

早稲田大学博士論文(論文冊子)		
	学位記	文科省報告
2010	5627	甲 3323

Isolation of pluripotent adult stem cells  
discovered from tissues derived from all three germ layers

三胚葉由来組織に共通した万能性体性幹細胞の探索

February 2011

Haruko Obokata  
小保方 晴子

早稲田大学大学院 先進理工学研究科

# 博士論文

## 論文題目

Isolation of pluripotent adult stem cells  
discovered from tissues derived from all three germ layers

三胚葉由来組織に共通した  
万能性体性幹細胞の探索

申請者

Haruko Obokata

小保方 晴子

生命医科学専攻 環境生命科学研究

2011年 2月

# CONTENTS

## 1. BACKGROUND

1.1 General Introduction .....	1
● Importance of stem cells	
● Unique properties of all stem cells	
1.2 Adult stem cell .....	7
● History of Adult stem cell research	
● Function of adult stem cells	
1.3 Research methods for identifying adult stem cells .....	10
● Normal differentiation pathways of adult stem cells.	
● Transdifferentiation.	
1.4 Pluripotent stem cells.....	14
● The similarities and differences between embryonic and adult stem cells	
● Embryonic stem cells	
A. Establish of embryonic stem cells grown in the laboratory	
B. Tests for identifying embryonic stem cells	
● Induced pluripotent stem cells	

1.5 Possibility of existence of adult pluripotent stem cells -----	20
--	----

- Spore-like stem cells

1.6 Hypothesis of this research -----	23
---------------------------------------	----

## 2. ISOLATION OF SMALL CELLS

2.1 Introduction -----	27
2.1.1 Stem cells and small cells	27
2.1.2 Stem cells and sphere formation	27
2.2 Experimental -----	28
2.2.1 Isolation of small cells	28
2.2.2 Characterization of small cells	29
2.3 Results -----	30
2.3.1 Sphere forming from isolated cells	30
2.3.2 Effect of trituration	30
2.3.3 Culture conditions of small cells	31
2.3.4 Sphere formation from bone marrow cells	31
2.3.5 Immature cell marker expression	32
2.3.6 Expression of embryonic like phenotypes	33
2.3.7 Quantitative PCR of Pluripotent markers	34
2.4 Summary of section 2 -----	35
2.5 Discussion -----	35
2.6 References -----	45

## 3. CHARACTERIZATION OF SMALL CELLS

3.1 Introduction	48
3.1.1 Differentiation potential of stem cells	
3.2 Experimental	48
3.2.1 Differentiation potential of cells <i>in vitro</i>	48
3.2.2 Differentiation potential <i>in vivo</i>	48
3.3 Results	49
3.3.1 Differentiation potential of cells <i>in vitro</i>	50
3.3.2 Differentiation potential of cells <i>in vivo</i>	51
3.4 Summary of section 3	51
3.5 Discussion	51
3.6 Referances	58

## 4. ISOLATION OF SMALL CELLS FROM TISSUES DERIVED FROM ALL THREE GERM LAYERS

4.1 Introduction	63
4.2 Experimental	63
4.2.1 Isolation of small cells from various tissues	64
4.2.2 Characterization of isolated small cells	64



4.2.3 Differentiation potential of sphere forming small cells	64
4.3 Results .....	65
4.3.1 Sphere formation from tissues derived from representative of the three germ layers	65
4.3.2 Immature gene expression of spheres derived from tissues representative of the three germ layers	66
4.3.3 Differentiation potential of Cells derived from tissues representative of the three germ layers	67
4.3.4 Differentiation potential of cells <i>in vivo</i>	68
4.4 Summary of section 4 .....	69
4.5 Discussion .....	69
4.6 References .....	76

## 5. CHIMERA MICE GENERATION FROM ISOLATED SMALL CELLS

5.1 Introduction .....	81
5.1.1 Adult stem cells and chimera mice	81
5.2 Experimental .....	81
5.2.1 Chimera mice generation with spheres	82
A. Aggregation method	
B. Injection method	
5.2.2 Genotyping .....	82

5.2.3 Immunohistological analyses .....	82
5.3 Results .....	83
5.3.1 Generation of chimera zygotes .....	83
5.3.2 Analyses of adult chimera mice .....	83
5.3.3 Analyses of chimera fetuses .....	83
5.4 Summary of section 5 .....	84
5.5 Discussion .....	85
5.6 References .....	93

