neural cells, muscle cells cells, hepatocytes. Neurons stained with βIII tubuline, Glias stained with GFAP and oligodendrocytes were stained with O4. Muscle cells stained with α-smooth muscle actin and Myosin and Desmin. Hepatocytes were stained with α-fetoprotein, Albumin and CK18.

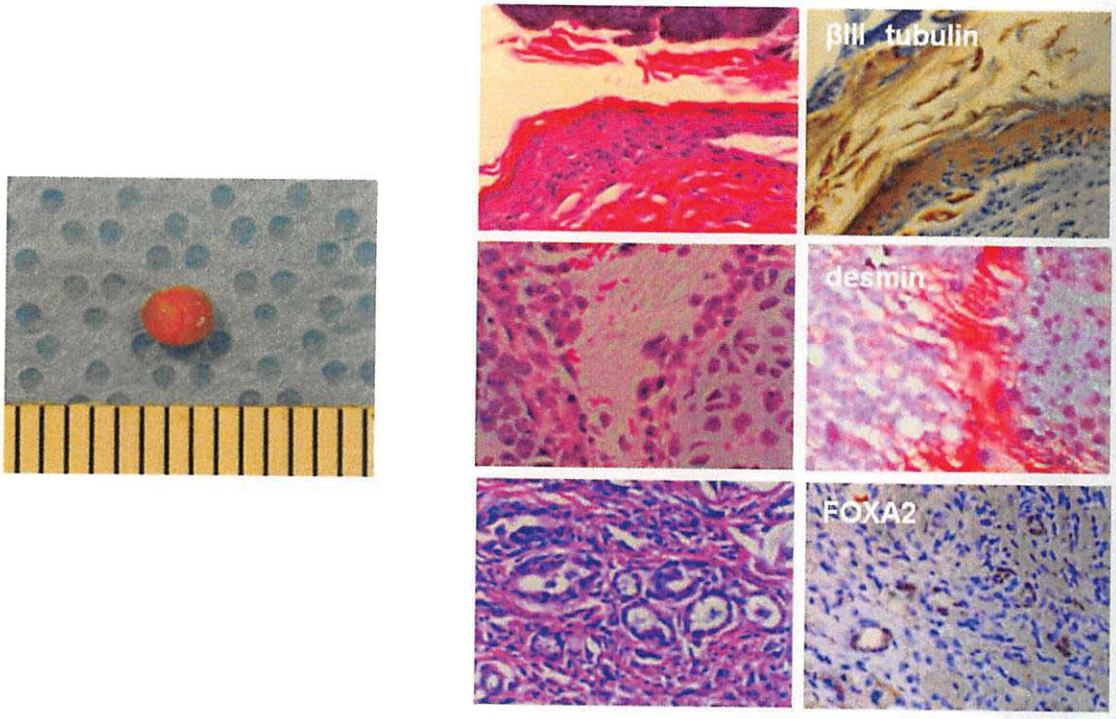


Figure 18 In vivo differentiation assay of sphere forming cells.

Teratoma like mass from spinalspheres contained nerve expressing betaIII-tubuline (ectoderm), muscle expressing desmin (mesoderm) and duct like structure expressing FOXA2 (endoderm).

