Chugai Succeeds in Establishing Stable Cell Lines of Cancer Stem Cell for the First Time

- A Step Forward in Elucidating the Mechanisms of Recurrence / Metastasis and Drug Resistance of Cancer and in the Development of a New Therapeutic Agent -

CHUGAI PHARMACEUTICAL CO., LTD.
Research Division
Masami Suzuki

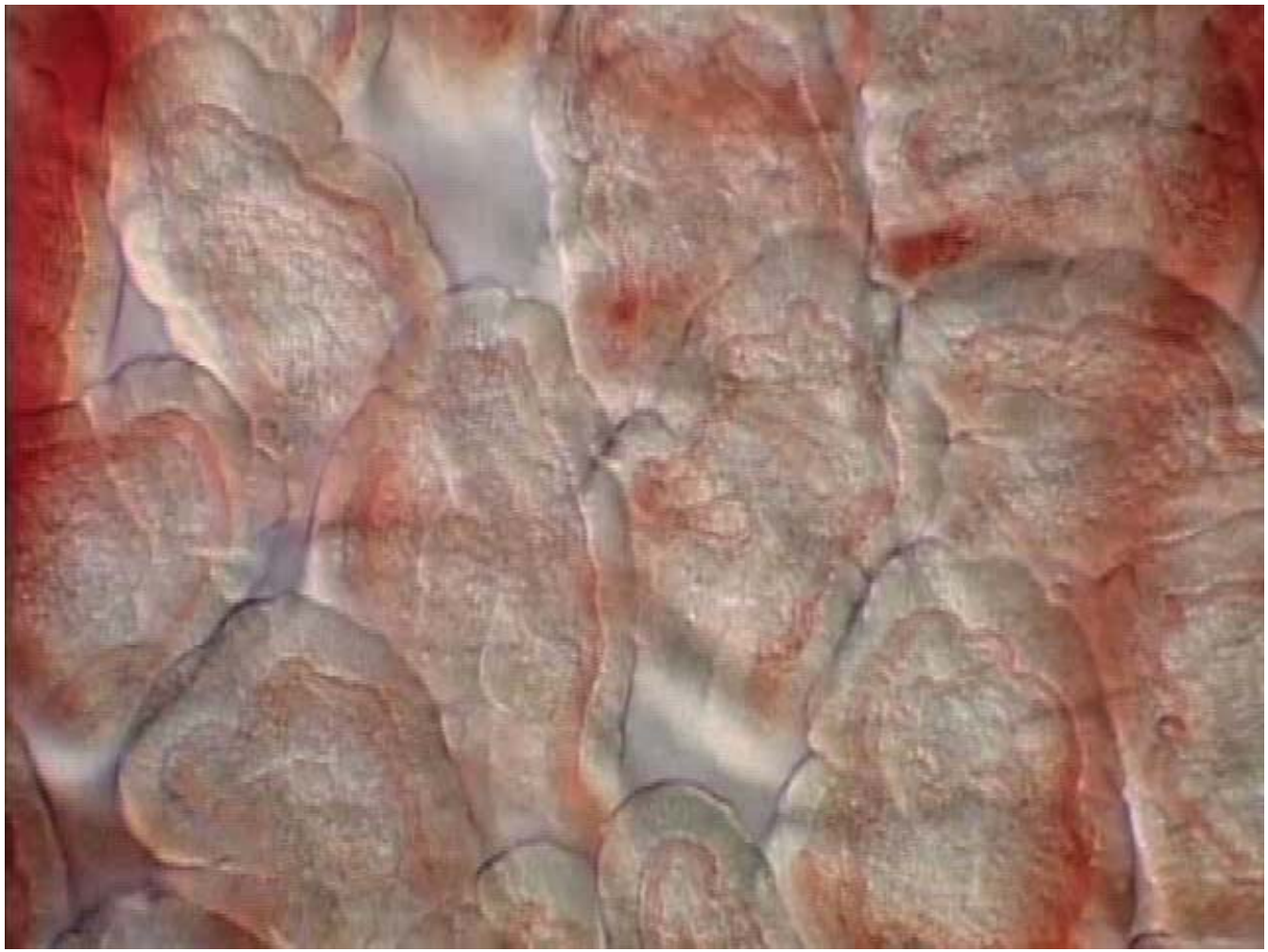
2012.11.16

Forward-Looking Statements

This presentation may include forward-looking statements pertaining to the business and prospects of Chugai Pharmaceutical Co., Ltd. (the "Company"). These statements reflect the Company's current analysis of existing information and trends. Actual results may differ from expectations based on risks and uncertainties that may affect the Company's businesses.

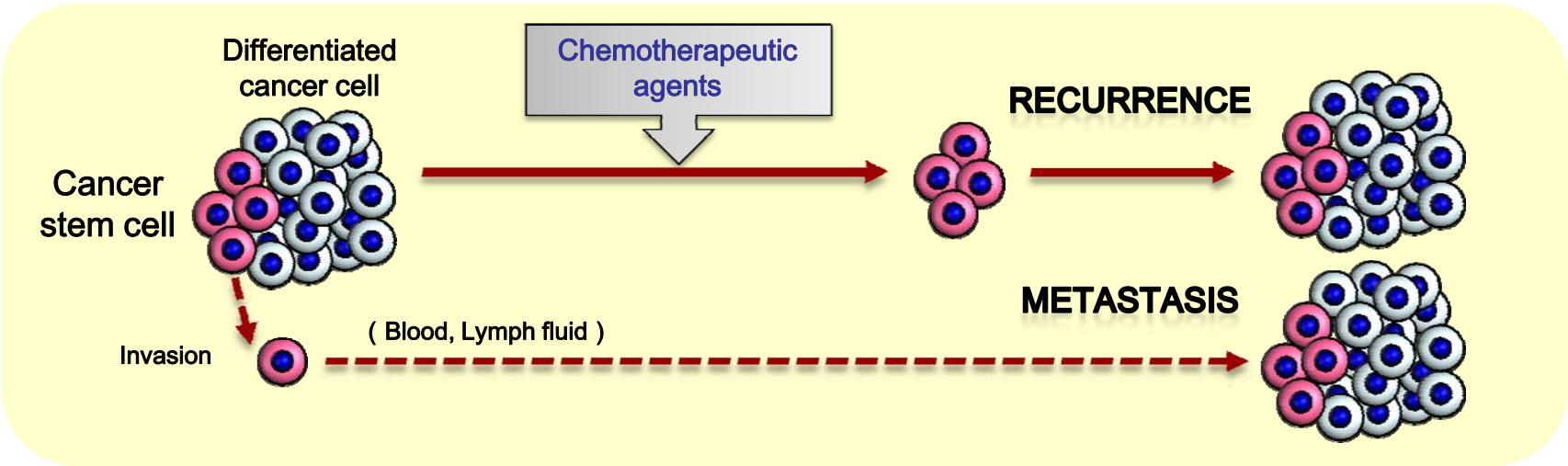
Stem Cells in Normal Small Intestine

- Epithelial cells that cover the surface of the intestine are renewed within 3 days.
- New epithelial cells are continuously generated from stem cells.



Cancer Stem Cells

- Definition (The American Association for Cancer Research, 2006)
  A cell within a tumor that possess the ability to self-renew and to generate progenies which commit to differentiation lineages, leading to a tumor hierarchy.
  → Tumor tissues are thought to arise from transformation of normal stem cells into cancer stem cells caused by mutations in the normal stem cells. The tumor tissues form a hierarchy of tumor cells consisting of a very small number of cancer stem cells and differentiated tumor cells.
  → The presence of cancer stem cells is considered to be one of the causes of resistance to anti-cancer agents and radiation therapy, and also of recurrence and metastasis.



Isolation of Cancer Stem Cells

Tumor tissues contain only a small number of cancer stem cells but a large number of differentiated cancer cells. Although several methods have been employed for isolation and identification of cancer stem cells, it remains difficult to establish cancer stem cell lines that can be maintained with high purity.

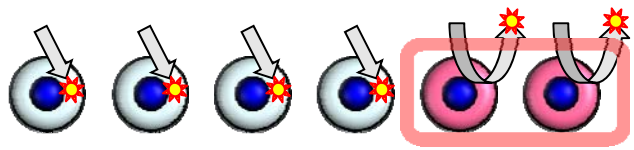
Previous approaches for isolation of cancer stem cells

◆Spheroid formation

Cancer stem cells are able to form spheroids from a single cell in a non-adherent culture condition. Cancer stem cells are concentrated in the spheroids.

◆Side population

Cancer stem cells are thought to actively export anti-cancer agents from inside the cells. The ability of cancer stem cell to eliminate anti-cancer agent can be used for isolation.



◆Cell surface markers

Antibodies against molecules that are preferably expressed in cancer stem cells can be used for isolation of cancer stem cells.

Our Recent Publication

We have established purified colon cancer stem cell lines and have unveiled characteristics of colon cancer stem cells.

LGR5-positive colon cancer stem cells interconvert with drug-resistant LGR5-negative cells and are capable of tumor reconstitution

STEM CELLS 2012; 30: 2631–2644

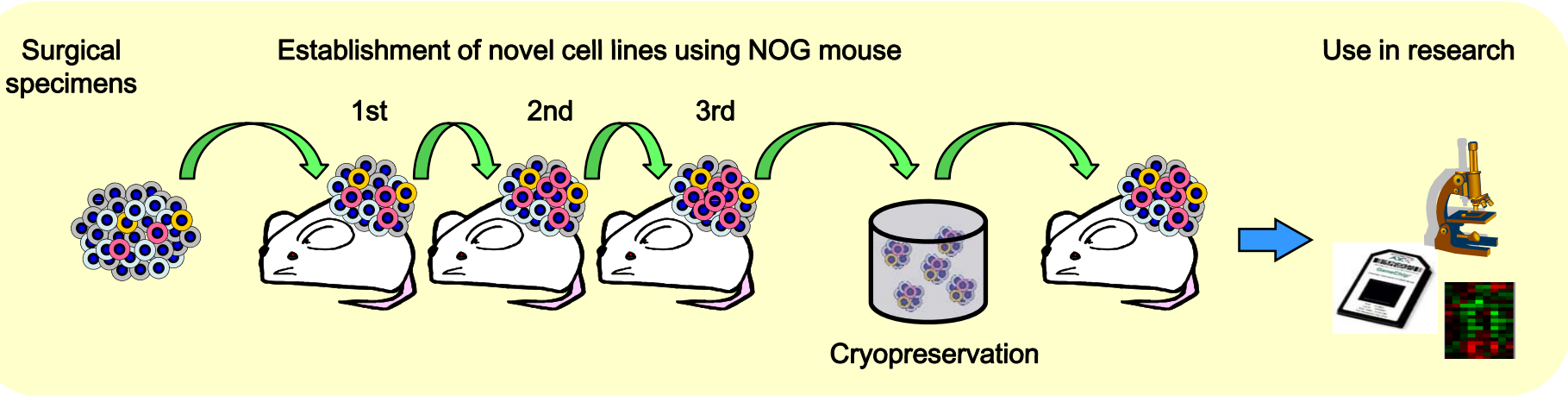
Background

- We found that when colon cancer tissues from patients were serially transplanted into NOG mice, tumors with the same morphology were repeatedly formed. This leads to the notion that cancer stem cells were always present in the transplanted cancer tissues. We also realized that the cancer stem cells were enriched during the passages of the cancer tissues in NOG mice. This prompted us to isolate cancer stem cells from the tumor tissues and establish a cancer stem cell line that can be stably cultured *in vitro*.
- After many attempts we were able to establish stable cancer stem cell lines by an unusual culture condition for stem cells, the adherent culture method.
- We hypothesized that cancer stem cells survive by changing their phenotypes depending on environment. So we examined the phenotypes caused by anti-cancer drug administration.