## 6. FUTURE PROSPECTS

6.1 General overview .....	95
6.2 Cell source for tissue engineering regenerative medicine .....	95
6.3 Future issues .....	96

## APPENDIX .....

97

- Material and method
- Primer sequences

## ACKNOWLEDGEMENTS.....

108

## CURRICULUM VITAE

# 1. BACKGROUND

## 1.1 General Introduction

- Importance of stem cells

Stem cells have the remarkable potential to develop into many different cell types in the body during early life and growth. In addition, in many tissues they serve as a sort of internal repair system, dividing essentially without limit to replenish other cells as long as the person or animal is still alive. When a stem cell divides, each new cell has the potential either to remain a stem cell or become another type of cell with a more specialized function, such as a muscle cell, a red blood cell, or a brain cell.

Stem cells are distinguished from other cell types by two important characteristics. First, they are unspecialized cells capable of renewing themselves through cell division, sometimes after long periods of inactivity. Second, under certain physiologic or experimental conditions, they can be induced to become tissue- or organ-specific cells with special functions. In some organs, such as the gut and bone marrow, stem cells regularly divide to repair and replace worn out or damaged tissues. In other organs, however, such as the pancreas and the heart, stem cells only divide under special conditions.



Until recently, scientists primarily worked with two kinds of stem cells from animals and humans: embryonic stem cells and non-embryonic "somatic" or "adult" stem cells. The functions and characteristics of these cells will be explained in this section. Scientists discovered ways to derive embryonic stem cells from early mouse embryos nearly 30 years ago, in 1981. The detailed study of the biology of mouse stem cells led to the discovery, in 1998, of a method to derive stem cells from human embryos and grow the cells in the laboratory. These cells are called human embryonic stem cells. The embryos used in these studies were created for reproductive purposes through *in vitro* fertilization procedures. When they were no longer needed for that purpose, they were donated for research with the informed consent of the donor. In 2006, researchers made another breakthrough by identifying conditions that would allow some specialized adult cells to be "reprogrammed" genetically to assume a stem cell-like state. This new type of stem cell, called induced pluripotent stem cells (iPSCs), will be discussed in a later part of this section.

Stem cells are important for living organisms for many reasons. In the 3- to 5-day-old embryo, called a blastocyst, the inner cells give rise to the entire body of the organism, including all of the many specialized cell types and organs such as the heart, lung, skin, sperm, eggs and other tissues. In some adult tissues, such as bone marrow, muscle, and brain, discrete populations of adult stem cells generate replacements for cells that are lost through normal wear and tear, injury, or disease.

Given their unique regenerative abilities, stem cells offer new potentials for treating diseases such as diabetes, and heart disease. However, much work remains to be done in the laboratory and the clinic to understand how to use these cells for cell-based therapies to treat disease, which is also referred to as regenerative or reparative medicine.

Laboratory studies of stem cells enable scientists to learn about the cells' essential properties and what makes them different from specialized cell types. Scientists are already using stem cells in the laboratory to screen new drugs and to develop model systems to study normal growth and identify the causes of birth defects.

Research on stem cells continues to advance knowledge about how an organism develops from a single cell and how healthy cells replace damaged cells in adult organisms. Stem cell research is one of the most fascinating areas of contemporary biology, but, as with many expanding fields of scientific inquiry, research on stem cells raises scientific questions as rapidly as it generates new discoveries

- The unique properties of all stem cells

Stem cells differ from other kinds of cells in the body. All stem cells—regardless of their source—have three general properties: they are capable of dividing and renewing themselves for long periods; they are unspecialized; and they can give rise to specialized cell types.

*Stem cells are capable of dividing and renewing themselves for long periods.* Unlike muscle cells, blood cells, or nerve cells—which do not normally replicate themselves—stem cells may replicate many times, or proliferate. A starting population of stem cells that proliferates for many months in the laboratory can yield millions of cells. If the resulting cells continue to be unspecialized, like the parent stem cells, the cells are said to be capable of long-term self-renewal.

Scientists are trying to understand two fundamental properties of stem cells that relate to their long-term self-renewal:

why can embryonic stem cells proliferate for a year or more in the laboratory without differentiating, but most non-embryonic stem cells cannot; and what are the factors in living organisms that normally regulate stem cell proliferation and self-renewal?