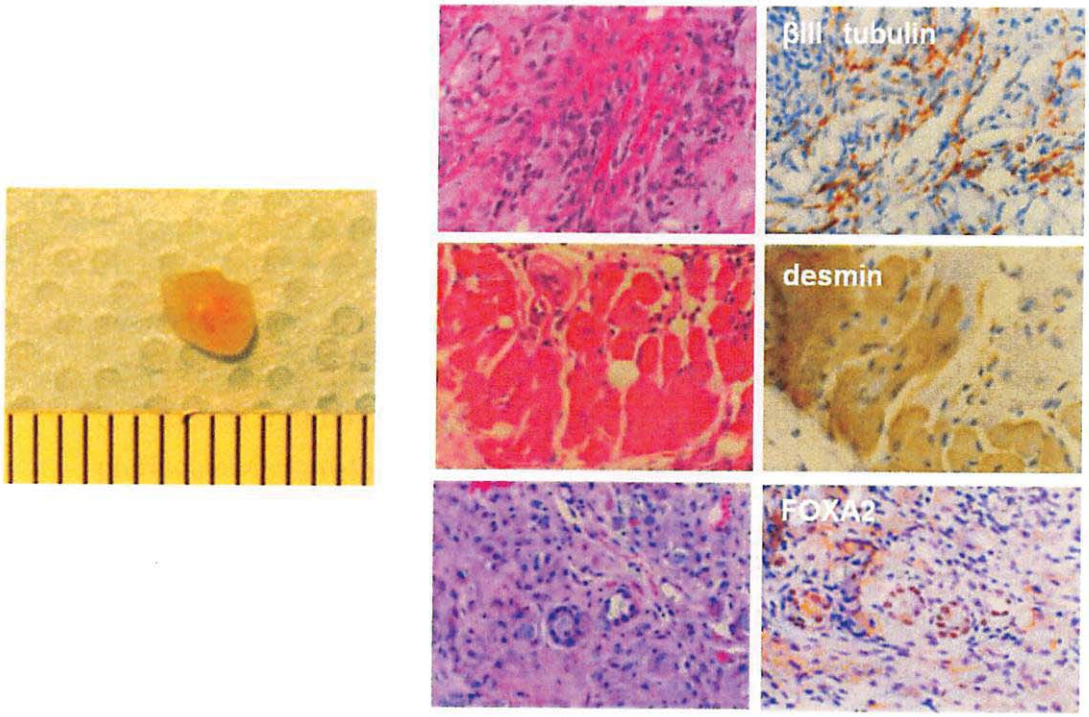


Figure 19 In vivo differentiation assay of sphere forming cells.

Teratoma like mass from pneumospheres contained epithelium expressing pancytokeratin (ectoderm), cartilage (Ct) demonstrating safranin O staining (mesoderm) and gland like structures expressing FOXA2 (endoderm). Scale bars: 50 μm

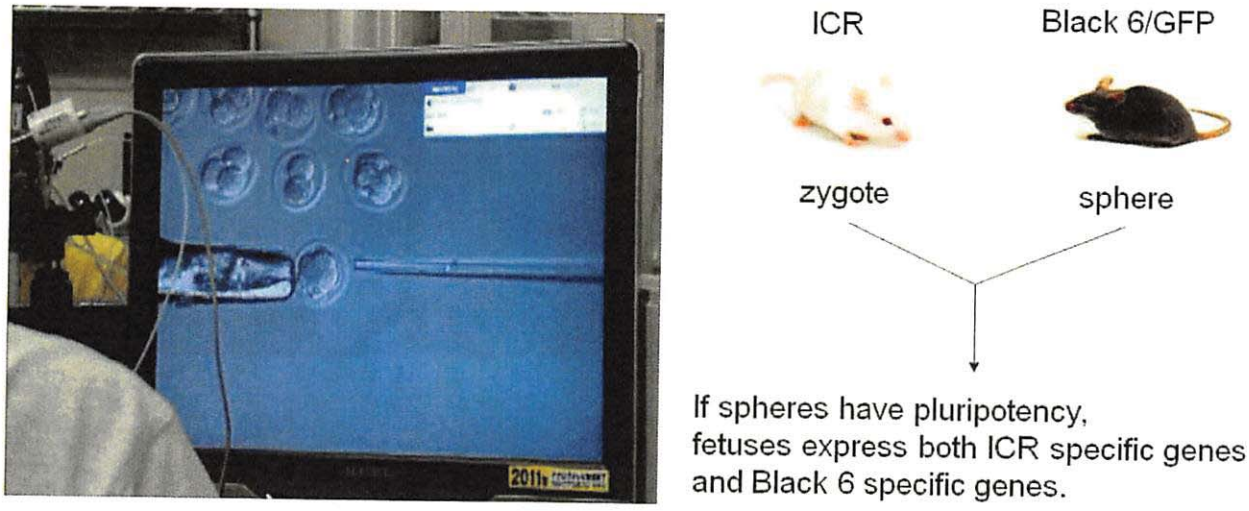


Figure 20 Chimera mice generation

Injection method (left). Scheme of chimera mice generation with spheres (right).

