Table 2. TCR Gene Rearrangements in H25-4, H254SeVT-3, or Rediffferentiated CD8$^+$ T Cells

Cell	Genome or mRNA	Productivity	Rearrangement			Sequence of Junctional Region		
			Vα		Jα	3′Vα	P(N)	5′Jα
TCRA								
H25-4	genome	productive	TRAV8-3*01		TRAJ10*01	TGTGCTGTGGGT	T	TCACGGGAGGAGGAAACAAACTC ACCTTTT
		unproductive[a]	TRAV13-1*01		TRAJ29*01	TGTGCAGCAA	TCC	TCAGGAAACACACCTCTTGTCTTT
H254SeVT-3	genome	productive	TRAV8-3*01		TRAJ10*01	TGTGCTGTGGGT	T	TCACGGGAGGAGGAAACAAACTC ACCTTTT
		unproductive[a]	TRAV13-1*01		TRAJ29*01	TGTGCAGCAA	TCC	TCAGGAAACACACCTCTTGTCTTT
reT-1	mRNA	productive	TRAV8-3*01		TRAJ10*01	TGTGCTGTGGGT	T	TCACGGGAGGAGGAAACAAACTC ACCTTTT
		unproductive[a]	TRAV13-1*01		TRAJ29*01	TGTGCAGCAA	TCC	TCAGGAAACACACCTCTTGTCTTT
reT-2.1	mRNA	productive	TRAV8-3*01		TRAJ10*01	TGTGCTGTGGGT	T	TCACGGGAGGAGGAAACAAACTC ACCTTTT
		unproductive[a]	TRAV13-1*01		TRAJ29*01	TGTGCAGCAA	TCC	TCAGGAAACACACCTCTTGTCTTT
reT-3	mRNA	productive	TRAV8-3*01		TRAJ10*01	TGTGCTGTGGGT	T	TCACGGGAGGAGGAAACAAACTC ACCTTTT
		unproductive[a]	TRAV13-1*01		TRAJ29*01	TGTGCAGCAA	TCC	TCAGGAAACACACCTCTTGTCTTT
			Vβ	Dβ	Jβ	3′Vβ	N1-Dβ-N2	5′Jβ
TCRB								
H25-4	genome	productive	TRBV7-9*01	TRBD1*01	TRBJ2-5*01	TGTGCCAGCAGCTTA	CGGGACAGGGTGCCG	GAGACCCAGTACTTC
		unproductive	germline	TRBD1*01	TRBJ2-7*01	TACAAAGCTGTAACATTGTG	GGGACAACT	CTACGAGCAGTACTTCGGGCCG
H254SeVT-3	genome	productive	TRBV7-9*01	TRBD1*01	TRBJ2-5*01	TGTGCCAGCAGCTTA	CGGGACAGGGTGCCG	GAGACCCAGTACTTC
		unproductive	germline	TRBD1*01	TRBJ2-7*01	TACAAAGCTGTAACATTGTG	GGGACAACT	CTACGAGCAGTACTTCGGGCCG
reT-1	mRNA	productive	TRBV7-9*01	TRBD1*01	TRBJ2-5*01	TGTGCCAGCAGCTTA	CGGGACAGGGTGCCG	GAGACCCAGTACTTC
reT-2.1	mRNA	productive	TRBV7-9*01	TRBD1*01	TRBJ2-5*01	TGTGCCAGCAGCTTA	CGGGACAGGGTGCCG	GAGACCCAGTACTTC
reT-3	mRNA	productive	TRBV7-9*01	TRBD1*01	TRBJ2-5*01	TGTGCCAGCAGCTTA	CGGGACAGGGTGCCG	GAGACCCAGTACTTC

PCR-amplified samples (H25-4: not shown; H254SeVT-3: shown in Figures 1J and 1K; reT-1, reT-2.1, and reT-3: shown in Figure 3B) were sequenced, then V, D, and J segment usages and junctional sequences in CDR3 were identified. Following reprogramming and redifferentiation, there were no alterations in gene rearrangement in either allele at the *TCRA* and *TCRB* gene loci. See Table S1 for additional data on another T-iPSC clone (TkT3V1-7).

[a]Out-of-frame junction (at CDR3).

