Tumor specific TGF-β insensitive CD8 + T cells augments the antitumor effect through inhibition of epithelial-mesenchymal transition in CD 105 + renal carcinoma stem cells

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Research Article

Keywords: transform growth factor-β, epithelial-mesenchymal transition, renal cell carcinoma, CD8+T, cancer stem cells, CD105

Posted Date: February 22nd, 2024

DOI: https://doi.org/10.21203/rs.3.rs-3972286/v1

Additional Declarations: No competing interests reported.

Abstract

Background The CD105+ cell clones isolated from renal cell carcinoma (RCC)were characterized as cancer stem cells. This study aimed to explore the antitumor mechanism of the transform growth factor-β(TGF-β) insensitive CD8+ T cells against CD105+ cells in vitro and vivo.

Methods The CD105+ cell clones were isolated from primary RCC cell lines and characterized by the immunofluorescence, qRT-PCR and western-blotting analysis. The expression levels of TGF-β1 were examined in 105 RCC tissues and correlation regression analysis were performed. The tumor specific TGF-beta insensitive CD8+ T cells were expanded ex vivo as previously described. The naïve CD8+ T cells and PBS as control. The humanized SCID mice were challenged with injection of CD105+ cells before adoptive transfer. The antitumor appraisal including survival analysis, tumor burden and bioluminescent imaging examination. The presence of pulmonary metastases was evaluated pathologically and epithelial-mesenchymal transition related molecular were analyzed.

Results The CD105+ cells were characterized with renal cancer stem cell for the high expression of Nanog, Oct4, Vimentin, Pax2 and high tumorigenicity. The TGF-beta-insensitive CD8+T cells showed the specific antitumor effect against CD105+ in vitro, were associated with suppressed pulmonary metastasis, and prolonged survival times, inhibited the epithelial-mesenchymal transition in tumor microenvironment.

Conclusion Our results demonstrate that the TGF-beta insensitive CD8+T cells show the tumor-specific antitumor effect including reduce tumor burden, inhibit pulmonary metastasis by blockade the EMT mechanism existed in CD105+CSCs. This study may provide a new perspective and method for the immunotherapy in RCC.

Introduction

Renal cell carcinoma (RCC) is the most common kidney tumor, representing 3% of total human malignancies, with a high metastatic index at the diagnosis and a high rate of relapse [1]. It has been reported that $CD105^+$ cells derived from RCC that display stem cell properties including clonogenic ability, generate epithelial and endothelial cells, high tumorigenicity [2–5]. Therapeutic approaches that specifically target the CSC population might substantially improve outcomes in patients with late-stage cancer. Therefore, targeting the $CD105^+$ CSCs is a promising antitumor strategy in RCC.

The epithelial-mesenchymal transition (EMT) is a developmental process, which is activated by members of the transforming growth factor beta (TGF-β) family and essential for tissue modeling, confers mesenchymal properties on epithelial cells and closely associated with the acquisition of aggressive traits by carcinoma cells [6–8]. TGF-β has a positive role on the CSC population promoting or sustaining stemness of the pool of CSCs, furthermore, it could induce CSCs EMT, providing a mechanistic link between mesenchymal phenotype, facilitate tumor invasion and metastasis [9–11]. Our studies have demonstrated that adoptive transfer blockade TGF-β pathway in $CD8^+$ T cells in tumor-bearing animal

could show potent antitumor effect in RCC [12], but whether this strategy still works in CSCs, which mechanism involved, remain unknown.

In this study, we isolated $CD105^+$ CSCs from primary RCC cell lines previous established. When cultured with $CD105^+$ CSCs, the blockade TGF-β pathway $CD8^+$ T cells as our previous studies reported (TGF-β insensitive $CD8^+$ T cells, TβRIIDN $CD8^+$ T) show the specific antitumor against CSCs in vitro. By using the SCID mouse model humanized with PBMC from RCC patients(hu-PBMC-SCID), we transfer the TβRIIDN $CD8^+$ T into $CD105^+$ tumor–bearing animals, our results show that TβRIIDN $CD8^+$ T could induce robust tumor-specific CTL responses, inhibit pulmonary metastasis, further experiments reveal that the EMT mechanism in $CD105^+$ CSCs were blocked.

Keywords: Renal cell carcinoma, Transform growth factor-β, Cancer stem cells, Epithelial-to-mesenchymal transition

Materials and Methods

Patients and cell lines

All patient samples were collected from the Department of Urology, Jinling Hospital, Nanjing, with written informed consent. The ethical approval was granted from Committees for Ethical Review in Jinling Hospital, Nanjing. This study involved two cohorts of RCC patients, cohort 1, 53 patients and cohort 2, 52 patients. All patients received no previous therapy and were followed until May 2023. All RCC samples are clear cell RCC which has been diagnosed by two pathologists, blinded to the clinical data. All cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum at 37°C with 5% CO2. TGF-β1-stimulation experiments were performed with recombinant human TGF-β1 (Peprotech, Rocky Hill, USA).

