Identification of a gene set that maintains tumorigenicity of the hepatocellular carcinoma cell line Li-7

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Identification of a gene set that maintains tumorigenicity of the hepatocellular carcinoma cell line Li-7

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Abstract:

The identification and development of therapeutic targets in cancer stem cells that lead to tumor development, recurrence, metastasis, and drug resistance is an important goal in cancer research. The hepatocellular carcinoma cell line Li-7 contains functionally different types of cells. Cells with tumour-forming activity are enriched in cancer stem cell-like CD13^+CD166^- cells and this cell population gradually decreases during culture in conventional culture medium (RPMI1640 containing 10% fetal bovine serum). When Li-7 cells are cultured in mTeSR1, a medium developed for human pluripotent stem cells, CD13^+CD166^- cells and their tumorigenicity are maintained. Here, we sought to identify the mechanisms of tumorigenicity in this sub-population. We compared gene expression profiles of CD13^+CD166^- cells with other cell sub-populations and identified nine overexpressed genes (ENPP2, SCGN, FGFR4, MCOLN3, KCNJ16, SMIM22, SMIM24, SERPINH1, and TMPRSS2) in CD13^+CD166^- cells. After transfer from mTeSR1 to RPMI1640 containing 10% fetal bovine serum, the expression of these nine genes decreased in Li-7 cells and they lost tumorigenicity. In contrast, when these genes of Li-7 cells were forcibly expressed in cultures using RPMI1640 containing 10% fetal bovine serum, Li-7 cells maintained tumorigenicity. A metabolome analysis using capillary electrophoresis-mass spectrometry showed that two metabolic pathways, “Alanine, aspartate and glutamate metabolism” and “Arginine biosynthesis”, were activated in cancer stem cell-like cells. Our analyses here showed potential therapeutic target genes and metabolites for treatment of cancer stem cells in hepatocellular carcinoma.

Key words: cancer stem cell, CD13 expression, hepatocellular carcinoma, RNA sequencing, tumorigenicity
**Introduction:**

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the second leading cause of cancer death in men[1]. Although six systemic therapies have been approved after phase III trials, namely atezolizumab plus bevacizumab, lenvatinib, regorafenib, cabozantinib, ramucirumab, and sorafenib, median overall survival of patients with advanced HCC is still low in the range 10.7 - 19.2 months[2–7]. Thus, the development of new treatments is desired.

The concept of cancer stem cells (CSCs) has deepened our understanding of the heterogeneity and complexity of cancer[8]. CSCs have the potential for self-renewal and multilineage differentiation and have a central role in tumorigenicity[8]. Since CSCs are resistant to chemotherapy and radiotherapy, they play important roles in tumor development, recurrence, and metastasis[9–13]. Overcoming these roles will require specific treatments that target CSCs.

We previously reported that the human HCC cell line Li-7 contains CSC-like cells, identified as CD13+CD166− cells, that have a high potential for tumorigenicity[14]. CD13+CD166− cells differentiate into CD13 CD166− and CD13 CD166+ cells during long-term in vitro culture in conventional RPMI 1640 culture medium supplemented with 10% fetal bovine serum (RPMI); this differentiation results in the loss of CSC-like cells and of tumorigenicity. We also reported that CSC-like cells in the Li-7 cell line could be maintained using the mTeSR1 medium developed for pluripotent stem cells. Culture in mTeSR1 significantly increased the tumorigenicity of the CSC-like cells and also influenced CD13/CD166 expressions[15]. Analysis of Li-7 cells cultured in RPMI or mTeSR1 provided an explanation for the reversible expression of CD13 and irreversible expression of CD166. In summary, the Li-7 line possessed three cell populations: (1) CD13+CD166− cells with strong tumorigenic activity and CSC characteristics; (2) CD13 CD166− cells able to revert to CD13+CD166− cells; and (3) CD13 CD166+ cells that are not tumorigenic and cannot revert to CD166− cells. However, the mechanisms of tumorigenicity and maintenance of CSC-like cells remain unknown. Elucidation of these mechanisms may reveal new therapeutic targets against CSCs in HCC.

In this study, we sought to identify genes that are associated with the tumorigenicity of Li-7 cells by comparing RNA sequences from CSC-like and other cells. Through this comparison we identified nine candidate genes and subjected these to further analyses. The nine genes functioned to maintain tumorigenicity when they were overexpressed in CD13+CD166− cells, but failed to recover it when overexpressed in other cell types. In addition, a metabolome analysis using capillary electrophoresis-mass spectrometry (CE-MS) identified metabolites that might be involved in the tumorigenicity of Li-7 cells.

**Materials and Methods:**

**Cell culture**
The human HCC cell line Li-7 (RCB1941) was provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. The cells were cultured in RPMI1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) (SIGMA) or mTeSR1 (STEMCELL Technologies). For culture in mTeSR1, cells were first seeded and cultured overnight in RPMI, washed once with PBS, and then switched to mTeSR1. Cells were incubated at 37°C with 5% partial pressure of CO₂ in a humidified atmosphere. Cells were passaged at approximately 70 to 80% confluency.

