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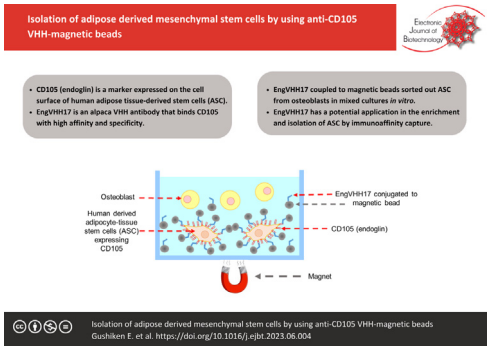
Short Communication

Isolation of adipose derived mesenchymal stem cells by using anti-CD105 VHH-magnetic beads

Eduardo Gushiken [a], Milagros Quintana [a], Luz Agueda Perez [a], Patricia Herrera-Velit [a,b], Elizabeth R. Balmayor [c], Martijn van Griensven [d], Jose R. Espinoza [a,e,*]

[a] Laboratorios de Investigación y Desarrollo, Facultad de Ciencias e Ingeniería, Universidad Peruana Cayetano Heredia, Lima 15102, Peru
[b] Departamento de Ciencias Celulares y Moleculares, Facultad de Ciencias e Ingeniería, Universidad Peruana Cayetano Heredia, Lima 15102, Peru
[c] Experimental Orthopaedics and Trauma Surgery, Department of Orthopaedic, Trauma, and Reconstructive Surgery, RWTH Aachen University Hospital, Aachen, Germany
[d] Department of Cell Biology-Inspired Tissue Engineering, MERLN Institute for Technology-Inspired Regenerative Medicine, Maastricht University, Maastricht, The Netherlands
[e] Departmento de Ingenieria, Facultad de Ciencias e Ingeniería, Universidad Peruana Cayetano Heredia, Lima 15102, Peru

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Human adipose tissue-derived stem cells (ASCs) are widely used in regenerative medicine and tissue engineering. Magnetic-activated cell sorting (MACS) with monoclonal antibodies (mAbs) that bind surface markers of stem cells such as CD105, CD73, and CD90 is currently applied for the enrichment and isolation of ASCs. Alternatives to mAbs are the variable domains of heavy chain antibodies (VHHs), which are naturally produced in camelids. We report the application of an anti-CD105 VHH conjugated to magnetic beads in the isolation of ASCs *in vitro*.

Results: Two identical anti-CD105 VHHs (EngVHH17) were screened by phage display from a VHH cDNA library constructed from the PBMCs of an alpaca immunized with a lysate of human bladder cancer cell line (T24 cells). EngVHH17 was cloned in pET22b(+), expressed in *Escherichia coli* BL21 and purified in Ni-NTA chromatography resulting in a ~15 kDa VHH antibody. EngVHH17 binds CD105 with high affinity (Kd = 3.9×10^{-10} M). The specific binding of EngVHH17 to CD105 was visualized by fluorescence inmunolabeling of phorbol-12-myristate-13-acetate (PMA)-differentiated THP1 cells using FITC labeled anti-6 × His Tag IgG1 mouse mAb. EngVHH17-magnetic beads selectively sorted human ASCs from osteoblasts in a mixed culture *in vitro*. The selective recovery of ASC cells using different ASC/Osteoblast ratios was higher than 85%.

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* Corresponding author
E-mail address: jose.espinoza@upch.pe (J.R. Espinoza).

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Conclusions: EngVHH17 coupled to magnetic beads binds CD105 expressed on the cell surface of ASCs and isolates them from osteoblasts in mixed cultures *in vitro* by application of an external magnetic field. EngVHH17 can be further evaluated for the isolation of MSCs by MACS.

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1. Introduction

Adipose tissue mesenchymal stem cells (ASCs) are a class of MSCs derived from the adipose-tissue stromal vascular fraction [1]. ASCs are widely used in tissue engineering and regenerative medicine. Methods of immunoaffinity-ASCs isolation are based on the binding of surface antigenic markers such as CD73 and CD105 [1,2] by specific monoclonal antibodies (mAbs), which are either linked to fluorescent dyes in fluorescent-activated cell sorting (FACS) or to magnetic microbeads in MACS [3,4].

VHHs are the variable domains of heavy chain antibodies that are naturally produced as a part of the immunoglobulin repertoire elicited against antigens in camelids [5]. VHHs are small and stable antibody domains that bind antigens with high affinity and specificity and can be produced in large quantities in *E. coli* or yeast by fermentation. These characteristics make VHHs appealing molecular tools that can replace mAbs in a variety of biomedical and biotechnological applications [6].

