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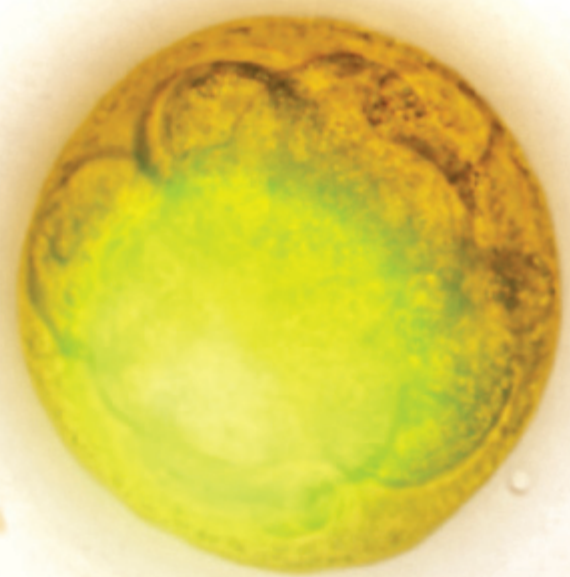
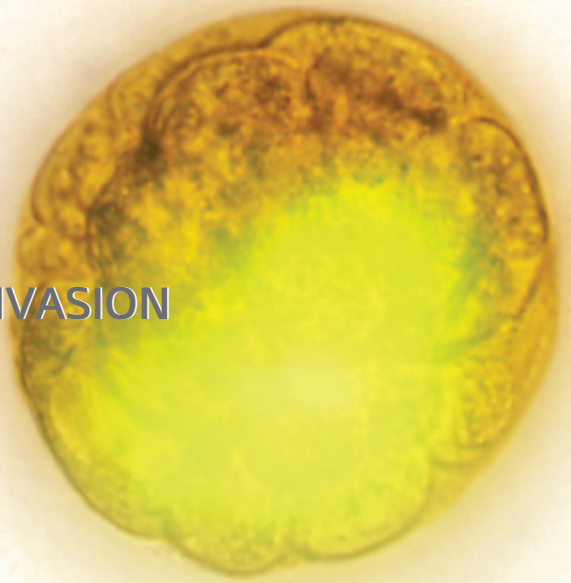
KEIO UNIVERSITY GRADUATE SCHOOL OF MEDICINE

GLOBAL COE PROGRAM

EDUCATION AND RESEARCH CENTER FOR STEM CELL MEDICINE

RESEARCH HIGHLIGHT COLLECTION 2009





TRANSGENIC TRANSMISSION

NATURAL-BORN HELPERS

SNAIL SETS PACE FOR TUMOR INVASION

THE PURE OF HEART

REPLENISHING NERVE CELLS FROM BONE MARROW

It is my great pleasure to welcome you to this collection of research highlights from Keio University's Education and Research Center for Stem-Cell Medicine Global Center of Excellence (GCOE) program. Stem cell research is a field of growing importance due to the near ubiquitous presence of stem cells in bodily organs. Stem cells span the lifetime of the organism, from the early embryonic stage to its death, and are closely linked to the pathogenesis and treatment of many diseases.

The purpose of the GCOE program is to create a world-class training program by reorganizing our graduate school to maximize our use of the human resources development strategy and research assets created by the Center for the Integration of Basic and Clinical Research in Stem-Cell Medicine and Immunology, a recently concluded 21st Century COE program. The GCOE program will form the basis for the creation of a new multidisciplinary academic field of 'stem-cell medicine'.

The goals set down for the human resource development program mirror key concepts in stem-cell research: 'self-renewality' of education and research through the establishment of a positive cycle of human resource development by which more experienced students mentor new students; 'multipotency' in research by developing a diverse pool of human resources; and 'mobility' through the exchange of human resources and ideas to develop an international network of collaborations.

Our strength lies in our many young investigators and the promotion of multidisciplinary research, and it is expected that the GCOE and its participants will make a valuable contribution to the overall improvement of education and research activities in the Graduate School of Medicine doctoral program.

This booklet contains a collection of five research highlights that introduce one outstanding paper from each of the five groups that make up the Education and Research Center for Stem-Cell Medicine. These articles have been carefully selected from the many published by our scientists in 2009. I hope that this brief introduction will give you a sense of the richness and depth of the work that we carry out, and provide an insight into the vital and exciting field of stem-cell medicine.