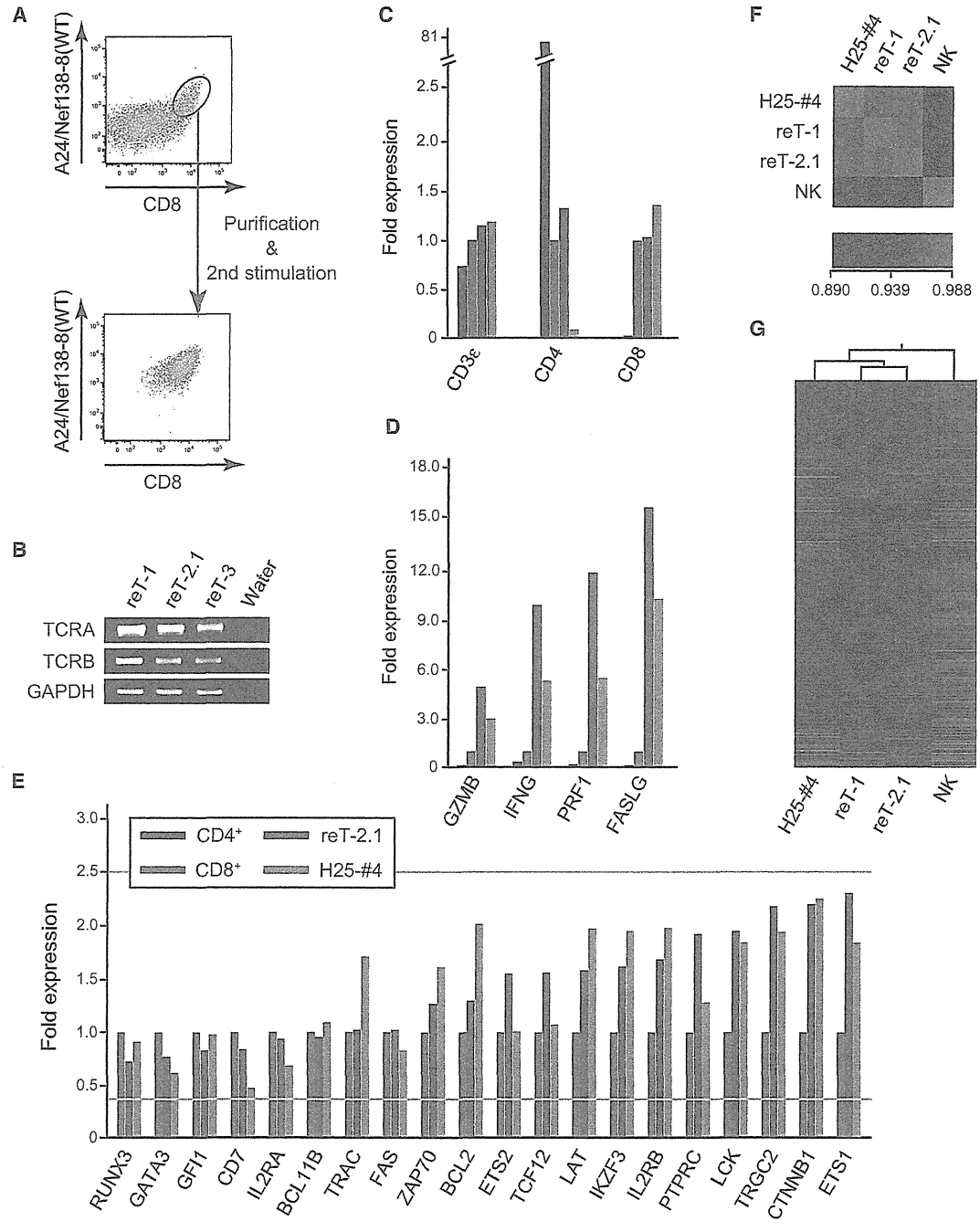

Figure 3. Characterizations of Redifferentiated T Cells as T Cells

(A) Recognition of A24/Nef-138-8(WT) tetramer at 50–60 days after starting redifferentiation, analyzed by flow cytometry (upper panel). Tetramer-positive cells were sorted by FACS or magnetically selected, then cultured for an additional 14 days, after which the expanded T cells were reanalyzed for tetramer (lower panel).

(B) TCR mRNAs were identified in a SMART-mediated cDNA library for reT-1, reT-2.1, and reT-3 cells. *GAPDH* is an internal control for PCRs.

(C–E) Quantitative PCR to compare the expression of major cell surface molecules (C), cell lytic molecules (D), and transcription factors and signal-transduction molecules (E) among PB $CD4^+$, PB $CD8^+$, reT-2.1, and H25-#4 cells. Individual PCR reactions were normalized against 18S rRNA.

(F and G) Global gene expression was analyzed using a cDNA microarray. Heat maps show the correlation coefficients between samples (F) and differential expression (>3-fold) of genes relative to NK cells (G). Red and green colorations indicate increased and decreased expression, respectively.

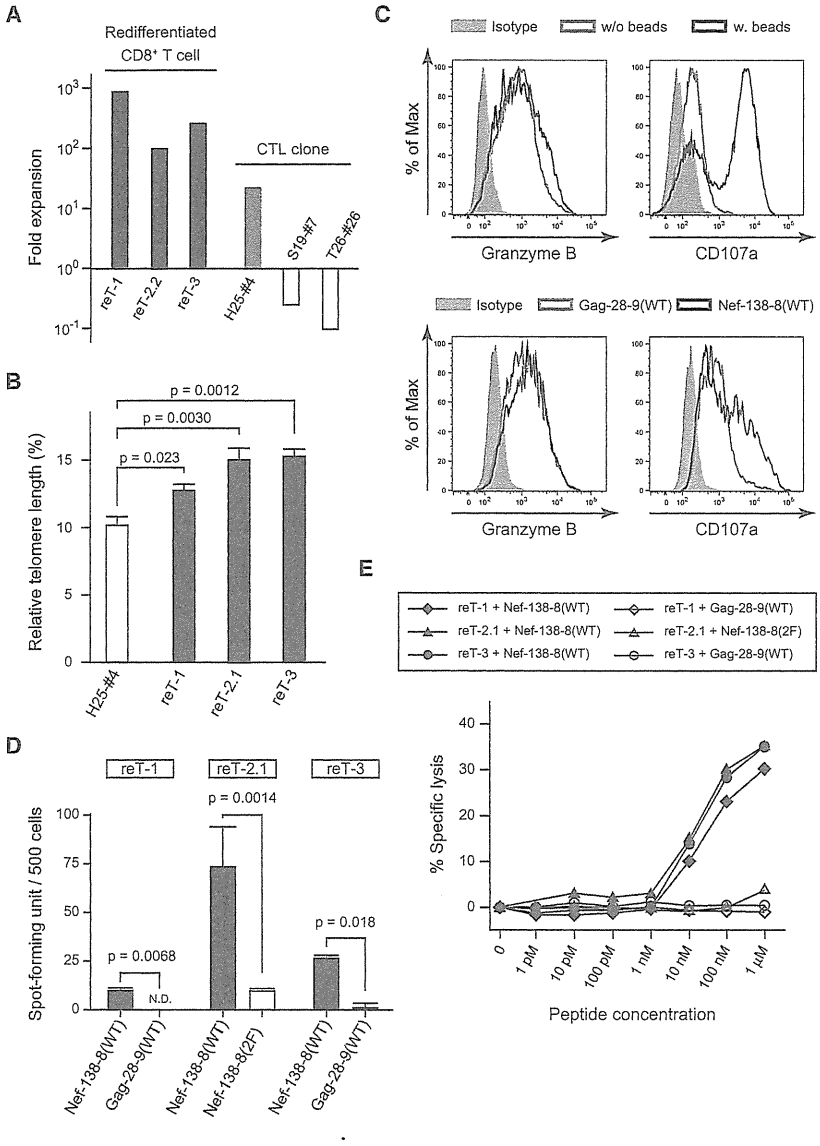

Figure 4. Redifferentiated T Cells Show T Cell Functionality and the Same Antigen Specificity as the Original CTL Clone

(A) Expansion ratios for reT-1, reT-2.2, and reT-3 cells elicited by PHA, IL-7, and IL-15 stimulation for 2 weeks. H25-#4 is the original clone. S19-#7 and T26-#26 were other Nef-138-8(WT)-specific CTL clones derived from different patients.

(B) Relative telomere length determined using flow-FISH. Data are presented as mean ± SEM.