In this work, we report the construction and screening of an anti-T24 cell lysate VHH cDNAs library by phage display, resulting in a VHH that binds to CD105 with high affinity and specificity. The anti-CD105 VHH coupled to magnetic ferric oxide beads binds ASCs *in vitro* and shows the capacity to isolate ASCs from osteoblast in mixed culture.

2. Materials and methods

2.1. Alpaca immunization

T24 cells (ATCC® HTB4™), a human bladder cancer cell line that expressing CD105 [7], were cultured in DMEM medium with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 mg/ml streptomycin until 70% confluence. Confluent T24 cells were trypsinized and washed in sterile PBS; after centrifugation, the cell pellet was resuspended in lysis buffer (20 mM TrisHCl, pH 8.0; 150 mM NaCl; 2 mM EDTA, 1% Triton X-100 with a cocktail of protease inhibitors) and centrifuged for 15 min at 14,000 RPM in a microfuge. The supernatant was saved and kept at −20°C until use. An alpaca received an intra-muscular injection of 500 mg of lysate protein with complete Freund's adjuvant, followed by weekly boosters of 300 mg of lysate protein with incomplete Freund's adjuvant for 9 weeks [8]. The immunization was followed by estimating the anti-T4 lysate and anti-CD105 IgG levels in serum by ELISA using recombinant CD105 (R&D Systems) and T4 protein lysate.

2.2. Library construction and phage display

At 10 weeks post-immunization, 100 ml of blood was drawn and PBMCs were isolated to obtain total RNA. cDNA synthesized by RT-PCR and VHH sequences amplified with primers: VHH-Sfi (5′TCGCGGCCCA GCCGGCCATGGCKCAGKTGCAGCTCGTGGAG TCN GG 3′) VHH-R1Not (5′TTGCGGCCGCTGGGGTCTTC GCTGTGGTGC G3′) and VHH-R2Not (5′ TTGCGGCCGCTTGTGGTTTTG GTGTCTTGG G 3′) annealing FR1, short and long hinge regions, respectively. PCR products were cloned into pHEN2 phagemid [9] and transformed *E. coli* TG1 by electroporation, producing a cDNA library of 1.4×10^8 clones with less than 0.01% of self-ligated transformants. The cDNA library was infected with VCS-M13 helper phage at the MOI value of 20 by incubation for 30 min at 37°C. Cells were precipitated at 2,800 × *g* for 10 min at room temperature. The pellet was resuspended in 50 ml of 2XYT medium containing 100 μg/ml of each ampicillin and kanamycin and incubated with 200 RPM shaking, at 30°C for 16 h. The culture was centrifuged at 3,200 × *g* for 15 min at 4°C. Phages in the supernatant were precipitated by adding 10 ml polyethylene glycol and recovered by centrifugation at 3,200 × *g* for 15 min at 4°C. The precipitate containing phages was resuspended in 1 ml PBS.

Phage display screening was performed in 3 consecutive rounds of panning with 1, 0.8 and 0.5 mg of recombinant CD105 (R&D Systems) using an initial input of 3.3×10^{13} phages; after three rounds of panning 2×10^7 phage library was produced. Each panning consisted of fixing CD105 into an Immulon® (Thermo Scientific) microtiter plate, incubated with phages in 3% BSA for 1 h at 37°C. Bound phages were eluted at high pH using triethylamine, followed by reinfection of *E. coli* TG1 and prepared for next panning. After third panning, 46 VHH-phages were randomly selected and subjected to phage ELISA with CD105 (R&D Systems). A positive anti-CD105 VHH-containing phage was selected by phage-ELISA, identified by sequencing, tagged as EngVHH17, and cloned into pET22b(+), containing a 6 × His tag, for expression in *E. coli* BL21. EngVHH17 was purified to homogeneity by nickel affinity chromatography using a Ni-NTA resin (Qiagen).

2.3. Estimation Kd of EngVHH17 binding to CD105

The saturation binding assay of EngVHH17 was performed with a range of 0, 1 ng, 4 ng, 5 ng, 10 ng, 25 ng, 50 ng, 100 ng, and 250 ng of CD105 dissolved in 100 μl of bicarbonate carbonate buffer pH 9.6, which were added to the microwells of an ELISA plate and incubated at room temperature for 2 h with gentle shaking for fixation. The content of the microwells was discarded and the plate was washed five times with 200 μl of ELISA wash buffer by gently shaking for 4.5 min, leaving it to stand for 30 s each time, and discarding the wash buffer. Then, 200 μl of ELISA blocking solution was added to each well, incubated at 37°C for 1 h, and washed as described above. A total of 100 μl of EngVHH17 (40 ng/μl) was added and incubated for 2 h at 37°C, and washed 5 times by adding 200 μl wash solution and shaking for 30 s each time. In addition, 100 μl of Penta His HRP conjugated antibody (Qiagen) diluted 1:2000 in blocking solution were added to each well, and incubated at 37°C for 1 h, and washed as described. Also, 100 μl of a fresh TMB substrate solution were added to each well, and incubated for 10 min in the dark. The reaction was stopped with 50 μl of 2 N H_2SO_4. The absorbance was read at 450 nm in a BioTek ELX800 Microplate reader. All reactions were performed in duplicate. Non-linear fit to one-site binding hyperbole and Scatchard plot linear regression of the data were performed using GraphPad