Hideyuki Okano
Program Leader
Education and Research Center for Stem-Cell Medicine
Global Center of Excellence program
Keio University Graduate School of Medicine

About the Education and Research Center for Stem-Cell Medicine

The Center strives to integrate research in basic and clinical medicine based on a comprehensive understanding of stem cells, and to create an outstanding and internationally competitive education and research center for the training of pioneering leaders. The centers conducts researchs in five key areas, each of which is highlighted in this collection:

Tissue stem-cell regulation and in vivo experimental medicine
Inflammation/immunological regulation and tissue regeneration
Development of new cancer treatments targeting cancer stem cells and EMT
Development of regenerative medicine for intractable diseases
Practical implementation of feasible regenerative medicine

Transgenic transmission

A new technique for generating genetically engineered monkeys could become invaluable to biomedical research

Tissue stem-cell regulation and in vivo *experimental medicine group*

Figure 1: A researcher holds twin transgenic marmoset infants Kei and Kou, who express eGFP in their forelimbs (see fluorescence micrographs, inset).

The generation of the first genetically engineered—or transgenic—non-human primates that can transmit a foreign gene to their offspring has been achieved in Japan by a collaborative group of researchers. The group, including several researchers from the Keio University of Medicine and the Central Institute for Experimental Animals, achieved this result using a novel technique that is described in the journal *Nature*[1].

According to the authors, the technique overcomes ethical and economic limitations of generating non-human primates, and is likely to become a valuable tool for investigating the mechanisms of human diseases and developing new therapies.

The technique involves injecting recombinant self-inactivating lentiviruses containing the enhanced green fluorescent protein (*eGFP*) gene into the embryos of marmoset monkeys, *Callithrix jacchus* (Fig. 1). Team member Hideyuki Okano notes that marmosets are more amenable to these techniques because they have a shorter gestation period and because females have more offspring that reach sexual maturity more quickly. The group used lentiviruses because they are integrated efficiently into the host genome, minimizing the number of eggs needed.

The researchers transferred embryos fertilized by *in vitro* fertilization and by natural intercourse into the wombs of surrogate mothers. Some were transferred immediately, while others were examined for expression of *eGFP* before the transfer. Several non-transferred embryos were also examined for expression of this gene. Seven of the surrogate mothers became pregnant; three miscarried, but the rest delivered five healthy offspring between them.

Upon birth, the researchers found that four of the five animals expressed the *eGFP* gene. The majority of these expressed the transgene in various tissues, including skin, hair and blood. The same tissues of these animals were tested again when they reached sexual maturity. In two individuals, Okano and colleagues detected expression of *eGFP* in the semen, so they collected sperm cells and used them to fertilize eggs. Some of the resulting embryos were also found to express *eGFP*.

Transgenic mice are created routinely, but differences in brain structure and function, biochemical pathways and drug sensitivity often make it difficult to extrapolate research findings to humans. The ability to generate a transgenic animal model more closely related to humans therefore has many advantages.

"We have now developed a non-invasive method of transferring the embryos to surrogate mothers, which will improve the 7% birth rate," explains Okano. "We are also planning to develop a marmoset model of Parkinson's disease." ■

1. Sasaki, E., Suemizu, H., Shimada, A., Hanazawa, K., Oiwa, R., Kamioka, M., Tomioka, I., Sotomaru, Y., Hirakawa, R., Eto, T., Shiozawa, S., Maeda, T., Ito, M., Kito, C., Yagihashi, C., Kawai, K., Miyoshi, H., Tanioka, Y., Tamaoki, N., Habu, S., Okano, H. & Nomura, T. Generation of transgenic non-human primates with germline transmission. *Nature* **459**, 523–528 (2009).

Natural-born helpers

A recently identified class of immune cells helps fight off parasites, but may also have a key role in asthma and allergy

Inflammation/immunological regulation and tissue regeneration group

Immune cells gain access to the peritoneal cavity within the abdomen through 'milky spots', which act as portals for lymphocyte entry from the circulatory system. However, many aspects of the function of these structures are not well understood, as demonstrated by recent findings from a team led by Keio University School of Medicine researchers Shigeo Koyasu and postdoctoral fellow Kazuyo Moro[1].

"Kazuyo started looking at the structure of the milky spot carefully, and noticed the presence of previously unreported lymphocyte clusters," says Koyasu. Preliminary analysis revealed notable structural differences between these and standard lymph nodes, and due to their direct contact with surrounding pockets of fat-storing cells called adipocytes, the researchers termed these cell groups 'fat-associated lymphocyte clusters' (FALCs). Immune cells are generally characterized by the protein markers expressed on their surface, and on this basis Koyasu's team confirmed that a fraction of FALC cells represent a population distinct from other known cell types.