Serial Passage of Colon Cancer Tissues in NOG Mice

- Serial passage of surgically resected colon cancer tissues was carried out at PharmaLogicals Research (Singapore).

Process for the establishment of colon cancer cell lines

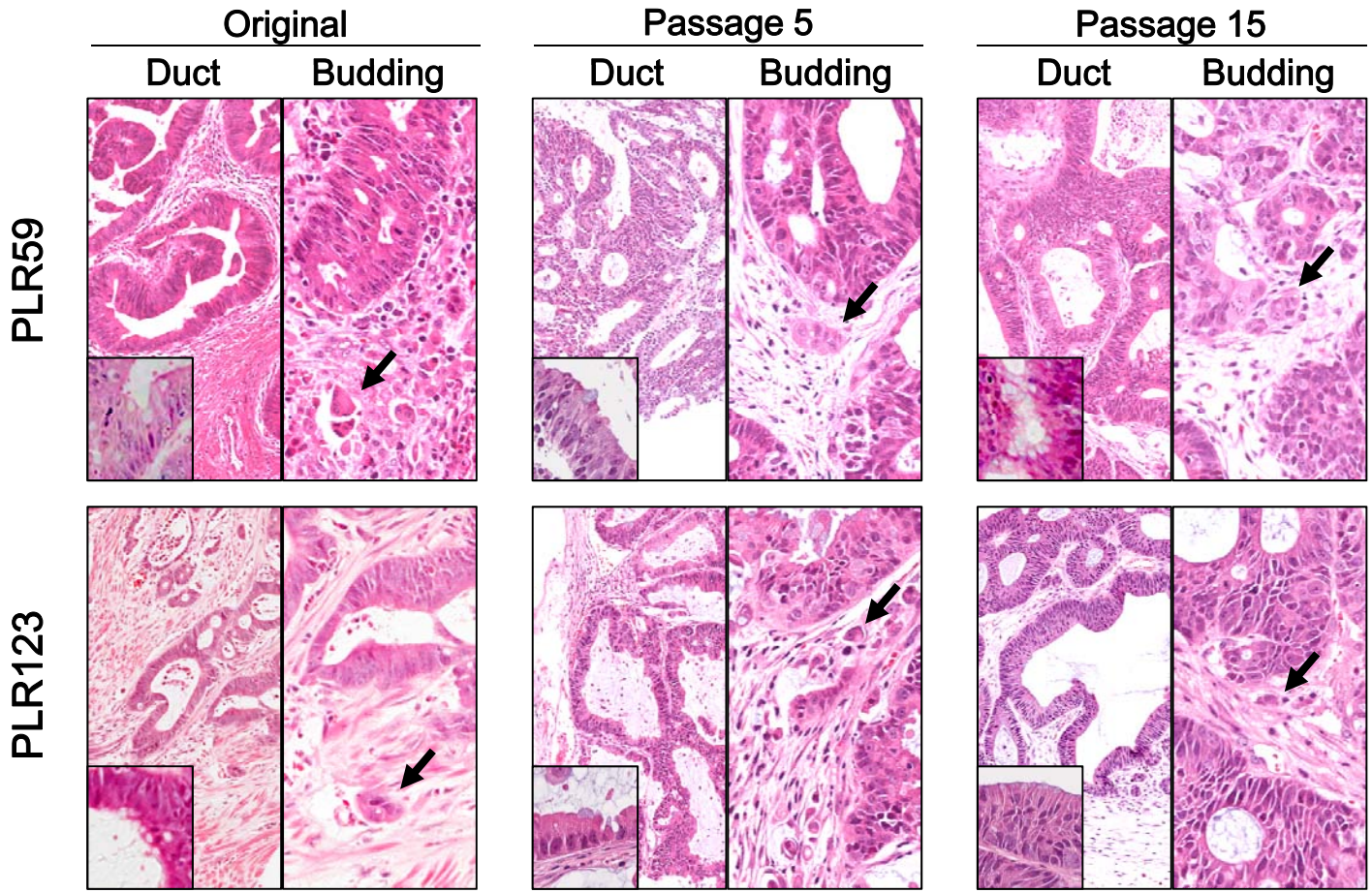


- Immunodeficient mice are key as a host animal for transplantation of cancer tissues and examination of the reconstitution of tumor hierarchy.
- Highly Immunodeficient mice (NOG mice) developed at the Central Institute for Experimental Animals were used for this study.

Histopathology of Colon Cancer Tissues Passaged in NOG Mice

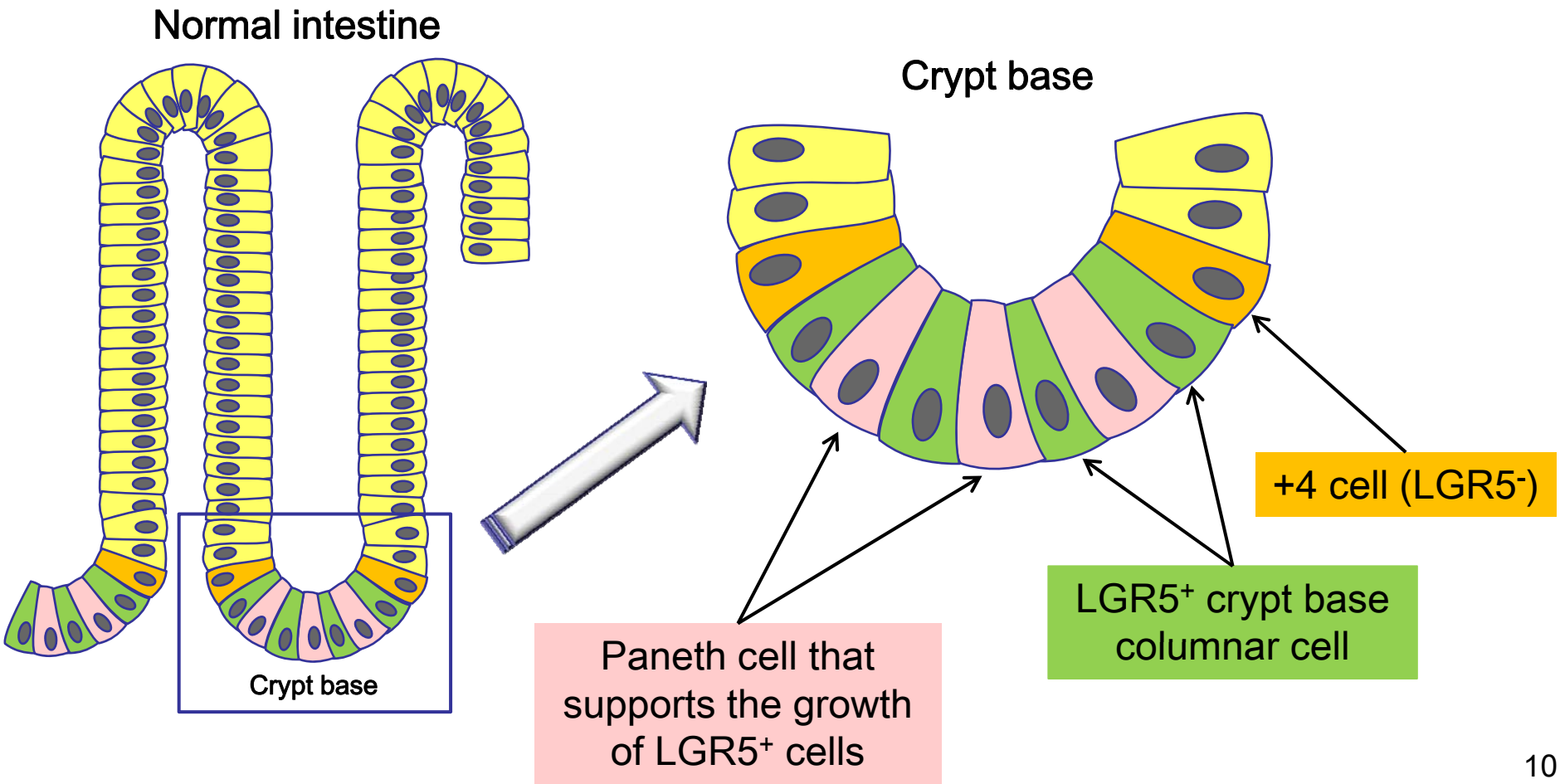
- The hierarchy and tumor tissue morphology, including tumor budding was maintained throughout several passages

  ⇒ possibility of the presence and maintenance of cancer stem cells



LGR5, a Marker for Normal Intestinal Stem Cells

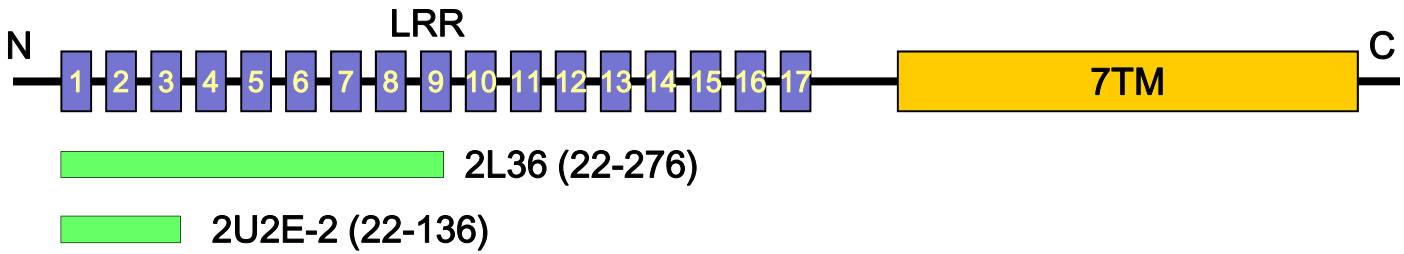
- Two types of stem cells have been reported: slow cycling stem cells (LGR5-negative) in the +4 position and proliferating stem cells (LGR5-positive) in the crypt base.



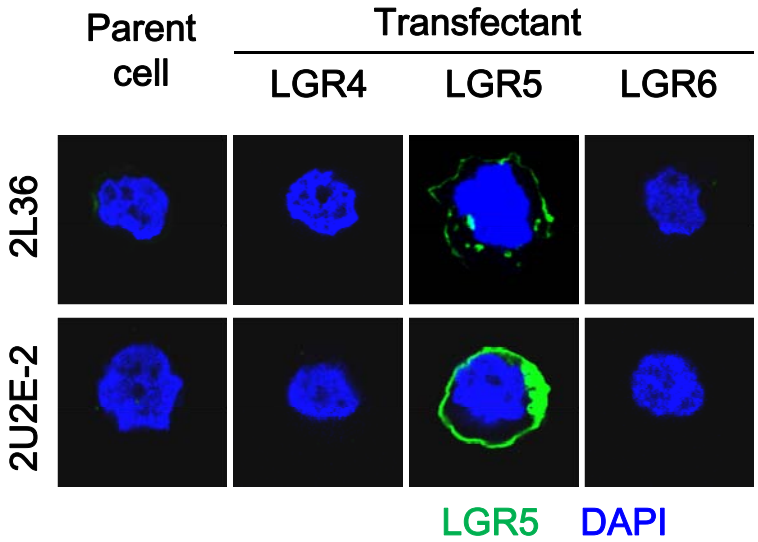
Specific Antibodies against LGR5

- Two anti-LGR5 antibodies, 2L36 and 2U2E-2, were generated. The antibodies are valuable tools for cancer stem cell research.

