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LETTER TO THE EDITOR

Claudin 6: a novel surface marker for characterizing mouse pluripotent stem cells

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Dear Editor,

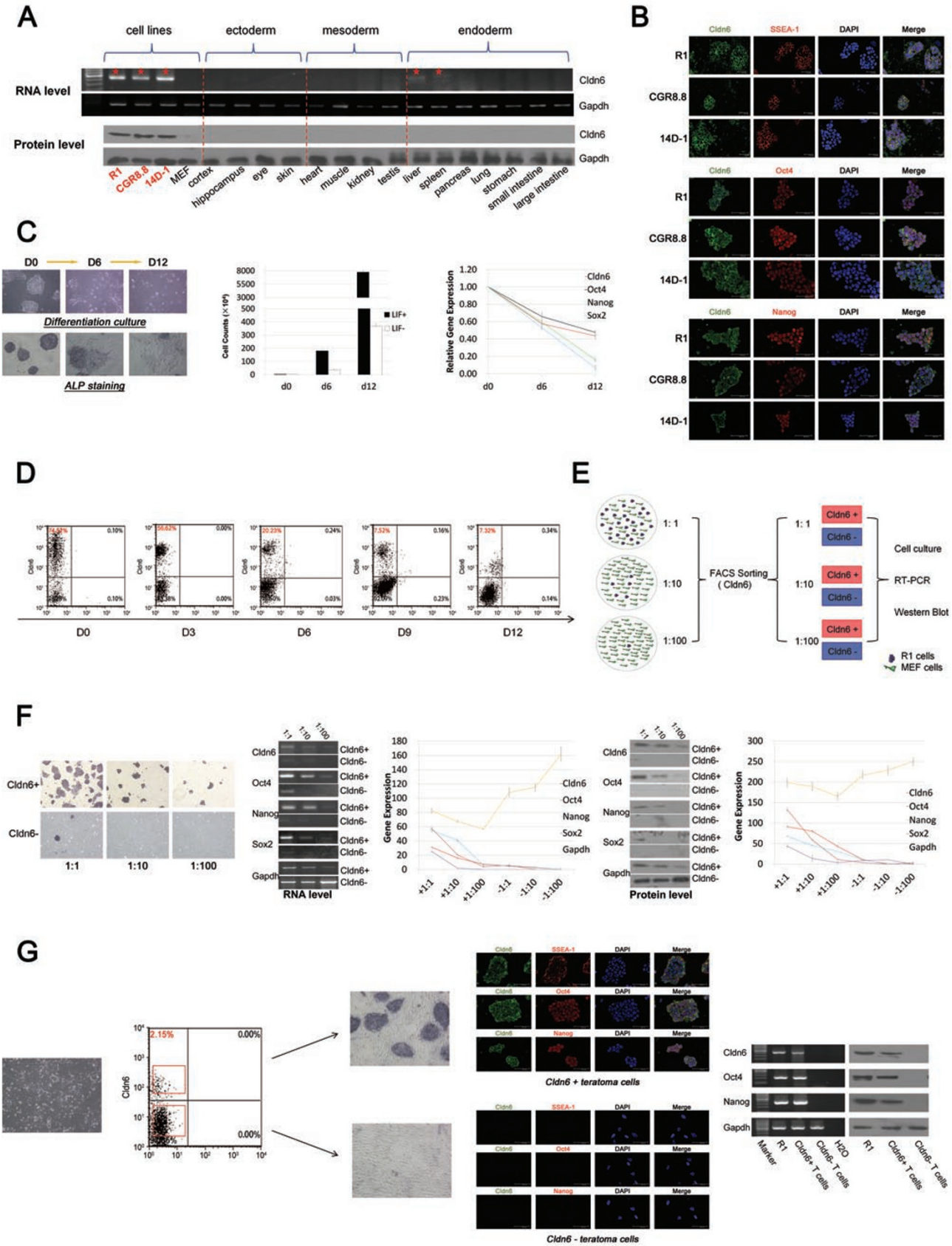
Stem cell surface proteins have been used as markers for isolating and purifying undifferentiated pluripotent stem cells (PSCs), and play important roles in regulating their pluripotency and differentiation. To date, very few specific, highly sensitive markers in PSCs have been identified [1-3]. This severely limits their utility in the analysis and recovery of functionally important PSCs. Therefore, it is necessary to expand the repertoire of stem cell markers and to dissect out the molecular mechanisms that regulate pluripotency and differentiation.