In vivo xenograft

All experiments involving mice were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the approval of the Institutional Animal Care and Use Committee (IACUC) at Jinling Hospital,Nanjing. Four-to six-week-old male SCID-beige mice were housed in sterile filter-top caged placed in a laminar backflow-cabinet under specific pathogen-free conditions. Autologous PBMCs purified from each patient's blood using a Ficoll-HyPaque (Pharmacia, New Jersey, USA) gradient after platelet depletion and washing, each mouse received 0.3 ml of the PBMCs (2×10^7cells) suspended in PBS via intra-peritoneal injection. One day before PBMCs injection, mice were sublethally irradiated with 3.5 Gy ([60Co] source Gammatron F 80S, Simens, Germany).

For in vivo limiting dilution assay, RCC primary cells were mixed with Matrigel (BD) at a ratio of 1:1 and injected subcutaneously at various cell doses per mouse. Kinetic of tumor formation was evaluated per week for 8 weeks. Frequency of CSCs was determined using ELDA software(http://bioinf.wehi.edu.au/software/elda/index.html).For pulmonary metastasis model, 1×10^6 CSCs were injected into the tail vein of humanized SCID mice. Mice were sacrificed 12 weeks later after

inoculation and consecutive sections of the whole pulmonary tissue were subjected to hematoxylin-eosin staining. All of the metastatic lesions in pulmonary were calculated microscopically to evaluate the development of pulmonary metastasis.

RNA isolation and RT–PCR analysis.

Total RNA was extracted by Trizol (Invitrogen, USA). Real-time quantitative PCR was performed on triplicate samples in a reaction mix of SYBR Green (Takara, China) by ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). The expression of indicated genes was normalized to endogenous reference control β-actin by using 2DDCt method.

Western blot

Cell lysates or retrieved proteins were analyzed by immunoblot with primary antibodies and IRDye 800 CW-conjugated secondary antibody (Rockland Immunochemicals, USA). The intensity of the fluorescence was scanned by Odyssey fluorescence scanner system (Li-Cor Biosciences, USA).

Generation of tumor specific TGF-β insensitive CD8 $^{+}$ T cells

With the use of $CD8^{+}$ Microbeads (Miltenyi Biotec), patient's $CD8^{+}$ T cells were positively selected from whole blood with a purity of > 98%. $CD8^{+}$ T cells was expanded with using autologous patient's DCs pulsed with the tumor lysate in the presence of recombinant human interleukin-2 (500U/mL;PeproTech) as previously described. There were two types of $CD8^{+}$ T cells used for experimentation: (a) tumor specific TGF-β insensitive $CD8^{+}$ T cells that were rendered insensitive to TGF-β by infection using a retrovirus containing dominant negative TGF-beta type II receptor and (b) naïve $CD8^{+}$ T cells isolated from PBMC (controls).

^{51} Chromium release assays

The two types of $CD8^{+}$ T cells were subjected to a standard ^{51}Chromium-release assay. $CD105^{+}$ cell lines and another irrelevant cell line, human prostate carcinoma cell line, PC-3 cells were used as targets. Briefly, target cells were labeled with 100 μ^{51}Cr. Different groups of $CD8^{+}$ T cells were added to U-bottom plates containing 5,000 cells /well with various effectors to target (E/T) ratios ranging from 1:1 to 100:1. Equal volumes of RPMI-1640 and 1 mol/L HCl were added to other wells as the negative and positive controls, respectively. After a 4-hour incubation, 100 μl of supernatants was harvested from each well and the 51Cr released was measured using a gamma-counter. The percent cell lysis was calculated according to the formula: percent specific ^{51}Cr-Release= (Experimental Release-Spontaneous Release) ×100/ (Maximum Release -Spontaneous Release).

Statistical analysis

All statistical analysis was performed using SPSS 22.0 software (SPSS Inc., USA). Numerical data were expressed as mean ± standard deviation (SD). The significance of mean values between two groups was

analyzed by two-tailed Student's t-test $P < 0.05$ was considered statistically significant. The Kaplan-Meier survival curve was analyzed by the log-rank test with the Graphad Prism v.6 software (Graphad Software Inc., San Diego, CA). Correlation was estimated by calculation of 2-tailed Pearson coefficients and significance. Data were transformed when needed to normalize variance. Symbols indicate statistical significance as follows: *p < 0.05, **p < 0.01, and ***p < 0.005

Results

Isolation and identification CD105$^+$ cells from primary RCC cells

Primary RCC cell lines were established as previously described [12]. We enriched CD105$^+$ cells by immunomicrobead sorting. When cultured in sphere-generating medium, the CD105$^+$ clones were able to grow in nonadhesive condition and to generate spheres(Fig. 1A)Immunofluorescence assay showed that the expression of stem cell associated-makers including the Nanog, Oct4, Vimentin, Pax2 were high whereas the CK,vWF expression were low Fig. 1B.D CD105$^+$ cells isolated from primary RCC cells lines were higher than other RCC cell lines(Fig. 1C). Three CD105$^+$ cell clones were injected subcutaneouslty in SCID mice. All CD105$^+$ clones were able to generate tumors even the number as few as 10^2 cells (Table 1). These data indicated that CD105$^+$ cell presented the characteristic of CSCs in RCC.

