行政院國家科學委員會專題研究計畫 成果報告

CD133 基因在口腔癌腫瘤化對於幹細胞特性維護及惡化進展之功能性研究

研究成果報告(精簡版)

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計 畫 主 持 人 ： 余承佳

計畫參與人員： 此計畫無其他參與人員

公 開 資 訊 ： 本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 101 年 01 月 15 日

中文摘要： 口腔癌(Oral Cancer)，是98年度台灣癌症死因的第六位。口腔癌的診斷及治療方式已經改善許多，然而口腔癌的預後成果及口腔癌病患存活率相當差。因此必須進一步研究口腔癌致癌細胞分子機轉。近年來有研究發現在高異質性癌症組織中有一子群細胞具有幹細胞特性，稱為癌症幹細胞或癌症起始細胞(cancer initiating cells)。在癌症研究上發現，癌症起始細胞證實對腫瘤的起始、增生、轉移扮演相當重要的角色。癌症起始細胞的存在可解釋為何病患經過化學治療或放射線治療後，癌症會有復發的情形。傳統醫療方式是以殺死癌細胞為主，但癌症起始細胞或許才是最主要的治療標的。口腔癌癌症起始細胞的存在已於近年的許多研究中被證實，然而，口腔癌症起始細胞相關分子機制仍須多方面的研究。先前本人利用無血清幹細胞篩選培養基研究從口腔癌細胞分離出口腔癌幹細胞球體(sphere body)。口腔癌幹細胞球體會高度表現幹細胞標記CD133且具備高腫瘤生成力。然而CD133對於口腔癌及口腔癌症起始細胞角色及分子機轉仍未知，本研究計畫目的主要將探討CD133基因在口腔癌腫瘤化對於幹細胞特性維護及惡化進展的功能。研究發現CD133表現抑制會抑制口腔癌癌幹細胞自我更能力及促進細胞凋亡及分化；而過度表現則會增進口腔癌細胞癌症幹細胞特性，在裸鼠實驗中，降低CD133表現可以減緩口腔癌腫瘤生成能力。進一步發現CD133可調控’上皮-間質細胞轉換過程’（Epithelial-mesenchymal transition，EMT）來影響幹細胞標記Oct4及Nanog量。綜上所述，本研究成果證實CD133在口腔癌腫瘤化生成過程對於癌症幹細胞特性之相關分子機轉，降低CD133表現量期待來消除口腔癌起始細胞特性而當作良好口腔癌治療標靶之設定。

中文關鍵詞： 口腔癌； CD133； 癌症起始細胞； 癌症幹細胞； 上皮細胞間質轉化

英文摘要：

英文關鍵詞：

行政院國家科學委員會補助專題研究計畫 ■成果報告 □期中進度報告

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計畫主持人：余承佳
計畫參與人員：

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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中　華　民　國　101 年　1 月　14　日

中文摘要

口腔癌(Oral Cancer)，是98年度台灣癌症死因的第六位。口腔癌的診斷及治療方式已經改善許多，然而口腔癌的預後成果及口腔癌病患存活率相當差。因此必須進一步研究口腔癌致癌細胞分子機轉。近年來有研究發現在高異質性癌症組織中有一子群細胞具有幹細胞特性，稱為癌症幹細胞或癌症起始細胞(cancer initiating cells)。在癌症研究上發現，癌症起始細胞證實對腫瘤的起始、增生、轉移扮演相當重要的角色。癌症起始細胞的存在可解釋為何病患經過化學治療或放射線治療後，癌症會有復發的情形。傳統醫療方式是以殺死癌細胞為主，但癌症起始細胞或許才是最主要的治療標的。口腔癌癌症起始細胞的存在已於近年的許多研究中被證實，然而，口腔癌症起始細胞相關分子機制仍須多方面的研究。先前本人利用無血清幹細胞篩選培養基研究從口腔癌細胞分離出口腔癌幹細胞球體(sphere body)。口腔癌幹細胞球體會高度表現幹細胞標記CD133且具備高腫瘤生成力。然而CD133對於口腔癌及口腔癌症起始細胞角色及分子機轉仍未知，本研究計畫目的主要將探討CD133基因在口腔癌腫瘤化對於幹細胞特性維護及惡化進展的功能。研究發現CD133表現抑制會抑制口腔癌癌幹細胞自我更能力及促進細胞凋亡及分化;而過度表現則會增進口腔癌細胞癌症幹細胞特性，在裸鼠實驗中，降低CD133表現可以減緩口腔癌腫瘤生成能力。進一步發現CD133可調控”上皮-間質細胞轉換過程”（Epithelial-mesenchymal transition，EMT）來影響幹細胞標記Oct4及Nanog量。綜上所述，本研究成果證實CD133在口腔癌腫瘤化生成過程對於癌症幹細胞特性之相關分子機轉，降低CD133表現量期待來消除口腔癌起始細胞特性而當作良好口腔癌治療標靶之設定。