Discovering the answers to these questions may make it possible to understand how cell proliferation is regulated during normal embryonic development or during the abnormal cell division that leads to cancer. Such information would also enable scientists to grow embryonic and non-embryonic stem cells more efficiently in the laboratory.

The specific factors and conditions that allow stem cells to remain unspecialized are of great interest to scientists. It has taken scientists many years of trial and error to learn to derive and maintain stem cells in the laboratory without them spontaneously differentiating into specific cell types. For example, it took two decades to learn how to grow human embryonic stem cells in the laboratory following the development of conditions for growing mouse stem cells. Therefore,

understanding the signals in a mature organism that cause a stem cell population to proliferate and remain unspecialized until the cells are needed. Such information is critical for scientists to be able to grow large numbers of unspecialized stem cells in the laboratory for further experimentation.

*Stem cells are unspecialized.* One of the fundamental properties of a stem cell is that it does not have any tissue-specific structures that allow it to perform specialized functions. For example, a stem cell cannot work with its neighbors to pump blood through the body (like a heart muscle cell), and it cannot carry oxygen molecules through the bloodstream (like a red blood cell). However, unspecialized stem cells can give rise to specialized cells, including heart muscle cells, blood cells, or nerve cells.

*Stem cells can give rise to specialized cells.* When unspecialized stem cells give rise to specialized cells, the process is called differentiation. While differentiating, the cell usually goes through several stages, becoming more specialized at each step. Scientists are just beginning to understand the signals inside and outside cells that trigger each step of the differentiation process. The internal signals are controlled by a cell's genes, which are interspersed across long strands of DNA, and carry coded instructions for all cellular structures and functions. The external signals for cell differentiation include chemicals secreted by other cells, physical contact with neighboring cells, and certain molecules in the microenvironment. The interaction of signals during differentiation causes the cell's DNA to acquire epigenetic marks that restrict DNA expression in the cell and can be passed on through cell division.

Many questions about stem cell differentiation remain. For example, are the internal and external signals for cell differentiation similar for all kinds of stem cells? Can specific sets of signals be identified that promote differentiation into specific cell types? Addressing these questions may lead scientists to find new ways to control stem cell differentiation in the laboratory, thereby growing cells or tissues that can be used for specific purposes such as cell-based therapies or drug screening.

Adult stem cells typically generate the cell types of the tissue in which they reside. For example, a blood-forming adult stem cell in the bone marrow normally gives rise to the many types of blood cells. It is generally accepted that a blood-forming cell in the bone marrow—which is called a hematopoietic stem cell—cannot give rise to the cells of a very different tissue, such as nerve cells in the brain. Experiments over the last several years have purported to show that stem cells from one tissue may give rise to cell types of a completely different tissue. This remains an area of great debate within the research community. This controversy demonstrates the challenges of studying adult stem cells and suggests that additional research using adult stem cells is necessary to understand their full potential as future therapies.

## 1.2 Adult stem cells

An adult stem cell is thought to be an undifferentiated cell, found among differentiated cells in a tissue or organ that can renew itself and can differentiate to yield some or all of the major specialized cell types of the tissue or organ. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found. Scientists also use the term somatic stem cell instead of adult stem cell, where somatic refers to cells of the body (not the germ cells, sperm or eggs). Unlike embryonic stem cells, which are defined by their origin (cells from the preimplantation-stage embryo), the origin of adult stem cells in some mature tissues is still under investigation.

Research on adult stem cells has generated a great deal of excitement. Scientists have found adult stem cells in many more tissues than they once thought possible. This finding has led researchers and clinicians to ask whether adult stem cells could be used for transplants. In fact, adult hematopoietic, or blood-forming, stem cells from bone marrow have been used in transplants for 40 years. Scientists now have evidence that stem cells exist in the brain and the heart. If the differentiation of adult stem cells can be controlled in the laboratory, these cells may become the basis of transplantation-based therapies.

- History of Adult stem cell research

The history of research on adult stem cells began about 50 years ago. In the 1950s, researchers discovered that the bone marrow contains at least two kinds of stem cells. One population, called hematopoietic stem cells, forms all the types of blood cells in the body. A second population, called bone marrow stromal stem cells (also called mesenchymal stem cells, or skeletal stem cells by some), were discovered a few years later. These non-hematopoietic stem cells make up a small proportion of the stromal cell population in the bone marrow, and can generate bone, cartilage, fat, cells that support the formation of blood, and fibrous connective tissue.