Table 1
Tumor generation of CD105 + cell clones in SCID-beige mice

Type	Cell clones		
	1×10^5	1×10^4	1×10^2
CD105+	10/10	10/10	10/10
CD105-	1/10	0/10	0/10

Table 2:Clinical Characteristic of RCC patients

RCC	Well differentiation (53)	Poorly differentiation (52)
Gender		
Male	28	31
Female	25	21
Age(years)	61.2 ± 5.7	58.5 ± 7.6
Tumor size (cm)	5.1 ± 2.4	7.6 ± 3.2
Furthmann grade		
I/II	53	15
III/IV	0	37
TNM stage		
I/II	50	11
III/IV	3	41
Distant metastasis		
Yes	0	13
No	53	39

TGF-β1 promotes the CD105 + clones EMT ability

As TGF-β1 is an important factor in the CSCs niches [13], we investigated the effect of TGF-β1 in the EMT process of $CD105^+$ clones. After 48h exposure to TGF-β1 (10 ng/ml), most $CD105^+$cells acquired mesenchymal-like alteration (Fig. 2A). Therefore, we examined the expression levels of key EMT markers, such as E-cadherin(E-cad), Vimentin (Vim), N-cadherin(N-cad) in TGF-β1 treated and untreated cells as control by immunofluorescence and western blot. As shown in Fig. 2B, C, a significant expression decreases in E-cad and increase Vim, N-cad were induced in TGF-β1 treated cells. After 12–15 days cultured in serum-free medium with TGF-β1 (10 ng/ml), the quantity and volume of the sphere in treated cells were significantly greater than the control cells ($P < 0.05$) (Fig. 2D, E). These data indicated that TGF-β1 can induce EMT in the $CD105^+$ CSCs in RCC.

Previously study showed that TGF-β1 was high expression in primary RCC cell lines [12]. In this study, we further examined the TGF-β1 expression in 105 RCC tissues. The average level of TGF-β1 was elevated in poorly-differentiated RCC compared with well-differentiated tumors. Correlation regression analysis revealed that high TGF-β1 expression in RCC tissues was associated with aggressive clinical features, which consistent with previous researches [13–15]. (Fig. 2F, Table 1). Moreover, patients with higher TGF-β1 levels exhibited worse overall survival and shorter time to recurrence. (Fig. 2G)

TGF-β insensitive $CD8^+$ T cells shows the specific antitumor effect in $CD105^+$ cells

We performed the ^{51}Chromium release assays to evaluate the tumor-killing ability in vitro of autologous TGF-β-insensitive (TβRIIDN) $CD8^+$ T cells in $CD105^+$ CSCs. When cultured with serum-free medium,

$CD105^+$ sphere cells exhibited impaired ability to form colonies and decreased expression of pluripotent transcription factors such as Oct4, Nanog, Pax2 in TβRIIDN $CD8^+$ T group compared with Naïve $CD8^+$ T and PBS group. (Fig. 4A, B, C, D)

After incubated with different type of $CD8^+$ T cells, the TβRIIDN $CD8^+$ T cells exhibited a 5.2-fold higher tumor-killing activity compared with naïve $CD8^+$ T cells [62.5% versus 12.0% at an effector/target (E/T) cell ratio of 100:1; Fig. 4, E-F. No apparent lytic activity was observed when the assay was done using the PC-3 negative control cell line.

These results indicated that TβRIIDN $CD8^+$ T cells show the specific antitumor effect in $CD105^+$ CSCs *in vitro.*

TGF-β insensitive $CD8^+$ T cells show potent antitumor activity in vivo

In vivo assays further confirmed the antitumor abilities of TβRIIDN $CD8^+$ T cells. The humanized SCID mouse model was established as previously described [12]. Therefore, these humanized mice were divided with three groups which injection with $CD105^+$ CSCs(1×10^6 cells) and treated with PBS, Naive $CD8^+$ and TβRIIDN $CD8^+$ T cells (1×10^7) cells specifically, twice a week after 14 days injection. As shown in Fig A-B, the bioluminescent imaging revealed that the TβRIIDN $CD8^+$ treated group showed lowest tumor burden. Interestingly, the treatment was discontinued after 28 days, but no tumor regrowth was observed in the TβRIIDN treated group even after 2 months. Survival analysis also showed that the TβRIIDN treated group significantly prolonged compared with others. The same result was observed in the volumes and weights of tumor. ($P < 0.05$; Fig. 4C and D).

Taken together, TGF-β insensitive $CD8^+$ T cells could show potent antitumor activity in vivo.