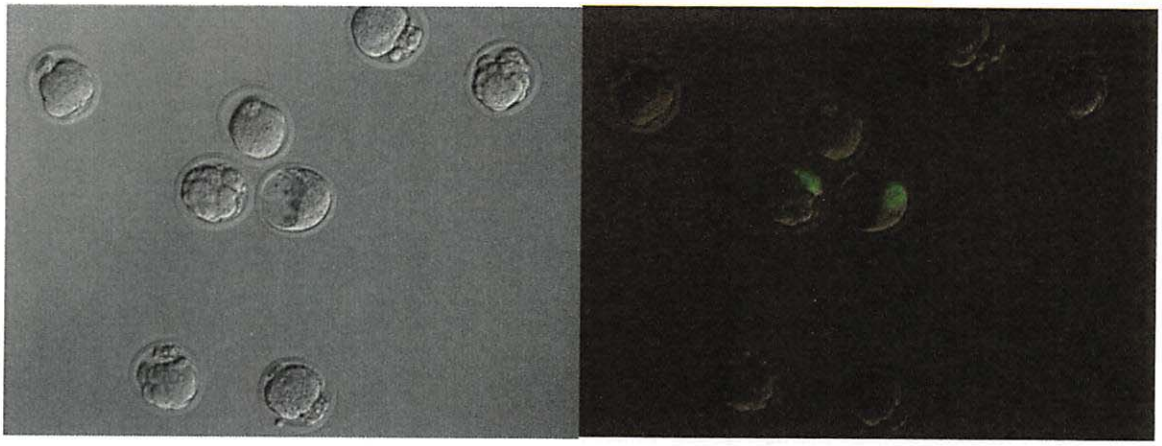


Figure 21 chimera zygote

GFP positive sphere cells were contributed to form blastcyst.

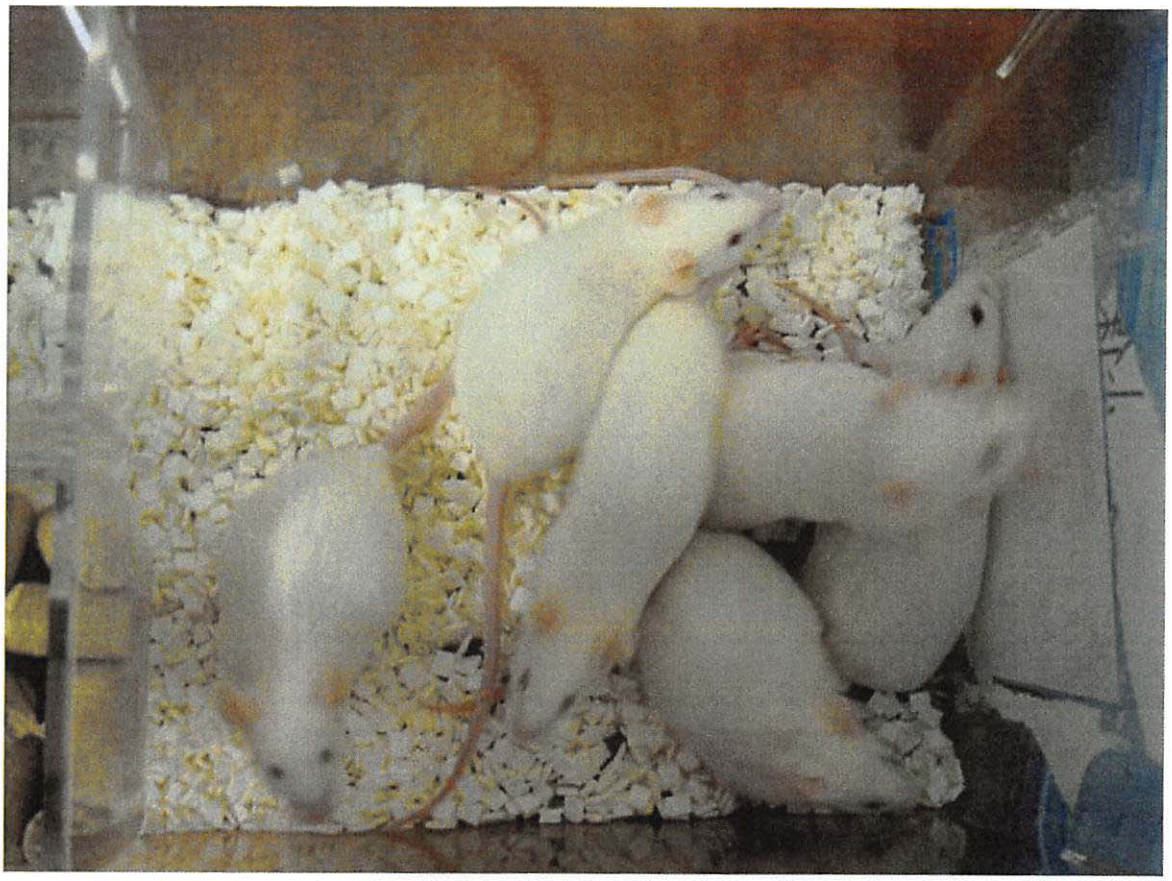


Figure 22 Adult chimera mice

Sphere derived hairs (black hair) were hardly observed in chimera mice coat.