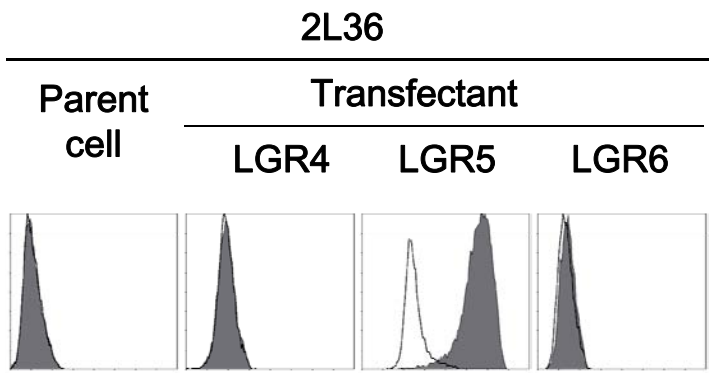
Epitopes of the anti-human LGR5 monoclonal antibodies



Immunocytochemistry



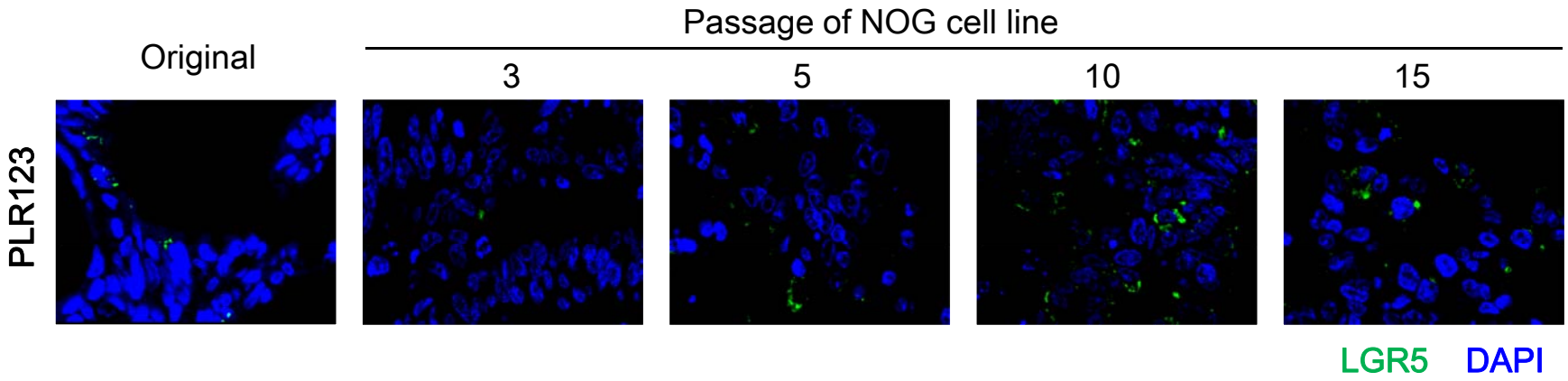
Flow cytometry analysis



Accumulation of LGR5+ Cells during Passages of Colon Cancer Tissues in NOG Mice

- LGR5+ cells were detected in the original human tumor tissues and in the xenotransplanted tumor tissues throughout the passages.

Immunostaining of LGR5 in the surgically resected tumors and xenografts



- In the xenotransplanted tumor tissues, the frequency of LGR5+ cells and tumor initiating activity increased during the passages.

Tumor initiating activity of the cells from xenografts of PLR123

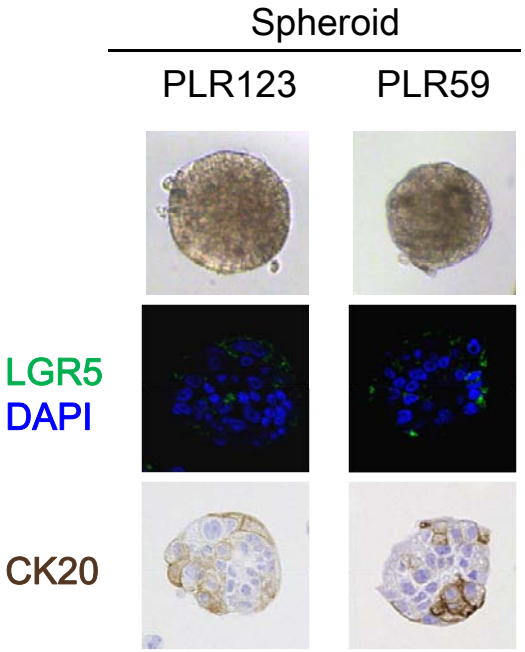
Passage	Cell number per inoculation site			Estimated CSC frequency
	1,000	100	10	
5	5/6	0/6	0/6	1/720
14	6/6	2/6	0/6	1/234

Tumor growth was determined 49 days after inoculation of tumor cells into NOG mice.

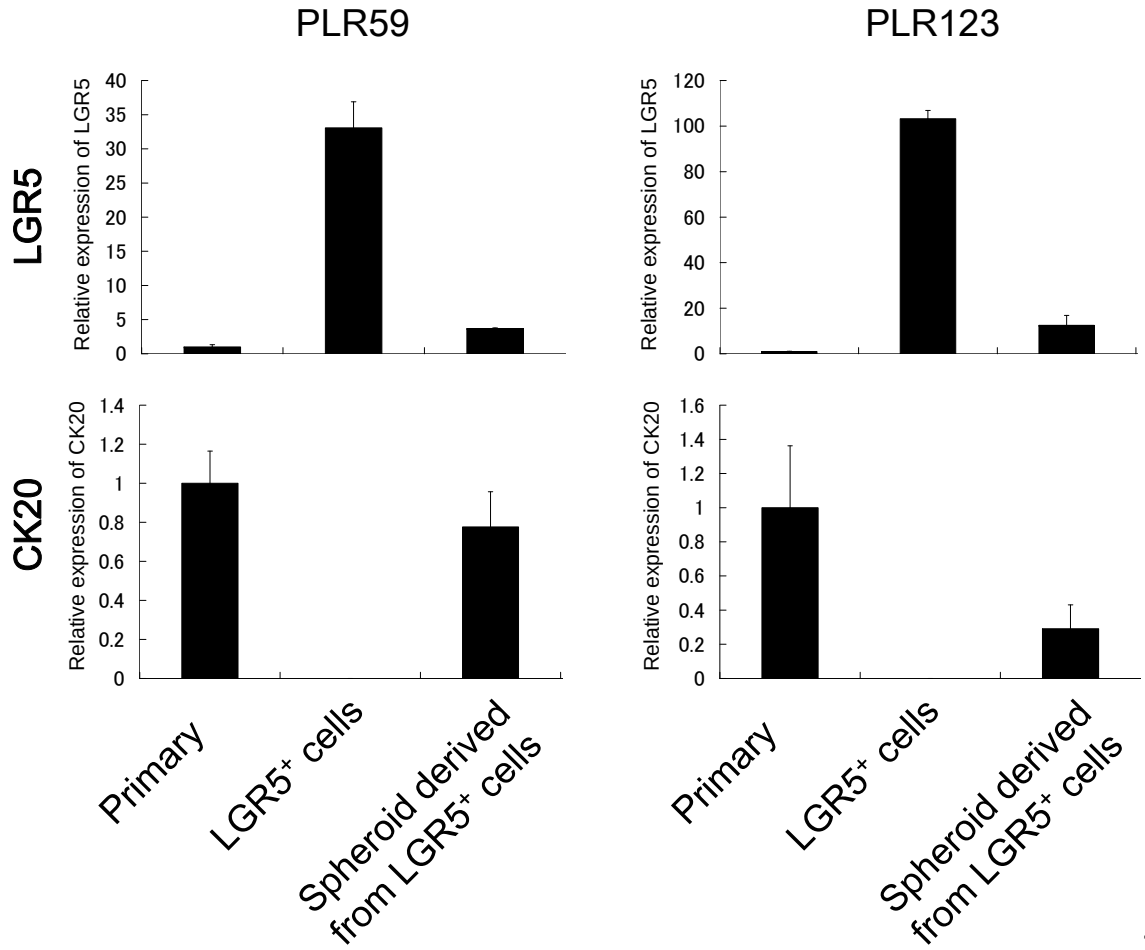
A Cell Line of Pure Cancer Stem Cells could not be Established by Spheroid Culture

- Spheroid contained only a few LGR5⁺ cells but more differentiated cells (CK20-positive cells).

Immunocytochemistry



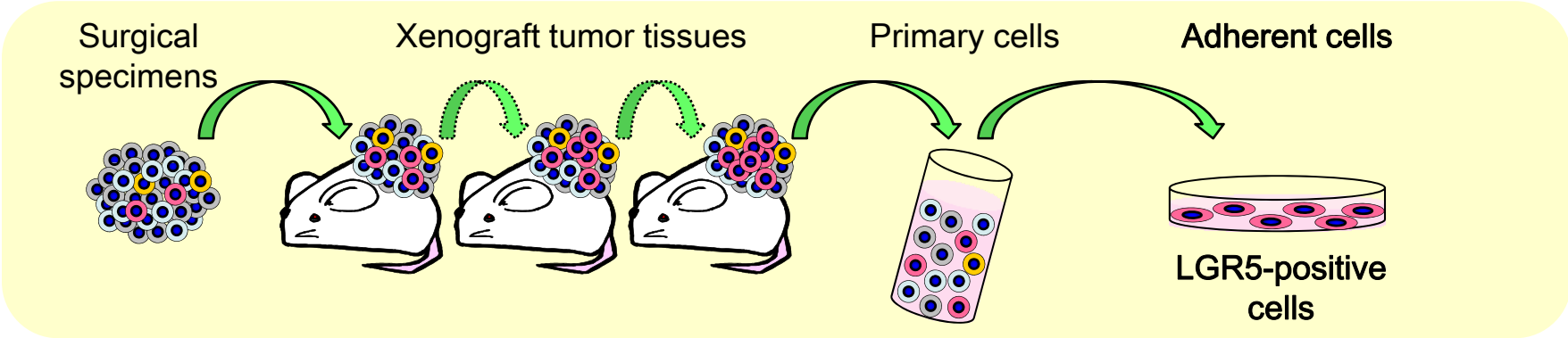
Expressions of LGR5 and CK20 mRNA



Establishment of Colon Cancer Cell Lines with Cancer Stem Cell (CSC) Properties

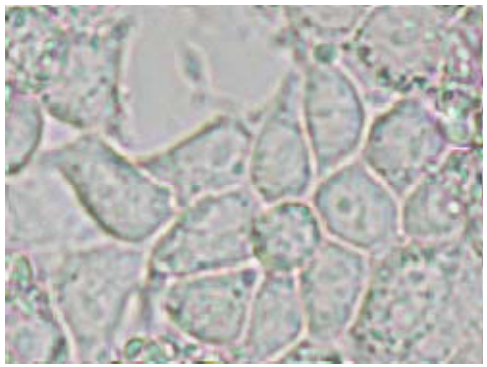
- Two human colon CSC lines were established after serial passages of xenografts in NOG mice and subsequent adherent culture of the cells.