TGF-β insensitive $CD8^+$ T cells inhibit $CD105^+$ CSCs EMT activity and pulmonary metastasis

We evaluated the therapeutic potential of TβRIIDN $CD8^+$ T cells in lung metastasis model. The humanized mice were divided with three groups which injection into tail vein with $CD105^+$ CSCs(1×10^6 cells) and treated with PBS, Naive $CD8^+$ and TβRIIDN $CD8^+$ T cells (1×10^7) cells specifically, twice a week after 14 days injection. As shown in Fig. 5A, B, TβRIIDN treated group showed the lowest metastasis burden compared with other groups. Furthermore, EMT has been reported to play critical roles in CSCs expansion in various cancers, we examined the expression levels of key EMT markers such as N-cad,E-cad,Snail ,Slug and Vim by qRT-PCR and western blot analysis in tumor tissues. As shown in Fig. 5A, B, high expression of E-cadherin, and decrease expression of N-cad, FN, Slug, Snail, Vim were observed in TβRIIDN $CD8^+$ treated group. Together, these results suggested that the EMT mechanism in tumor tissue was inhibited by TβRIIDN $CD8^+$ T cells.

Discussion

CSCs are considered as the root of cancer initiation, metastasis and relapse [16–17]. Studies showed that CD105 are well-accepted as renal CSCs markers [2–5, 18, 19]. Our development of a method for enrichment of $CD105^+$ CSCs through primary RCC cell lines showed the higher expression CSC markers such as Nanog, Pax2, Vimentin, Oct4 and tumorigenicity compared with other RCC cell lines, displayed the characteristic of CSCs in vitro and vivo.

Solid tumors such as RCC possess an immunosuppressive microenvironment that can reduce the antitumor function of $CD8^+$T cells [20, 21]. Studies showed that TGF-β, as an immunosuppressive cytokine in the tumor microenviroment, has been associated with poor prognosis short overall survival and recurrence [22, 23]. We also found that the high expression of TGF-β1 in RCC tissues significantly correlated with poor prognosis. Furthermore, when cultured with TGF-β1, $CD105^+$ CSCs have acquired EMT alteration including downregulation of E-cad expression, upregulation of Vim and N-cad expression, and increased sphere formation population. These results indicated that TGF-β play important role in RCC and promote the EMT in CSCs, which related with recurrence and metastasis. Accumulating evidences have shown the connection between the EMT and CSCs in human cancers, and TGF-β plays pivotal role not only in the stemness of CSCs but also in the immune cells in tumor microenvironment [24–26]. TGF-β can control adaptive immunity by promoting the expansion of Treg cells directly, regulating the regulatory $CD4^+$ T cell response, controlling the function of effector T cells. Therefore, interfering with TGF-β signaling in immune cells is a rational strategy for cancer immunotherapy [27–30].

Gorelik et al reported that blockade of TGFβ pathway in T lymphocytes could elicit a potent anti-tumoral response [31]. A strategy that has been evaluated in the context of T-cell engineering is expression of a dominant-negative TGF-β receptor that led to improved efficacy of prostate-specific membrane antigen-specific CAR-T cells in murine xenograft model [33]. In previous studies, we demonstrated that the TGF-β insensitive $CD8^+$ T cells show the potent antitumor effect in RCC. However, in $CD105^+$ CSCs, due to the tumor heterogeneity, whether this strategy still work remains unknown. Therefore, we focused on CSCs as a target of cancer immunotherapy to achieve more efficient anti-tumor responses. In vitro experiment, we found that TβRIIDN $CD8^+$ T cells show the specific tumor-killing activity against $CD105^+$CSCs. In vivo experiment showed that TβRIIDN $CD8^+$ T cells could inhibit $CD105^+$ CSC growth, reduce tumor burden and pulmonary metastasis in vivo, further experiment revealed that this effect was mainly achieved by blockade the EMT mechanism in CSCs.

Redirect and reprogram T cells to enhance the T cell toxicity ability is also important in the process of tumor rejection. Eyquem J et al reported that using the CRISPR/Cas9 genome editing technique targeting the CAR to the TRAC locus in T cell could avert tonic CAR signaling and establish effective internalization and re-expression of the CAR following delaying effector T-cell differentiation and exhaustion [34]. Zheng F et al reported that combined α-CTLA-4/α-PD-L1 and CSC-DC vaccine could enhance the number of circulating functional $CD8^+$ T cells, enhanced CSC-DC vaccine-induced host immune responses [35]. However, in this study, we did not perform the experiment about the effect of PD/L-1 drugs against

CD105 + CSCs. We also didn't quantify the affection of the absolute number of CD8 + T cells in blood circulation and the tumor parenchyma. Although it will take time and effort to illuminate this issue, it maybe provides new insights into the mechanisms of this immunotherapy.

In conclusion, our findings demonstrate that the TGF-β insensitive $CD8^+$ T cells show the tumor-specific antitumor effect including reduce tumor burden, inhibit tumor growth and pulmonary metastasis by blockade the EMT mechanism existed in $CD105^+$CSCs. This study may provide a new perspective for the immunotherapy in RCC.