(C) Intracellular production of granzyme B (left panel) and CD107a mobilization (right panel) induced by stimulation of reT-2.1 cells with α-CD3/CD28 beads or Nef-138-8(WT). Shaded plot: stimulated cells, isotype antibody; gray line: unstimulated cells, granzyme B or CD107a antibody; black line: stimulated cells, granzyme B or CD107a antibody.

(D) IFN-γ production in the presence of Nef-138-8(WT) measured using ELISPOT. Data are presented as mean ± SD. N.D., not determined.

(E) Standard ^{51}Cr release assay performed using the indicated concentrations of Nef-138-8(WT). Effector:target = 5:1.

See Figure S6 for additional data.

"rejuvenation." Throughout the experiments, neither autonomous cell expansion nor aberrant cell survival without cytokines as leukemia cells was observed (data not shown). Taken together, these data indicate that by passing through the T-iPSC state, cloned cytotoxic T cells can become "rejuvenated" to central memory-like T cells with excellent potential for proliferation and survival.

Redifferentiated CD8$^+$ T Cells Exhibit Antigen-Specific T Cell Functionality

To determine whether redifferentiated CD8$^+$ T cells exerted cytotoxic effects upon recognition of specific peptides in the context of an MHC, we performed functional assays using HLA-A24-positive B-LCL cells as antigen-presenting cells. Gag-28-9(WT) (KYKLKHIVW) is an antigenic peptide (aa 28–36) from the HIV-1 Gag protein (Altfeld et al., 2006), whereas Nef-138-8(2F) (RFPLTFGW) is a Tyr-to-Phe-substituted single-residue mutant form of Nef-138-8(WT). Both peptides were presented on HLA-A24 cells.

One of the major mechanisms by which CTLs induce cytotoxity is the secretion of cytolytic molecules triggered by TCR signaling. Intracellular staining revealed that the cytolytic molecule granzyme B was produced and stored in the granules of redifferentiated CD8$^+$ T cells (Figure 4C, left column). CD107a, also known as lysosomal-associated membrane protein 1 (LAMP1), is a granulocyte membrane protein that transiently appears at the cell surface and is coupled to degranulation (secretion of cytolytic molecules) of the stimulated CTLs, after which CD107a returns to the cytoplasm (Rubio et al., 2003). CD107a molecules on the cell surface were captured by a fluorochrome-conjugated antibody when redifferentiated CD8$^+$ T cells were stimulated with α-CD3/28 beads or Nef-138-8(WT) peptide, but not in the absence of the beads or Gag-28-9(WT) peptide (Figure 4C, right column). In the second experiment, we used the enzyme-linked immunosorbent spot (ELISPOT) assay to assess cytokine productivity per cell and confirmed that redifferentiated CD8$^+$ T cells produced significant levels of IFN-γ in response to stimulation by its specific antigen, Nef-138-8(WT) (Figure 4D). In a separate experiment, we used a ^{51}Cr release assay to investigate cytolytic capacity and found that redifferentiated CD8$^+$ T cells lysed ^{51}Cr-incorporated B-LCLs only when Nef-138-8(WT) was presented on B-LCLs (Figure 4E).

These results are highly indicative that redifferentiated CD8$^+$ T cells can release cytotoxic molecules and kill antigen-expressing target cells in an antigen-specific manner. Moreover,

monoclonal TCRs mediate highly precise cell targeting that should broaden the therapeutic window for antigen-specific T cell therapy by avoiding the troublesome mispairing TCRs that can occur with the commonly used exogenous TCR transfer technique for inducing antigen-specific T cells from hematopoietic stem cells or peripheral mature T cells (Bendle et al., 2010; Brenner and Okur, 2009).

DISCUSSION

Using a HIV-1-epitope-specific CTL clone as a model, we demonstrated here that the reprogramming into pluripotency of a T cell clone and the subsequent redifferentiation to mature functional $CD8^+$ T cells are possible. These redifferentiated $CD8^+$ T cells are highly proliferative naive cells with elongated telomeres, and they exert T cell functions in the same HIV-1-epitope-specific manner, permitting the inference that this process of reprogramming and redifferentiation can rejuvenate mature antigen-specific T cells.

Generation of iPSCs from T cells was initially difficult. On the basis of reports by Seki et al. (2010), we also found that SeV is suitable for the reprogramming of aged and exhausted fibroblasts, as well as of T cells. We also found that coexpression of SV40 large T antigen acted synergistically with the classic Yamanaka factors in enhancing the reprogramming efficiency of T cells. Therefore, SV40 large-T antigen introduction using the SeV vector system was also included in the protocol. Worth noting is that c-*MYC* is a known oncogene, and when it is inserted into the genomic DNA by the retroviral vector, it may become a risk for tumorigenesis in the generation of iPSCs. The same concern does not apply to SeV vector systems, given that the genomic RNA could be removed from the cytosol after reprogramming. Therefore, the utilization of SeV vectors both improved reprogramming efficiency and shielded redifferentiating cells from oncogene- or provirus-mediated tumorigenesis (Kohn et al., 2003).

In the redifferentiation experiments, mimicking TCR signaling led to CD8-linage specification without reassembly of *TCRA* genes. Preassembled TCR genes are a distinctive feature of T-iPSCs not found on other pluripotent stem cells. TCR$\alpha\beta$ is aberrantly expressed on redifferentiating CD4/CD8 DN cells, and the TCR signaling evoked results in the cessation of *RAG* expression. Serwold and colleagues reported that aberrantly early expression of TCR from preassembled *Tcra* and *Tcrb* following TCR signaling in murine thymocytes drives later lymphomagenesis (Serwold et al., 2010). They cautioned that T-iPSCs might confer risk for TCR-mediated lymphomagenesis. Therefore, the redifferentiation method will need to be further optimized and confirmed for clinical safety before application in practical treatments. This may be achieved by the use of an inducible suicide-gene system for eliminating unwanted tumors after injections (Hara et al., 2008; Veldwijk et al., 2004).

Immunological assays found that the redifferentiated $CD8^+$ T cells exerted T cell functions such as cytolytic activity, IFN-γ secretion, and degranulation in a normal manner when stimulated with their specific antigens. The most striking difference was in their proliferation capacity and elongated telomeres, which correlates with the central-memory T cell phenotype. Stem cell-like memory T cells (T_{SCM}) were recently identified as a subpopulation of T cells that has the capacity for self-renewal and that is multipotent and able to generate central memory, effector memory, and effector T cells (Gattinoni et al., 2011; Turtle et al., 2009). In a humanized mouse model, T_{SCM} cells reconstituted the T cell population more efficiently than other known memory subsets while mediating a superior antitumor response. It was found that inhibition of GSK3β enhances the generation of T_{SCM} in culture. Combining T-iPSC-mediated T cell rejuvenation with GSK3β inhibition may therefore enable efficient generation of T_{SCM} cells and permit highly effective immunotherapy along with the reconstitution of a normal T cell immune system.