They found that FALC cells actively produce cytokine interleukin-5 (IL-5), a signaling factor that supports cells involved in innate immune response—an essential first wave of defense against microbial infection. They also secrete a second cytokine, interleukin-13 (IL-13), which helps the body to mount its defense against parasitic worms. Accordingly, genetically modified mice lacking FALCs showed impaired immune responses when infected with the intestinal parasite *Nippostrongylus brasiliensis*.

Based on their apparently important role in facilitating the 'natural' immune response, which is launched as an immediate reaction to infectious threats and lacks the adaptive component seen in subsequent encounters with the same pathogen, Koyasu and Moro named the FALC cells 'natural helper' (NH) cells (Fig. 1).

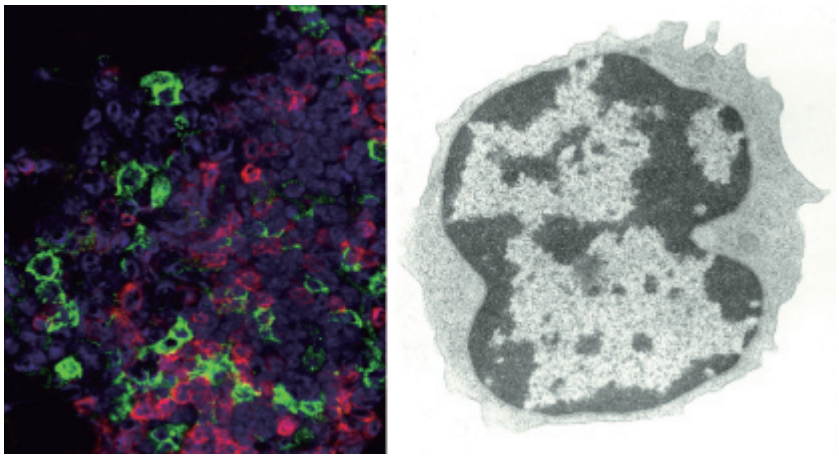


Figure 1: Fat-associated lymphocyte clusters (FALCs) of the peritoneal cavity (left) contain 'natural helper' immune cells (green), which express surface molecules that distinguish them from known T cells (red). The natural helper cells (right) appear to participate in elimination of certain parasites, but may also contribute to allergy and asthma.

IL-5 and IL-13 are both examples of TH2 cytokines, which guide responses against diverse extracellular pathogens but can also cause problems of their own. "IL-5 and IL-13 are produced rapidly during asthma, but people did not know which cells are responsible for such rapid production of TH2 cytokines," says Koyasu. "We suspect that NH cells have important roles in the pathophysiology of not only parasitic infection but also many allergic diseases."

Given how long FALCs have essentially remained hidden in plain sight, a great deal remains to be determined about the functional role of NH cells—and Moro, Koyasu and colleagues clearly have their work cut out for them. "We are interested in the developmental pathway of this newly identified lymphocyte and how this population is related to already identified immune cells," says Koyasu "We also plan to analyze how NH cells behave during infection and allergic reaction." ■

1. Moro, K., Yamada, T., Tanabe, M., Takeuchi, T., Ikawa, T., Kawamoto, H., Furusawa, J.I., Ohtani, M., Fujii, H. & Koyasu, S. Innate production of TH2 cytokines by adipose tissue-associated c-Kit+Sca-1+ lymphoid cells. *Nature* **463**, 540–544 (2010).

Snail sets pace for tumor invasion

As tumors enter their metastatic phase, they also interfere directly with the immune pathways that might thwart their invasive growth