Declarations

Author Contribution

Longxin Wang participate in the entire process of experiment preparation, experimental implementation, and manuscript writing ,Chao Zhang prepared figures 1-4,Dezhou Yue prepared figure 5 and table 1-2,Jie Dong is responsible for experimental implementation, coordination, and manuscript preparation.All authors reviewed the manuscript.

Acknowledgement: This study was funded with the National Natural Science Foundation of China No.81372741,81472392.

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Figures

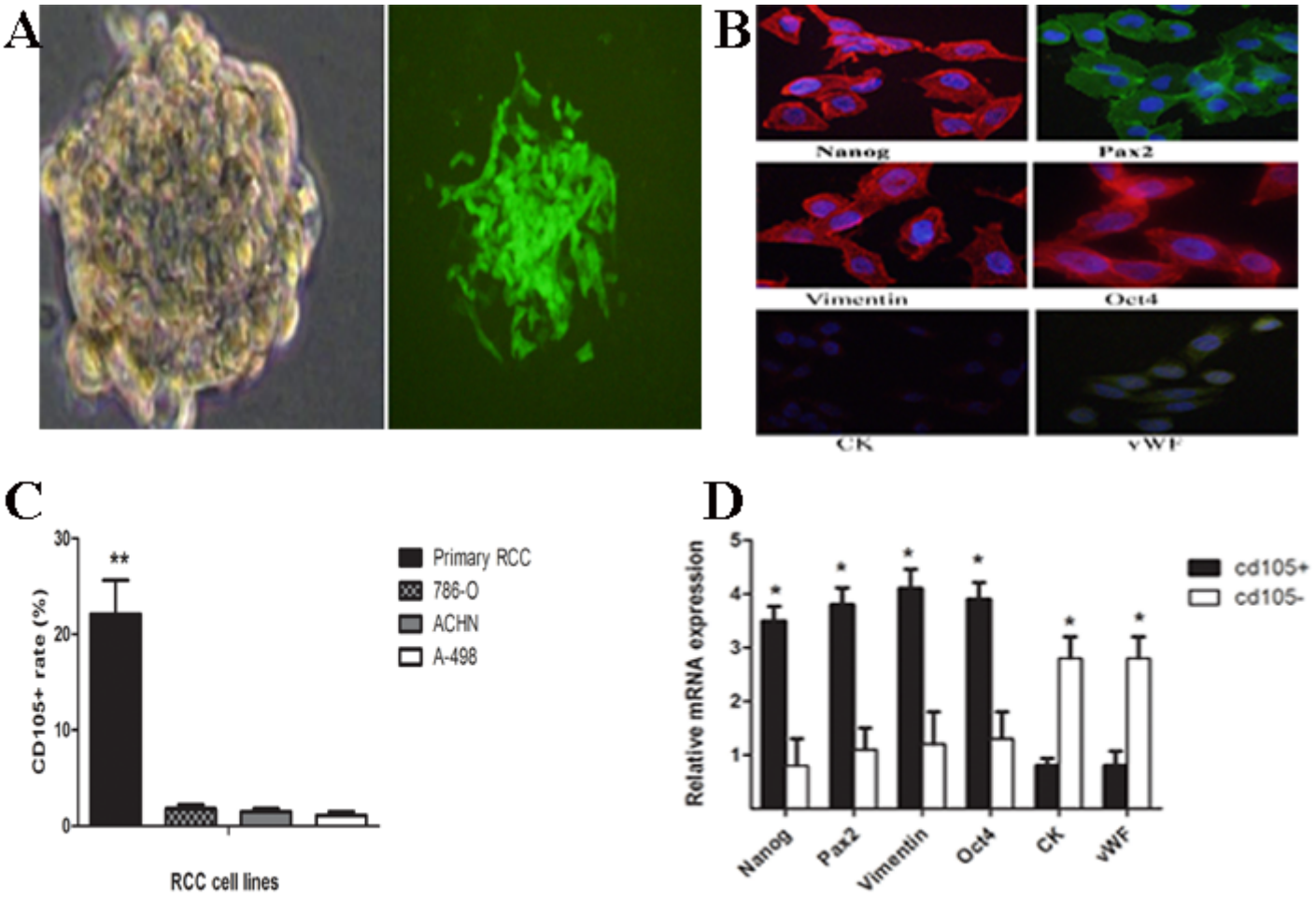

Figure 1

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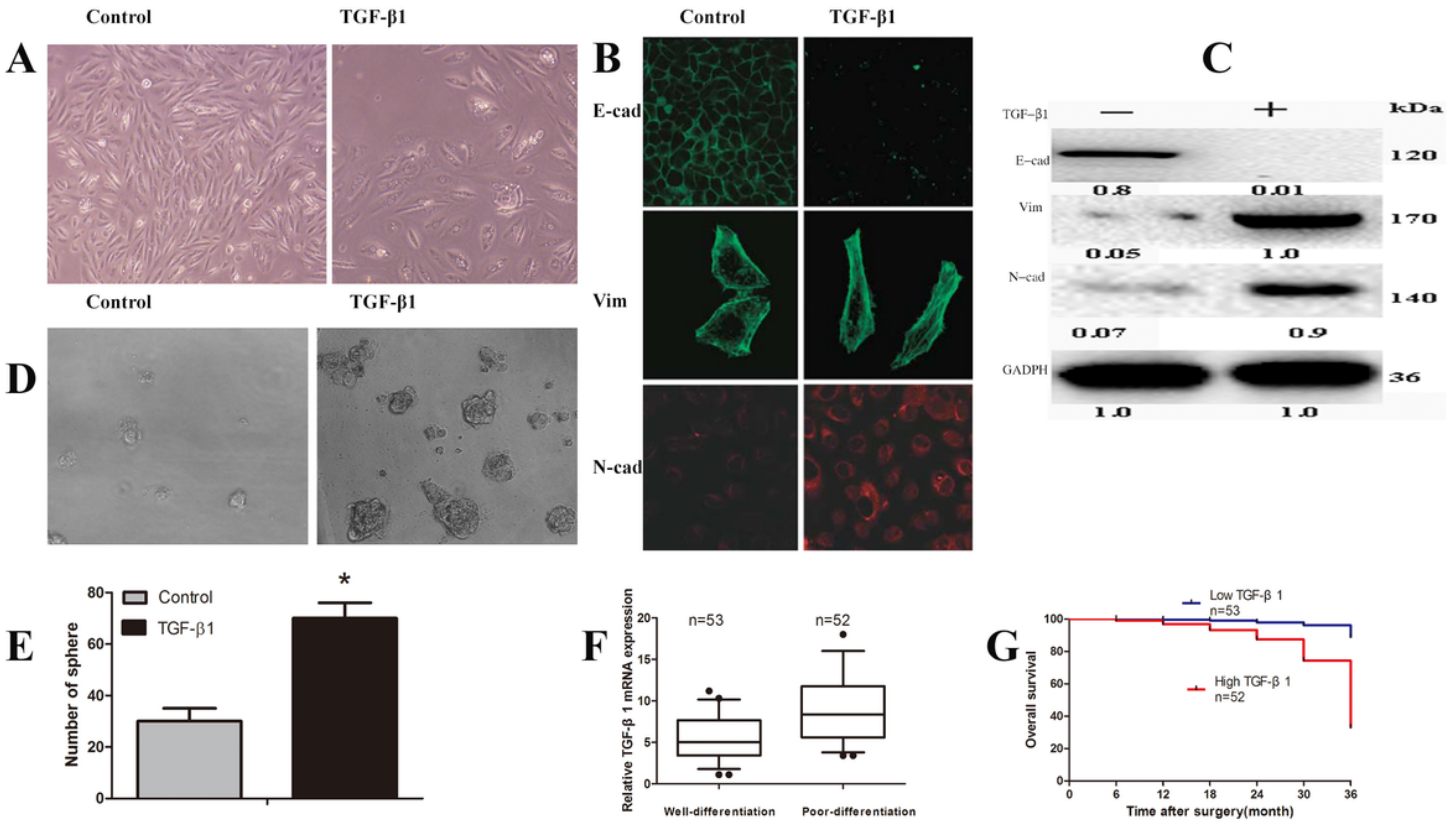