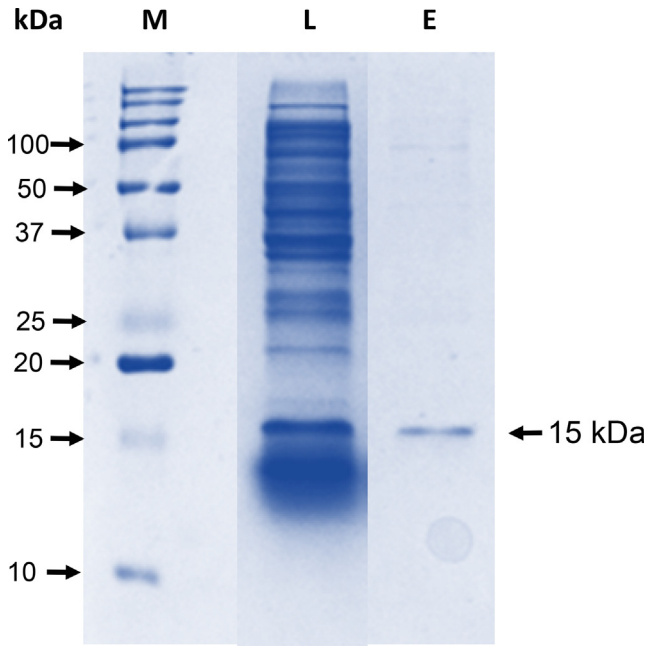

Fig. 1. Anti-CD105 ELISA with pure EngVHH17. Purification of recombinant EngVHH17 by affinity chromatography in a Ni-NTA column. M: markers; L: *E. coli* lysate; E: EngVHH17. Markers Kaleidoscope Prestained Standards (BioRad).

Prism version 5.0 for Windows, GraphPad Software, San Diego, California USA.

2.4. Western blot

T24 lysate proteins were separated by SDS-10% PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Merck) by electrophoresis at 1000 mA at 4°C in a Mini Protean chamber (Biorad-Laboratories, Inc.). The PVDF membrane was blocked in 2% fat-free dry milk-0.05% tween 20 in PBS solution for 1 h at 37°C, incubated with EngVHH17 (1/50 dilution) and bound VHH was visualized with horseradish peroxidase-labelled anti-Penta His antibody (Qiagen) (1/1,000 dilution) and the substrate hydrogen peroxide and 4-chloro-1-naphtol. The reaction was stopped by washing the PVDF membrane in distilled water.

2.5. Immunofluorescence staining of CD105 in PMA-treated THP1 cells

The human THP1 cell line (ATCC® TIB-202™) was used for the visualization of EngVHH17 binding to CD105. THP1 cells (2.5×10^4/well) were cultured in a complete medium (RPMI + 10% fetal bovine serum), and differentiated to induce CD105 expression with 65 nM phorbol-12-myristate-13-acetate (PMA) for 72 h in lab-tek chamber slides (Nunc International). Undifferentiated THP1 cells were attached to coverslips by drying a PBS drop containing 2.5×10^4 cells. For labeling, cells were fixed in cold 4% formaldehyde-PBS for 15 min and washed twice with 2% BSA in PBS. Fixed cells were incubated either with 50 μg/ml of EngVHH17 or 10 μg/ml of anti-CD105 mouse IgG2a monoclonal antibody (MEM-226, Invitrogen) diluted in 2% BSA –PBS for 1 h at room temperature. Cells were washed three times in PBS and then incubated for 1 h with the fluorescently labeled secondary antibody: 1.5 μg/ml FITC anti-6 × His Tag mouse monoclonal antibody (Invitrogen) or 4 μg/ml Alexa Fluor™ 594 goat anti-mouse IgG2a (Invitrogen).