In the 1960s, scientists who were studying rats discovered two regions of the brain that contained dividing cells that ultimately become nerve cells. Despite these reports, most scientists believed that the adult brain could not generate new nerve cells. It was not until the 1990s that scientists agreed that the adult brain does contain stem cells that are able to generate the brain's three major cell types—astrocytes and oligodendrocytes, which are non-neuronal cells, and neurons, or nerve cells.



- Function of Adult stem cells

Adult stem cells have been identified in many organs and tissues, including brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, teeth, heart, gut, liver, ovarian epithelium, and testis. They are thought to reside in a specific area of each tissue (called a "stem cell niche"). In many tissues, current evidence suggests that some types of stem cells are pericytes, cells that compose the outermost layer of small blood vessels. Stem cells may remain quiescent (non-dividing) for long periods of time until they are activated by a normal need for more cells to maintain tissues, or by disease or tissue injury.

Typically, there is a very small number of stem cells in each tissue, and once removed from the body, their capacity to divide is limited, making generation of large quantities of stem cells difficult. Scientists in many laboratories are trying to find better ways to grow large quantities of adult stem cells in cell culture and to manipulate them to generate specific cell types so they can be used to treat injury or disease. Some examples of potential treatments include regenerating bone using cells derived from bone marrow stroma, developing insulin-producing cells for type 1 diabetes, and repairing damaged heart muscle following a heart attack with cardiac muscle cells.

### 1.3 Research methods for identifying adult stem cells

Scientists often use one or more of the following methods to identify adult stem cells: (1) label the cells in a living tissue with molecular markers and then determine the specialized cell types they generate; (2) remove the cells from a living animal, label them in cell culture, and transplant them back into another animal to determine whether the cells replace (or "repopulate") their tissue of origin.

Importantly, it must be demonstrated that a single adult stem cell can generate a line of genetically identical cells that then gives rise to all the appropriate differentiated cell types of the tissue. To confirm experimentally that a putative adult stem cell is indeed a stem cell, scientists tend to show either that the cell can give rise to these genetically identical cells in culture, and/or that a purified population of these candidate stem cells can repopulate or reform the tissue after transplant into an animal.

As indicated above, scientists have reported that adult stem cells occur in many tissues and that they enter normal differentiation pathways to form the specialized cell types of the tissue in which they reside.

- Normal differentiation pathways of adult stem cells.

In a living animal, adult stem cells are available to divide, when needed, and can give rise to mature cell types that have characteristic shapes and specialized structures and functions of a particular tissue. Hematopoietic stem cells give rise to

all the types of blood cells: red blood cells, B lymphocytes, T lymphocytes, natural killer cells, neutrophils, basophils, eosinophils, monocytes, and macrophages.

Mesenchymal stem cells give rise to a variety of cell types: bone cells (osteocytes), cartilage cells (chondrocytes), fat cells (adipocytes), and other kinds of connective tissue cells such as those in tendons.

Neural stem cells in the brain give rise to its three major cell types: nerve cells (neurons) and two categories of non-neuronal cells—astrocytes and oligodendrocytes.

Epithelial stem cells in the lining of the digestive tract occur in deep crypts and give rise to several cell types: absorptive cells, goblet cells, paneth cells, and enteroendocrine cells.

Skin stem cells occur in the basal layer of the epidermis and at the base of hair follicles. The epidermal stem cells give rise to keratinocytes, which migrate to the surface of the skin and form a protective layer. The follicular stem cells can give rise to both the hair follicle and to the epidermis.

- **Transdifferentiation.**

A number of experiments have reported that certain adult stem cell types can differentiate into cell types seen in organs or tissues other than those expected from

the cells' predicted lineage (i.e., brain stem cells that differentiate into blood cells or blood-forming cells that differentiate into cardiac muscle cells, and so forth). This reported phenomenon is called transdifferentiation.