Process for the establishment of colon CSC lines

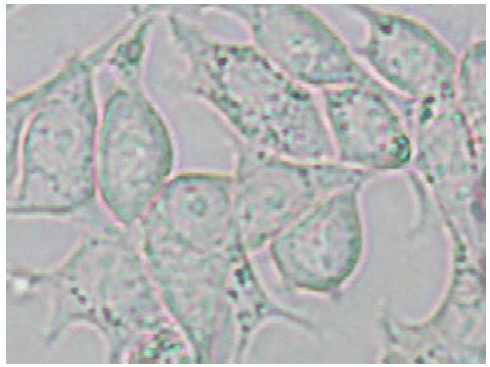


Phase contrast microscopy of the LGR5⁺ cell lines

PLR59



PLR123



Confirmation of Cancer Stem Cell Properties

- Requirements for the definition of cancer stem cell

1. Tumor initiating activity
2. Self-renewal by symmetrical division
3. Generation of progeny that commits to differentiation lineage by asymmetrical division

4. Resistance to anti-cancer drugs

Tumor Initiating Activity (TIA) of LGR5$^+$ Cells

- Adherent culture more efficiently enriched the cells possessing TIA.

TIA of the cells from the primary, spheroid and adherent

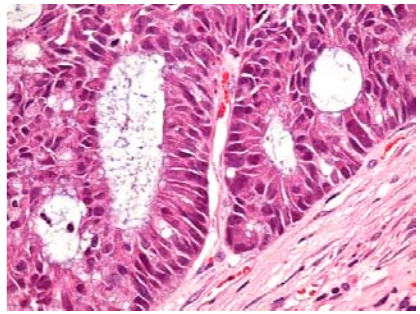
Line	Preparation	Cell number per inoculation site				Estimated density of CSCs
		1,000	100	10	1	
PLR59	Primary	12/12	5/12	0/12	-	1/195
	Spheroid formed from primary cell	6/6	6/6	1/6	-	1/31
	Adherent (LGR5$^+$ CSCs)	**6/6**	**6/6**	**6/6**	**1/12**	**1/4**
PLR123	Primary	12/12	6/12	0/12	-	1/161
	Spheroid formed from primary cell	6/6	5/6	2/6	-	1/45
	Adherent (LGR5$^+$ CSCs)	**6/6**	**6/6**	**6/6**	**2/12**	**1/4**

Tumor growth was determined 70 days after inoculation of tumor cells into NOG mice.

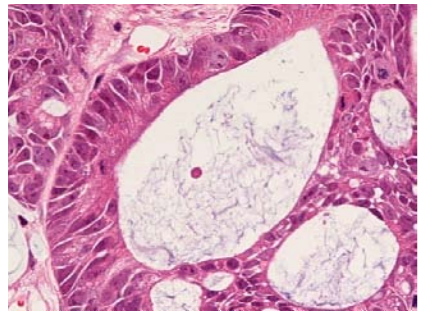
- The histopathological morphology of the tumors derived from the adherent cultured cells was almost the same as the original tumors.

Histopathology of xenograft derived from the adherent cultured cells (PLR123)

10 cells

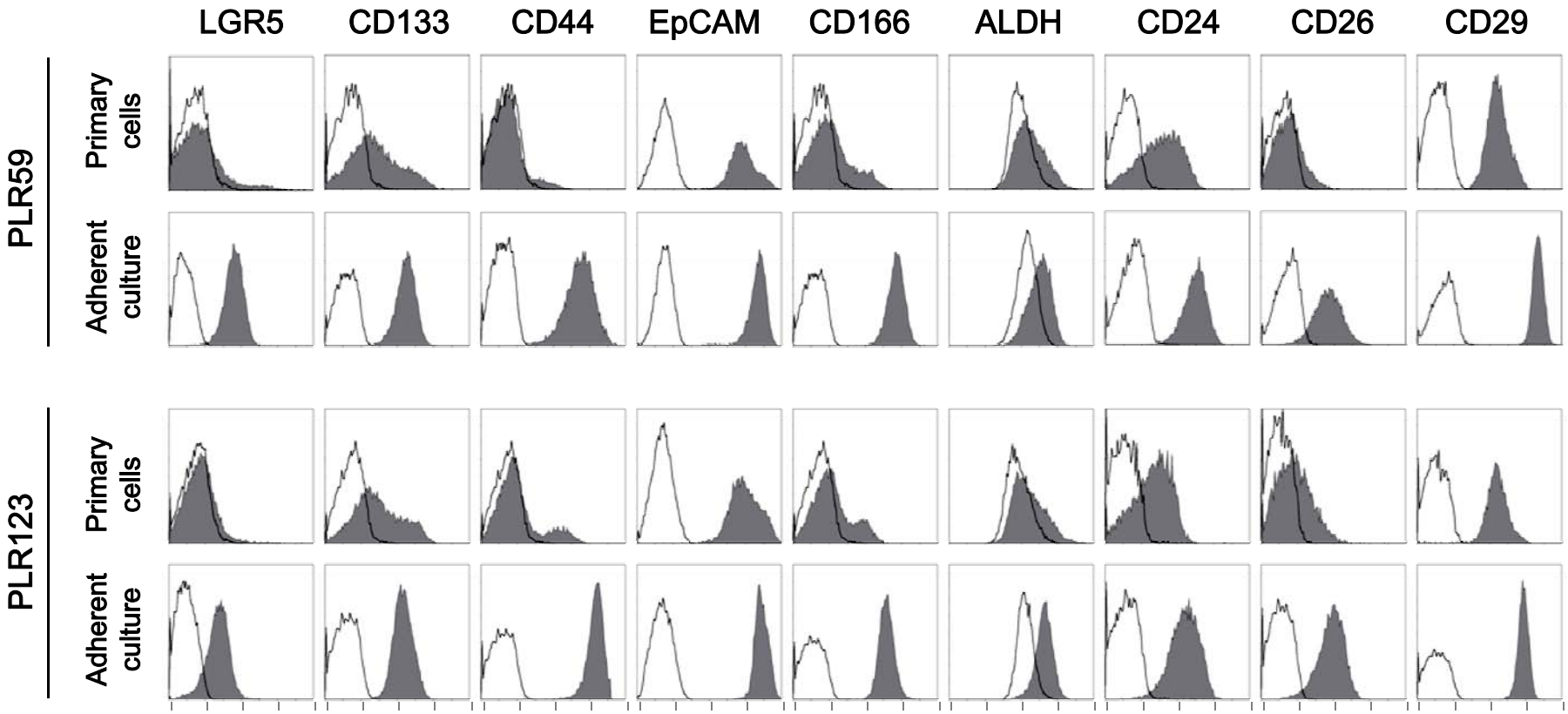


Single cell



Cell Surface Markers of the LGR5+ Cells

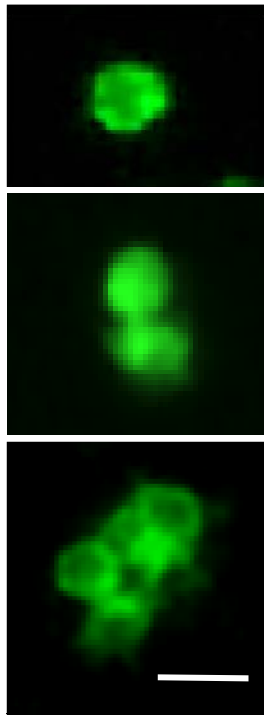
- The adherent cultured cells were clearly positive for all known colon CSC markers.



Symmetrical and Asymmetric Cell Division of LGR5$^+$ Cells *in vitro*

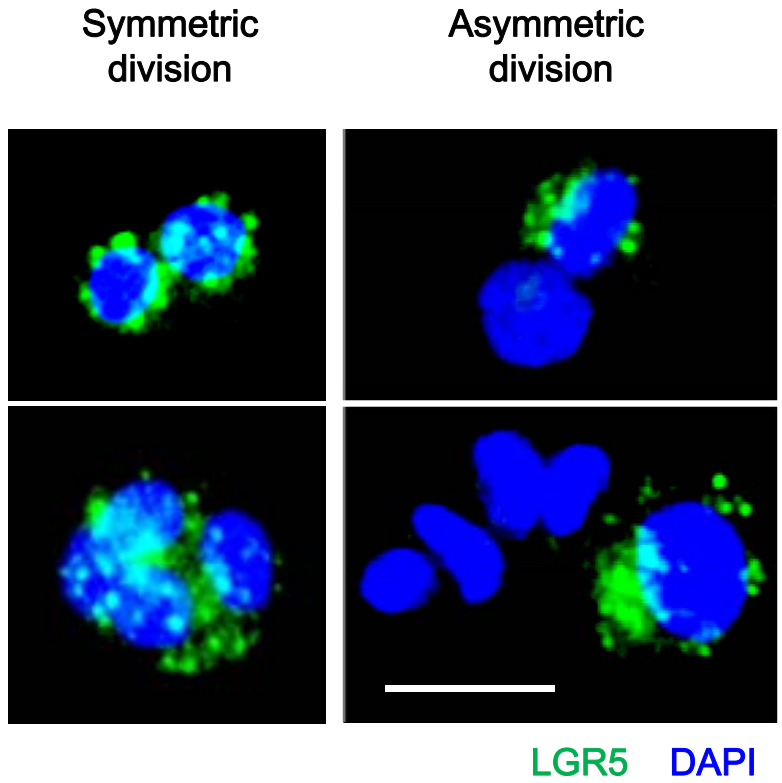
- The LGR5$^+$ cells divided symmetrically under the adherent culture conditions, but they underwent asymmetrical cell divisions in the presence of matrigel and FBS.

Symmetrical division



Symmetrical division of the LGR5$^+$ CSCs stained with PKH67 dye (PLR123)

Symmetrical and asymmetrical divisions



Symmetrical and asymmetrical divisions of the LGR5$^+$ CSCs in the presence or absence of matrigel and FBS (PLR123)

Confirmation of Cancer Stem Cell Properties

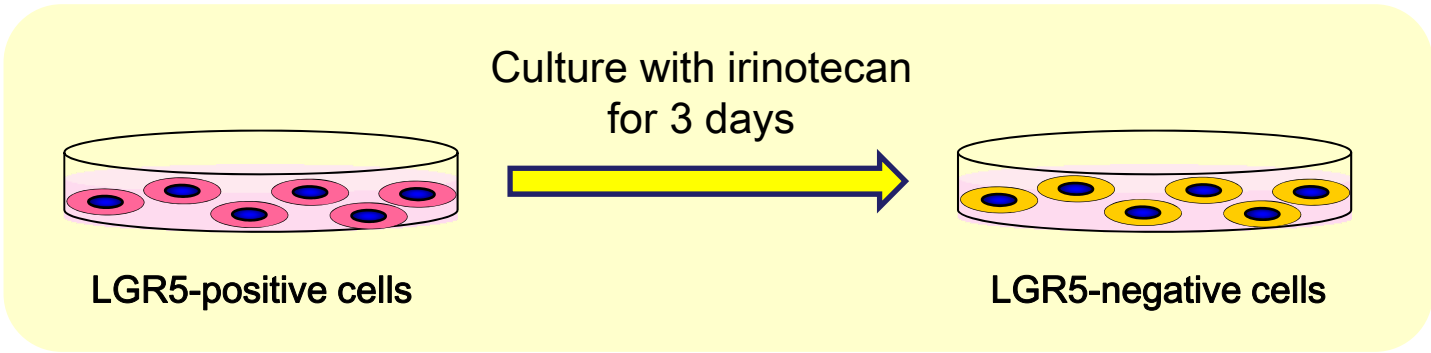
- Requirements for the definition of cancer stem cell