Although these data suggest that rejuvenated T cells enjoy an advantage over the original T cell clone, it remains unclear whether these HIV-epitope-specific rejuvenated T cells are effective in improving the overall status of HIV infection. This is because the role of $CD8^+$ T cells in HIV infection appears to vary depending on the disease stage (Appay et al., 2000; Borrow et al., 1994; Brodie et al., 1999; Day et al., 2006; Koup et al., 1994). Evasion of the immune response through CTL escape is another important factor in HIV pathogenesis, and the escaped virus is a substantial hurdle for HIV therapies (Phillips et al., 1991). Therefore, this system may work best instead against tumors such as a melanoma, for which certain antigenic epitopes are known, or against viral infections other than HIV, for which the roles of $CD8^+$ cytotoxic T cells are more established. Nonetheless, the system described in our study will make it possible to preserve and to supply highly proliferative, functional $CD8^+$ T cells specific to a variety of HIV epitopes without worrying about exhaustion. It may also act as a valuable tool in better understanding the role of adoptive immunity in HIV infection.

Here, we have presented a proof of concept of $CD8^+$ T cell rejuvenation. The concept is not limited only to $CD8^+$ cytotoxic T cells. It may also be applied to $CD4^+$ helper or regulatory T cells to control desired or undesired immune reactions in the context of malignancies, chronic viral infections, autoimmune diseases, or transplantation-related immune disorders, if optimization of redifferentiation conditions can be achieved. Biological and technical challenges lie ahead, but the data presented in this work open new avenues toward antigen-specific T cell therapies that will supply unlimited numbers of rejuvenated T cells and will regenerate patients' immune systems.

EXPERIMENTAL PROCEDURES

Generation of Antigen-Specific CTL Clones

Nef138-8(WT)-specific CTL lines were induced from PBMCs of a patient chronically infected with HIV-1 who is positive for HLA-A24, as described (Kawana-Tachikawa et al., 2002). Each CTL line was expanded from a single-cell sorted $tetramer^+$ T cell, and the cells in every CTL line were confirmed for expression of only one kind of TCR$\alpha\beta$. For more details of CTL-clone establishment, see the Supplemental Experimental Procedures.

Generation of T-iPSCs

Human iPSCs were established from PB T cells or a CTL clone as described (Takayama et al., 2010), slightly modifying the culture conditions. In brief, T cells were stimulated by α-CD3/CD28 antibody-coated beads (Miltenyi Biotec) or by 5 μg/ml PHA-L (Sigma-Aldrich). The activated cells were transduced with reprogramming factors via retroviral or SeV vectors and were cultured in RH10 medium (RPMI-1640 supplemented with 10% human AB Serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 ng/ml streptomycin), which was

gradually replaced with human iPSC medium (Dulbecco's modified Eagle's medium/F12 FAM supplemented with 20% knockout serum replacer, 2 mM L-glutamine, 1% nonessential amino acids, 10 μM 2-mercaptoethanol, and 5 ng/ml basic fibroblast growth factor [bFGF]). The established iPSC clones were transfected with small interfering RNA L527 (Nishimura et al., 2011) using Lipofectamine RNAi Max (Invitrogen) for removal of SeV vectors from the cytoplasm.

Analysis of TCR Gene Rearrangement in Genomic DNA

Genomic DNA was extracted from approximately 5×10^6 cells using QIAamp DNA kits (QIAGEN) according to the manufacturer's instructions. For *TCRB* gene rearrangement analysis, PCR was performed according to BIOMED-2 protocols (van Dongen et al., 2003). For *TCRA* gene rearrangement analysis, PCR was performed using the primers shown in Figure S2 and LA Taq HS (TaKaRa). The PCR protocol entailed three amplification cycles (30 s at 95°C, 45 s at 68°C, and 6 min at 72°C); 15 amplification cycles (30 s at 95°C, 45 s at 62°C, and 6 min at 72°C); and 12 amplification cycles (15 s at 95°C, 30 s at 62°C, and 6 min at 72°C). The dominant band within the expected size range was purified using a QIAquick gel-extraction kit (QIAGEN) and was then sequenced. V, D, and J segment usages were identified by comparison to the ImMunoGeneTics (IMGT) database (http://www.imgt.org/) and by using an online tool (IMGT/V-QUEST) (Lefranc, 2003). Gene-segment nomenclature follows IMGT usage.

Analysis of TCR Gene Rearrangement in mRNA

A method based on the "switch mechanism at the 5′-end of the reverse transcript (SMART)" (Du et al., 2006) was used to synthesize double-stranded cDNAs (Super SMART cDNA synthesis kit; BD Clontech). Reverse transcription was conducted with the 3′ SMART CDS primer, SMART II A oligonucleotides (Super SMART cDNA synthesis kit), and PrimeScript Reverse Transcriptase (TaKaRa) for 90 min at 42°C. Double-stranded cDNA was then synthesized and was amplified with 5′ PCR Primer II A (Super SMART cDNA synthesis kit), and reagents were provided in an Advantage 2 PCR Kit (BD Clontech). The PCR protocol entailed 20 cycles of 5 s at 95°C, 5 s at 65°C, and 3 min at 68°C. The amplified double-stranded cDNA was used as templates in TCRA- or TCRB-specific amplification reactions. With forward primer (2^{nd}_5′-SMART) and reverse primer (3′-TRAC for *TCRA* or 3′-TRBC for *TCRB*), 25 cycles of amplification were performed (30 s at 94°C, 30 s at 55°C, and 1 min at 72°C). PCR products were cloned into pGEM-T Easy Vector (Promega) and were sequenced.