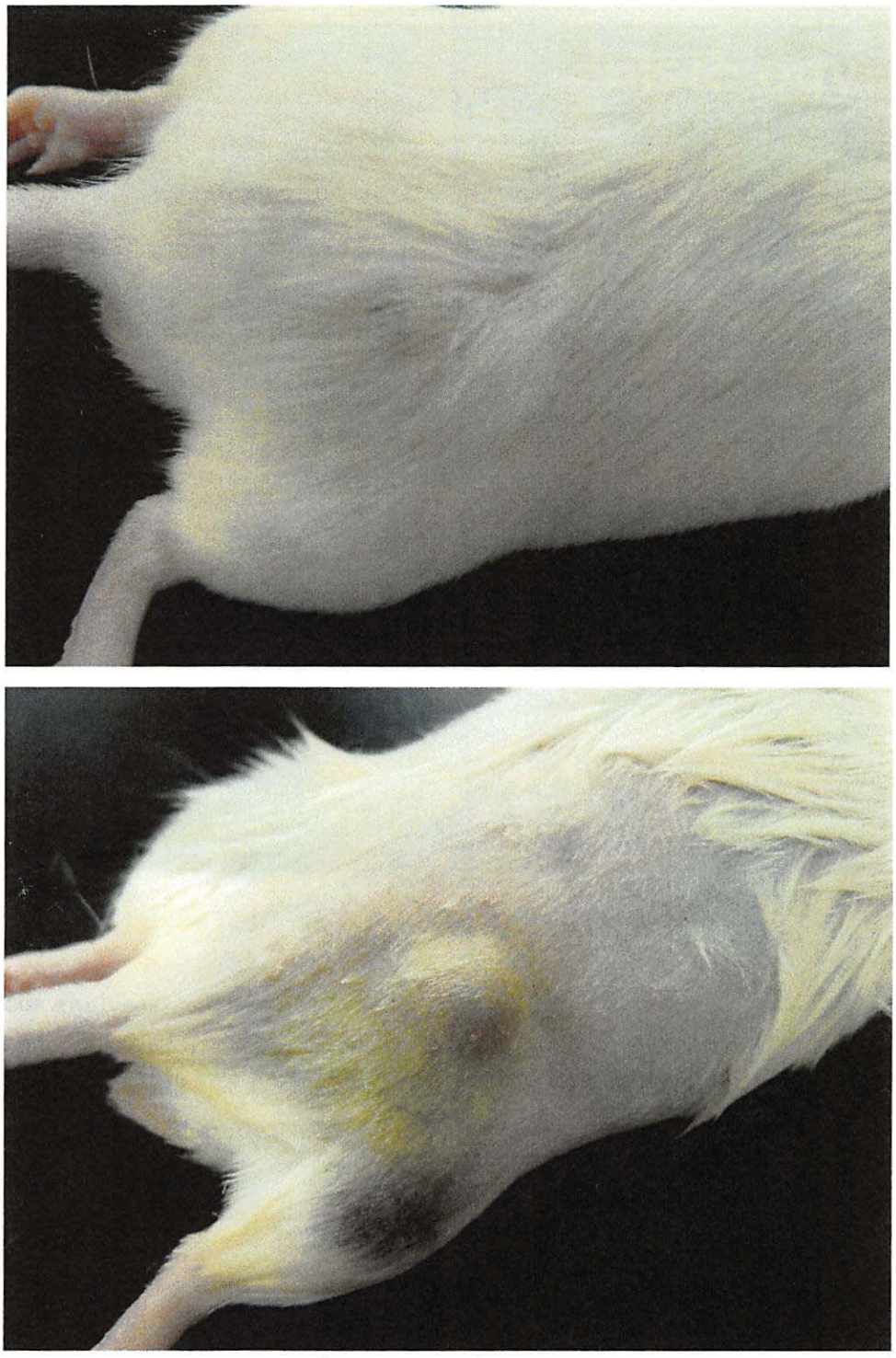


Figure 23 Chimera skin contributed by sphere cells

Sphere cells derived cells were found in adult mice's skin.