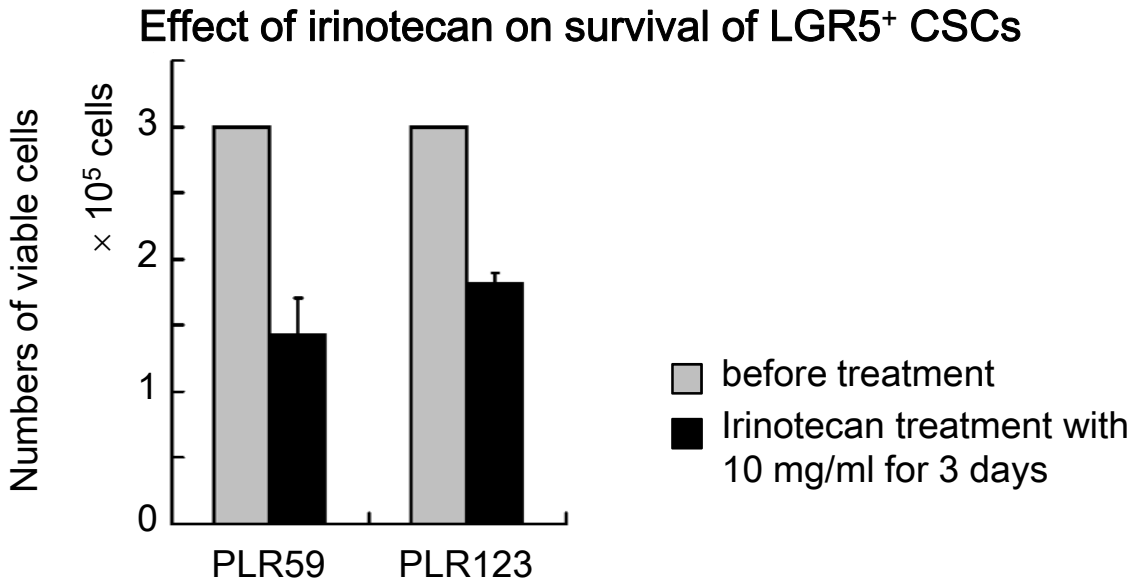
1. Tumor initiating activity
2. Self-renewal by symmetrical division
3. Generation of progeny that commits to differentiation lineage by asymmetrical division

4. Resistance to anti-cancer drugs

Drug-resistant LGR5$^-$ CSCs Induced by Irinotecan

- After treatment of the LGR5$^+$ CSCs with irinotecan, drug resistant LGR5$^-$ CSCs appeared.





Cells were cultured for 3 days under an adherent condition.

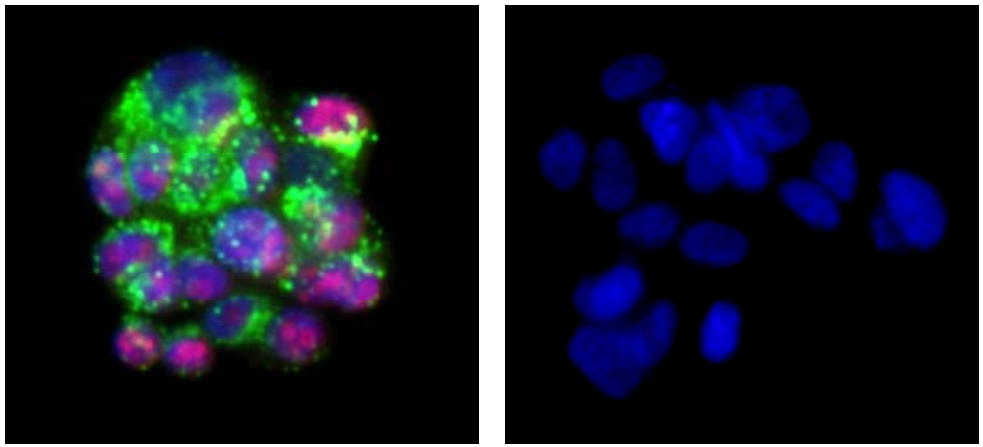
Drug-resistant LGR5^{-} CSCs Induced by Irinotecan

- The drug-resistant LGR5^{-} CSCs showed a non-proliferating state, and retained undifferentiated characteristics.

Ki67 staining of the LGR5^{+} and LGR5^{-} CSCs (PLR123)

Irinotecan treatment

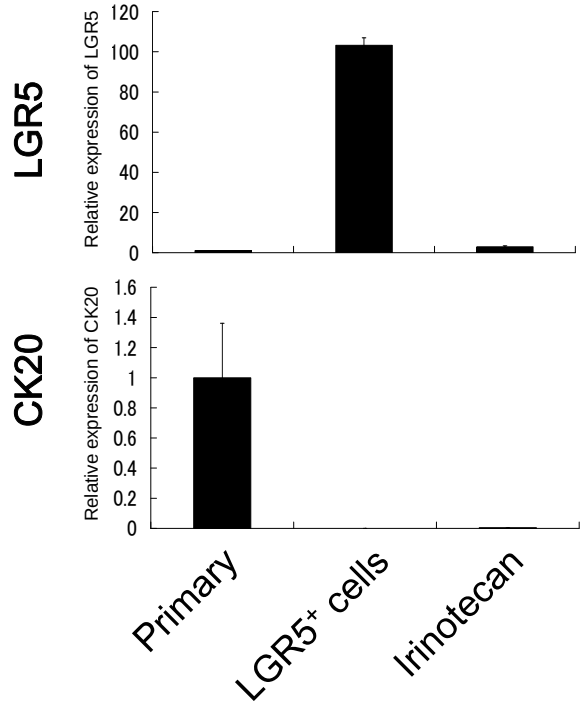
Before | After



Ki67 LGR5 DAPI

10 μg/mL of irinotecan for 3 days

Expressions of LGR5 and CK20 mRNA (PLR123)

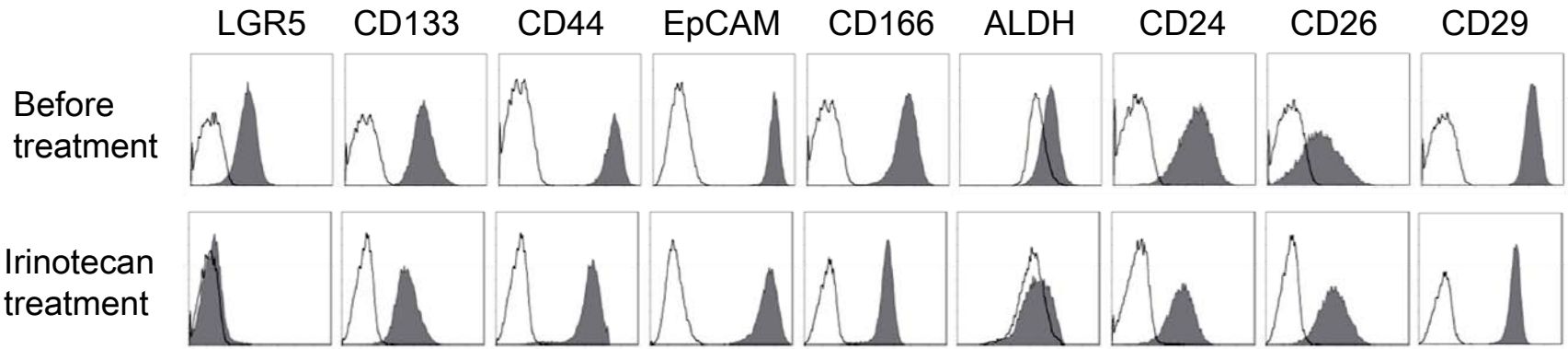


10 μg/mL of irinotecan for 3 days

Properties of the Drug-resistant LGR5$^-$ CSCs

- The LGR5$^-$ CSCs were positive for other colon CSC markers.

Flow cytometry analysis of CSC markers (PLR123)



10 μg/mL of irinotecan for 3 days

- The LGR5$^-$ CSCs retained tumor initiating activity.

Tumor initiating activity of LGR5$^-$ CSCs

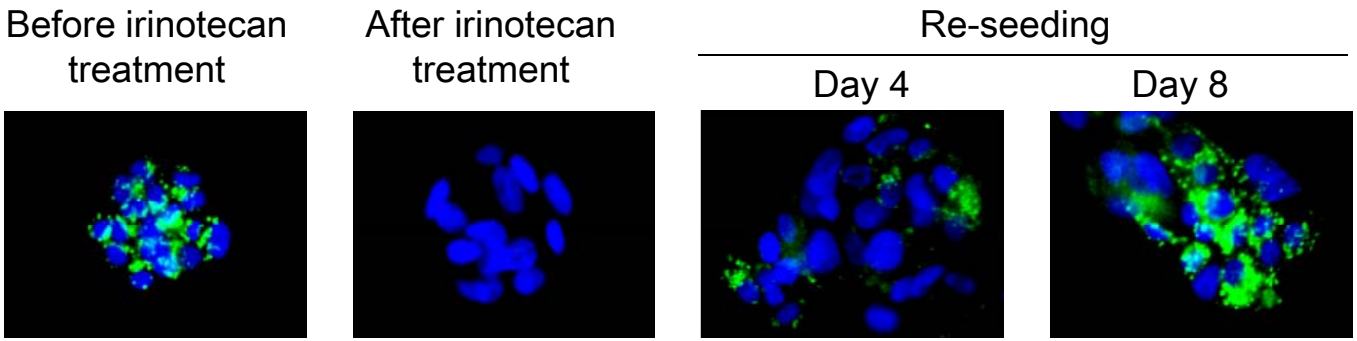
Line	Cell number per inoculation site		
	1,000	100	10
PLR59	6/6	6/6	2/6
PLR123	6/6	6/6	1/6

Tumor growth was determined 49 days after inoculation of tumor cells into NOG mice.

Transition from LGR5⁻ State to LGR5⁺ State *in vitro*

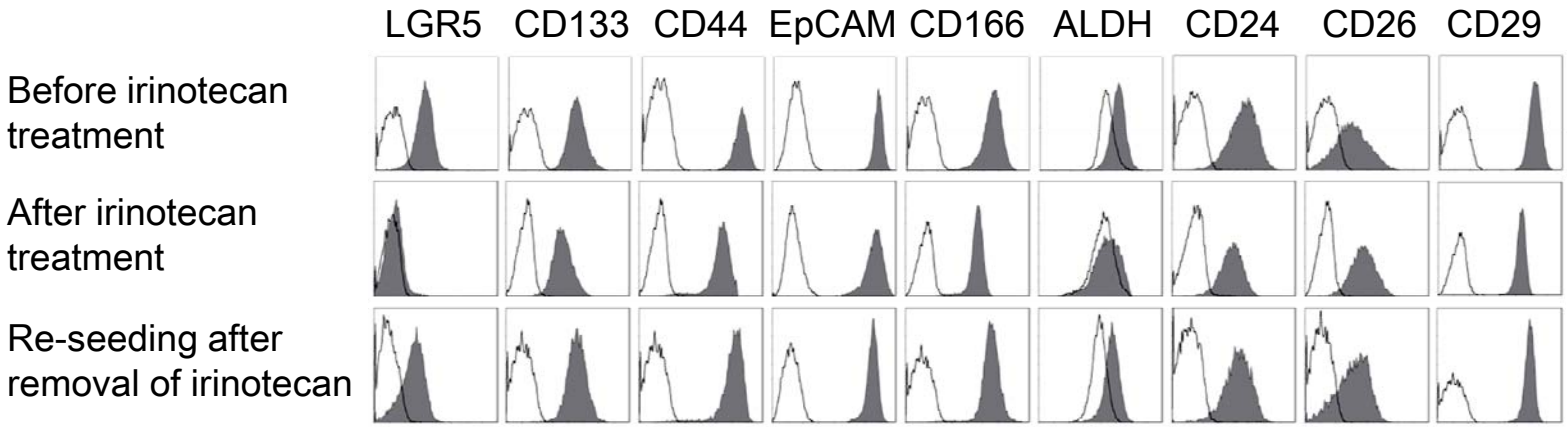
- The LGR5⁻ CSCs did not grow even after irinotecan was removed from the culture medium. They became LGR5⁺ and resumed proliferation after re-seeding.