Flow cytometric analysis and cell sorting
The following antibodies were used for flow cytometric analysis and cell sorting: allophycocyanin-conjugated anti-human CD13 (BD Biosciences); phycoerythrin-conjugated anti-human CD166 (BD Biosciences); phycoerythrin-Vio770-conjugated anti-human CD166 (Miltenyi Biotech). Cells were stained with fluorescent dye-conjugated antibodies in PBS supplemented with 5% FBS at 4°C for 30 minutes. The cells were washed once with PBS/5% FBS and re-suspended in 7-AAD (BD Biosciences) in PBS/5% FBS to detect dead cells. Aggregated cells were excluded using an FSC-H/FSC-W dot plot; isotype controls were used to determine negative cell populations. FACS Verse (BD Biosciences) and FACS SORP Aria (BD Biosciences) were used for analysis and cell sorting, respectively.

RNA sequencing and expression analysis
We collected 1 x 10⁶ cells of three cell populations (CD13⁺CD166⁻ cells, CD13⁻CD166⁺ cells, and CD13⁻CD166⁻ cells) cultured in RPMI and one cell population cultured in mTeSR1 (CD13⁺CD166⁻ cells) by cell sorting. The purity of each population was over 95%. RNA extraction, RNA sequencing, and expression analysis were performed by Hokkaido System Science (Sapporo, Japan). Illumina HiSeq 2500 platform (Illumina) was used for RNA sequencing with a 100-bp read length. The raw sequence data was converted to FastQ format and was mapped to the human genome using TopHat. The data was then normalized and analyzed using Cufflinks.

Cloning and transduction
Total RNA was extracted from Li-7 cells using TRizol Reagent (Invitrogen) and reverse transcribed to cDNA using SuperScript IV First-Strand Synthesis System (Invitrogen). PCR was performed using Tks Gflex DNA Polymerase (Takara Bio) and extracted genes were directly cloned into an entry vector using a pENTR/D-TOPO Cloning kit (Invitrogen). The cloning primers are listed in Table S1. The entry clones for SERPINH1 (HGE022338/W01A055O02) and TMPRSS2 (HGE007275/W01A018D03) were provided by RIKEN BRC through the National BioResource Project of MEXT, Japan[16–19]. Entry clones were converted to lentiviral
vectors containing the Tet-On advanced inducible gene expression system by an LR reaction using Gateway LR Clonase II Enzyme mix (Invitrogen). Lentivirus was produced by co-transfection of lentiviral vector and the plasmids pCMV-VSV-G-RSV-Rev (RDB04393) and pCAG-HIF gp (RDB04393), which are required for packaging into 293T cells by transfection using FuGENE HD Transfection Reagent (Promega). Candidate genes were introduced into Li-7 cells using lentivirus with auxiliary transfection reagent polybrene (nacalai tesque); the transduced cells were cultured with 10 µg/ml blasticidin (Gibco) to select cells with successful transduction. Doxycycline (1 µg/ml; SIGMA) was added to the culture medium to induce overexpression of the genes. All the procedures were performed according to manufacturer’s instructions where appropriate.

Quantitative real-time PCR
Total RNA was extracted from cells using TRIzol Reagent and reverse transcribed to cDNA using the SuperScript IV First-Strand Synthesis System. TB Green Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio) and a Thermal Cycler Dice Real Time System TP800 (Takara Bio) were used for quantitative real-time PCR according to the manufacturer’s instructions. Expression levels of the genes were calculated using the delta-delta Ct method by comparison with the control housekeeping genes GAPDH, ACTB, and B2M. The primers used are listed in Table S1.

Tumor formation in mice
Five-week-old female BALB/c nu/nu nude mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). We made suspensions of 5 x 10^6 cells in 200 µl of RPMI and injected these subcutaneously into both sides of a recipient mouse. The mice were sacrificed when tumors appeared or at 12 weeks after injection.

Spheroid formation assay
We seeded 1 x 10^4 cells into each well of a 96-well NanoCulture plate-MS (ORGANOGENIX) with 100 µl of NanoCulture medium R-type supplemented with 10% FBS-R (ORGANOGENIX). Half of the medium was replaced with new medium twice a week. The number of spheroids with a diameter greater than 100 µm or 200 µm was counted on day 15 using a microscope equipped with a digital camera (DP25, Olympus).

Chemosensitivity and cell proliferation assay
We performed a chemosensitivity assay by seeding 5 x 10^3 cells into a 96-well flat bottom plate and incubating the plate overnight at 37°C. The medium was replaced with fresh medium containing different concentrations of 5-FU and cell viabilities were measured 72 hours later using a Cell Counting Kit-8
Absorbance at 450nm was measured using a 2030 Multilabel Reader (ARVO X3; PerkinElmer).

Cell viability was assessed by seeding 5 x 10^3 cells into a 96-well flat bottom plate; a Cell Counting Kit-8 was used to assay cell proliferation at 2, 24, 48, 72, and 96 hours of culture in the same manner as the chemosensitivity assay.