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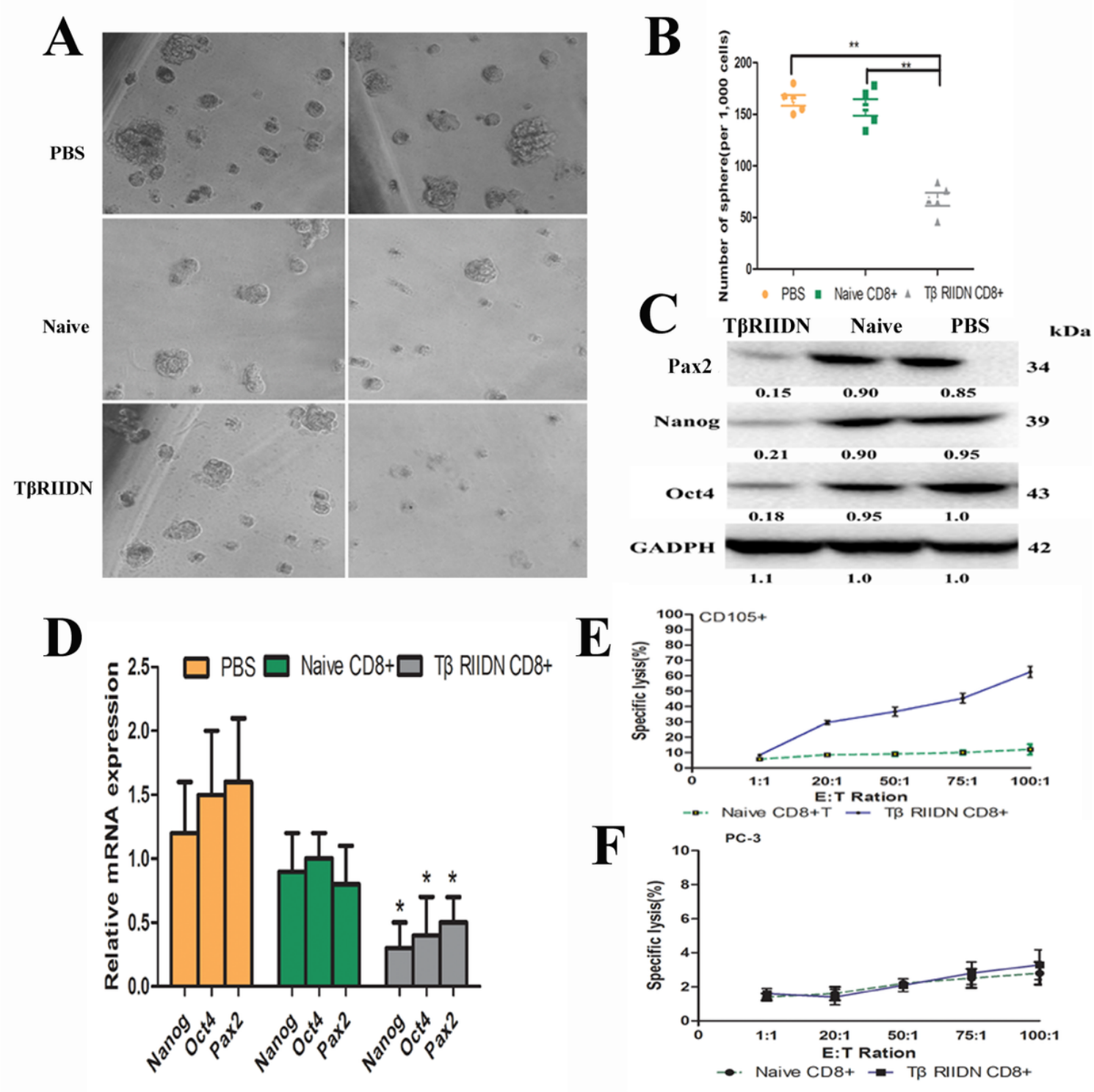

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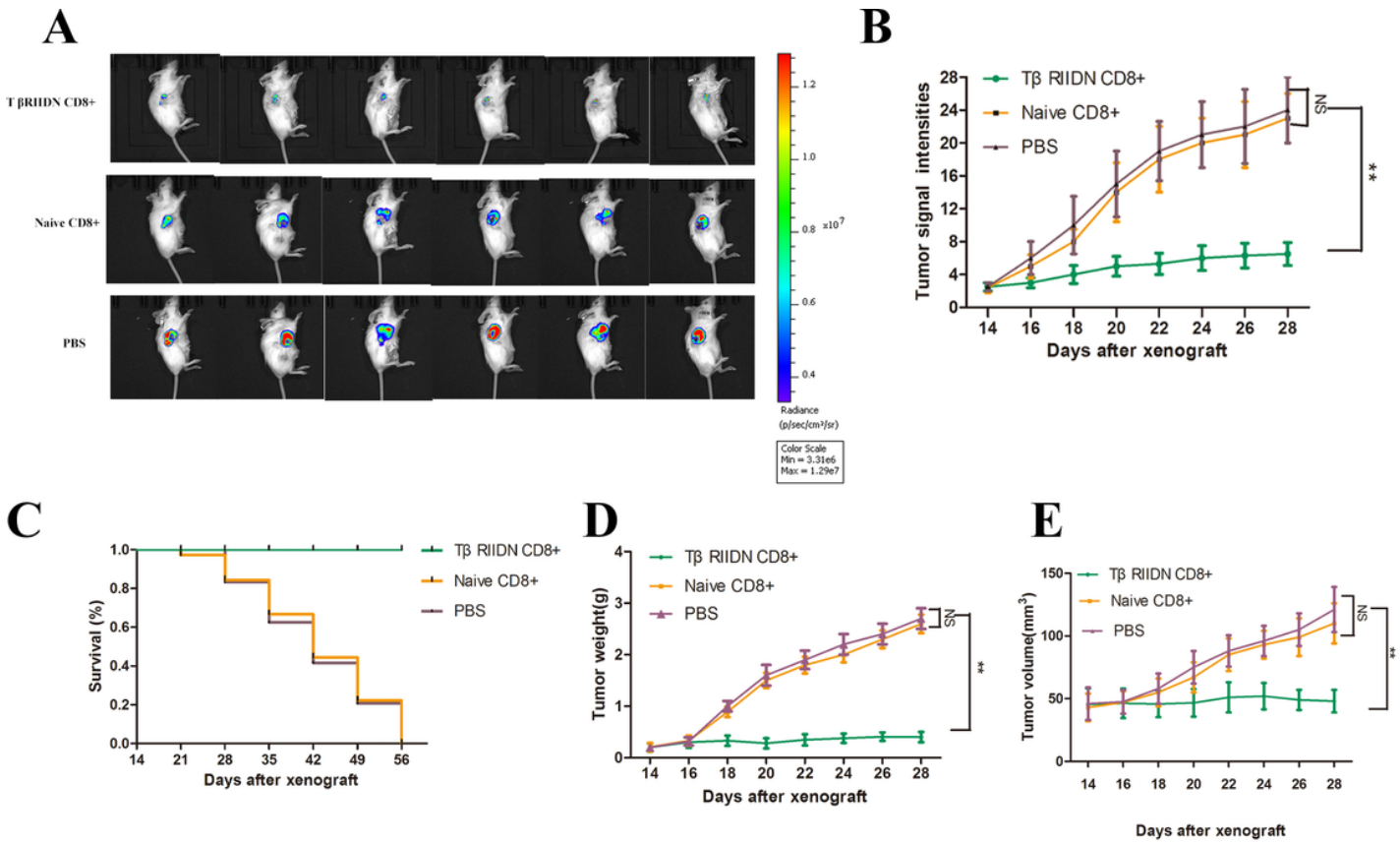