T Cell Differentiation from T-iPSCs

To differentiate human iPSCs into hematopoietic cells, we slightly modified a previously described protocol (Takayama et al., 2008). Small clumps of iPSCs (<100 cells) were transferred onto irradiated C3H10T1/2 cells and cocultured in EB medium (Iscove's modified Dulbecco's medium supplemented with 15% fetal bovine serum [FBS] and a cocktail of 10 μg/ml human insulin, 5.5 μg/ml human transferrin, 5 ng/ml sodium selenite, 2 mM L-glutamine, 0.45 mM α-monothioglycerol, and 50 μg/ml ascorbic acid) in the presence of VEGF, SCF, and FLT-3L. Hematopoietic cells contained in iPSC sacs were collected and were transferred onto irradiated OP9-DL1 cells (provided by RIKEN BRC through the National BioResource Project of the Ministry of Education, Culture, Sports, Science, and Technology [MEXT]) (Watarai et al., 2010). The hematopoietic cells underwent T lineage differentiation on OP9-DL1 cells during coculture in OP9 medium (αMEM supplemented with 15% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 ng/ml streptomycin) in the presence of FLT-3L and IL-7. The T lineage cells were then harvested, mixed with irradiated HLA-A24$^-$ PBMCs, and cocultured in RH10 medium in the presence of IL-7 and IL-15.

Intracellular Staining

For intracellular staining of granzyme B, T cells were stimulated by α-CD3/28 beads or peptide-loaded HLA-A24$^+$ B-LCLs. After 2 hr, brefeldin A (5 μg/ml; Invitrogen) was added, with incubation for 4 hours more. Cells were then harvested and fixed in Fixation/Permeabilization solution (BD Biosciences). Intracellular staining was performed as per the manufacturer's protocol using Perm/Wash buffer (BD Biosciences) and fluorescein isothiocyanate (FITC)-conjugated granzyme B antibody (BD Biosciences). For capturing CD107a transiently expressed on cell surfaces, T cells were incubated with α-CD3/28 beads or peptide-loaded HLA-A24$^+$ B-LCLs and were cultured with FITC-conjugated CD107a antibody (BioLegend) for 6 hr. Harvested cells were fixed and stained as described above. Data were acquired on FACSAria II equipment (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Measurement of Telomere Length by Flow-FISH

Telomere length was measured using a Telomere PNA Kit/FITC (DAKO) as previously described (Neuber et al., 2003).

ELISPOT and ^{51}Cr Release Assays

The antigen-specific responses of T cells were measured using an ELISPOT assay for IFN-γ and a standard ^{51}Cr release assay as described (Kawana-Tachikawa et al., 2002; Tsunetsugu-Yokota et al., 2003). HLA-A24$^+$ B-LCLs were used as antigen-presenting cells.

Statistics

All data are presented as mean ± SD. All statistics were performed using Excel (Microsoft) and Prism (GraphPad software) programs, applying two-tailed Student's t test. Values of $p < 0.05$ were considered significant. For additional details, see the Supplemental Experimental Procedures.

ACCESSION NUMBERS

The Gene Expression Omnibus accession number for microarray data reported in this paper is GSE43136.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2012.11.002.

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SALL4, a Stem Cell Biomarker in Liver Cancers

Tsunekazu Oikawa[1,7], Akihide Kamiya[2,10], Mikio Zeniya[3], Hiromi Chikada[2], Ahn Dong Hyuck[4], Yuji Yamazaki[4], Eliane Wauthier[7], Hisao Tajiri[1], Lance D. Miller[5], Xin Wei Wang[6], Lola M. Reid[7-10] and Hiromitsu Nakauchi[4,10]

[1]Division of Gastroenterology and Hepatology, Department of Internal Medicine, Jikei University School of Medicine, 3-19-18, Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan

[2]Institute of Innovative Science and Technology, Tokai University, 143 Shiomokasuya, Isehara, Kanagawa 259-1193, Japan

[3]Gastroenterology, Jikei University Graduate School of Medicine, 3-19-18, Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan

[4]Division of Stem Cell Therapy, Center for Stem Cell and Regenerative Medicine, The Institute of Medical Science, University of Tokyo, 4-6-1 Shirokane-dai, Minato-ku, Tokyo 108-8639, Japan

[5]Department of Cancer Biology, Wake Forest School of Medicine, Winston Salem, North Carolina 27157

[6]Laboratory of Human Carcinogenesis, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland 20892

[7]Department of Cell Biology and Physiology, [8]Program in Molecular Biology and Biotechnology, and [9]Full member, UNC Lineberger Comprehensive Cancer Center of the University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

[10]co-equal senior authors

Tsunekazu Oikawa: oitsune@jikei.ac.jp
Akihide Kamiya: kamiyaa@tokai-u.jp
Mikio Zeniya: zeniya@jikei.ac.jp
Hiromi Chikada: chikadahiromi@tokai-u.jp
Ahn Dong Hyuck: ahndh@ims.u-tokyo.ac.jp
Yuji Yamazaki: yuji@ims.u-tokyo.ac.jp
Eliane Wauthier: elaine_wauthier@med.unc.edu
Hisao Tajiri: tajiri@jikei.ac.jp
Lance D. Miller: ldmiller@wakehealth.edu
Xin Wei Wang: xw3u@nih.gov
Lola M Reid: Lola.M.Reid@gmail.com
Hiromitsu Nakauchi: nakauchi@ims.u-tokyo.ac.jp

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Corresponding Authors:
Akihide Kamiya, PhD. Institute of Innovative Science and Technology, Tokai University, 143 Shiomokasuya, Isehara, Kanagawa 259-1193, Japan. E-mail: kamiyaa@tokai-u.jp, Tel: (81) 463-93-1121 (ext 2783), Fax: (81) 463-95-3522
And
Hiromitsu Nakauchi, MD, PhD. Division of Stem Cell Therapy, Center for Stem Cell and Regenerative Medicine, The Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. E-mail: nakauchi@ims.u-tokyo.ac.jp, Tel: (81) 3-5449-5330, Fax: (81) 3-5449-5451.
And
Lola M. Reid, PhD. Department of Cell Biology and Physiology and Program in Molecular Biology and Biotechnology. Full Member of the Lineberger Cancer Center. UNC School of Medicine, Chapel Hill, NC 27599. Email: Lola.M.Reid@gmail.com. Tel: 919-966-0346. Fax: (919) 966-6112.