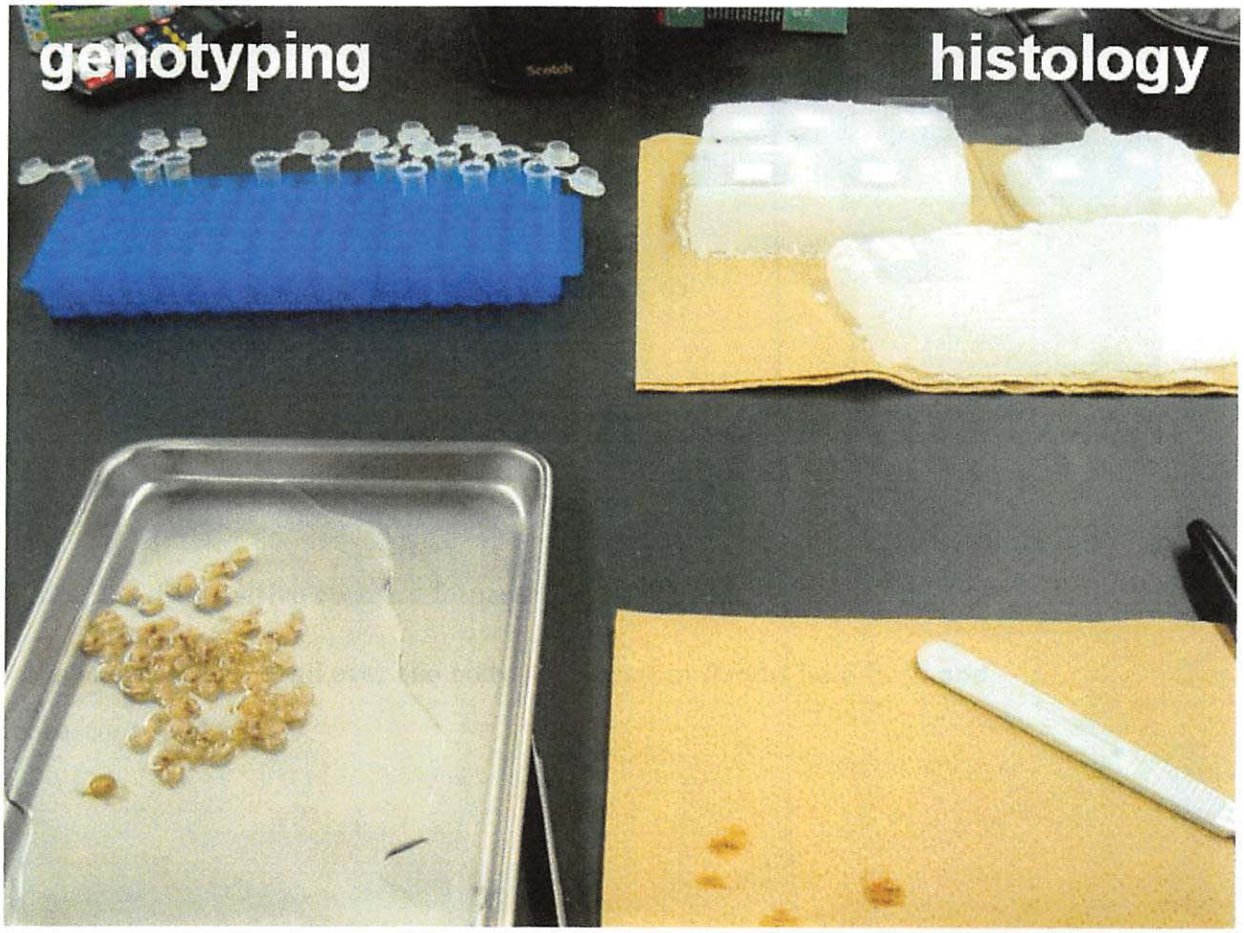


Figure 24 Chimera fetus analyses

Chimera fetuses were dissected into two parts. One was utilized for genotyping. Another was utilized for histological analyses.