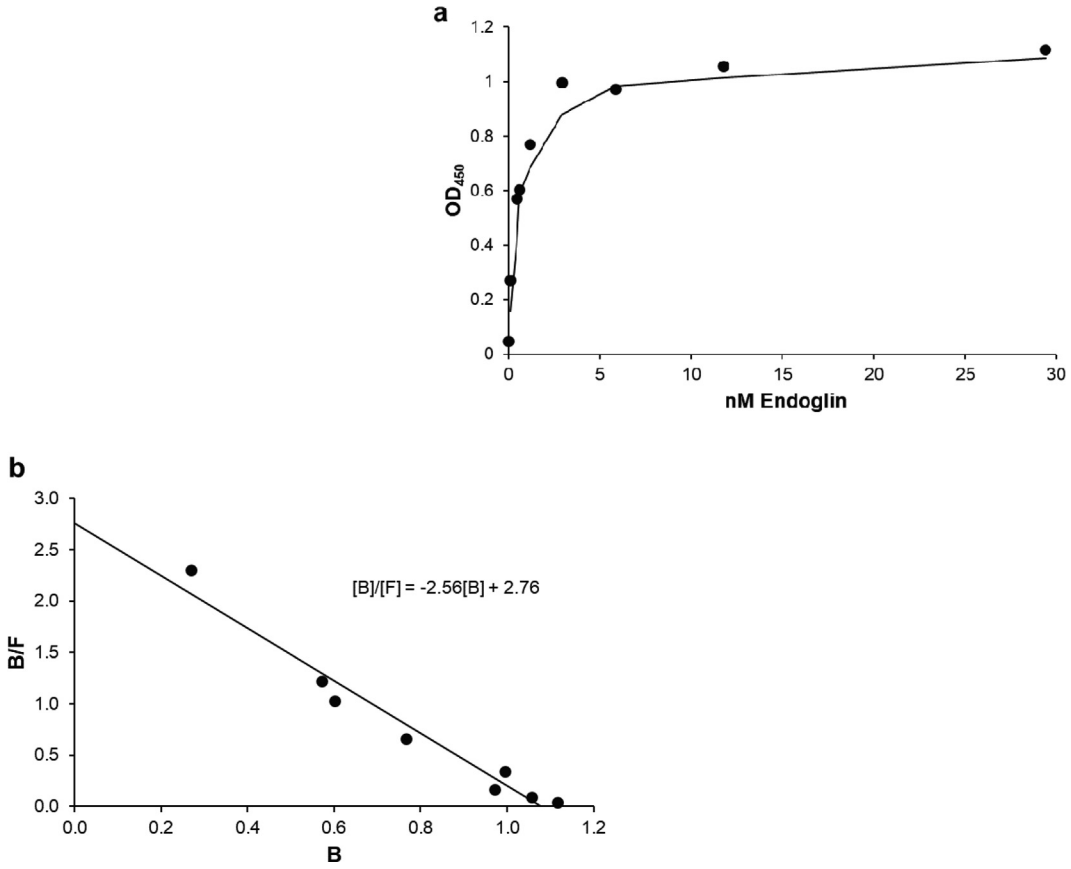

Fig. 2. (a) A saturation binding curve with a concentration range of CD105 by EngVHH17-ELISA. Each point is the mean of OD_{450} values of duplicate assay. One-site hyperbole fit (R^2 = 0.99). (b) The Scatchard plot of the binding EngVHH17 to CD105. The Scachard equation **[B]/[F] = -2.56[B] + 2.76** was fitted by a linear regression (R^2 = 0.98), a dissociation constant value (Kd) of 3.9×10^{-10} M was estimated for the binding of CD105 to EngVHH17.