**CE-MS analysis**

CD166^+^ cells in Li-7 cell cultures were removed using biotin-conjugated anti-human CD166 (Miltenyi Biotech) and Dynabeads M-280 Streptavidin (Invitrogen). Subsequently, 1 x 10^7 CD166^+^ cells were cultured in mTeSR1 for 1, 4, and 7 weeks and then analyzed. The cultured cells were collected in 2 ml plastic tubes, washed with saline, and pelleted. Metabolome analysis was performed as previously described[20]. Briefly, 1 x 10^7 cells from each sample were added to 500 µl methanol containing 8 µM of two reference compounds (methionine sulfone for cation and camphor 10-sulfonic acid for anion analysis) and were extracted using a Retsch mixer mill MM310 at a frequency of 27 Hz for 1 min with 5mm zirconia beads. The extracts were centrifuged at 20400 x g for 3 min at 4°C. Five hundred µl of the methanol solution was then transferred to a new tube. Data was then converted to a csv file and processed using the online software MetaboAnalyst 5.0. After data processing and normalization, correlation analyses, principal component analyses (PCAs), and heatmap clustering analyses were performed. PC1 principal loadings were then calculated for a pathway analysis of the top 30 metabolites.

**Statistics**

In Fig. 4B Student’s t-test was performed to identify statistically significant differences. In Fig. 5 Log-rank test was performed to identify statistically significant differences. A value of p<0.05 was considered significant. Analyses were performed with BellCurve for Excel (Social Survey Research Information Co., Ltd.). Normalization, correlation heatmap analysis, PCA, heatmap clustering analysis, and pathway analysis for CE-MS metabolites were performed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca). “Homo sapiens (KEGG)” library was selected for pathway analysis. FDR < 0.05 and Impact > 0.2 were considered as significant.

**Results:**

**RNA-sequence**

We performed RNA sequencing to identify genes associated with tumorigenicity in the Li-7 cell line. Three cell populations that had been cultured in RPMI, CD13^+^CD166^+^ cells (sample 1), CD13^+^CD166^+^ cells (sample 2), and CD13^+^CD166^+^ cells (sample 3), and a cell population that had been cultured in mTeSR1,
CD13+CD166- cells (sample 4), were collected by cell sorting (Fig. 1A). We previously determined that tumorigenicity from high to low was in the order of samples 4, 1, 2, and 3[14,15]. Therefore, we searched for genes that were more abundantly expressed in the order “sample 4 > sample 1 > sample 2 > sample 3”. As a result, 2234 genes were identified. Next, we searched for genes showing significantly different expression levels between the cell populations with highest (sample 1) and lowest (sample 3) in RPMI culture. We identified 11 genes with significant differences of expression between these two groups (Fig. 1B, Table 1).

**Overexpression of nine genes in CD13-CD166- cells and CD13-CD166+ cells**

Initially, we tried to overexpress all 11 genes to determine whether they were associated with tumorigenicity. However, two genes, CACNA1D and CDKN2B-AS1, could not be isolated for some technical reason. The remaining nine genes could be isolated and were separately or collectively transduced into Li-7 cells using lentivirus vectors. As is shown in Fig. 2, expression of the transduced genes was inducible by doxycycline (Dox).

Li-7 cells transduced with a single gene and Li-7 cells transduced with all nine genes (9g-Li-7) were cultured in RPMI with Dox (RPMI+Dox) or without Dox (RPMI-Dox). Overexpression of ENPP2, FGFR4, MCOLN3, and TMPRSS2, and of all nine genes simultaneously caused delayed cell proliferation but had no significant effect on tumorigenicity in mice, spheroid formation, in chemosensitivity assays, or the expression pattern of CD13 and CD166 (Table S2). The nine genes did not enhance tumorigenicity when overexpressed in Li-7 cells that had been cultured in RPMI over a long period (Table 2A and 2B). Thus, the nine genes did not recover tumorigenicity in CD13-CD166- cells or CD13-CD166+ cells in the Li-7 line.

**Overexpression of the nine genes in CSC-like cells (CD13+CD166- cells)**

In spite of the results described above, we speculated that the nine genes might still be associated with maintaining tumorigenicity if their expression had decreased with time after changing from mTeSR1 to RPMI (Fig. 3). Switching to RPMI caused the disappearance of CD13+CD166- and CD13-CD166- cell populations and decreased tumorigenicity in Li-7 cells (Fig. S1A, S1B).

In the 9g-Li-7 culture, a significant CD166- population remained after culture with RPMI+Dox compared to RPMI-Dox at 10 weeks after changing the medium to RPMI (Fig. 4A and 4B). To confirm the roles of the transduced genes in maintaining tumorigenicity, cells were cultured with RPMI+Dox or RPMI-Dox for 6, 8, and 10 weeks and then subcutaneously transplanted into recipient mice. The tumorigenicity of cells cultured with RPMI+Dox was comparable to that of cells cultured with mTeSR1. On the other hand, cells cultured with RPMI-Dox formed fewer tumors and required a longer time period to form tumors (Fig. 5).
Other characteristics of CSCs such as high spheroid forming ability, high 5-FU resistance, and slow proliferation were also maintained after 10 weeks of culture with RPMI+Dox (Fig. S2A-D). These results clearly indicate that overexpression of the nine genes resulted in the maintenance of tumorigenicity of Li-7 cells presumably due to the maintenance of a CSC-like cell population.