Development of new cancer treatments targeting cancer stem cells and EMT group

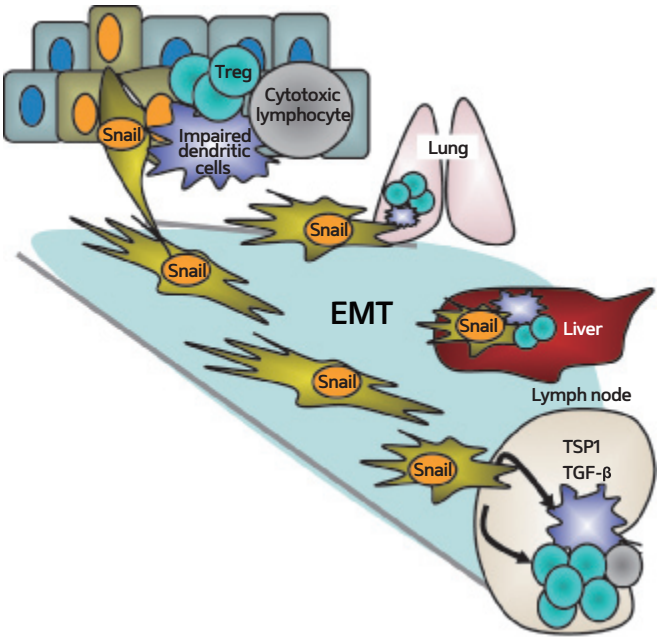


Figure 1: Tumor cells undergoing epithelial-mesenchymal transition (EMT) suppress immune response via multiple mechanisms. Snail expression increases in EMT cells (yellow and orange, top) as a prelude to metastatic invasion of tissues throughout the body (pale blue area), and this process is facilitated by inhibition of immune response at both the effector level by establishing resistance to anti-tumor cytotoxic T cells (top center) and the induction level by impairing T-cell activation (bottom) (TGF-β, EMT inducer).

Cancerous cells often express surface markers that distinguish them from their healthy counterparts, making them potential targets for recognition by the immune system. Scientists have achieved some success in developing drugs that stimulate anti-cancer immune responses, but tumors also have defense mechanisms in place that limit the effectiveness of such strategies.

Keio University School of Medicine researcher Yutaka Kawakami has spent much of the past decade investigating immune responses to human cancer including tumor-mediated immunosuppression, and a recent study from Chie Kudo-Saito and others in his laboratory has uncovered a crucial link to epithelial-mesenchymal transition (EMT)—a process in which cells undergo a dramatic morphological and functional transformation as a prelude to invasive growth and metastasis[1].

According to Kudo-Saito, the transcription factor known as Snail acts as a master switch for many of these changes. "Correlation of Snail expression with poor prognosis of patients has been reported in human cancers," she says, "including breast and colon cancers." Their team also demonstrated striking immunoregulatory effects for this factor; spleen cells cultured alongside Snail-expressing melanoma cells tend to develop into Treg cells, which actively keep immune responses in check. This effect appears to be partly mediated by increased production of signaling factor thrombospondin-1 (TSP1) by Snail-expressing cells.

Snail also exploits other, parallel modes of immunosuppression (Fig. 1). Certain populations of bone marrow cells develop into mature dendritic cells in the presence of cancerous cells, subsequently stimulating T-cell proliferation. However, co-culture with Snail-expressing melanoma cells instead triggers the development of regulatory dendritic cells, which constrain this proliferation. Remarkably, Snail also appears to promote resistance of tumor cells to destruction by cytotoxic T lymphocytes during the course of immunotherapy. "The most surprising finding was that many cellular and molecular mechanisms are involved in this EMT-induced immunosuppression," says Kawakami.

Accordingly, strategies that induce the targeted down-regulation of either Snail or TSP1 effectively limited tumor spread in mice implanted with Snail-expressing melanoma cells, leading to increased tumor targeting by immune cells, reduced metastatic growth and markedly improved survival rates.

The researchers believe this represents an important link between two mechanisms underlying poor cancer outcome. "EMT is associated with metastasis not only thorough increased invasive ability of cancer cells, but also through induction of immunosuppression," says Kudo-Saito. "In other words, metastasis is accelerated by immune evasion." Kawakami's team has observed similar behavior in a variety of cancer cell types, and future work will focus on further untangling the complicated mechanisms by which metastatic tumors execute their immunosuppressive sabotage. ■

1. Kudo-Saito, C., Shirako, H., Takeuchi, T. & Kawakami, Y. Cancer metastasis is accelerated through immunosuppression during Snail-induced EMT of cancer cells. *Cancer Cell* **15**, 195–206 (2009).

The pure of heart

Fluorescent labeling and purification of heart muscle cells could herald cell-based therapies for heart disease

Development of regenerative medicine for intractable diseases group

Researchers have long viewed stem cells—when matured into heart muscle cells—as an unlimited source of cells for transplantation to restore heart function lost as a result of heart disease, but have had limited success in achieving efficient maturation. Attempts to develop better therapies for heart disease have also been thwarted by the possibility of accidental transplantation of immature stem cells into the heart leading to the formation of tumors.