關鍵字：口腔癌; CD133; 癌症起始細胞; 癌症幹細胞; 上皮細胞間質轉化

Abstract

Oral Cancer is a human lethal cancer with clinical, pathological, phenotypical and biological heterogeneity. Caner initiating cells (CICs), which are responsible for tumor growth and coupled with gain of epithelial-mesenchymal transition (EMT), have been identified. CD133 (prominin-1), a 5-transmembrane glycoprotein, is originally recognized as a hematopoietic stem cells marker, and has been considered as an important cell surface marker to represent the subpopulation of cancer-initiating cells. Previously, we enriched a subpopulation of oral cancer-derived cancer initiating cells (OC-CICs) with up-regulation of CD133 and enhancement of EMT. Others demonstrate that Src kinase interacts with and phosphorylates the cytoplasmic domain of CD133. However, the physiological function of CD133/Src signaling in OSCCs has not been uncovered. Initially, down-regulation of CD133 significantly reduced the self-renewal ability and expression of stemness genes, and promoted the differentiation and apoptotic capability of OC-CICs. Additionally, knockdown of CD133 in OC-CICs also lessened both in vitro malignant properties including cell migration/cell invasiveness/anchorage independent growth, and *in vivo* tumor growth by nude mice xenotransplantation assay. In opposite, over-expression of CD133 enhanced the stemness properties and tumorigenic ability of OSCCs. Lastly, up-regulation of CD133 increased phosphorylation of Src coupled with EMT transformation in OSCCs, on the contrary, silence of CD133 or treatment of Src inhibitor inversely abrogated above phenotypic effects, which were induced by CD133 up-regulation in OSCCs or OC-CICs. Our present results suggested that CD133/Src signaling is a regulatory switch to gain of EMT and of stemness properties in OSCC. Finally, CD133/Src axis might be a potential therapeutic target for OSCC by a potential therapeutic target for OSCC by eliminating OC-CICs. The future works, the downstream signaling pathway of

CD133/Src induction, Ras/MAPK, PI3K/AKT and STAT3 will be investigated. Furthermore, the role of CD133 extracellular domain in CD133/Src signaling also will be investigated.

Key words: oral cancer; CD133; cancer initiating cells; cancer stem cells; epithelial-mesenchymal transformation

備註：本計畫已於 2011 年刊登於PLoS One雜誌(PLoS One 6: e28053: 1-12)

Introduction

Accumulating data demonstrate that tumor formation is driven by a subpopulation of cells that exhibit self-renewal capacity–the purported cancer stem cells (CSCs) or cancer initiating cells (CICs) 【1,2】. CICs have been shown to have the capacity to promote tumor progression and metastasis, and also contribute to radio-resistance and chemo-resistance【3】. Recently, we and others have verified the existence of CICs in OSCC (OC-CICs) 【4,5,6,7】. However, there is lack of evidence how cell surface signaling modulating the intracellular stemness properties or tumorigenicity of OC-CICs. CD133 (prominin-1), a 5-transmembrane glycoprotein, was originally recognized as a hematopoietic stem cells marker【8】. Consequently, CD133 has been considered as an important cell surface marker to represent the subpopulation of CICs in brain tumors, colon carcinoma, prostate carcinoma, hepatocellular carcinoma, thyroid carcinoma and head and neck cancer 【9,10,11,12,13,14,15】. Previously, we have demonstrated that the up regulation of CD133 in OC-CICs, further, the up-regulation of CD133 in OSCC cancerous tissue is negatively correlated with the survival prognosis of OSCC patients【6】. Recent reports also suggest that expression of CD133 in tumor tissues could serve as a prognostic indicator for tumor re-growth, malignant progression, and patient survival. Nevertheless, the CD133 mediated molecular mechanisms in regulating CICs in OSCC is still unclear. The detailed molecular mechanisms involved in the regulatory links between EMT and stem cell–related genes such as CD133 and Src are still poorly understood. Herein, we demonstrate a critical role of CD133 in the enhancement of stemness, gain of EMT, and promoting tumorigenicity of OC-CICs. Additionally, down-regulation of CD133 or inhibition of CD133 induced Src activation lessens stemness properties and tumorigenicity of OSCCs both *in vitro* and *in vivo*. Ultimately, we demonstrate the significance of CD133/Src signaling on EMT process in OSCC.

Purpose

Previously, we enriched a subpopulation of oral cancer-derived cancer initiating cells (OC-CICs) from oral squamous carcinoma cells (OSCCs) with up-regulation of CD133. In this proposal, the physiological function of CD133 and the molecular mechanisms mediated by CD133 in OC-CICs will be elucidated.