**CE-MS-based metabolome analysis of Li-7 cells cultured in mTeSR1**

The loss of tumorigenicity of Li-7 cells after culture for several weeks in RPMI was largely recovered depending with time after replacement of RPMI with mTeSR1, i.e., the proportion of CD13-positive cells increased immediately after replacing RPMI with mTeSR1 and by Day7 almost all cells changed to CD13-positive cells (Fig. S3). Recovery of tumorigenicity required culture with mTeSR1 for at least 10 days (Table 3). Metabolome analyses of the CD166+ cell population in the Li-7 line after culture for 1, 4, and 7 weeks with mTeSR1 were performed to identify activated metabolic pathways in the CSC-like cells. A total of 144 metabolites was detected in the CE-MS analysis (Fig. 6A). A heatmap clustering analysis and PCA score plots identified differences in metabolites depending on the incubation period with mTeSR1 (Fig. 6B and 6C). The principal component loadings of PC1 in PCA were calculated (Fig. 6D) and the top 30 metabolites were extracted (Table 4). Pathway analysis of the top 30 metabolites in the PC1 principal loadings, the metabolites enriched in cells cultured with mTeSR1 for 4 or 7 weeks, revealed many pathways showing significant activation (Table 5). Among these pathways, “Alanine, aspartate and glutamate metabolism” and “Arginine biosynthesis” had FDR values < 0.05 and Impact values > 0.2.

**Discussion:**

CSCs are important targets in treatment strategies for cancers. We previously reported the presence of CSC-like cells in HCC cell line Li-7 with the decrease in number during culture in RPMI, the medium used during generation of Li-7[14]. In a subsequent study, we searched for a method to maintain CSC-like cells in cultures of Li-7 cells and found that a medium used for human pluripotent stem cell culture, mTeSR1, maintained CSC-like cells and their tumorigenicity[15]. Here, we sought to identify candidate genes associated with tumorigenicity of HCC cells using the Li-7 cell line.

As described here, we extracted genes that are highly expressed in CSC-like cells and overexpression of the nine genes resulted in the maintenance of tumorigenicity of CSC-like cells in Li-7 cell line. One of the nine genes FGFR4 has been previously implicated in HCC, and the FGF19-FGFR4 pathway in HCC has attracted attention as a potential oncogenic pathway. The combined use of FGFR4 inhibitors and sorafenib suppresses FGFR4/ERK signaling and enhances tumor suppression in mice[21]. Moreover, FGF19-FGFR4 signaling facilitates self-renewal of liver CSCs by stimulating the FGF19/stimulated store-operated Ca²⁺
entry/nuclear factors of activated T cells (NFAT)-c2 signaling circuit[22]. FGFR4 may play a pivotal role for
tumorigenicity and maintenance of CSC-like cells in the Li-7 line.

Deletion of the gene ENPP2 results in a reduction of both fibrosis and HCC in mice[23]; SERPINH1 can
function as a biomarker for early-stage HCC[24] and is associated with cell proliferation, migration, and
invasion in HCC cell lines[25]. These observations suggest that both of these genes may be related to
tumorigenicity of CSC-like cells in the Li-7 line. Although the relevance to HCC is not known, potassium
channels, including KCNJ16, are involved in cell cycle, proliferation, cell migration, apoptosis, and
angiogenesis in various cancers[26,27]. TMPRSS2 is also involved in other types of cancer, such as prostate
cancer, through its important role in cell invasion, tumor growth, and metastasis[28]. There is thus a
possibility that TMPRSS2 is also associated with liver cancer. To the best of our knowledge, there are no
reports on the possible involvement of SCGN, MCOLN3, SMIM22, or SMIM24 with HCC or CSCs; moreover,
the functions of SMIM22 and SMIM24 have not yet been identified. In a future study, we shall narrow
down the identification of essential genes associated with tumorigenesis.

We performed a metabolome analysis here to obtain insights on the tumorigenicity of Li-7 cells.
Through this CE-MS analysis, altered metabolites were identified that depended on the culture conditions
with mTeSR1. We found that “Alanine, aspartate and glutamate metabolism” and “Arginine biosynthesis”
showed significant activation in cells from long term cultures with mTeSR1 that were tumorigenic. A PCA
indicated that the top 30 metabolites in the PC1 loading values associated with these metabolic pathways
were L-asparagine, gamma-aminobutyric acid (GABA), L-glutamine, citrate, D-glucosamine 6-phosphate,
L-citrulline, and L-ornithine. Of these 7 metabolites, L-asparagine and GABA are frequently reported in
relation to tumorigenicity. L-asparagine is reported as an important regulator of cancer cell proliferation
and amino acid homeostasis[29]. Decrease of de novo intracellular asparagine synthesis suppresses tumor
growth in a mouse xenograft model[30]. GABA has been reported to be involved in the growth of various
cancers including HCC[31]. Decreased expression of gamma-aminobutyrate aminotransferase leads to
increased levels of GABA and tumor growth via the Ca\(^2+\)-NFAT1 axis[32]. Furthermore, silencing the GABA-
A receptor subunit Pi reduces ERK1/2 phosphorylation and suppresses migration[33]. Thus, L-asparagine
and GABA may be involved in the tumorigenicity of Li-7 cells.