Now, transplantation of healthy heart muscle cells, derived from stem cells, is a possibility. Reporting in *Nature Methods*[1], a team of Japanese researchers led by Fumiyuki Hattori and Keiichi Fukuda from Keio University in Tokyo has developed a novel way to purify heart muscle cells from the stem cells of three different species.

Until now, methods to purify stem cell-derived heart muscle cells from immature stem cells have involved genetic modification of the cells, which could have unwanted effects.

Hattori, Fukuda and colleagues have found that a fluorescent dye that labels mitochondria—the energy-generating organelle prevalent in heart muscle cells—is taken up at high levels by heart muscle cells. They observed that this fluorescent dye could label these cells in living hearts, as well as when the cells were dissociated in a culture dish.

The researchers took advantage of this fact by using a fluorescence-activated cell sorter to separate out the unlabeled cells from the fluorescently labeled cells. Using this technique, they achieved close to 100% purity of the heart muscle cells (Fig. 1). In fact, they observed that these cells began beating in the culture dish, just as heart muscle cells would beat within the organ itself. According to Hattori, because the fluorescent label was gone from the cells within a day, the dye did not seem to affect the cells in any adverse way.

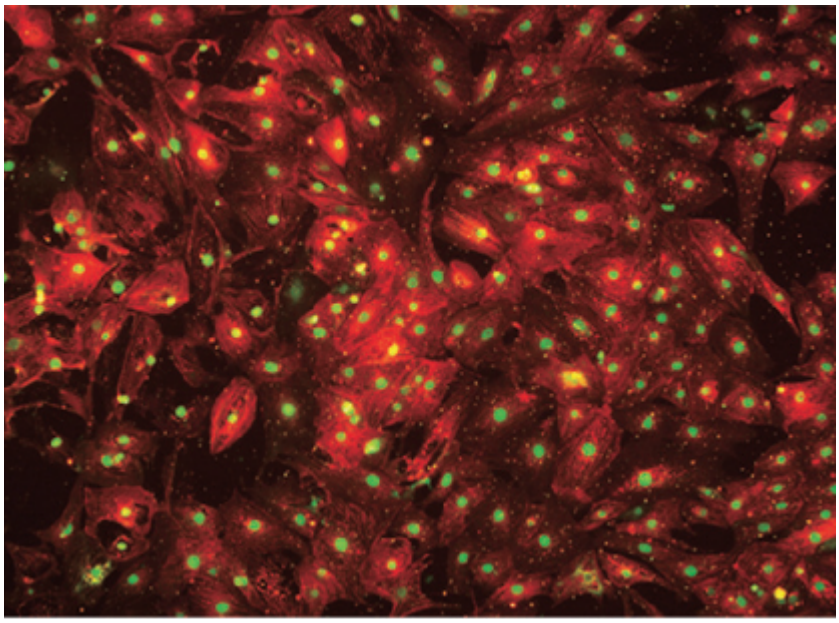


Figure 1: Purified heart muscle cells in culture. Cells were derived from human stem cells.

When the researchers transplanted unpurified stem cells into mice, tumors formed. However, no tumors arose after transplantation of the fluorescently labeled and purified heart muscle cells. When transplanted into the heart, the cells survived poorly, but did much better when they were aggregated in culture prior to transplantation. Hattori says the findings have important implications for future cell therapies aimed at labeling, purifying and transplanting heart muscle cells for the treatment of heart disease.

As the next step in their research, Hattori says, "we aim to integrate the grafts into the heart to see if they modify heart function in the host." ■

1. Hattori, F., Chen, H., Yamashita, H., Tohyama, S., Satoh, Y., Yuasa, S.,Li, W., Yamakawa, H., Tanaka, T., Onitsuka, T., Shimoji, K., Ohno, Y., Egashira, T., Kaneda, R., Murata, M., Hidaka, K., Morisaki, T., Sasaki, E., Suzuki, T., Sano, M., Makino, S., Oikawa, S. & Fukuda, K. Nongentic method for purifying stem cell-derived cardiomyocytes. *Nature Methods* **7**, 61–66 (2009).