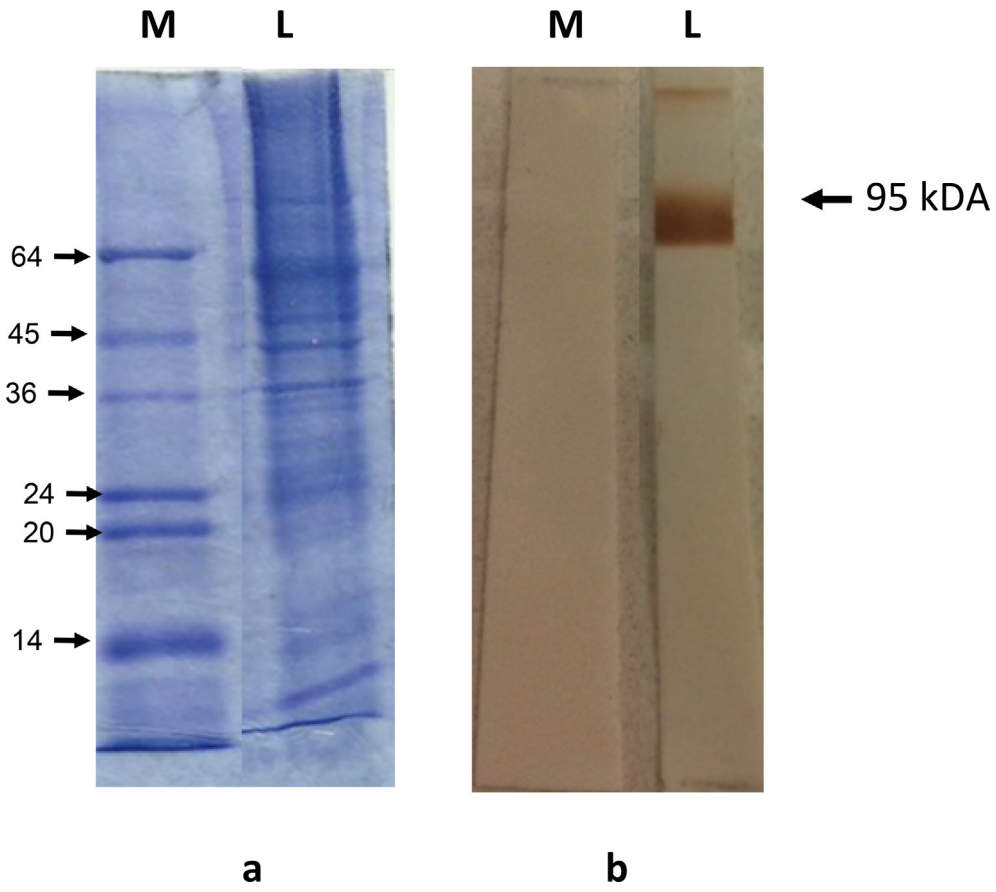

Fig. 3. Western blot with T24 cell lysate and EngVHH17. (a) T24 lysate resolved in 15% SDS-PAGE. M: molecular weight markers; L: T24 cell lysate. (b) Western blot with EngVHH17 showing a ~ 95 kDa band corresponding to immune detection of the CD105 monomer present in the T24 cell lysate.

Immunolabeled cells were washed three times in PBS. The chamber slides and coverslips were mounted in Antifade Mounting Medium with DAPI (Vector Laboratories, H-1200-10). Images of fluorescence-immunolabeled THP1 cells were obtained at 20× magnification using a Zeiss LSM 880 confocal microscope.

2.6. ASC isolation

Fresh adipose tissue from patients, who underwent a fat resection, was collected. Patients provided informed consent. The local ethical committee granted permission to collect the tissue. All procedures were carried out in accordance with the Declaration of Helsinki in its latest amendment.

For the isolation of adipose-derived mesenchymal stem cells (ASCs), the fat was cut into small pieces (at a range of 1–3 mm) by using a scalpel and forceps. PBS was added and the mixture was centrifuged (430 × *g* 10 min, room temperature). Next, an equal volume of collagenase solution (0.5 mg/ml in DPBS; 355 U/mg) was added to the upper-fat layer and incubated at 37°C for 1 h. The remaining cell pellet containing the ASCs was resuspended and filtered through a cell strainer (40 μm). ASCs were transferred into a T175 culture flask and cultivated in a humidified environment at 37°C and 5% CO_2 as described in detail [10]. A medium change was performed twice a week. Cells of the third passage were used.

2.7. Osteoblasts isolation

Osteoblasts were isolated from cancellous bone from the femoral heads of patients receiving a total hip endoprosthesis. Patients provided informed consent. The local ethical committee granted permission to collect the tissue. All procedures were carried out in accordance with the Declaration of Helsinki in its latest amendment.

Cancellous bone from femoral heads was used for osteoblast isolations. Bone samples were mechanically shredded into small bone fragments by using a Luer forceps and transferred into a cell culture flask. Culture medium (DMEM with 10% FCS, 1% P/S and 50 μg L-ascorbate-2-phosphate) was added and the flask was incubated for 7 days, with no further medium changes, at 37°C and 5% CO_2 in a humidified environment. Thereafter, the cell culture medium was changed to osteoblast expansion medium (Low glucose D MEM, 5% FCS, 1% penicillin/streptomycin, 10 mM β-glycerol-phosphate disodium salt hydrate, 1.56 mM $CaCl_2$, 0.025 M HEPES, 100 nM dexamethasone and 0.2 mM L-ascorbic acid 2-phosphate). Cells were expanded until reaching passage 3 to ensure homogeneity of the culture and adequate purity for usage in the recovery experiments.

2.8. ASC recovery using EngVHH17

The coupling reaction of EngVHH17 and magnetic beads (Fe_3O_4) proceeded by incubation of 10 mg of EngVHH17 and 1 × 10^6 Fe_3O_4 nanoparticles in PBS pH 7.4 at room temperature for 2 h [3].