Regarding CSC metabolism, metabolic alterations such as in the AKT-mTOR pathway, glutamine
metabolism, and fatty acid metabolism regulate self-renewal of stem cells and their function[34]. With
regards to lipid metabolism, de novo synthesis of fatty acids from citrates is increased in cancer cells to
satisfy energy demands[34]. The expression of enzymes involved in fatty acid de novo synthesis or fatty
acids uptake, such as ATP-citrate lyase, acetyl-CoA carboxylase, fatty acid synthase, and fatty acid
transporter CD36, are elevated in the CSCs of HCC[34]. These four lipid-related enzymes have been found
to be most highly expressed in CD13^+CD166^- cells cultured with mTeSR1 among the four cell samples used for RNA sequencing analysis (data not shown). This information and the finding of increased intracellular citrate concentrations in the CE-MS analysis, suggest that lipid metabolism may be involved in the mechanisms maintaining CSC-like cells in the Li-7 line. The results obtained from metabolome analysis may be specific for cell line. However, identifying metabolic pathways that are activated in cells with high tumorigenicity may provide clues to controlling CSCs in vivo. There is a possibility that the metabolic pathways including the metabolites identified from Li-7 are involved in tumorigenicity of HCC. However, further analyses of primary cancer cells are necessary to confirm this hypothesis. Therefore, analyzing primary cancer cells must be an important next step to further elucidate the metabolic pathways involved in tumorigenicity of HCC.

Our study was initiated to identify genes associated with the tumorigenicity of CSC-like cells in the HCC cell line Li-7. As a result, nine genes were found to be associated with maintenance of CSC-like cells and their tumorigenicity. In addition, some metabolites seemed to be associated with tumorigenicity. Although the relationship between the genes and metabolites requires further study, it is interesting to note that there are reports on Ca^{2+}/NFAT1 (or NFATc2) axis and ERK signaling for the gene FGFR4 and the metabolite GABA. In our next study, we shall investigate these nine genes further with the aim of identifying targets for therapy against CSCs in HCC. It is possible that other genes, in addition to the nine described here, are associated with the maintenance of CSCs and tumorigenicity in HCC. The Li-7 cell line will be valuable to identify other such genes in future research.

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Author Contributions:
Y.S. designed the research, performed the experiments, analyzed data and wrote manuscripts. S.H. and Y.H. made substantial contributions to the conception of the work. R.S., M.Y.H. performed the CE-MS analysis and wrote manuscript. T.Y., T.H., and K.T. supervised the experiments. K.S. designed the research, performed the experiments, wrote manuscripts, supervised the experiments and edited manuscript. I.H. and Y.N. supervised the experiments and edited manuscript. All authors approved the final version of the manuscript to be published.
Declarations:

Funding Information

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Conflict of Interest

I.H. serves as a consultant to Asahi Kasei, Ono, Chugai, Taiho, and Eisai pharmaceutical companies. The other authors have no conflict of interest.

Ethics approval

Research protocol was approved by Institutional Review Board. All animal experiments were approved by the Institutional Animal Care and Use Committee of RIKEN Tsukuba Branch.

Informed consent

Not applicable.

References


17. Otsuki T, Ota T, Nishikawa T, Hayashi K, Suzuki Y, Yamamoto JI, et al. Signal sequence and keyword trap in silico for selection of full-length human cDNAs encoding secretion or membrane proteins from


Figure Legends

Fig. 1: Samples for RNA sequencing and the method for extracting candidate genes.

(A) RNA sequencing was performed in three cell fractions that had been cultured in RPMI, namely, CD13^+CD166^- cells (sample 1), CD13^-CD166^- cells (sample 2), and CD13^-CD166^+ cells (sample 3), and in one cell fraction that had been cultured in mTeSR1, CD13^-CD166^- cells (sample 4). We collected 1 x 10^6 cells of each cell fraction by cell sorting.

(B) Previous work established that tumorigenicity was in the order sample 4 > sample 1 > sample 2 > sample 3; gene expression levels were extracted in this order. In total, 2234 genes were identified. In addition, the samples with the highest and lowest tumorigenic activity in RPMI, i.e., samples 1 and 3, respectively, were compared and 11 candidate genes with differential gene expression using q-value < 0.05 were identified.

Fig. 2: Relative expression levels of transduced genes in 9g-Li-7 cells.

(A) Gene expression levels in 9g-Li-7 cells were examined by qPCR with the delta-delta Ct method. The expression levels of each gene after the addition of Dox were quantified with respect to the expression levels of each gene without the addition of Dox. GAPDH, ACTB, and B2M were used as housekeeping gene controls, respectively, i.e., the delta-delta Ct method was applied three times separately with each housekeeping gene. (B) Expression profile of CD13 and CD166 on 9g-Li-7 cells cultured with or without Dox was analyzed with time by flow cytometry.

Fig. 3: Relative expression levels of nine candidate genes in cells switched from mTeSR1 to RPMI and cultured for different periods.