Immunostaining of LGR5 after treatment of the LGR5⁺ CSCs with irinotecan (PLR123)



10 μg/mL of irinotecan for 3 days

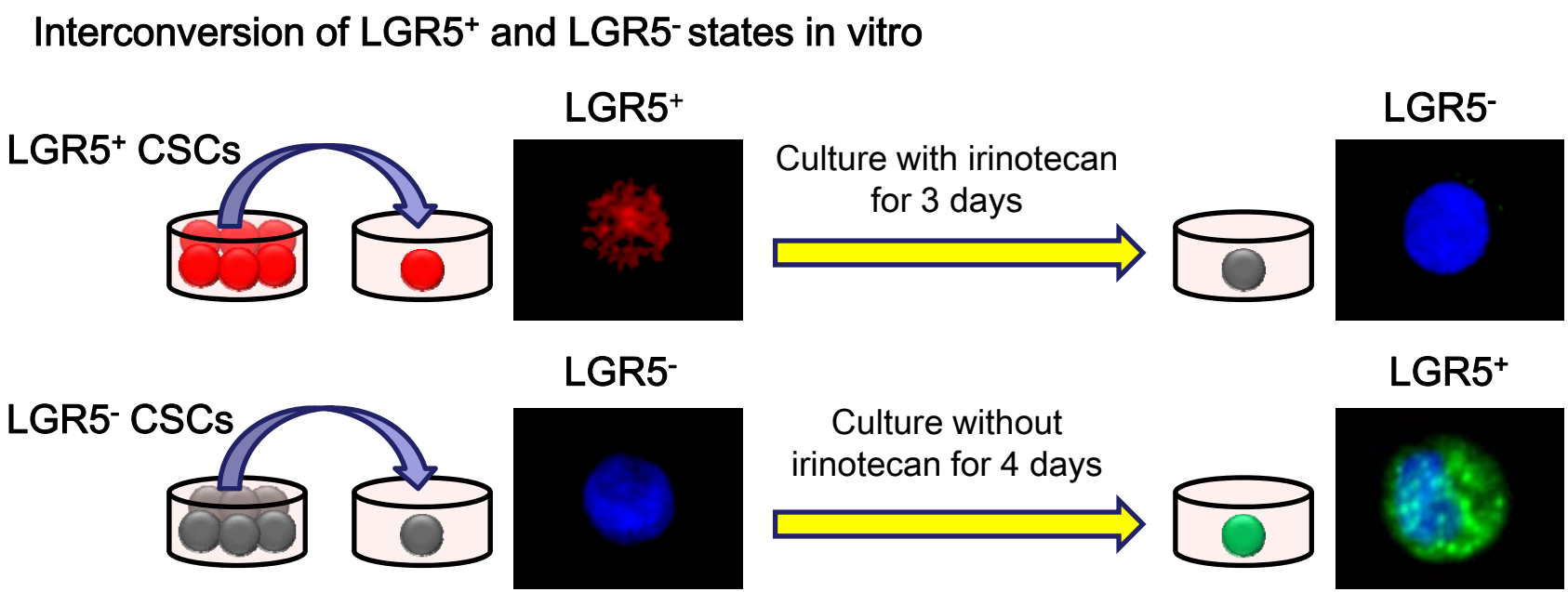
CSC markers of the LGR5⁺ CSCs before and after treatment with irinotecan (PLR123)



10 μg/mL of irinotecan for 3 days

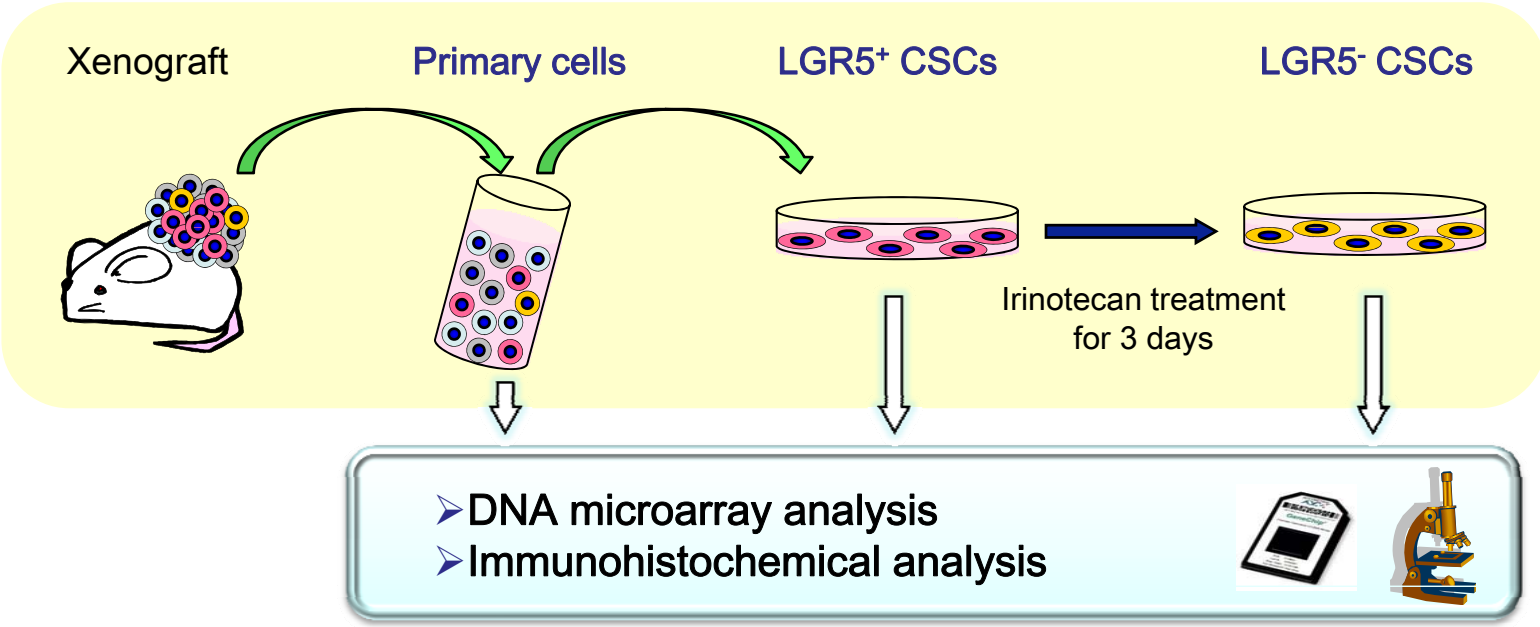
Interconversion between LGR5^{+} and LGR5^{-} States *in vitro*

- The transition from the Lgr5^{+} state to an Lgr5^{-} state and vice versa was confirmed by single cell culture.



Molecules that can Identify LGR5^{-} CSCs

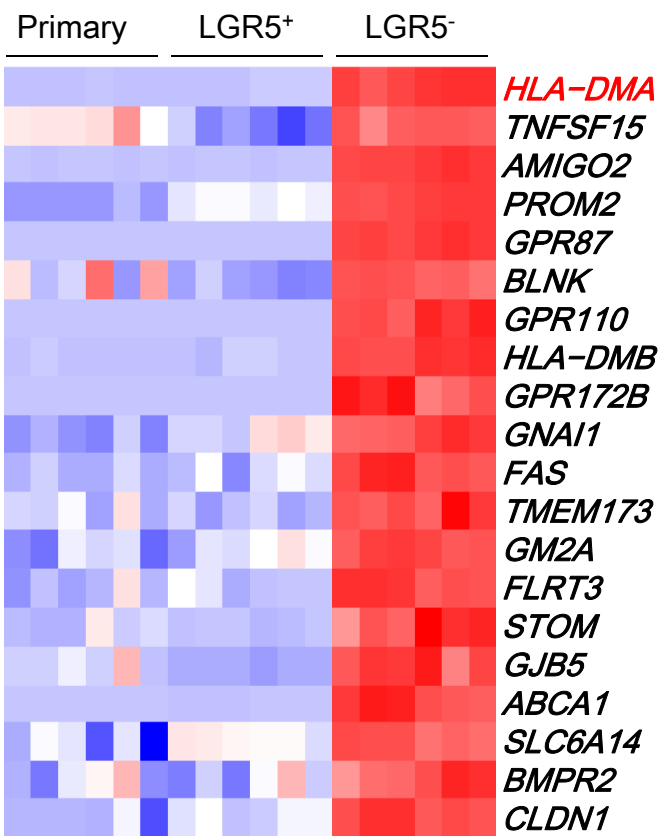
- To obtain marker molecules for identification of LGR5^{-} CSCs, DNA microarray analysis and immunohistochemical analysis were performed.



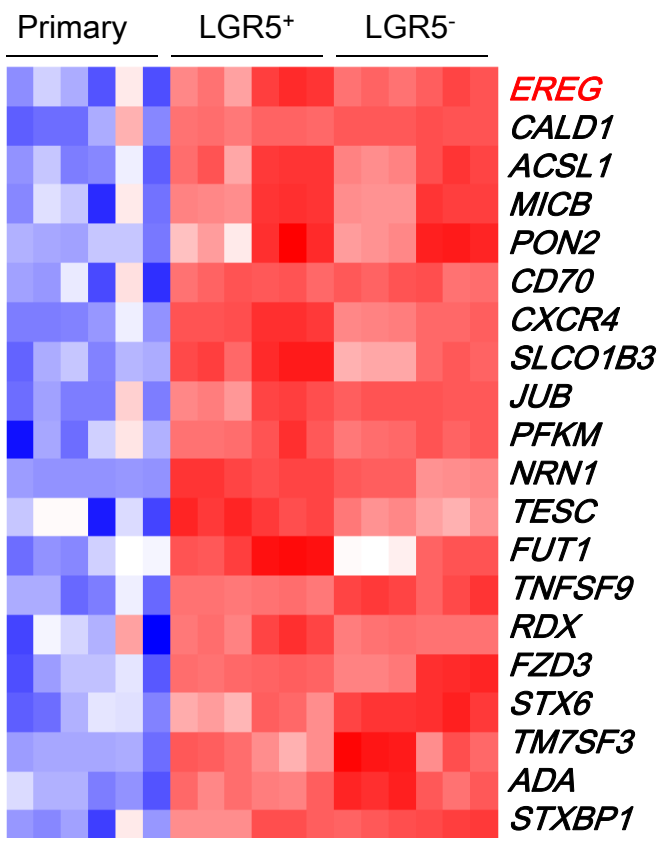
Molecules that can Identify LGR5^{-} CSCs

- HLA-DMA was specifically expressed in LGR5^{-} CSCs and epiregulin (EREG) was expressed in both LGR5^{+} and LGR5^{-} CSCs.