Materials and Methods

Stable overexpression of CD133 in OSCC cells

Human CD133 gene was amplified from human fetal lung and spleen cDNA template obtained from Biosettia Inc. (Cat. No. cDNA-hsa-09; San Diego, CA, USA) and then cloned into pCDH1-MCS1-EF1-copGFP (System Biosciences, Cat. No: CD511A-1; Mountain View, CA, USA). The sequences of oligos used for CD133 PCR amplification are 5'-ACCGTCTAGAATGGCCCTCGTACTCGGCTCCCTGTTGCTG-3' and 5'- ATCAAAGCTTATTGAAGCTGTTCTGCAGGTGAAGAG- tgcc-3'. Lentivirus production was

performed by co-transfection of plasmid DNA mixture with lentivector plus helper plasmids (VSVG and Gag-Pol) into 293T cells (American Type Culture Collection, Manassas, VA) using Lipofectamine 2000 (LF2000, Invitrogen, Calsbad, CA, USA). The lentivirus M.O.I titer is determined by flow cytometry (average of 5 x 10^4 and 2 x 10^5 TU/ml). To generate the stable cell lines, sub-confluent OSCCS cells were infected with lentivirus in the presence of 8 μg/ml polybrene (Sigma-Aldrich, St Louis, MO, USA). The green fluorescence protein (GFP), which was co-expressed in lentiviral-infected cells, was served as a selection marker to indicate the successfully infected OSCCs.

Construction of Lentiviral-mediated RNAi for silencing CD133

The pLV-RNAi vector, which co-expressing GFP protein in infected host cells, was purchased from Biosettia Inc. (Biosettia, San Diego, CA, USA). The method of cloning the double-stranded shRNA sequence is described in the manufacturer's protocol. Lentiviral vectors expressing shRNA that targets human CD133 (oligonucleotide sequence: Sh-CD133-1:5'-AAAAGGACAAGGCGTTCACAGATTTGGATCCAAATCTGTGAACGCCTTGTCC-3'; Sh-CD133-2:5'-AAAAGGATACACCCTACTTACTATTGGATCCAATAGTAAGTAGGGTGTATCC-3') were synthesized and cloned into pLVRNAi to generate a lentiviral expression vector. Sh-Luc:5'-CCGGACTTACGCTGAGTACTTCGAACTCGAGTTCGAAGTACTCAGCGTAAGTTTTTG-3' was utilized for experimental control. Lentivirus production was performed as above.

Side population analysis

For side population analysis, single HNSCC cells suspension at 1×10^6/ml was prepared in pre-warmed DMEM medium with 2% fetal bovine serum (FBS). Hoechst 33342 dye was then added at a final concentration of 5μg/ml in the presence or absence of fumitremorgin C (FTC) (10 μM; Sigma, St Louis, MO, USA) and was incubated at 37°C for 90 min with intermittent shaking. The cells were washed with ice-cold HBSS with 2% FBS and centrifuged at 4°C, and re-suspended in the same buffer. Propidium iodide at a final concentration of 2μg/ml was added for gating viable cells. The Hoechst 33342 dye was excited at 357 nm and its fluorescence was dual-wavelength analyzed (blue, 402–446 nm; red, 650–670 nm). Analyses were done on a FACS Vantage (BD, San Diego, CA, USA).

***In vitro* cell migration and invasion Assay**

For transwell migration assays, 2 x 10^5 cells were plated into the top chamber of a transwell (Corning, Acton, MA) with a porous membrane (8.0 μm pore size). Cells were plated in medium with lower serum (0.5% FBS), and medium supplemented with higher serum (10% FBS) was used as a chemoattractant in the lower chamber. The cells were incubated for 24 h at 37^oC and cells that did not migrate through the pores were removed by a cotton swab. Cells on the lower surface of the membrane were stained with Hoechst 33258 (Sigma-Aldrich) to show the nuclei; fluorescence was detected at a magnification of 100x using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Soft agar colony forming assay

Each well (35 mm) of a six-well culture dish was coated with 2 ml bottom agar (Sigma-Aldrich) mixture (DMEM, 10% (v/v) FCS, 0.6% (w/v) agar). After the bottom layer was solidified, 2 ml top agar-medium mixture (DMEM, 10% (v/v) FCS, 0.3% (w/v) agar) containing $2x10^4$ cells was added, and the dishes were incubated at 37^oC for 4 weeks. Plates were stained with 0.005% Crystal Violet then the colonies were counted. The number of total colonies with a diameter ≥100 μm was counted over five fields per well for a total of 15

fields in triplicate experiments.