The medium used for culturing CD13^-CD166^- cells was changed from mTeSR1 to RPMI and expression levels of the nine candidate genes were examined weekly using qPCR.

Fig. 4: Maintenance of a CSC-like cell population in 9g-Li-7 cells following overexpression of the transduced nine genes.

(A) CD13^-CD166^- 9g-Li-7 cells were switched from mTeSR1 to RPMI-Dox (upper columns) or RPMI+Dox (lower columns) medium and cultured for 10 weeks. Expression of CD13 and CD166 was analyzed with time by flow cytometry. (B) Based on flow cytometric analysis, the percentage of CD13-positive cells in RPMI-culture with or without Dox were calculated. The data are means +/- SD of three separate experiments. *P<0.05.
Fig. 5: Overexpression of the set of nine genes maintained tumorigenicity in 9g-Li-7 cells in RPMI culture.

CD13^+CD166^− 9g-Li-7 cells were switched from mTeSR1 to RPMI-Dox or RPMI+Dox and 5 x 10⁶ cells obtained from different time periods of culture were injected subcutaneously to both lateral sides of recipient mice. The proportion of mice with tumor formation at different intervals after injection of CD13^+CD166^− 9g-Li-7 cells was assessed. Tumor formation was examined weekly (n = 12). **P<0.01

Fig. 6: CE-MS analysis

CE-MS analysis detected 144 metabolites that changed in Li-7 cells during culture in mTeSR1 medium.

(A) Correlation map and (B) heatmap of metabolites detected by CE-MS. The degree of change is marked with red (up-regulation) or blue (down-regulation).

(C) PCA score plot. Red, 1 week culture in mTeSR1; green, 4 weeks culture in mTeSR1; blue, 7 weeks culture in mTeSR1.

(D) Principal component loading of PC1.

ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine 5′-triphosphate; CDP, cytidine diphosphate, CML, [protein-N(epsilon)-(carboxymethyl)]lysine CMP, cytidine monophosphate; CTP, cytidine 5′-triphosphate; dCDP, 2′-deoxycytidine 5′-diphosphate; dGDP, 2′-deoxyguanosine 5′-diphosphate; dTMP, thymidine 5′-monophosphate; dTTP, deoxythymidine triphosphate; GMP, guanosine-monophosphate; GDP, guanosine diphosphate; GTP, guanosine 5′-triphosphate; IMP, inosinic acid; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PRPP, phosphoribosyl pyrophosphate; UDP, uridine 5′-diphosphate; UMP, uridine monophosphate
Fig. 1

(A) CD13 vs. CD166

1. RPMI
   1: CD13⁺CD166⁻
   2: CD13⁻CD166⁻
   3: CD13⁻CD166⁺

2. mTeSR1
   4: CD13⁺CD166⁻

(B) RNA-seq 4 sample

2234 genes

gene expression

4 > 1 > 2 > 3

11 genes

gene expression

1 > 3 (q-value < 0.05)
Fig. 2

(A) Relative gene expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>ENPP2</th>
<th>SCGN</th>
<th>FGFR4</th>
<th>MCOLN3</th>
<th>KCNJ16</th>
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<td>5.7</td>
<td>8.5</td>
<td>18.4</td>
<td>196.7</td>
<td>137.2</td>
<td>14.1</td>
<td>58.5</td>
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<tr>
<td>ACTB</td>
<td>46.9</td>
<td>187.4</td>
<td>5.9</td>
<td>8.8</td>
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<td>205.1</td>
<td>143.0</td>
<td>14.7</td>
<td>61.0</td>
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<tr>
<td>B2M</td>
<td>49.2</td>
<td>196.7</td>
<td>6.2</td>
<td>9.3</td>
<td>20.1</td>
<td>215.3</td>
<td>150.1</td>
<td>15.5</td>
<td>64.0</td>
</tr>
</tbody>
</table>

(B) Flow cytometry analysis

CD13 vs CD166
Relative gene expression

Fig. 3
Fig. 4

(A) mTeSR1

(B) CD13+/CD166- cells (%)
Fig. 5

- mTeSR
- mTeSR-RPMI 6wks
- mTeSR-RPMI 8wks
- mTeSR-RPMI 10wks

Tumor-free mouse (%) vs. wks post injection for different conditions and time points.
Fig. 6

(A) Principal component loading values

(B) Metabolites

(C) Source Plot

(D) Principal component loading values
Table 1: The 11 candidate genes selected after RNA sequencing