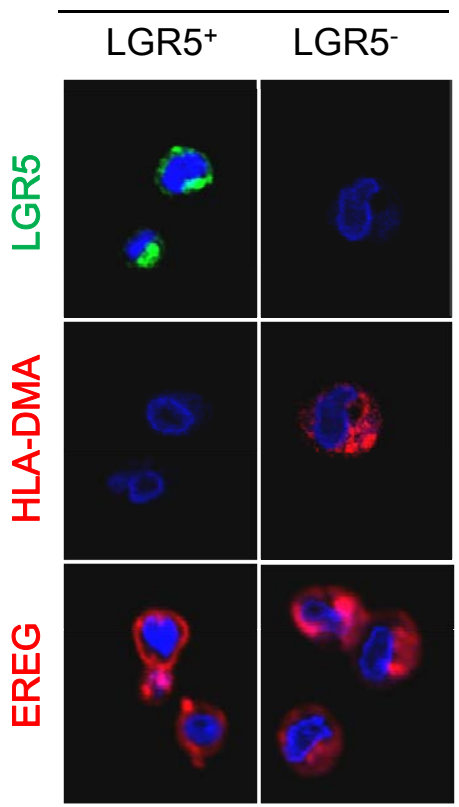
Upregulated in LGR5^{-} CSCs



Upregulated in LGR5^{+} and LGR5^{-} CSCs



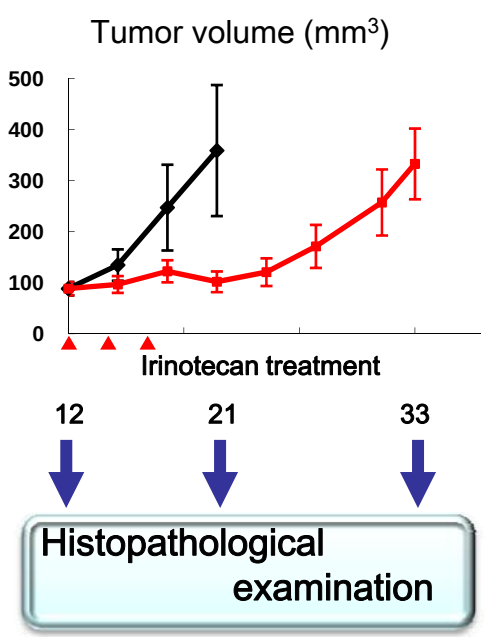
Expression of HLA-DMA and EREG in CSCs

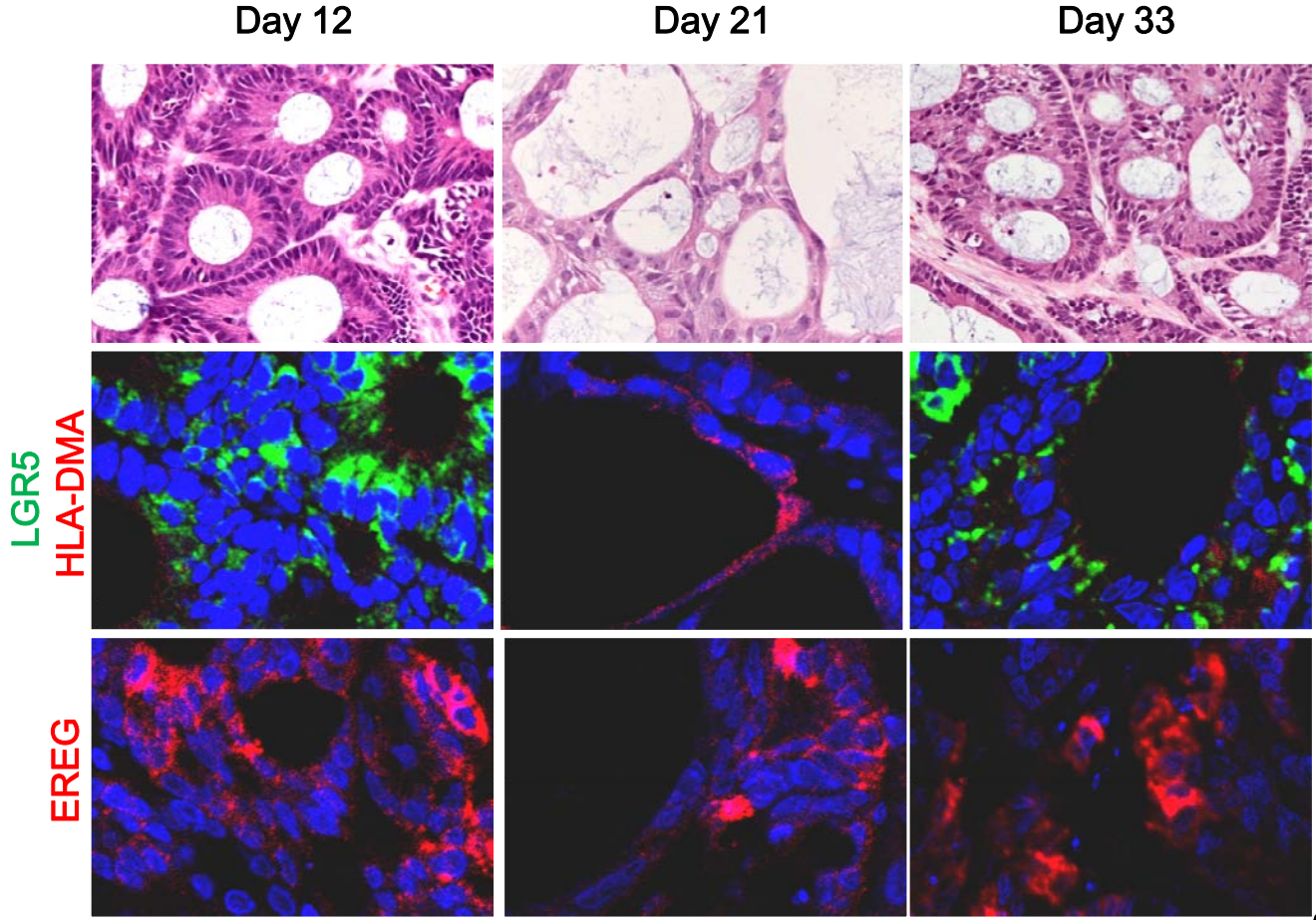


Conversion from LGR5^{+} CSCs to LGR5^{-} CSCs *in vivo*

- Anticancer drug diminished LGR5^{+} CSCs and increased LGR5^{-} CSCs *in vivo*. LGR5^{+} CSCs re-appeared after termination of drug treatment.

Histopathology and immunostaining of the tumors after treatment of irinotecan (PLR123)

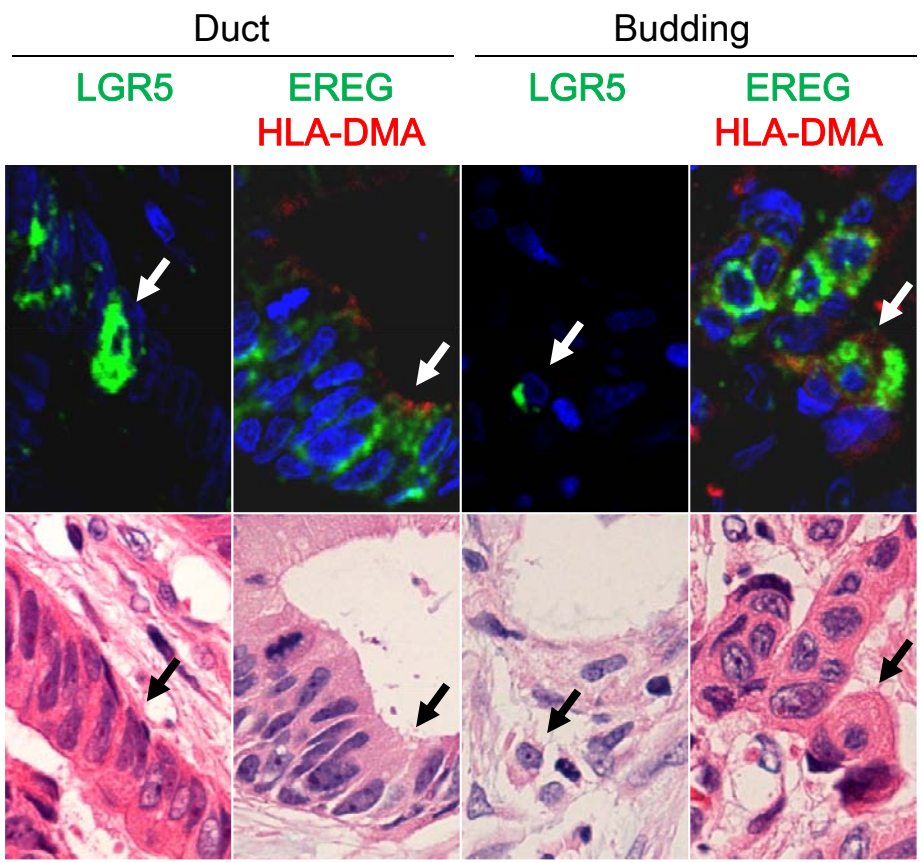




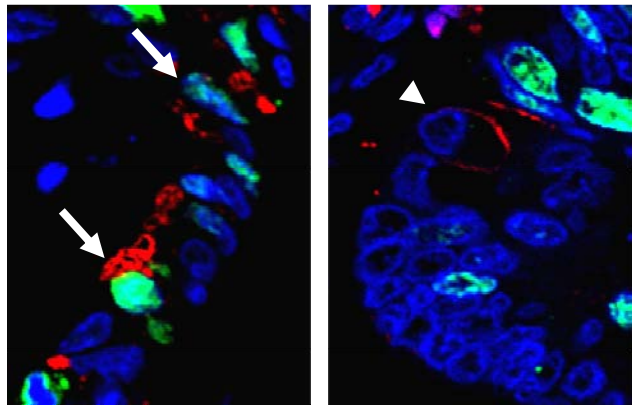
Existence of LGR5^{+} and LGR5^{-} CSCs in Human Colon Cancers

- LGR5^{+} CSCs (Ki67^{+}) and LGR5^{-}/HLA-DMA^{+}/EREG^{+} CSCs (Ki67^{-}) were present in colon cancer tissues from patients.
- Percentages of the LGR5^{+} and LGR5^{-} CSCs in 12 specimens ranged between 0.003-1.864% for the LGR5^{+} CSCs and 0.001-0.243% for the LGR5^{-} CSCs.