To investigate new stem cell markers, we compared gene expression profiles between stem cells (mESCs and iPSCs) and differentiated cells such as mouse embryonic fibroblasts (MEFs), bone marrow mesenchymal stem cells, embryonic bodies, osteoblasts, neurons, bone marrow mononuclear cells and others (Supplementary information, Tables S1 and S2). We identified one particularly interesting molecule, Claudin 6 (Cldn6), which was consistently expressed at elevated levels in stem cells relative to non-stem cells (Supplementary information, Table S3). Cldn6, a member of the tight junction family, has been reported to play an important role during mouse embryonic epithelium formation [4] and the development of endodermal tissues [5]. The level of Cldn6 expression is correlated with dedifferentiation and malignant proliferation of epithelial cells [6, 7]. However, little is known about the characteristics and function of Cldn6 in mouse PSCs [8].

To determine whether Cldn6 expression was specific to stem cells, we initially examined differences of Cldn6 expression levels between stem cells and somatic cells at both the RNA and protein levels. It appeared that Cldn6 was highly expressed in the stem cells, while the mouse adult tissues showed very limited, if any, expression (Figure 1A). These results were consistent with the findings from gene expression studies that Cldn6 showed the higher expression levels in stem cells compared with differentiated cells. Using flow cytometry analysis, Cldn6 was detected in the SSEA-1-positive R1 cells, but not in MEF cells (Supplementary information, Figure S1A). Furthermore, Cldn6 was localized on the surface of stem cells that concurrently expressed the collection of established stem cell markers including SSEA-1, Oct4 and Nanog (Figure 1B). This strongly suggests that Cldn6 is specifically expressed in undifferentiated stem cells.

To further determine whether Cldn6 expression indicated the undifferentiated state of stem cells, we next analyzed Cldn6 expression throughout the course of stem cell differentiation. Undifferentiated R1 cells were allowed to spontaneously differentiate by culturing them in the absence of LIF on gelatin-coated dishes for 12 days. As shown in Figure 1C, R1 cells gradually lost their pluripotency, as indicated by cell morphology changes, decreased cell proliferation rates, alkaline phosphatase (ALP)-negative staining and reduced expression of the established pluripotency markers. During stem cell differentiation (e.g., R1), Cldn6-positive cells drastically decreased from 74.52% to 7.32% (Figure 1D), as estimated by flow cytometry analysis. SSEA-1 expression was showed at high levels during the first 3 days of stem cell differentiation, and subsequently showed a gradual decline in expression from the fourth day onward [9]. Decreasing expression of Cldn6 appeared to be a sensitive indicator of early differentiation, as its expression levels were significantly lower in the first few days and expression was reduced by 20% on the third day (Figure 1D). Similar differentiation-dependent reductions were observed for the pluripotency markers Oct4, Nanog and Sox2 using RT-PCR and ICC assays (Figure 1C and Supplementary information, Figure S1B). These findings further support the notion that Cldn6 downregulation in R1 cells accompanies the loss of pluripotency and indicates an early differentiation state of stem cells.

To demonstrate the utility of Cldn6 as a selective marker for pluripotent cells, we tested our ability to recover PSCs using artificially mixed cells. Specifically, we mixed R1 and MEF cells in different ratios from 1:1 to 1:100 and isolated the Cldn6-positive cells by FACS from these artificial cell mixtures. ALP staining, RT-PCR and western blotting were performed to characterize the


A
cell lines
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Cldn6
SSEA-1
DAPI
Merge
Oct4
Nanog
R1
CGR8.8
14D-1
C
D0
D6
D12
Differentiation culture
ALP staining
Cell Counts (×10⁶)
LIF+
LIF-
Relative Gene Expression
Cldn6
Oct4
Nanog
Sox2
D
D0
D3
D6
D9
D12
E
1:1
1:10
1:100
FACS Sorting
(Cldn6)
Cldn6 +
Cldn6 -
Cell culture
RT-PCR
Western Blot
R1 cells
MEF cells
F
Cldn6+
Cldn6-
RNA level
Protein level
Gene Expression
G
2.15%
0.00%
Cldn6 + teratoma cells
Cldn6 - teratoma cells
Marker
R1
Cldn6+ T cells
Cldn6- T cells
H2O

recovered cells (Figure 1E). After FACS sorting using Cldn6 as the marker, the selected cells showed higher ALP activity than the negative cells, and the number of ALP-staining colonies was directly correlated with the original R1:MEF ratios (Figure 1F). The known pluripotency markers were examined at both the RNA and protein levels in the cultured Cldn6-positive cells (Figure 1F).