Abbreviations: ABCG2, ATP-binding Cassette-G2; AFP, alpha-fetoprotein; ALB, albumin; BD, bile duct; CASP3, caspase-3; CC, cholangiocarcinoma; CK19,

cytokeratin19; CSCs, cancer stem cells; DAPI, 4',6-diamidino-2-phenylindole; DP, ductal plate; EMT, epithelial–mesenchymal transition; EpCAM, epithelial cell adhesion molecules; FACS, fluorescent-activated cell sorter; FL-HCC, fibrolamellar hepatocellular carcinoma; 5-FU, 5-fluorouracil; hBTSCs, human biliary tree stem cells, hHBs, human hepatoblasts; HCC, hepatocellular carcinoma; HC-CC, combined hepatocellular and cholangiocarcinoma; HNF4α, hepatocyte nuclear factor 4-alpha; hHpSCs, human hepatic stem cells; PBGs, peribiliary glands; PT, portal tract; qRT-PCR, quantitative real-time polymerase chain reaction; shRNA, short hairpin RNA; TICs, tumor-initiating cells; TTR, transthyretin; UGT2B7, UDP-glucuronosyltransferase-2B7

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Author Contributions: The project was originally conceived and experimentally designed by T. Oikawa, A. Kamiya and H. Nakauchi. T. Oikawa and A. Kamiya did the collection and assembly of data, data analyses and interpretation of the data, especially that on mechanistic studies with respect to SALL4. H. Chikada, Y. Yamaszaki and A.D. Hyuck helped with collection and assembly of data. E. Wauthier and L. M. Reid established the bank of normal fetal, neonatal, pediatric and adult human livers, biliary tree tissue, and pancreatic tissues and that of surgical specimens of hepatocellular carcinomas (HCCs), cholangiocarcinomas (CCs) the combined hepatocellular and cholangiocarcinoma (HC-CC) and the fibrolamellar hepatocellular carcinoma (FL-HCC). They also established cultures and transplantable tumor lines of some of the HCCs, CCs and the FL-HCC. The management and funding of these studies on human tissues were done by L. M. Reid. L. M. Reid and T. Oikawa designed the experiments; T. Oikawa collected the data; and T. Oikawa and L. M. Reid together did data analyses and interpretation of data. X. W. Wang and L. D. Miller performed the bioinformatics analyses correlating SALL4 expression in liver cancers with patient survival. The manuscript was drafted and edited by T. Oikawa, A. Kamiya, L. M. Reid, and they handled responses to reviewers. M. Zeniya, H. Tajiri helped with editing of the manuscript. H. Nakauchi also did management and interpretation of the data and helped with writing and editing of the manuscript.

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Abstract

Liver cancers, hepatocellular carcinomas (HCCs), cholangiocarcinomas (CCs) and fibrolamellar HCCs (FL-HCCs), are among the most common cancers worldwide and are associated with a poor prognosis. Investigations of genes important in liver cancers have focused on Sal-like protein 4, SALL4, a member of a family of zinc finger transcription factors. It is a regulator of embryogenesis, organogenesis, pluripotency, can elicit reprogramming of somatic cells, and is a marker of stem cells. We found it expressed in normal murine hepatoblasts, normal human hepatic stem cells, hepatoblasts and biliary tree stem cells, but not in mature parenchymal cells of liver or biliary tree. It was strongly expressed in surgical specimens of human HCCs, CCs, a combined hepatocellular and cholangiocarcinoma, a FL-HCC and in derivative, transplantable tumor lines in immune-compromised hosts. Bioinformatics analyses indicated that elevated expression of SALL4 in tumors is associated with poor survival of HCC patients.

Experimental manipulation of SALL4's expression results in changes in proliferation versus differentiation in human HCC cell lines *in vitro* and *in vivo* in immune-compromised hosts. Virus-mediated gene transfer of SALL4 was used to do gain and loss of function analyses in the cell lines. Significant growth inhibition *in vitro* and *in vivo*, accompanied by an increase in differentiation occurred with down-regulation of SALL4. Over-expression of SALL4 resulted in increased cell proliferation *in vitro,* correlating with an increase in expression of cytokeratin19 (CK19), EpCAM, and ATP-binding cassette-G2 (ABCG2).

SALL4's expression is an indicator of stem cells, a prognostic marker in liver cancers, correlates with cell and tumor growth, with resistance to 5-FU, and its suppression results in differentiation and slowed tumor growth. SALL4 is a novel therapeutic target for liver cancers.

Introduction

Liver cancers, comprised primarily of hepatocellular carcinomas (HCCs), cholangiocarcinomas (CCs), and fibrolamellar HCCs (FL-HCCs), are the fifth most common cancer and the third leading cause of cancer mortality in the world(1). Cancers have a subpopulation of cancer stem cells (CSCs) or tumor-initiating cells (TICs), which have properties shared with normal stem cells(2,3). CSCs and TICs have highly aggressive phenotypes in oncogenesis and are resistant to chemotherapies and radiation therapies. Expression of membrane pumps, ATP-binding cassette-G2 (ABCG2), account for the resistance to chemotherapies and are responsible for elimination of DNA-binding dyes causing the cells to be displayed as a side fraction, a "side population (SP)"(4,5). Epithelial cell adhesion molecule (EpCAM), a key factor in the Wnt signaling pathway, was reported as a specific cell surface markers of human hepatic stem cells (hHpSCs), of some, but not all, subpopulations of human biliary tree stem cells (hBTSCs)(6-8) and liver TICs(9). CD133 (prominin), CD90 (Thy-1), CD44 (hyaluronan receptor), and CD13 (alanine aminopeptidase) have also been found in liver TICs(10-12). In parallel, CD133 and CD90 have been found on angioblasts or other mesenchymal cells tightly associated with hHpSCs(13), and so, some data discussing CD90 or CD133 may actually be interpreted as relevant to the mesenchymal cell components of the tumors. Several lines of evidence implicate genetic alternations during hepatocarcinogenesis, particularly the Wnt signaling pathway, p53 and alterations in matrix-degrading enzyme secretion(14-20).