As stated above, ASCs and osteoblasts were independently cultured. Cells at the same passage (passage 3) were harvested and mixed to obtain 1 ml of heterogenous cell suspension at different osteoblasts/ASCs ratios: 1:1, 1:2, 2:1, 1:10, 10:1. The heterogenous cell suspention with EngVHH17-magnetite beads was incubated in non-adherent plates with 300 RPM shaking for 20 min at 4°C. The

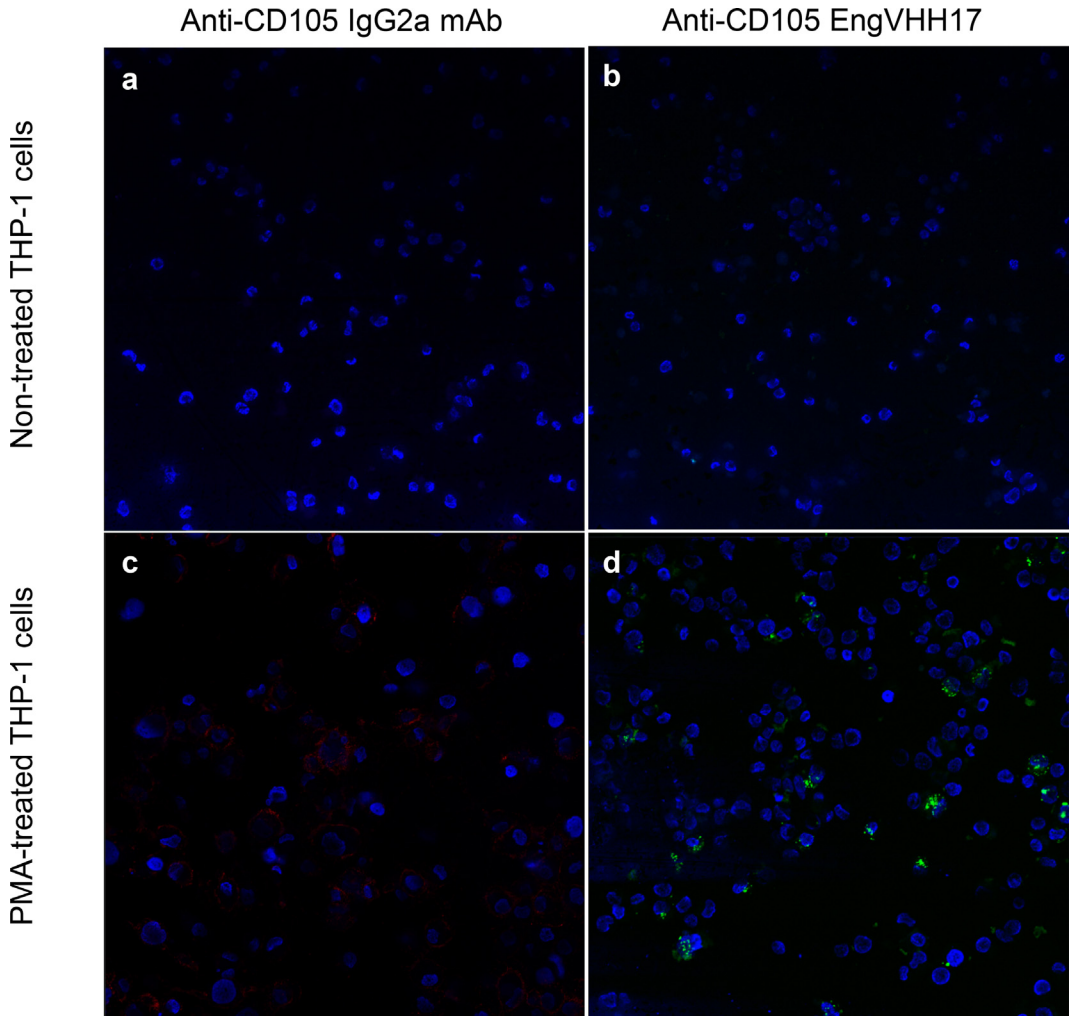

Fig. 4. Visualization of CD105 on the cell surface of differentiated THP1 cells. Human THP-1 cells were treated with 65 nM phorbol 12-myristate 13-acetate (PMA) for 72 h. THP1 (2.5×10^4 cells/well) were fixed and CD105 immunolabeled by using anti-CD105 IgG2a mAb and secondary antibody Alexa Fluor™ 594 labeled goat anti-mouse IgG2a, or anti-CD105 EngVHH17 and FITC anti-6 × His Tag mouse mAb, and DAPI. Undifferentiated THP1 cells do not show immunofluorescence labeling with anti-CD105 mouse IgG2a mAb (**panel a**); anti-CD105 EngVHH17 (**panel b**); PMA-treated THP1 cells for 72 h showed immunofluorescence labeling on the cell surface with anti-CD105 mouse IgG2a mAb (**panel c**); anti-CD105 EngVHH17 (**panel d**); Nuclear DNA of THP1 cells was fluorescence stained with DAPI (blue). Images were taken on a Zeiss LSM 880 confocal microscope at 20× magnification.