Although isolated instances of transdifferentiation have been observed in some vertebrate species, whether this phenomenon actually occurs in humans is under debate by the scientific community. Instead of transdifferentiation, the observed instances may involve fusion of a donor cell with a recipient cell. Another possibility is that transplanted stem cells are secreting factors that encourage the recipient's own stem cells to begin the repair process. Even when transdifferentiation has been detected, only a very small percentage of cells undergo the process.

In a variation of transdifferentiation experiments, scientists have recently demonstrated that certain adult cell types can be "reprogrammed" into other cell types in vivo using a well-controlled process of genetic modification (see Section VI for a discussion of the principles of reprogramming). This strategy may offer a way to reprogram available cells into other cell types that have been lost or damaged due to disease. For example, one recent experiment shows how pancreatic beta cells, the insulin-producing cells that are lost or damaged in diabetes, could possibly be created by reprogramming other pancreatic cells. By "re-starting" expression of three critical beta-cell genes in differentiated adult pancreatic exocrine cells, researchers were able to create beta cell-like cells that can secrete insulin. The reprogrammed cells were similar to beta cells in appearance, size, and shape; expressed genes characteristic of beta cells; and were able to partially restore blood sugar regulation in mice whose own beta cells had been chemically destroyed. While

not transdifferentiation by definition, this method for reprogramming adult cells may be used as a model for directly reprogramming other adult cell types.

In addition to reprogramming cells to become a specific cell type, it is now possible to reprogram adult somatic cells to become like embryonic stem cells (induced pluripotent stem cells, iPSCs) through the introduction of embryonic genes. Thus, a source of cells can be generated that are specific to the donor, thereby avoiding issues of histocompatibility, if such cells were to be used for tissue regeneration. However, like embryonic stem cells, determination of the methods by which iPSCs can be completely and reproducibly committed to appropriate cell lineages is still under investigation

## 1.4 Pluripotent stem cells

- The similarities and differences between embryonic and adult stem cells

Human embryonic and adult stem cells each have advantages and disadvantages regarding potential use for cell-based regenerative therapies. One major difference between adult and embryonic stem cells is their different abilities in the number and type of differentiated cell types they can become. Embryonic stem cells can become all cell types of the body because they are pluripotent. Adult stem cells are thought to be limited to differentiating into different cell types of their tissue of origin.

Embryonic stem cells can be grown relatively easily in culture. Adult stem cells are rare in mature tissues, so isolating these cells from an adult tissue is challenging, and methods to expand their numbers in cell culture have not yet been worked out. This is an important distinction, as large numbers of cells are needed for stem cell replacement therapies.

Scientists believe that tissues derived from embryonic and adult stem cells may differ in the likelihood of being rejected after transplantation. We don't yet know whether tissues derived from embryonic stem cells would cause transplant rejection, since the first phase 1 clinical trial testing the safety of cells derived from hESCs has only recently been approved by the United States Food and Drug Administration (FDA).

Adult stem cells, and tissues derived from them, are currently believed less likely to initiate rejection after transplantation. This is because a patient's own cells could be

expanded in culture, coaxed into assuming a specific cell type (differentiation), and then reintroduced into the patient. The use of adult stem cells and tissues derived from the patient's own adult stem cells would mean that the cells are less likely to be rejected by the immune system. This represents a significant advantage, as immune rejection can be circumvented only by continuous administration of immunosuppressive drugs, and the drugs themselves may cause deleterious side effects

- Embryonic stem cells

A. What stages of early embryonic development are important for generating embryonic stem cells?

Embryonic stem cells, as their name suggests, are derived from embryos. Most embryonic stem cells are derived from embryos that develop from eggs that have been fertilized *in vitro*—in an *in vitro* fertilization clinic—and then donated for research purposes with informed consent of the donors. They are *not* derived from eggs fertilized in a woman's body.

A. Establish of embryonic stem cells grown in the laboratory

Growing cells in the laboratory is known as cell culture. Human embryonic stem cells (hESCs) are generated by transferring cells from a preimplantation-stage embryo into a plastic laboratory culture dish that contains a nutrient broth known as culture medium. The cells divide and spread over the surface of the dish. The



inner surface of the culture dish is typically coated with mouse embryonic skin cells that have been treated so they will not divide. This coating layer of cells is called a feeder layer. The mouse cells in the bottom of the culture dish provide the cells a sticky surface to which they can attach. Also, the feeder cells release nutrients into the culture medium. Researchers have devised ways to grow embryonic stem cells without mouse feeder cells. This is a significant scientific advance because of the risk that viruses or other macromolecules in the mouse cells may be transmitted to the human cells.