Results

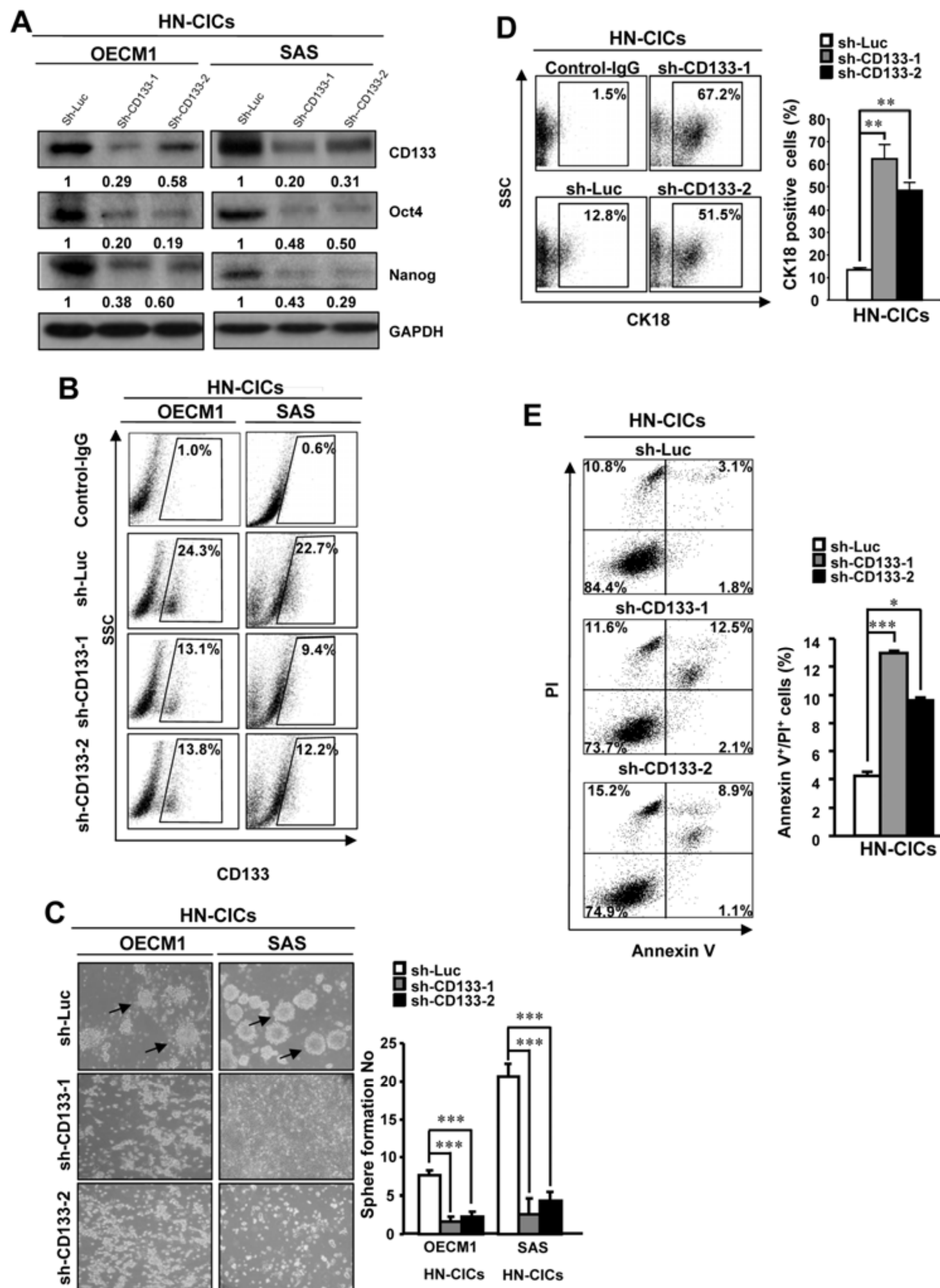

Figure 1. Depletion of CD133 impairs self-renewal property but inversely increases differentiation and apoptotic activity of OC-CICs.

(A) Single cell suspension of OC-CICs was transduced with sh-Luc or sh-CD133 lentivirus for 3 days. Total proteins prepared from infected cells were prepared and analyzed. The silencing effect of CD133 shRNA in OC-CICs was validated translationally by western blotting (OECM1 (*left panel*) and SAS (*right panel*)). Immunoblotting against anti-Oct-4, anti-Nanog, or anti-GAPDH antibodies was performed as indicated. The amount of GAPDH protein of different crude cell extracts was referred as loading control, and for further quantification. (B) Cell surface CD133 of sh-CD133-1, sh-CD133-2 and sh-Luc OC-CICs was analyzed by flow cytometry (C) OC-CICs were first infected with sh-CD133-1, sh-CD133-2 or sh-Luc lentivirus for 3 days, and then further cultivated under the serum-free defined selection medium. The cellular morphology of

OC-CICs treated with sh-Luc or CD133-shRNA lentivirus was examined. Arrows indicated the sphere cells. The expression profile of CK18 (D) or Annexin V vs. PI positive staining (E) of OC-CICs cells infected with sh-CD133-1, sh-CD133-2 or sh-Luc lentivirus was assessed, respectively, by flow cytometry. The percentage of Annexin V^{+}/PI^{+} double positive cells was recorded (E; right panel). The control IgG was used to define the baseline signal in (B) and (D). The experiments were repeated three times and the representative results were shown. Results are means ± SD (*, p< 0.05; ***, p< 0.001).