plates were placed above a magnet and washed with cold PBS to remove unbound cells, fresh culture medium was added and incubated at 37°C overnight. Recovered cells were stained by the alkaline phosphatase method, then observed and counted by microscopy.

3. Results

3.1. EngVHH17 production in E. coli

Phage display screening through three rounds of panning with CD105 resulted in 2×10^7 phage library, from which 46 phages were picked at random. A single anti-CD105 VHH tagged as EngVHH17 showed high affinity binding to CD105 by phage-ELISA. EngVHH17 was cloned for expression in pET22(+)b/*E. coli* BL21 and purified from the culture supernatant by affinity chromatography using a Ni-NTA column; EngVHH17 appeared as a ~15 kDa single band in SDS-10% PAGE (Fig. 1). The yield of pure recombinant VHHEng17 produced in *E. coli* was ~3.6 mg/L of culture.

3.2. Estimation of dissociation constant (Kd) and Western blot

The saturation binding curve with a CD105 concentration range and 4 μg of EngVHH17 showed a one-site binding hyperbole fit ($R^2 = 0.99$) according to the presence of a single CD105 binding site per EngVHH17 molecule. The Scatchard plot, fitted by linear regression ($R^2 = 0.98$), estimated a dissociation constant value (Kd) of 3.9×10^{-10} M for the binding of CD105 to EngVHH17 (Fig. 2).

Western blotting of T24 cell lysate proteins resolved by SDS-PAGE with EngVHH17 showed a single 95 kDa band corresponding to a monomer of CD105 expressed in T24 cells (Fig. 3).

3.3. Visualization of CD105 on the cell surface of differentiated THP1 cells

THP1 cells expressed CD105 upon differentiation with PMA, while non-differentiated cells did not express CD105 on the cell membrane [8]. Accordingly, undifferentiated THP1 cells did not show fluorescence when immunolabeled either with anti-CD105 mouse IgG2a mAb (Fig. 4a) or EngVHH17 (Fig. 4b). THP1 cells dif-

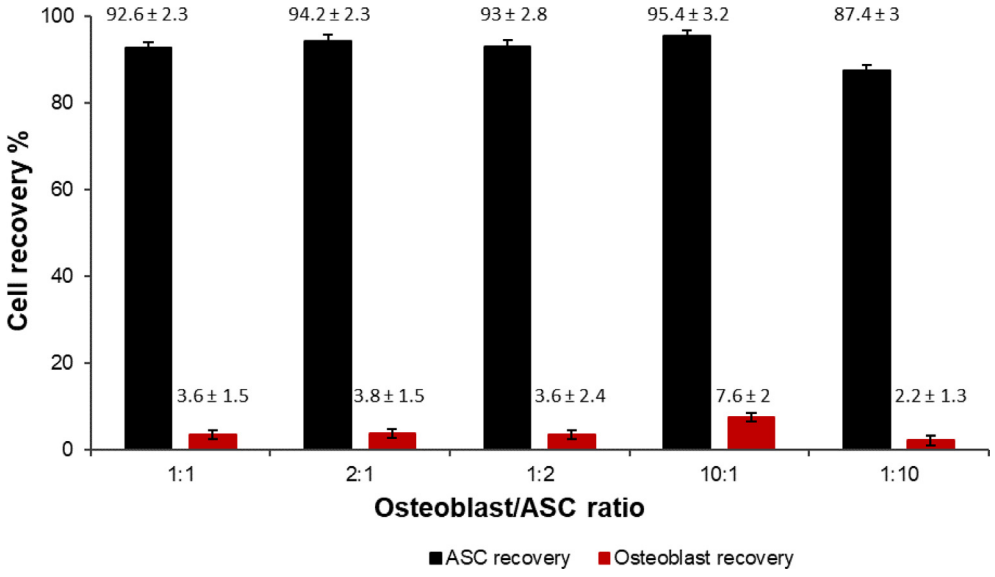

Fig. 5. Recovery of ASCs with EngVHH17-magnetic beads. The ASCs were recovered by using EngVHH17-magnetic beads from different concentration ratios of osteoblasts/ASCs mixed culture *in vitro*. Each bar represents the average ± standard deviation.

ferentiated with PMA for 72 hours were fluorescently immunolabeled with either anti-CD105 mouse IgG2a mAb and goat anti-IgG2a conjugated to Alexa-Fluor 594 (Fig. 4c) or with EngVHH17 and anti-6 × -His Tag IgG1 mouse mAb conjugated to FITC (Fig. 4d).