Next, we investigated the feasibility of using Cldn6 as a surface marker to purify stem-like cells from complicated samples such as teratomas. Teratoma tissues were incised and dissociated into a suspension of single cells. Cldn6-positive cells were selected by FACS sorting and cultured in the presence of LIF (Figure 1G). These Cldn6-positive cells expressed the pluripotency markers, indicative of ES-like cells, while Cldn6-negative teratoma cells lacked expression of pluripotency genes (Figure 1G). Similar results were found using other ESCs or pluripotent iPSC cell lines (such as those derived using the Yamanaka method [10]), further demonstrating the feasibility of using Cldn6 for the isolation of PSCs from mixed cell populations. Therefore, Cldn6 successfully served as a selection marker that can separate and purify stem cells from a mixed population of cells both *in vitro* and *in vivo*, indicating that Cldn6 is a promising marker for the isolation and characterization of stem cells.

To further understand its biological function in mouse stem cells, experiments were conducted in which endogenous Cldn6 expression was inhibited by siRNA (Supplementary information, Figure S2A). Inhibitory expression of Cldn6 in stem cells significantly affected Nanog expression at both the RNA and protein levels, whereas no statistically significant changes were observed for Oct4 and Sox2 (Supplementary information, Figure S2A). In addition, the differentiation capacity of Cldn6-null mouse embryonic stem cells was estimated by generation of teratomas (Supplementary information, Figure S2B). Although tissues from all the three germ layers were formed, endodermal genes (*Sox17*, *Foxa2*, *Gata4* and *Afp*) were found to be downregulated, while mesodermal genes (*Myod1*, *Gata2* and *Flk1*) were upregulated (Supplementary information, Figure S2C). These preliminary findings support the need for further studies that focus on the role of Cldn6 in stem cell pluripotency and differentiation. Further research identifying the network genes surrounding Cldn6 in mouse stem cells, which are involved in stem cell maintenance and differentiation, are essential to confirm Cldn6 as an undisputed marker of stem cells.

Herein, we showed that Cldn6 was specifically expressed in undifferentiated stem cells and its expression decreases coincidently with a downregulation of the pluripotent factors Oct4, Nanog and Sox2 upon early differentiation. This makes Cldn6 a much-needed addition to the collection of known PSC markers. Undifferentiated stem cells can be isolated both *in vitro* and *in vivo* from mixed cellular populations using Cldn6 as the marker, demonstrating the utility of Cldn6 as a marker for identification of mouse PSC.

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Figure 1 Cldn6 was expressed specifically in undifferentiated stem cells and could be used as a marker to purify stem-like cells from mixed populations. **(A)** RNA and protein expression levels were higher in PSCs compared with different kinds of somatic cells. Stars indicate the expression of Cldn6. **(B)** R1, CGR8.8 and 14D-1 were double incubated with anti-Cldn6 (green) and anti-SSEA-1 (red, top), anti-Oct4 (red, middle) or anti-Nanog (red, bottom), respectively. Cldn6 was stained in the membrane of these PSCs and appeared to be co-localized in the pluripotent marker-positive cells. Scale bars in top panels are 25 μm, while in middle and bottom panels are 50 μm. **(C)** R1 cells were cultured under differentiation conditions without LIF; cell morphology (upper in left panel), ALP staining (lower in left panel), cell counts (middle panel) and pluripotency marker expression (right panel) showed the differentiation of R1 cells. Cell counts were assayed by trypan blue exclusion. Cells were magnified 400× in the left panel. **(D)** Cldn6 expression diminished during early differentiation determined by flow cytometry. **(E)** Using Cldn6 as a marker to quantitatively recover stem cells from mixed populations. R1 and MEF cells were mixed in different ratios, then sorted by FACS and divided into $Cldn6^+$ and $Cldn6^-$ subgroups. ALP staining, RT-PCR and western blotting were performed to analyze the pluripotency of these sorted cell groups. **(F)** After sorting the cell mixtures, $Cldn6^+$ and $Cldn6^-$ cells were reanalyzed for pluripotent features including ALP staining (left panel) and expression of pluripotency genes (middle and right panels). Cells were magnified 200× in the left panel. **(G)** Cldn6 as a marker for isolating cells from teratomas. Cells from teratomas were expanded in the presence of LIF and separated by FACS. After 3-day culture, isolated $Cldn6^+$ and $Cldn6^-$ cells were assayed for ALP activities and expression of pluripotent genes. $Cldn6^+$ and $Cldn6^-$ T cells indicate Cldn6-positive teratoma cells and Cldn6-negative teratoma cells, respectively. Cells were magnified 400× in bright field and the scale bars in fluorescent field are 50 μm.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)