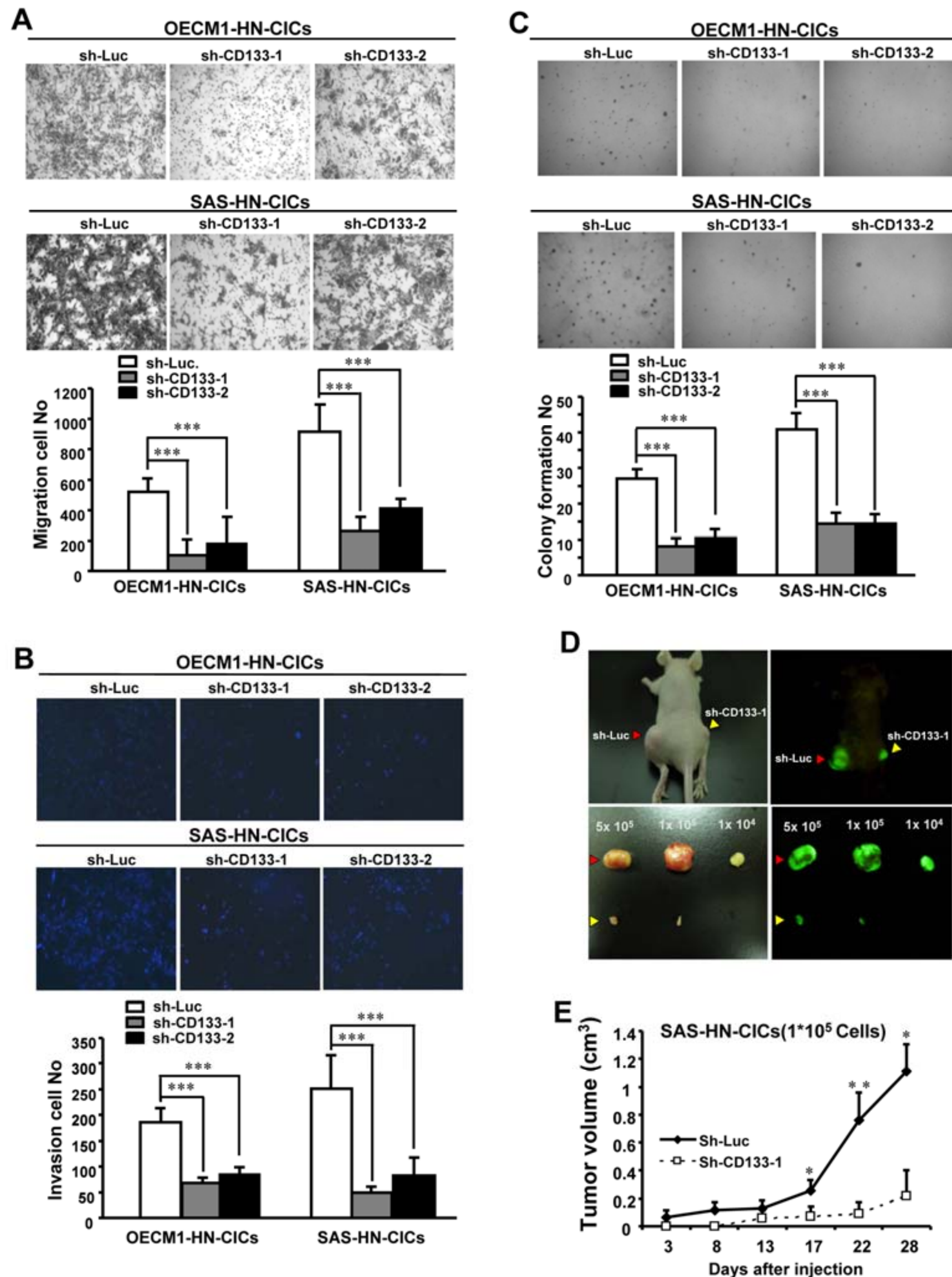

Figure 2. CD133 knockdown reduces migration/invasiveness/clonogenicity and impairs *in vivo* tumorigenic properties of OC-CICs.

To elucidate the capability of cell migration (A), cell invasiveness (B) and anchorage independent growth (C) of OC-CICs (OECM1 (*upper panel*), SAS (*lower panel*)) with CD133 down-regulation, single cell suspension of OC-CICs infected with CD133-specific shRNA or control sh-Luc lentivirus for three days were plated onto transwell, transwell coated with matrigel and soft agar, respectively, and analyzed as described in Materials and Methods. Results are means ± SD of triplicate samples from three experiments. (D) Representative

tumors of control OC-CICs and of CD133-knockdown SAS-derived OC-CICs were generated, and the tumors were then dissected from the subcutaneous space of recipient mice (n= 3)(Phase contrast: *left two panels*; GFP imaging*: right two panels*) (Red arrows: sh-Luc OC-CICs; Yellow arrows: sh-CD133-1 OC-CICs). (E) Tumor volume was measured, respectively, after inoculation of CD133-knockdown or sh-Luc–expressing SAS-derived OC-CICs. Error bars correspond to SD. (*, p< 0.05; ***, p< 0.001)