SALL4, a homologue of the Drosophila homeotic gene spalt, is a zinc finger transcription factor required for proliferation and maintenance of pluripotency through interactions with OCT3/4, SOX2 and NANOG. It is found at high levels in embryonic stem cells (ESCs)(21-26), and is one of the genes capable of eliciting reprogramming of somatic cells to become induced pluripotent stem cells (iPSCs)(27,28). Mutations in

SALL4 cause Okihiro syndrome, known as an autosomal dominant disorder and characterized by multiple organ defects(29). Recent studies have demonstrated that SALL4 is constitutively expressed in hematopoietic stem cells and a potent regulator of their expansion(30,31). SALL4 transgenic mice exhibit symptoms like myelodysplastic syndrome (MDS) and subsequently develop acute myeloid leukemia (AML). Primary AML and MDS patients have higher SALL4 expression levels than that in controls indicating that SALL4 plays a major role in leukemogenesis. Furthermore, SALL4 contributed to the maintenance of SP cells and chemosensitivity in leukemia by regulating the ABC drug transporter genes(31-33). Solid tumors, such as germ cell tumors, breast and alpha-fetoprotein (AFP)-producing gastric cancers also express SALL4(34-37). Taken together, these data suggest that SALL4 is a novel stem cell marker, a gene involved in embryogenesis and organogenesis and a putative stem cell gene associated with CSCs. We now report that SALL4 expression occurs in diverse liver cancers including HCCs, CCs and FL-HCCs, and that SALL4 increases growth and blocks differentiation in liver cancer cell lines.

Materials and Methods

Cell Proliferation and Chemo-resistance Assays

Liver cancer cell lines were infected with retroviruses or lentivirus at multiplicity of infection of 40 in the presence of 10 μg/mL protamine sulfate. After infection, cells were cultured for 3 days. Cells then were collected and isolated using a MoFlo™ fluorescence-activated cell sorter (FACS) (DAKO, Glostrup, Denmark). Then, 2×10^3 cells were seeded into 96 well plates and cultured in the presence or absence of 2 μg/ml 5-fluorouracil (5-FU) for 3 to 7 days. Cell proliferation was evaluated in triplicate using the Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan). After incubation at 37°C for 2 h, the absorbance at 450 nm was measured.

Immunohistochemistry

The tissues were embedded in paraffin and cut into 5 μm sections. After deparaffinization, antigen retrieval was performed with sodium citrate buffer for EpCAM, CK19, or ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0) for SALL4 in a steamer for 20 min. Endogenous peroxidases were blocked by incubation for 30 min in 0.3% H_2O_2. After blocking, primary antibodies (**Supplementary Table 3**) were applied at 4ºC overnight. M.O.M immunodetection kit (Vector Laboratories, Burlingame, CA) was used for detecting primary mouse anti-human SALL4 antibody on mouse xenotransplant FL-HCC tumor to avoid the inability of the anti-mouse secondary antibody to endogenous mouse immunoglobulins in the tissue. Sections were incubated for 30 min at room temperature with ImmPRESS peroxidase-micropolymer staining kits and 3,3'-diaminobenzidine substrate (Vector Laboratories). For double immunostaining, MACH2 peroxidase- and alkaline phosphatase-polymer detection kit, 3,3'-diaminobenzidine and Warp Red chromogen kit (Biocare Medical, Concord, CA) were used. Sections were lightly counterstained with hematoxylin.

Xenograft Transplantation

Each transplant consisted of 1×10^6 cells of each of the cell lines stably expressing-shRNA against *SALL4* or *luciferase* suspended in 200 μl Dulbecco's modified Eagle medium (DMEM) and Matrigel (1:1). The cells were transplanted into Non-obese diabetic, severe combined immunodeficient (NOD/SCID) mice (6-week-old, male) under anesthesia. Control and SALL4-knockdown cells were implanted into the subcutaneous space on the right and left sides of the backs of recipient mice, respectively. For 8 weeks, the mice were examined for tumor formation.

SALL4 Profiling Analyses in HCCs

SALL4 expression data were derived from cDNA microarray analysis of 139 HCC specimens described previously(38). The microarray data, with NCI's Human Array-Ready Oligo Set microarray platform (GPL1528), are publically available at the Gene Expression Omnibus (GEO;http;://www.ncbi.nlm.nih.gov/geo) with accession numbers GSE1898 and GSE4024. High and low SALL4 groups were dichotomized according to the median SALL4 expression in tumors. Kaplan-Meier survival analysis was used to compare patient survival based on dichotomized SALL4 expression, using GraphPad Prism software 5.0 (GraphPad Software, San Diego, CA) with statistical P values generated by the Cox-Mantel log-rank test. Survival data linking to this cohort were kindly provided by Dr. Snorri Thorgeirsson at NCI.

Other material and methods can be found in the online supplement.

Results

SALL4 Expression in Human Normal Liver and Biliary Tree Tissues in situ and in vitro

We have previously reported that SALL4 is expressed in murine hepatoblasts (mHBs) but not adult murine hepatocytes and plays a critical role in their differentiation(39). In these studies, we analyzed SALL4 expression in normal human liver tissues. Immunohistochemical analyses showed that SALL4 is diffusely expressed in the nuclei of liver cells from both fetuses and neonates. Neonatal hepatocytes were more weakly positive for SALL4 than parenchymal cells in fetal livers and some had lost SALL4 expression altogether. In contrast, SALL4 expression was not detected in mature hepatocytes and cholangiocytes in adult livers (**Fig.1A-C**). Double immunostaining of EpCAM and cytokeratin19 (CK19) show clearly that EpCAM and CK19 strongly co-stain

the cytoplasm of ductal plate cells, now recognized to comprise hHpSCs, and human hepatoblasts (hHBs) in fetal and neonatal livers. It is found also in hBTSCs within peribiliary glands (PBGs), the stem cell niches of the biliary tree, in neonatal livers (**Fig.S1A**) and in adult livers(40). We found that SALL4 co-expressed with EpCAM+/CK19+ ductal plate cells, known to comprise hHpSCs (***arrows***), and the adjacent hHBs (***arrowheads***). It also was found in multiple subpopulations of hBTSCs within PBGs located within livers or biliary tree tissue from all donor ages and included cellular subpopulations that are EpCAM-/CK19+, EpCAM-/CK19-, EpCAM+/CK19- and EpCAM+/CK19+ cells. Shown are ones from fetal or neonatal livers (**Figs.1D,S1A**). We also found that SALL4, NCAM and EpCAM co-expressed in colonies of hHpSCs and in colonies of hBTSCs (**Fig.1E-F,S1B**). These results suggest that SALL4 is found only in early lineage stage parenchymal cells, such as hHpSCs, hBTSCs, hHBs, and to less extent in committed progenitors, but not in later lineage stages of parenchymal cells of either liver or biliary tree.