The process of generating an embryonic stem cell line is somewhat inefficient, so lines are not produced each time cells from the preimplantation-stage embryo are placed into a culture dish. However, if the plated cells survive, divide and multiply enough to crowd the dish, they are removed gently and plated into several fresh culture dishes. The process of re-plating or subculturing the cells is repeated many times and for many months. Each cycle of subculturing the cells is referred to as a passage. Once the cell line is established, the original cells yield millions of embryonic stem cells. Embryonic stem cells that have proliferated in cell culture for a prolonged period of time without differentiating, are pluripotent, and have not developed genetic abnormalities are referred to as an embryonic stem cell line. At any stage in the process, batches of cells can be frozen and shipped to other laboratories for further culture and experimentation.

## B. Tests for identifying embryonic stem cells

At various points during the process of generating embryonic stem cell lines, scientists test the cells to see whether they exhibit the fundamental properties that make them embryonic stem cells. This process is called characterization.

Scientists who study human embryonic stem cells have not yet agreed on a standard battery of tests that measure the cells' fundamental properties. However, laboratories that grow human embryonic stem cell lines use several kinds of tests, including:

Growing and subculturing the stem cells for many months. This ensures that the cells are capable of long-term growth and self-renewal. Scientists inspect the cultures through a microscope to see that the cells look healthy and remain undifferentiated.

Using specific techniques to determine the presence of transcription factors that are typically produced by undifferentiated cells. Two of the most important transcription factors are Nanog and Oct4. Transcription factors help turn genes on and off at the right time, which is an important part of the processes of cell differentiation and embryonic development. In this case, both Oct 4 and Nanog are associated with maintaining the stem cells in an undifferentiated state, capable of self-renewal.

Using specific techniques to determine the presence of particular cell surface markers that are typically produced by undifferentiated cells.

Examining the chromosomes under a microscope. This is a method to assess whether the chromosomes are damaged or if the number of chromosomes has changed. It does not detect genetic mutations in the cells.

Determining whether the cells can be re-grown, or subcultured, after freezing, thawing, and re-plating.

Testing whether the human embryonic stem cells are pluripotent by 1) allowing the cells to differentiate spontaneously in cell culture; 2) manipulating the cells so they will differentiate to form cells characteristic of the three germ layers; or 3) injecting the cells into a mouse with a suppressed immune system to test for the formation of a benign tumor called a teratoma. Since the mouse's immune system is suppressed, the injected human stem cells are not rejected by the mouse immune system and scientists can observe growth and differentiation of the human stem cells.

Teratomas typically contain a mixture of many differentiated or partly differentiated cell types—an indication that the embryonic stem cells are capable of differentiating into multiple cell types. As long as the embryonic stem cells in culture are grown under appropriate conditions, they can remain undifferentiated (unspecialized). But if cells are allowed to clump together to form embryoid bodies, they begin to differentiate spontaneously. They can form muscle cells, nerve cells, and many other cell types. Although spontaneous differentiation is a good indication that a culture of embryonic stem cells is healthy, it is not an efficient way to produce cultures of specific cell types.

So, to generate cultures of specific types of differentiated cells—heart muscle cells, blood cells, or nerve cells, for example—scientists try to control the differentiation of embryonic stem cells. They change the chemical composition of the culture medium, alter the surface of the culture dish, or modify the cells by inserting specific genes. Through years of experimentation, scientists have established some basic protocols or "recipes" for the directed differentiation of embryonic stem cells into some specific cell types.

If scientists can reliably direct the differentiation of embryonic stem cells into specific cell types, they may be able to use the resulting, differentiated cells to treat certain diseases in the future. Diseases that might be treated by transplanting cells generated from human embryonic stem cells include Parkinson's disease, diabetes, traumatic spinal cord injury, Duchenne's muscular dystrophy, heart disease, and vision and hearing loss.

- Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) are adult cells that have been genetically reprogrammed to an embryonic stem cell–like state by being forced to express genes and factors important for maintaining the defining properties of embryonic stem cells. Although these cells meet the defining criteria for pluripotent stem cells, it is not known if iPSCs and embryonic stem cells differ in clinically significant ways. Mouse iPSCs were first reported in 2006, and human iPSCs were first reported in late 2007. Mouse iPSCs demonstrate important characteristics of pluripotent stem cells, including expressing stem cell markers, forming tumors containing cells from

all three germ layers, and being able to contribute to many different tissues when injected into mouse embryos at a very early stage in development. Human iPSCs also express stem cell markers and are capable of generating cells characteristic of all three germ layers.

Although additional research is needed, iPSCs are already useful tools for drug development and modeling of diseases, and scientists hope to use them in transplantation medicine. Viruses are currently used to introduce the reprogramming factors into adult cells, and this process must be carefully controlled and tested before the technique can lead to useful treatments for humans. In animal studies, the virus used to introduce the stem cell factors sometimes causes cancers. Researchers are currently investigating non-viral delivery strategies. In any case, this breakthrough discovery has created a powerful new way to "de-differentiate" cells whose developmental fates had been previously assumed to be determined. In addition, tissues derived from iPSCs will be a nearly identical match to the cell donor and thus probably avoid rejection by the immune system. The iPSC strategy creates pluripotent stem cells that, together with studies of other types of pluripotent stem cells, will help researchers learn how to reprogram cells to repair damaged tissues in the human body.

#### 1.5 Possibility of existence of adult pluripotent stem cells

Many important questions about adult stem cells remain to be answered. They include:

How many kinds of adult stem cells exist, and in which tissues do they exist?

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?

As described above, stem cells were discovered by studies of development. In development, germ-layer differentiation is a critical point. All adult stem cells discovered until today are known to be generated after germ-layer differentiation. By this reason, adult stem cells cannot differentiate crossing germ layers.

Whereas, some scientists suggested that "left over" embryonic cells still reside in adult body.

For example, MAPC, spore-like stem cells, MIAMI cells, VSESC and MUSE cells.

They suggest those theories because some phenomena can not be explained by knowledge from development study. Adult stem cells have generally been felt to be limited to multipotency and unable to cross germ layer lineages as they develop. However, first, a part of mesenchymal stem cells were derived from ectoderm not mesoderm. Second, a part of mesenchymal stem cells can differentiate into cells derived from ectoderm. Thus, some of adult stem cells cross the germ layers. If the origin of germ layer is critical for determining stem cells' fate, these phenomena can not be explained. This is the reason why researchers suggest that "left over" embryonic cells still reside in adult body.

Especially spore-like stem cell research suggested a very unique theory.

- Spore-like stem cells (Figure 2)

While the existence of adult stem cells has been reported for more than a decade, our research team has advanced the theory that common adult stem cells reside in all body tissues, they possess small figure and stress-tolerant property. These cells were named spore-like stem cells.



## 1.6 Hypothesis of this research

- Sphere formation

Sphere formation is recognized as one of the isolation methods for adult stem cells.

Because stem cells should hold a strong proliferative potential and self-renewal potency, sphere formation is recognized as a result of those potencies.

Interestingly, as presented by neurospheres, some sphere-forming stem cells show gene expression overlapping with ES cells.

- Immature adult stem cells

If stem cells which can cross the germ layer lineages exist in the adult body, we assume that they should be very distinct from ES cells because ES cells were not native cells existing in native organisms. ES cells are artificial.

- Hypothesis

Considering all the various factors together,

1. Adult stem cells stem which can cross germ layers (very immature adult stem cells) may exist.
2. They are very small, and stress-tolerant
3. They form spheres

In this study, we characterized cells isolated from three adult tissues (lung, muscle and spinal cord) representative of the three different germ layers (endoderm, mesoderm and ectoderm) and from bone marrow. The cells were triturated to break mature cells and propagated as non-adherent clusters or spheres in a serum-free culture medium. We found that cells from each source, initially expressed many of the markers associated with ESCs and demonstrated differentiation potential into all three germ layers at a time that neural lineage markers had not yet been expressed. Ultimately cells from each tissue, differentiated into cells representative of all three germ layers *in vitro*. The isolation initially contained a significant amount of floating debris, non-adherent cells, insoluble proteins or fibers, and other extraneous materials, all of which appeared to participate in the formation of non-adherent spherical clusters that contained the cells. The cellular make-up of individual spheres was not identical; that is, spheres were composed of heterogeneous populations of cells, even when the spheres were generated from cells procured from the same tissue at the same time. Similarities or differences seen in the cell content of different spheres, were believed to be secondary to the environment in which they were cultured.