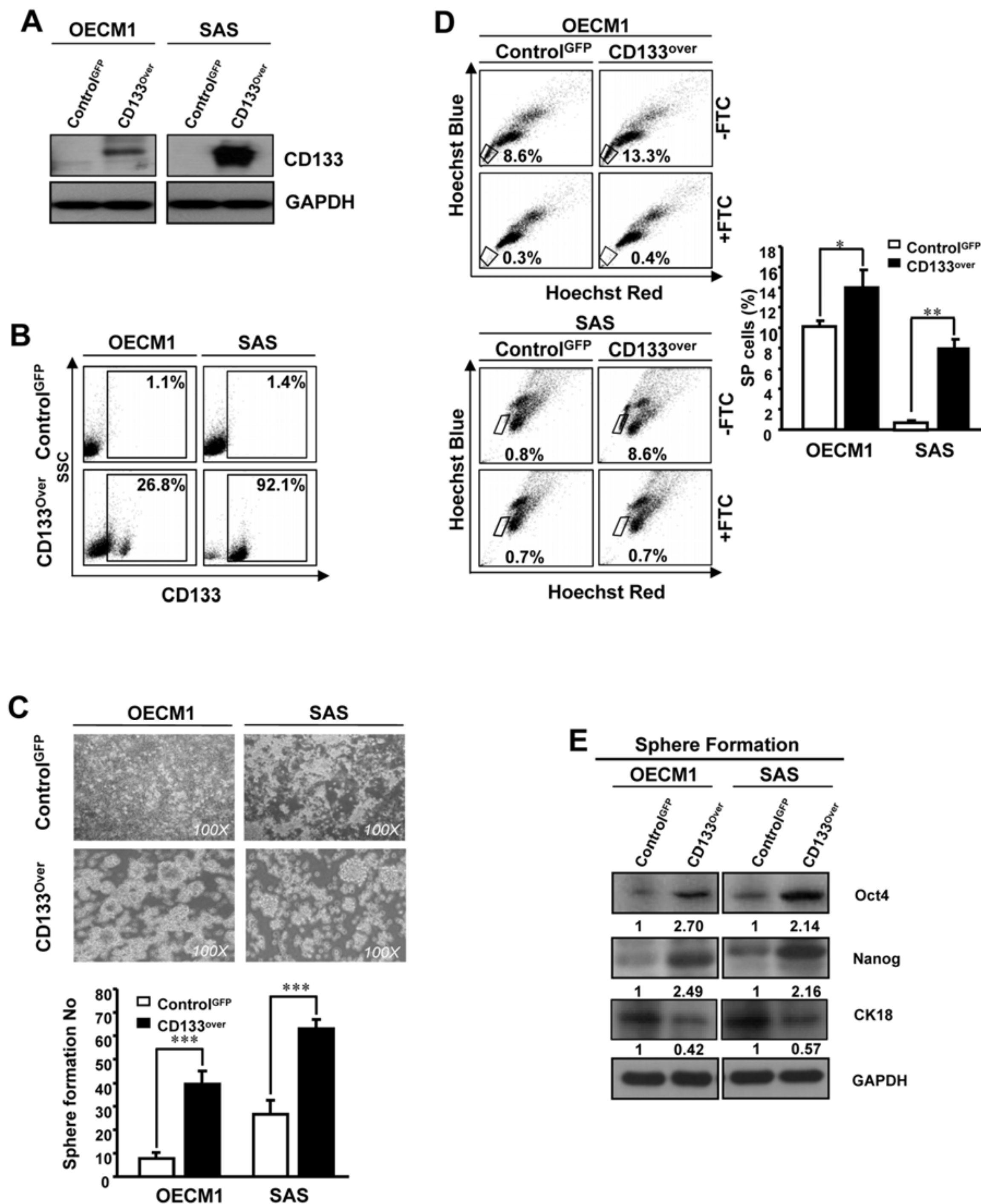

Figure 3. Overexpression of CD133 in OSCCs promotes stemness properties.

(A) Expression of CD133 protein in OSCCs infected with either CD133-overexpressing or control GFP lentiviruse was examined by western blot. The amount of GAPDH protein was referred as loading control. (B) Cell surface CD133 expression in CD133-overexpressing or control OSCCs was analyzed by flow cytometry. (C) Representative images of tumor sphere formation ability of control-GFP or CD133-overexpressing

OSCCs. (D) Single cell suspensions of stable CD133-overexpressing and control GFP-expressing OSCCs were incubated with Hoechst 33342 in the presence or absence of 10 μM fumitremorgin C (FTC), then, analyzed by flow cytometry to identify the SP cells. (E) Total crude cell extracted proteins from control-GFP or CD133-overexpressing OSCCs under cultivation with defined serum-free medium for 2 weeks were prepared and immunoblotted against anti-Oct-4, anti-Nanog, anti-CK18 or anti-GAPDH antibodies as indicated. The amount of GAPDH protein of different crude cell extracts was referred as loading control for further quantification.