ID, symbol, official full name of the candidate genes, and whether (0) or not (x) they are protein coding.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene symbol</th>
<th>Official full name</th>
<th>Protein coding</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENSG000000157388</td>
<td>CACNA1D</td>
<td>Calcium voltage-gated channel subunit alpha1 D</td>
<td>◯</td>
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<tr>
<td>ENSG000000136960</td>
<td>ENPP2</td>
<td>Ectonucleotide Pyrophosphatase/Phosphodiesterase 2</td>
<td>◯</td>
</tr>
<tr>
<td>ENSG00000079689</td>
<td>SCGN</td>
<td>Secretagogin, EF-hand calcium binding protein</td>
<td>◯</td>
</tr>
<tr>
<td>ENSG00000160867</td>
<td>FGF4</td>
<td>Fibroblast Growth Factor Receptor 4</td>
<td>◯</td>
</tr>
<tr>
<td>ENSG00000055732</td>
<td>MCOLN3</td>
<td>Mucolipin 3</td>
<td>◯</td>
</tr>
<tr>
<td>ENSG00000153822</td>
<td>KCNJ16</td>
<td>Potassium voltage-gated channel subfamily J member</td>
<td>◯</td>
</tr>
<tr>
<td>ENSG00000267795</td>
<td>SMIM22</td>
<td>Small integral membrane protein 22</td>
<td>◯</td>
</tr>
<tr>
<td>ENSG00000095932</td>
<td>SMIM24</td>
<td>Small integral membrane protein 24</td>
<td>◯</td>
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<td>ENSG00000149257</td>
<td>SERPINH1</td>
<td>Serpin Family H Member 1</td>
<td>◯</td>
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<tr>
<td>ENSG00000184012</td>
<td>TMPRSS2</td>
<td>Transmembrane Serine Protease 2</td>
<td>◯</td>
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<tr>
<td>ENSG00000240498</td>
<td>CDKN2B-AS1</td>
<td>Cyclin dependent kinase inhibitor 2B – antisense 1</td>
<td>x</td>
</tr>
</tbody>
</table>
Table 2: Tumorigenicity of cells transduced with either a single candidate gene or all nine genes (9g-Li-7)

Recipient mice were injected subcutaneously to both lateral sides with $5 \times 10^6$ cells transduced with either one or all nine candidate genes and cultured in RPMI-Dox or RPMI+Dox. The number of mice showing tumor formation was determined weekly.

(A) Preliminary experiment to compare tumorigenicity in different cell samples ($n = 2$ mice per treatment group).

(B) Reproducibility of tumorigenicity in $KCNJ16$ or 9g-Li-7 cells ($n = 6$ and $n = 4$ respectively).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene expression</th>
<th>4w</th>
<th>8w</th>
<th>12w</th>
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<tr>
<td>ENPP2</td>
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<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>On</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>SCGN</td>
<td>-</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>On</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>FGFR4</td>
<td>-</td>
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<td>0/2</td>
<td>0/2</td>
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<tr>
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<td>0/2</td>
<td>0/2</td>
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<tr>
<td>MCOLN3</td>
<td>-</td>
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<td>0/2</td>
<td>0/2</td>
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<tr>
<td></td>
<td>On</td>
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<td>0/2</td>
<td>0/2</td>
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<tr>
<td>KCNJ16</td>
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<td>SMIM22</td>
<td>-</td>
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<td>0/2</td>
<td>0/2</td>
</tr>
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<td>9 genes</td>
<td>-</td>
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<td>0/2</td>
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<tr>
<td></td>
<td>On</td>
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<td>On</td>
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</table>
Table 3: Change in tumorigenicity with time after switching cells from RPMI to mTeSR1

Recipient mice were injected subcutaneously on both lateral sides with $5 \times 10^6$ Li-7 cells cultured for different times in mTeSR1. The mice were screened weekly for tumor formation ($n = 2$ or $n = 6$ were used per treatment group).

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>1w</th>
<th>2w</th>
<th>3w</th>
<th>4w</th>
<th>5w</th>
<th>6w</th>
<th>7w</th>
<th>8w</th>
<th>9w</th>
<th>10w</th>
<th>11w</th>
<th>12w</th>
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</thead>
<tbody>
<tr>
<td>RPMI</td>
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<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>2/6</td>
<td>2/6</td>
<td></td>
</tr>
<tr>
<td>RPMI→mTeSR1 1day</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
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<td></td>
</tr>
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<td>RPMI→mTeSR1 3days</td>
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<td>0/2</td>
<td>0/2</td>
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<td>0/2</td>
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<td></td>
</tr>
<tr>
<td>RPMI→mTeSR1 7days</td>
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<td>0/2</td>
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<td>0/2</td>
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<tr>
<td>RPMI→mTeSR1 10days</td>
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</tr>
<tr>
<td>RPMI→mTeSR1 14days</td>
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<td>0/2</td>
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<td>0/2</td>
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<td></td>
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<tr>
<td>RPMI→mTeSR1 21days</td>
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</tr>
</tbody>
</table>