Replenishing nerve cells from bone marrow

Cell transplantation therapies could benefit from a newly identified source of stem cells

Practical implementation of feasible regenerative medicine group

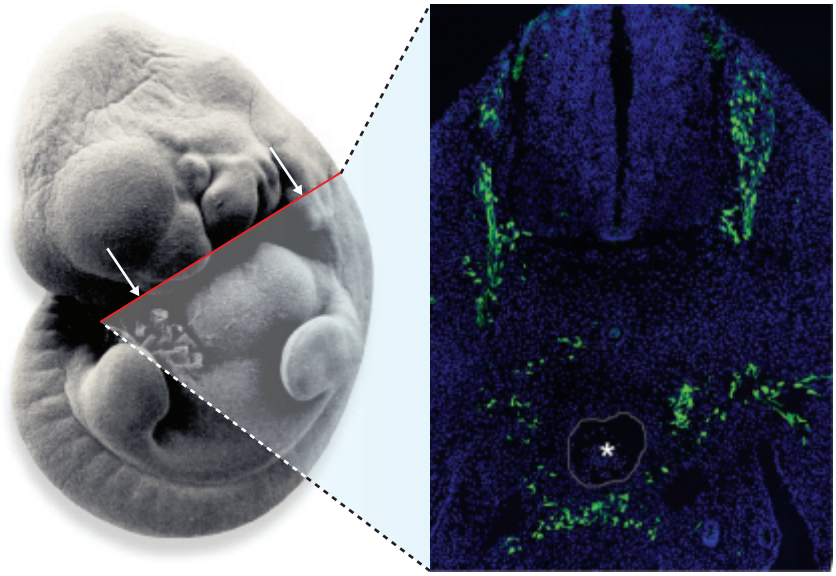


Figure 1: Migration pathway of neural crest cells to the bone marrow in an embryonic mouse. As captured in a transverse cross-section (main image) of the embryo (top left), GFP-labeled neural crest cells (green) migrate from the dorsal neural tube to the aorta (starred) and then continue migrating through the blood vessels to the bone marrow.

Stem cells derived from an embryonic tissue called the neural crest could be used to develop cell transplantation for neurological diseases, according to a study by researchers from the Keio University School of Medicine[1].

The neural crest is a transient population of cells that migrates from the dorsal aspect of the neural tube during neural development. Neural crest cells undertake a number of different migratory paths and, upon reaching their destination, differentiate to produce diverse cell types and tissues including neurons, glial cells, the cartilage in the face and the smooth muscle of the heart and blood vessels.

Other research had previously suggested that neural crest-derived stem cells (NCSCs) are present in adult mice. However, these cells had not been characterized properly. Narihito Nagoshi, the lead author of the study, and his colleagues therefore generated transgenic mice that express green fluorescent protein (GFP) in migrating crest cells so that they could trace the cells.

Histological analysis of the animals showed that NCSCs were indeed present in various adult tissues, including the bone marrow of the tibia, the whisker pads and the dorsal root ganglia, which are located just outside the spinal cord and contain the cell bodies of primary sensory neurons.

Further investigation revealed that NCSCs reach the bone marrow by migrating through the blood stream, following the same migratory pathway taken by hematopoietic stem cells, which form blood cells (Fig. 1).

NCSCs from all tissues divided to renew themselves, but retained their 'multipotency' allowing them to differentiate into neurons, glia and smooth muscle when isolated from the animals and placed into culture dishes. For reasons that are still unclear, however, the proportion of cells from each source that could do so varied greatly—almost 75% of cells obtained from dorsal root ganglia remained multipotent, but only 3% obtained from bone marrow displayed this ability.

Nevertheless, the findings open new avenues for the development of cell transplantation therapies, notes Nagoshi. They suggest that stem cells could be harvested from, and then transplanted back into, the same patient. This would overcome the problem of graft rejection by the immune system, a major obstacle to researchers trying to develop such therapies.

"The identification of NCSCs in accessible tissues provides a possible new source of cells that can be utilized to treat nerve injury," says Nagoshi, "and this overcomes the ethical concerns associated with embryonic and fetal tissue-derived stem cells. We are now doing experiments to determine if NCSCs can be used to treat mice with spinal cord injuries." ■

1. Nagoshi, N., Shibata, S., Kubota, Y., Nakamura, M., Nagai, Y., Satoh, E., Morikawa, S., Okada, Y., Mabuchi, Y., Katoh, H., Okada, S., Fukuda, K., Suda, T., Matsuzaki, Y., Toyama, Y. & Okano, H. Ontogeny and multipotency of neural crest-derived stem cells in mouse bone marrow, dorsal root ganglia, and whisker pad. *Cell Stem Cell* **2**, 392–403 (2008).

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