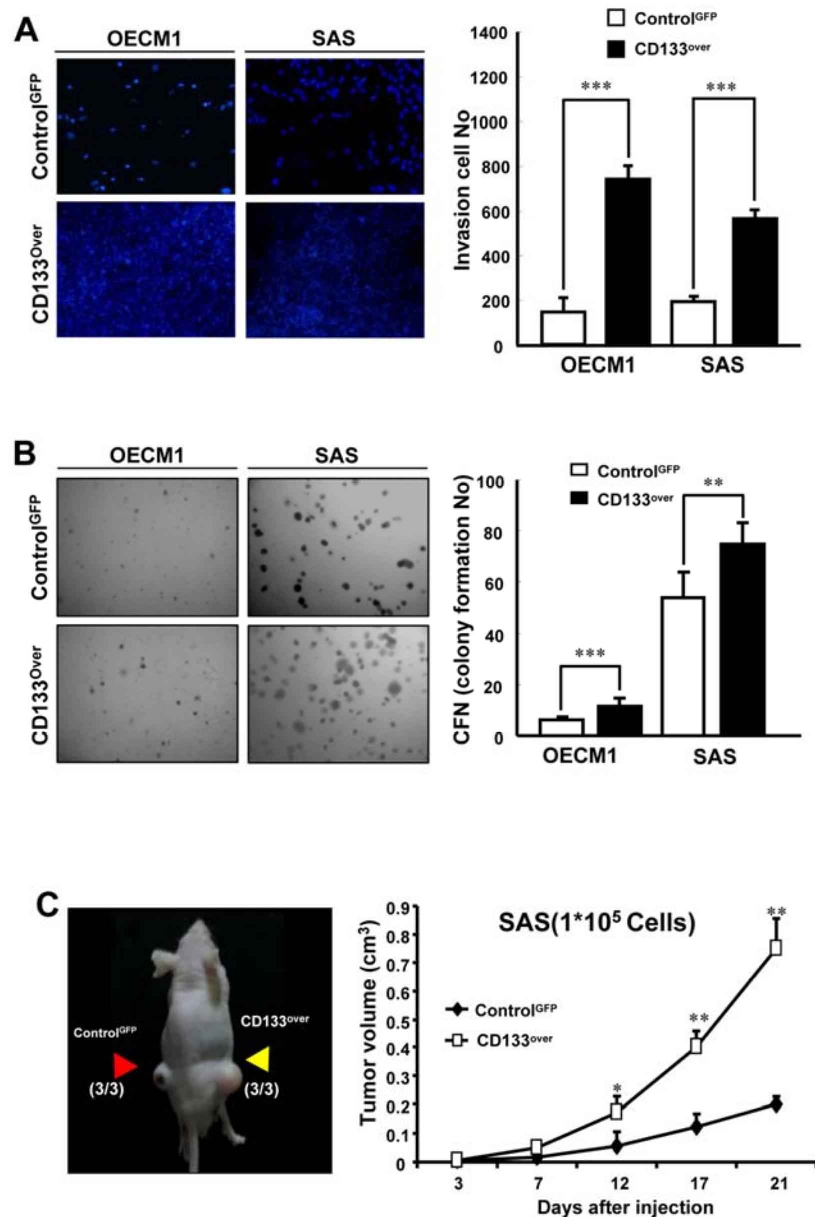

Figure 4. Overexpression of CD133 in OSCCs enhances malignant activities.

(A) The invasiveness ability of control-GFP or CD133-overexpressing OSCCs were examined as described in materials and methods (*, $p< 0.05$; ***, $p< 0.001$). (B) Anchorage-independent growth of control-GFP or CD133-overexpressing OSCCs was analyzed (**, $p< 0.01$; ***, $p< 0.001$). (C) Representative tumor growth of control-GFP or CD133-overexpressing OSCCs ($1X10^5$ cells) in the subcutaneous space of recipient mice (Red arrows: control-GFP OSCCs; Yellow arrows: CD133-overexpressing OSCCs)(*left panel*). Tumor volume was measured after inoculation of control-GFP (n=3) or CD133-overexpressing OSCCs (n=3), respectively (*right panel*). Error bars correspond to SD (*, $p< 0.05$; **, $p<0.01$).