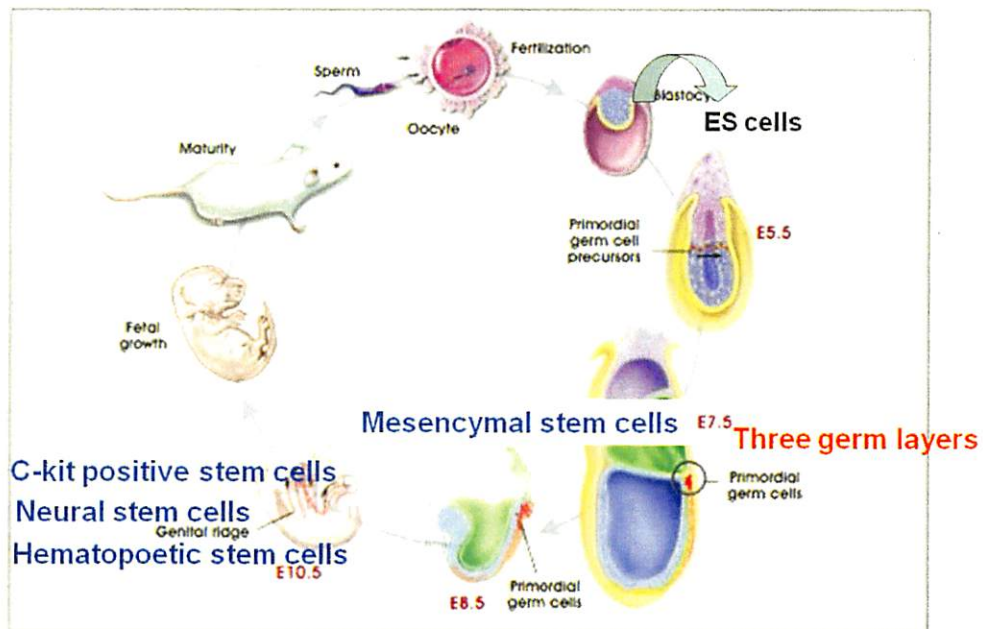


Figure 1 Development and adult stem cells

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.

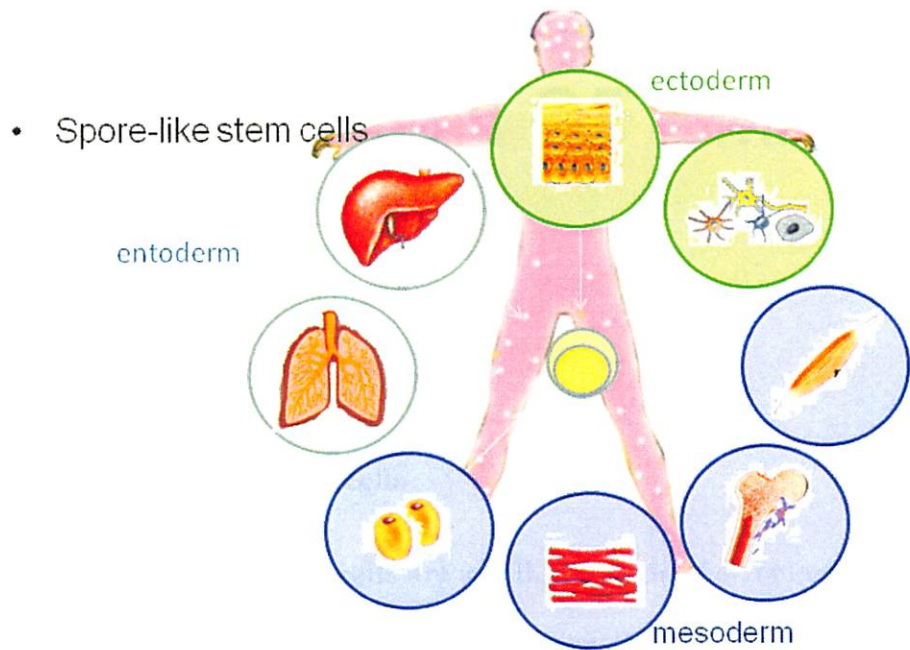


Figure 2 Hypothesis of this study.

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