SALL4 Expression in Human Liver Cancers

We analyzed SALL4 expression in surgical specimens of non-cancerous liver tissue and in liver cancers. SALL4 was not detected in chronic hepatitis but faintly detected in bile ductules and in hepatocytes at the interface of parenchymal and stromal cells in liver cirrhosis (**Fig.S2A-B**). Seventeen of 20 HCC specimens were positive for SALL4 in the nuclei of the tumor cells, whereas 3 specimens showed no SALL4 expression. In some cases, biliary epithelial cells, presumptive hBTSCs, around the tumors expressed SALL4 (**Figs.2A-C,S2C-D**). Four of 5 CC specimens expressed SALL4. We found that SALL4 is expressed in combined hepatocellular and cholangiocarcinoma (HC-CC) and in a transplantable human tumor line derived from a FL-HCC (**Fig.2D-F**). Double immunostaining showed that SALL4+/EpCAM+/CK19+ cancer cells were observed in

CC, which strongly expressed EpCAM and CK19 in serial sections (**Fig.S2E-F**). These results suggest that SALL4 expression indicates selection for stem cells as a minor cell population in normal tissue and cirrhotic tissues and as a dominant cell population in liver cancers.

SALL4 Expression in Human Liver Cancer Cell Lines

To investigate the functions of SALL4 in liver cancers, we used liver cancer cell lines, Huh7 and PLC/PRF/5 cells. The quantitative real time-polymerase chain reaction (qRT-PCR) analyses showed that both cell lines expressed SALL4A mRNA. SALL4 protein was also detected using immunocytochemistry (**Fig.3A**).

Regulation of Cell Proliferation by SALL4

To examine whether SALL4 regulates tumor growth of liver cancer cell lines, we used a SALL4A-over-expressing retroviral vector(28). Over-expression of SALL4A was verified using qRT-PCR. Transduction of SALL4A into the cells significantly increased SALL4A mRNA and also protein levels by Western blots and immunocytochemistry (**Figs.3B,S3**). SALL4A-over-expressing liver cancer cells had enhanced cell proliferation (**Fig.3C**).

Next, we conducted SALL4 expression knockdown studies using a lentiviral vector expressing-short hairpin RNA (shRNA)(32, 39). Transduction efficiency was estimated using FACS revealing that the percentage of cells infected with lentiviruses expressing-shRNA against *luciferase* or *SALL4* was more than 90% (**Fig.S4**). Transduction of shRNA into the cells significantly decreased both mRNA and protein production of SALL4 (**Fig.3D**). We observed growth inhibition in SALL4-knockdown liver cancer cells in culture (**Figs.3E,S5**). Therefore, SALL4 regulates the proliferative potential of liver cancer cell lines *in vitro*.

SALL4 Regulates Cell Proliferation through Cyclin D1 and D2 Expressions

To analyze molecular mechanisms regulating SALL4-induced proliferation of liver cancer cell lines, cell-cycle analyses were examined. Cell-cycle analyses using flow cytometry showed that over-expression of SALL4 induced the decrease of the G1 phase in liver cancer cells (**Fig.4A**). Next, Cyclin D1 and D2 mRNA expressions were examined using qRT-PCR. Consistent with the flow cytometry analysis, Cyclin D1 and D2 levels were induced by SALL4A over-expression. In contrast, their levels were decreased by SALL4 knockdown (**Fig.4B**), implicating a correlation of Cyclin levels to those of cell proliferation. Though we also analyzed expression of cyclin inhibitors, significant changes were not observed (data not shown).

To exclude the possibility that shRNA-knockdown of SALL4 expression inhibited cell proliferation by means of an induction of apoptosis, we analyzed the effect of viral infection on apoptosis of the liver cancer cell lines. The qRT-PCR analyses showed that caspase-3 (CASP3) expression, an early stage marker of apoptosis, did not change in SALL4-knockdown liver cancer cells (**Fig.4C**). Apoptosis was also evaluated using flow cytometric analyses. The number of Annexin-V+ cells did not change by SALL4 knockdown, suggesting that inhibition of cell proliferation was not due to apoptosis (**Fig.4D**).

SALL4 Expression is Inversely Correlated with Differentiation Markers

Given that hepatocytic maturation was suppressed by SALL4 over-expression in mHBs(39), we hypothesized that SALL4 could affect the differentiation of liver cancer cell lines. To explore this, we analyzed mRNA expression for hepatocytic differentiation marker genes using qRT-PCR. Expression of albumin (ALB), transthyretin (TTR), and UDP-glucuronosyltransferase-2B7 (UGT2B7) were suppressed

by SALL4 over-expression. In contrast, their levels were significantly enhanced in SALL4-knockdown liver cancer cells (**Figs.5A,S6A,S7**). These results suggested that SALL4 inhibits hepatocytic differentiation in mHBs and also human liver cancer cell lines. Hepatocyte nuclear factor 4-alpha (HNF4α), a key transcriptional factor regulating differentiation of HBs into hepatocytes with acquisition of mature liver functions, did not decrease in SALL4-over-expressing liver cancer cells, indicating that SALL4 inhibits hepatocytic differentiation through a pathway independent of HNF4α (**Fig.S6A**). As shown above, CK19 and EpCAM are expressed in normal hHpSCs, hHBs, and cholangiocytes in livers of all donor ages but not adult hepatocytes, and EpCAM is also a TIC marker for liver cancer. Over-expression of SALL4 in liver cancer cells induced expression of CK19 and EpCAM (encoded by TACSTD1 gene), indicating a correlation between SALL4 and CK19. Down-regulation of SALL4 suppressed the expression of CK19 but not EpCAM in liver cancer cells. SALL4-over-expressing PLC/PRF/5 cells had up-regulated POU5F1 (OCT3/4) and CD90 (**Figs.5B,S6B,S7**). Similarly, ABCG2, a multidrug resistance gene found in normal hHpSCs as well as in CSCs and responsible for chemo-resistance, was significantly increased in SALL4-over-expressing Huh7 cells. In contrast, SALL4 knockdown of liver cancer cells resulted in lowered ABCG2 levels (**Fig.5B**). These results suggest that SALL4 either plays a role controlling maintenance of stemness and TIC marker genes or is a biomarker for stem cell phenotypic traits.

SALL4 Increases Expression of EMT Genes but does not Influence Cell Invasion

Epithelial–mesenchymal transition (EMT) phenomena occurs in invasion and metastasis of cancer cells and is also associated with the acquisition of stem cell-like characteristics. To investigate whether SALL4 regulates EMT, we analyzed its effects on EMT-related genes in liver cancer cell lines. The mRNA expression of CXCR4 and