3.4. ASCs recovery using EngVHH17 conjugated to magnetic beads

EngVHH17 was conjugated to magnetic beads to evaluate the capacity to capture ASCs *in vitro*. A suspension of 6×10^6 magnetic (Fe_3O_4) beads coated with 10 mg/ml EngVHH17 in a solution of 1×10^6 cells/ml showed the capacity of retaining ASCs under the application of an external magnetic field, while non-coated magnetic beads showed very poor or null ASC retention. EngVHH17—magnetic beads selectively capture ASCs in the presence of osteoblasts that do not express CD105. The selective recovery of ASCs using different osteoblast/ASC ratios was higher than 90%, except when the ratio was 1:10, which showed 87% recovery (Fig. 5).

4. Discussion

We report the cloning and production of a single-domain antibody EngVHH17 that binds to human CD105, which is a marker of stem cells [1,2]. EngVHH17 was selected from a cDNA library constructed from PBMC of an alpaca immunized with a lysate of the T24 cell line. T24 is a transformed cell line derived from a bladder tumor that expresses CD105 and a large number of proteins related or not to the transformed phenotype [7].

Screening the anti-T24 lysate VHH cDNA library for anti-CD105 VHHs was challenging because the high homology between alpaca and human (CD105 homology > 90%) and the low fraction of the antigen in the cell lysate could result in poor or absent antigenicity. However, anti-CD105 IgG antibodies were detected in the alpaca serum, reaching a peak at 10 weeks post-immunization. Screening of the anti-lysate T24 VHH cDNA library by phage display and phage-ELISA identified a single anti-CD105 VHH, which was cloned and expressed in *E. coli* and termed EngVHH17.

EngVHH17 is a ∼15 kDa antibody that shows good expression in *E. coli* and binds to CD105 in ELISA, Western blot and cells in culture, as similarly observed in anti-CD105 monoclonal antibodies used for the isolation of MSCs or for the generation of chimeric antibodies [11,12,13]. EngVHH17 binds CD105 with high affinity (Kd value = 3.9×10^{-10} M) as other reported anti-CD105 VHHs [11], and high specificity since EngVHH17 recognizes a single band corresponding to CD105 in a Western blot assay with T24 lysate proteins resolved by SDS-15% PAGE. EngVHH17 binds to CD105 expressed on the cell surface of PMA-differentiated THP1 cells in culture, but not to undifferentiated THP1 cells [8].

The EngVHH17-magnetic beads were able to selectively capture ASCs from a mixture with osteoblasts at different cell concentration ratios with recoveries higher than 90%. Similar results were obtained with an anti-CD105 mAb used to isolate ASCs [3]. EngVHH17 is stable in aqueous solutions and binds antigens with high affinity and specificity. VHHs are advantageous over mouse mAbs as they can be produced in large quantities in *E. coli* and the relatively small size of VHHs (∼15 kDA) may favor both access to epitopes that bulky mAbs (∼150 kDA) cannot reach and a better distribution through the porous materials of scaffolds. In addition, VHHs can enhance their binding capacity to ASC and scaffolds by incorporating sequences and functional groups into the primary protein [6].

The results suggest that EngVHH17 conjugated to magnetic beads can be used for ASC isolation by immunoaffinity capture. Nevertheless, it is still required to evaluate whether the capture of ASCs by EngVHH17 affects the stem cell characteristics such as viability, proliferation, and differentiation of ASCs.

Authors contribution

Study conception and design: J.R. Espinoza, M. van Griensven.
Data collection: E. Gushiken, M. Quintana, L.A Perez, E.R. Balmayor, P. Herrera-Velit.
Analysis and interpretation of results: J.R. Espinoza, P. Herrera-Velit, E.R. Balmayor.
Draft manuscript preparation: J.R. Espinoza.
Revision of the results and approval of the final version of the manuscript: J.R. Espinoza, M. van Griensven.

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Conflict of interest

The results and data were generated as a part of the graduate research activities of Eduardo Gushiken, Milagros Quintana, and L. Agueda Perez under the co-supervision of Dr. Elizabeth R. Balmayor and Dr. Patricia Herrera-Velit and supervision of Dr. Martijn van Griensven and Dr. Jose R. Espinoza.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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