Presence of LGR5^{+} and LGR5^{-} CSCs in colon cancer tissues from patients



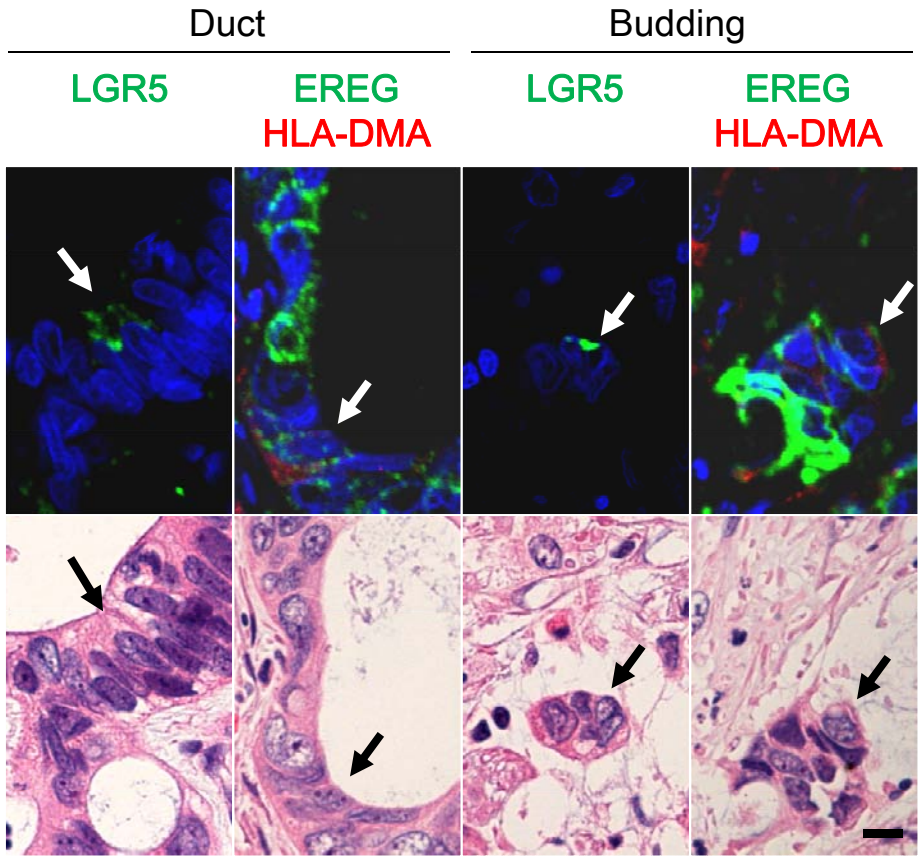
Ki67 staining of the LGR5^{+} and the LGR5^{-} / HLA-DMA^{+} CSCs in colon cancer tissues from patients



LGR5^{+} and LGR5^{-} CSCs in Metastatic Colon Cancer Tissues

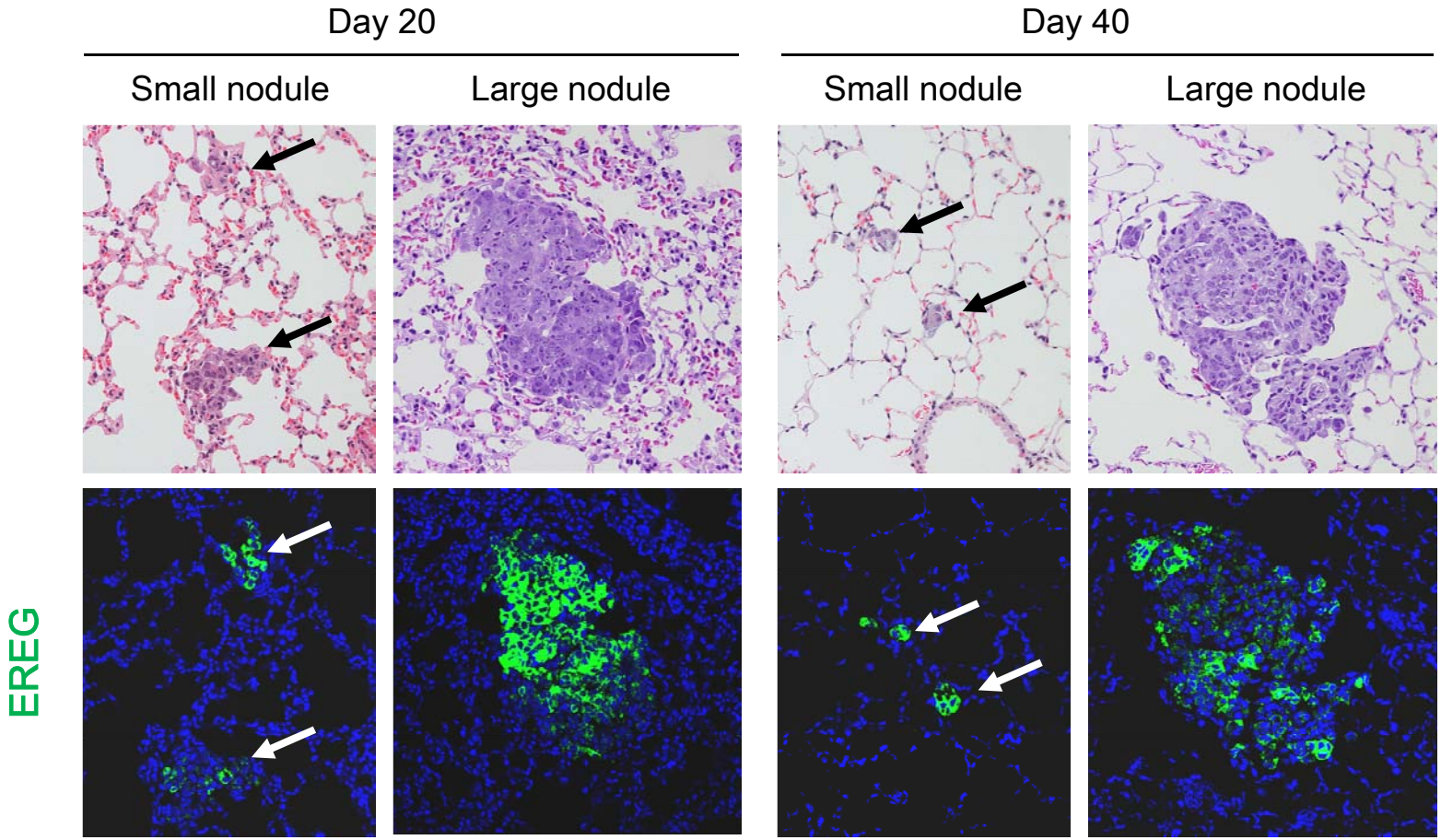
- LGR5^{+} and LGR5^{-}/HLA-DMA^{+}/EREG^{+} CSCs were also present in metastatic colon cancer tissues from patients.

Presence of LGR5^{+} and LGR5^{-} CSCs in liver metastatic tumors from patients



Expression of EREG in Metastasized Tumors in a Mouse Model

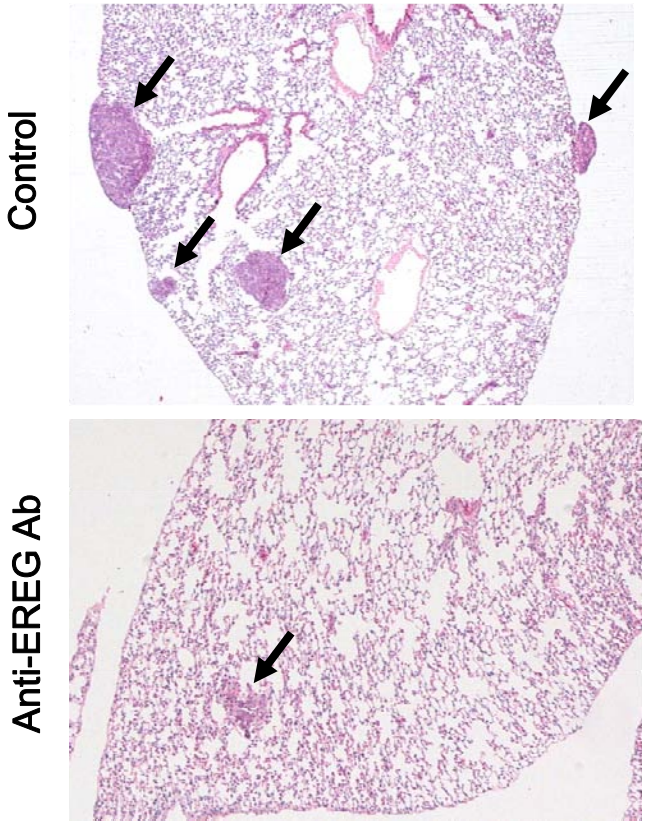
- For tumors developed in the lung, the majority of tumor cells were EREG positive at both 20 and 40 days after the injection of LGR5^{+} cells.



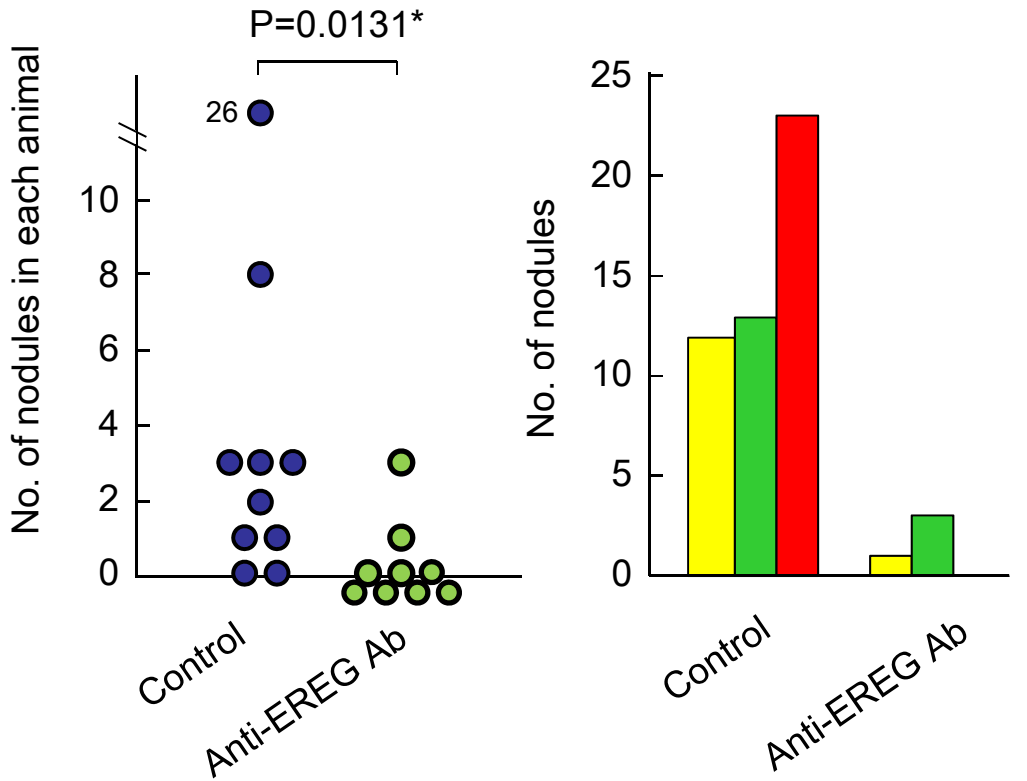
Antitumor Activity of Anti-EREG Antibody in a Metastasis Model

- The number of tumors was significantly decreased as compared to the control mice. The size of each tumor was also markedly reduced in the antibody treated mice.

Histopathology of tumors with or without administration of anti-EREG antibody



Antitumor activity of anti-EREG antibody



Yellow column; under 100 μm,
Green column; 100 – 200 μm,
Red column; over 200 μm

Conclusions

- Stable cell lines having cancer stem cell properties were established from human colon cancer.
- Colon cancer stem cells were found to interconvert between a proliferating and a drug-resistant, non-proliferating state depending on the environment.
- The existence of both proliferating and drug-resistant, non-proliferating cancer stem cells were also found in tumor tissues of colon cancer patients.
- Epiregulin was a molecule expressed on cancer stem cells in both proliferating and drug-resistant, non-proliferating states and is thought to be a potential target for anti-cancer stem cell therapy.

- These results provide new biological insights into drug resistance of cancer stem cells and new therapeutic options for cancer treatment.

Contacts: Corporate Communications Dept.

Corporate Communications Group

Tel: +81 (0)3-3273-0881 Fax: +81 (0)3-3281-6607
e-mail: pr@chugai-pharm.co.jp

Hitoshi Aikawa, Koichi Kawahara, Kae Miyata, Hiroshi Araki

Investor Relations Group

Tel: +81 (0)3-3273-0554 Fax: +81 (0)3-3281-6607
e-mail: ir@chugai-pharm.co.jp

Mac Uchida, Yusuke Tokita, Chisato Kitamura, Yuka Minoshima