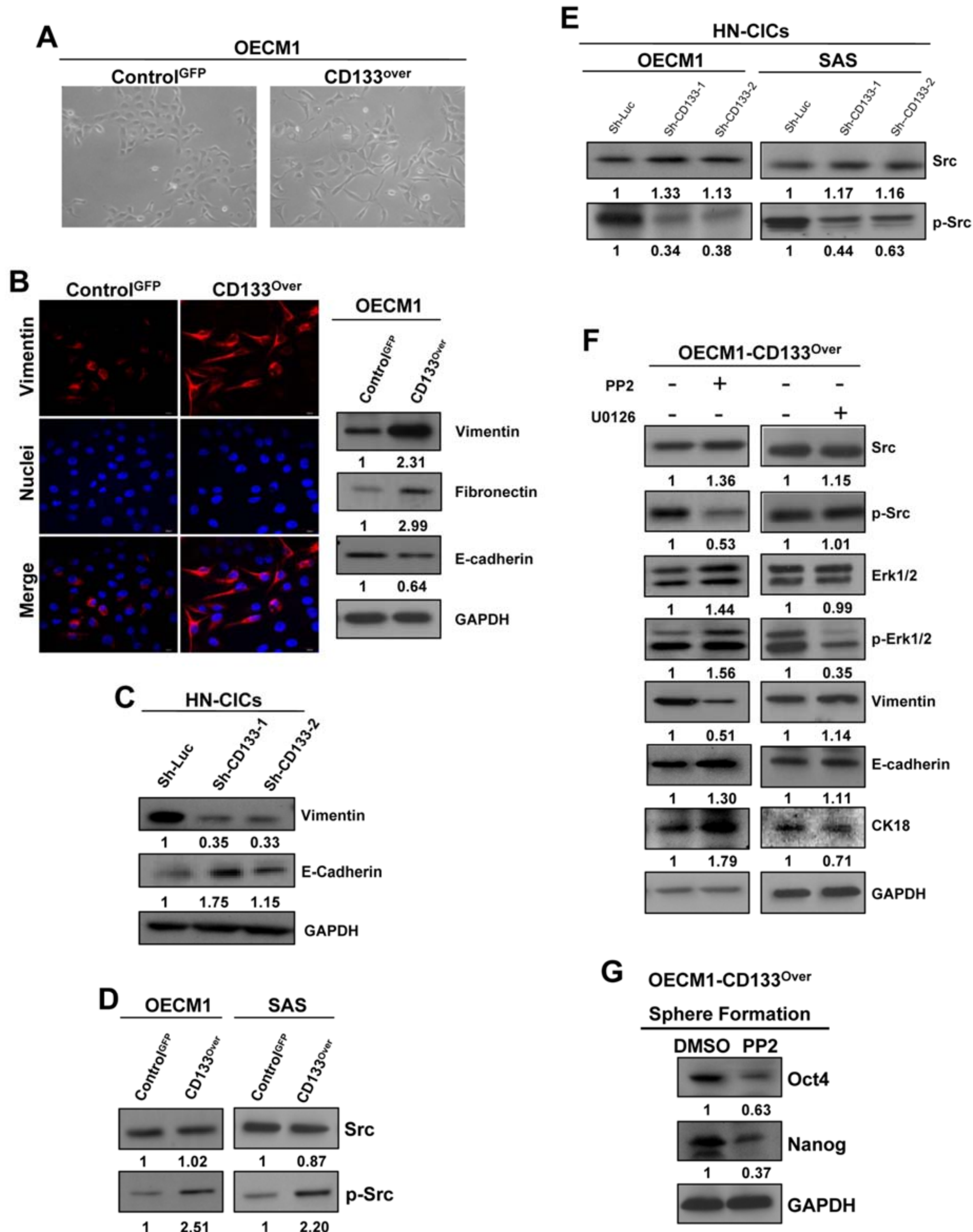

Figure 5. CD133/Src signaling regulates the mesenchymal transformation in OSCCs and OC-CICs.

(A) Morphological difference between control-GFP and CD133-overexpressing OSCCs. (B) Immunoblotting analysis (*right panel*) and confocal immunofluorescent staining (*left panel*) of EMT-related markers in control-GFP or CD133-overexpressing OSCCs were analyzed. (C) The protein levels of Vimentin and E-cadherin in the indicated OC-CICs were analyzed by western blot. (D) Protein level of Src or p-Src in control-GFP or CD133-overexpressing OSCCs were analyzed by immunoblotting. (E) Single cell suspension of OC-CICs was infected with sh-Luc-expressing or shRNAi CD133 lentirus, respectively, and the expression

of Src or p-Src in above OC-CICs was analyzed by western blot. (F) CD133-overexpressing OSCCs were first treated with 10μM PP2 (Src inhibitor) or 10μM U0126 (Erk inhibitor) for 24 hours. The expression of Src, p-Src, Erk1/2, p-Erk1/2, vimentin, E-caherin, or CK-18 of above treated cells was evaluated by western blot analysis with GAPDH being an internal loading control. (G) CD133-overexpressing OSCCs were first cultured with defined serum-free medium for 2 weeks along with the addition of PP2, and the expression of Oct-4, Nanog, or GAPDH proteins in control (DMSO) or PP2 treated cells was analyzed by immunoblotting.

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國科會補助計畫衍生研發成果推廣資料表

日期:2011/12/13

國科會補助計畫	計畫名稱: CD133 基因在口腔癌腫瘤化對於幹細胞特性維護及惡化進展之功能性研究
	計畫主持人: 余承佳
	計畫編號: 100-2314-B-040-001- 學門領域: 牙醫學

無研發成果推廣資料

100 年度專題研究計畫研究成果彙整表

計畫主持人：余承佳			計畫編號：100-2314-B-040-001-				
計畫名稱：CD133 基因在口腔癌腫瘤化對於幹細胞特性維護及惡化進展之功能性研究							
成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	1	1	100%	篇	研究成果發表於(2011) PLoS One 6: e28053: 1-12 (SCI, 為該文通訊作者)
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

With the emerging research interest of cancer initiating cells (CICs) in cancer research, to characterize the biology of OC-CICs could provide a new insight to the carcinogenesis and therapy of OC. Our research plan will further provide the molecular mechanisms of the carcinogenic role of CD133 in the tumorigencicty and maintenance of oral cancer and oral cancer-derived cancer initiating cells. The results of this project will provide the basic knowledge of therapeutic potential of targeting CICs in oral cancer therapy. Of note, CD133 might be used for a potential therapeutic target for OSCC by eliminating OC-CICs.