Table 4: Top 30 metabolites of PC1 principal component loading values

dTTP, deoxythymidine triphosphate; PC, Principal component

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>KEGG ID</th>
<th>Principal component loading values of PC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyproline</td>
<td>C01015</td>
<td>0.97737451</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>C00064</td>
<td>0.96889786</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>C00188</td>
<td>0.96274028</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>C00407</td>
<td>0.95298413</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>C00152</td>
<td>0.92819391</td>
</tr>
<tr>
<td>Kynurenine</td>
<td>C00328</td>
<td>0.91843776</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>C00073</td>
<td>0.91627861</td>
</tr>
<tr>
<td>2-Aminobutyrate;2-Aminoisobutyrate</td>
<td>C02261</td>
<td>0.91515905</td>
</tr>
<tr>
<td>Hippurate</td>
<td>C01586</td>
<td>0.91132056</td>
</tr>
<tr>
<td>Carnosine</td>
<td>C00386</td>
<td>0.90988113</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>C00123</td>
<td>0.89164832</td>
</tr>
<tr>
<td>3-Methyl-L-histidine</td>
<td>C01152</td>
<td>0.88844959</td>
</tr>
<tr>
<td>Creatinine</td>
<td>C00791</td>
<td>0.88109249</td>
</tr>
<tr>
<td>beta-Alanine</td>
<td>C00099</td>
<td>0.86245984</td>
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<tr>
<td>L-Serine</td>
<td>C00065</td>
<td>0.83615021</td>
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<tr>
<td>Homolysine</td>
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<td>0.81711772</td>
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<tr>
<td>Citrulline</td>
<td>C00327</td>
<td>0.8042428</td>
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<tr>
<td>Isocitrate</td>
<td>C00311</td>
<td>0.78713754</td>
</tr>
<tr>
<td>GABA</td>
<td>C00334</td>
<td>0.77563008</td>
</tr>
<tr>
<td>D-Glucosamine 6-phosphate</td>
<td>C00352</td>
<td>0.75552601</td>
</tr>
<tr>
<td>2-Isopropylmalate;3-Isopropylmalate</td>
<td>C02504, C04411</td>
<td>0.73385456</td>
</tr>
<tr>
<td>Oxidized glutathione</td>
<td>C00127</td>
<td>0.7299361</td>
</tr>
<tr>
<td>S-Adenosyl-L-methionine</td>
<td>C00019</td>
<td>0.72375454</td>
</tr>
<tr>
<td>Ophthalmate</td>
<td>C21016</td>
<td>0.70069164</td>
</tr>
<tr>
<td>Diethanolamine</td>
<td>C06772</td>
<td>0.68768877</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>C00250</td>
<td>0.68500982</td>
</tr>
<tr>
<td>Putrescine</td>
<td>C00134</td>
<td>0.67714093</td>
</tr>
<tr>
<td>Citrate</td>
<td>C00158</td>
<td>0.6647938</td>
</tr>
<tr>
<td>Ornithine</td>
<td>C00077</td>
<td>0.66321042</td>
</tr>
<tr>
<td>dTTP</td>
<td>C00459</td>
<td>0.62677679</td>
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</tbody>
</table>
Table 5: Pathway analysis of the top 30 metabolites in PC1 principal component loading values

Pathways of metabolites with raw values of \( p < 0.05 \) are listed in the table. The total number of compounds in the pathways (Total), the expected number of matching compounds in the pathways (Expected), the matched number of compounds (Hits), the original \( p \) value calculated from the enrichment analysis (Raw \( p \)), the \( p \) value adjusted by the Holm-Bonferroni method (Holm \( p \)), the \( p \) value adjusted using False Discovery Rate (FDR), and the pathway impact value calculated from a pathway topology analysis (Impact) of each metabolic pathway are shown. FDR < 0.5 and Impact > 0.2 are considered to be significant.

<table>
<thead>
<tr>
<th>Metabolic pathways</th>
<th>Total</th>
<th>Expected</th>
<th>Hits</th>
<th>Raw ( p )</th>
<th>-log10(( p ))</th>
<th>Holm ( p )</th>
<th>FDR</th>
<th>Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoacyl-tRNA biosynthesis</td>
<td>48</td>
<td>0.92903</td>
<td>7</td>
<td>2.07E-05</td>
<td>4.6837</td>
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<td>0.0017402</td>
<td>0.16667</td>
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<tr>
<td>Alanine, aspartate and glutamate metabolism</td>
<td>28</td>
<td>0.54194</td>
<td>5</td>
<td>0.00013829</td>
<td>3.8592</td>
<td>0.011478</td>
<td>0.0058082</td>
<td>0.20032</td>
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<tr>
<td>Valine, leucine and isoleucine biosynthesis</td>
<td>8</td>
<td>0.15484</td>
<td>3</td>
<td>0.00034365</td>
<td>3.4639</td>
<td>0.02818</td>
<td>0.0096223</td>
<td>0</td>
</tr>
<tr>
<td>Arginine biosynthesis</td>
<td>14</td>
<td>0.27097</td>
<td>3</td>
<td>0.0020644</td>
<td>2.6852</td>
<td>0.16722</td>
<td>0.043353</td>
<td>0.28934</td>
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<tr>
<td>Glyoxylate and dicarboxylate metabolism</td>
<td>32</td>
<td>0.61935</td>
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<td>0.0028159</td>
<td>2.5504</td>
<td>0.22527</td>
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<td>Cysteine and methionine metabolism</td>
<td>33</td>
<td>0.63871</td>
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<td>0.0031613</td>
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<td>Arginine and proline metabolism</td>
<td>38</td>
<td>0.73548</td>
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<td>0.54194</td>
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<td>1.4271</td>
<td>1</td>
<td>0.31421</td>
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</tbody>
</table>
Supplementary Files

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- SupplementalTables.pdf
- SupplementalFigures.pdf