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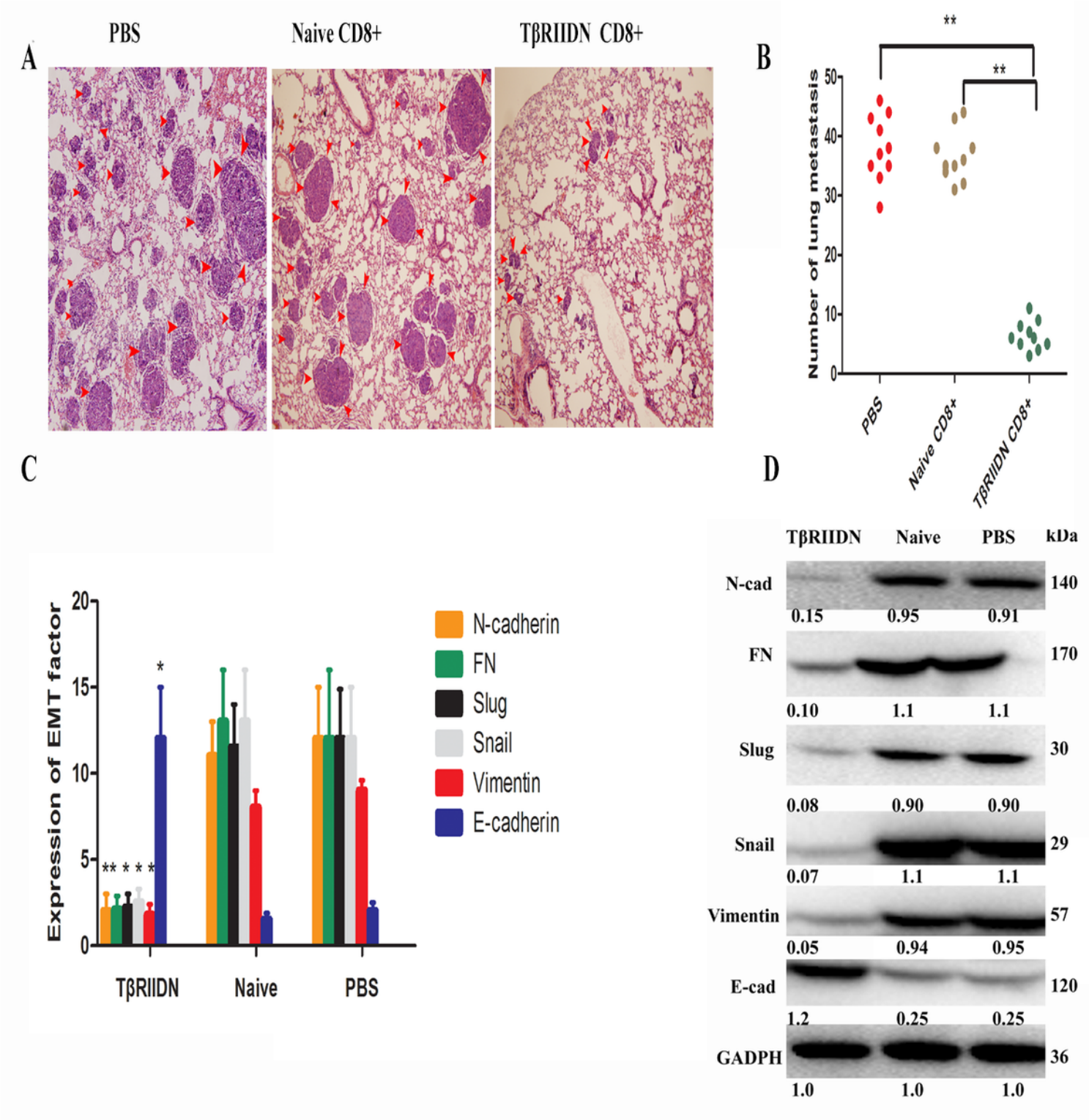

Figure 5

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