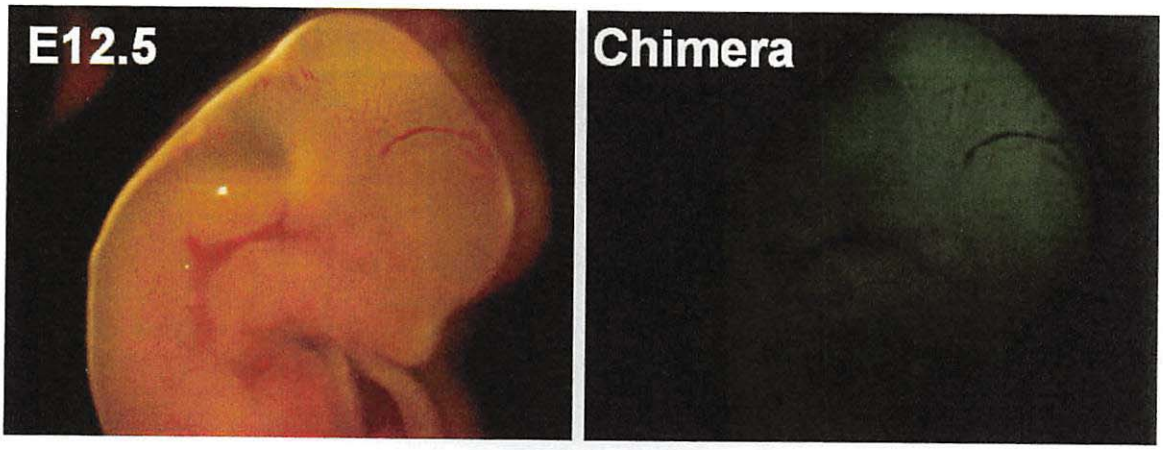


Figure 25 GFP positive chimera fetuses

Fetus expressed GFP all over the body. Observed in Bright field (left) and fluorescent field (right).

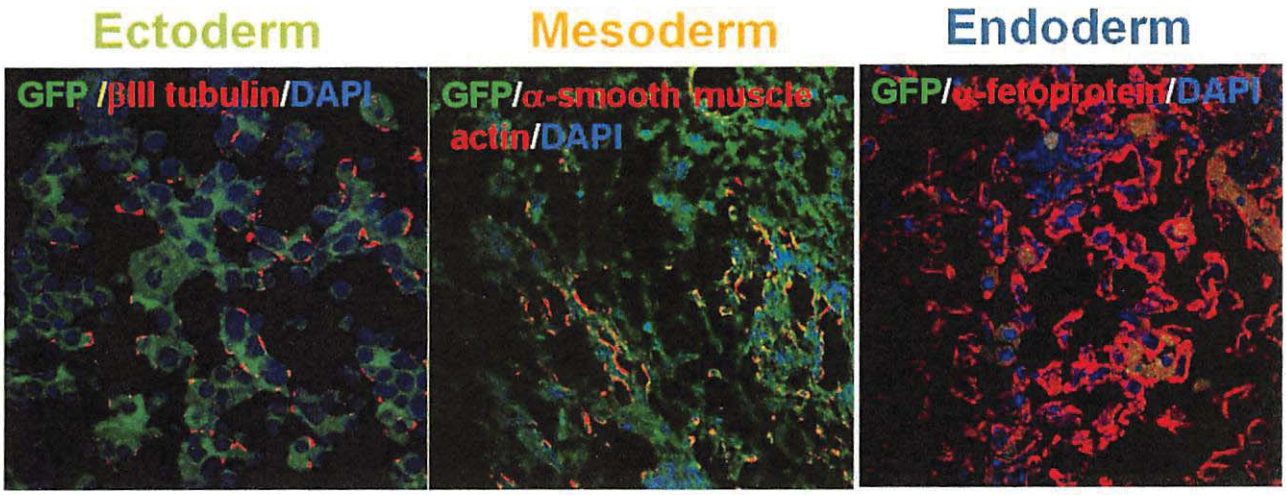


Figure 26 Histoloigical analyses of GFP positive chimera fetuses

GFP positive cells in chimera fetuses differentiated into mature cells derived from three germ layers. βIII tubulin expressing GFP positive cells (left). α-smooth muscle actin expressing GFP positive cells (middle). α-fetoprotein expressing GFP positive cells (right).