ONLINE DATA SUPPLEMENT:

Derivation of Lung Epithelium From Human Cord Blood-Derived Mesenchymal Stem Cells

Viranuj Sueblinvong, Roberto Loi, Philip L. Eisenhauer, Ira M. Bernstein, Benjamin T. Suratt, Jeffrey L. Spees, and Daniel J. Weiss

Methods:

Animals

Adult (6-12 weeks) NOD-SCID mice (NOD.CB17-*Prkdc*^{scid}/J Jackson Laboratories) were utilized as recipients of human cord blood mesenchymal stem cells (CB-MSCs) administration. All studies were subject to Institutional Animal Care and Use Committee (IACUC) review at the University of Vermont (UVM) (Burlington, VT) and conformed to institutional and AAALAC standards for humane treatment of laboratory animals.

Isolation, culture, expansion and characterization of CB MSCs

A total of 31 human umbilical cord blood (CB) samples were obtained from term, normal deliveries at the UVM. All studies were subjected to Institutional Review Board (IRB) review at the UVM and informed consent was obtained from all donors. CB samples were collected into syringes pre-filled with citrate anticoagulation (5 ml in 60 ml syringes). CB mononuclear cells were isolated by Ficoll® density gradient centrifugation (Fisher BioReagents), re-suspended in a 1:1 mixture of CB Basal Medium (a-MEM (GIBCO BRL), 10% FBS, 100 U/mL Penicillin, 100 µg/mL Streptomycin and 2 mM L-Glutamine) (1) and MSC conditioned medium obtained from established adult bone marrow derived-MSCs culture, and plated at $1x10^6$ cells/cm^2 in standard 75 cm^2 tissue culture dishes (Corning®, NY). Samples with gross blood contamination were incubated in 0.83% NH_4Cl for RBC lysis prior to final re-suspension (2). Cells were cultured overnight at 37°C with 5% humidified CO_2 and the medium changed the next day

to remove non-adherent cells. Plastic adherent cells were subsequently cultured in a 1:1 mixture of CB basal medium and MSCs conditioned medium until colonies were established. Subsequent cultures were maintained in CB basal medium at no more than 50% confluence. The cells were harvested and re-plated at a density of 100-250 cells/cm^2 for expansion.

After 2-3 weeks culture, passage 2-4 adherent cells were harvested with 0.025% Trypsin in 0.033% EDTA, resuspended at 10^7 cells/ml, incubated with antibodies to cell surface proteins including: CD 29, CD 44, CD 49 b,c,f, CD 59, CD 73a, CD 90, CD 105, CD 117, CD 147, CD 166, CD 19, CD 34, CD 36, CD 45, CD 79a, CD 106, CD 116 and CD 271, and assessed by flow cytometry for presence of these markers. The result was compared with adult bone marrow-derived MSCs (3).

In parallel, passage 2-4 CB adherent cells were incubated in the specific differentiation media for adipocytes (CB basal medium supplemented with 0.5 µM dexamethasone (Sigma), 0.5 µM isobutylmethylzanthine (IBMX, Sigma), and 50 µM indomethacin (Sigma) (3)), osteoblasts (CB basal medium supplemented with 1 nM dexamethasone, 50 µM L-ascorbic acid-2 phosphate, and 20 mM ß-glycerol phosphate (DAG) (4)) and chondroblasts (utilizing micromass culture system in DMEM high glucose supplemented with 500 ng/mL bone morphogenetic protein-6 (R&D Systems), 10 ng/ml transforming growth factor (TGF) ß-3, 10^{-7} M dexamethasone, 50 µg/ml ascorbic acid-2-phosphate, 40 µg/ml proline, 100

µg/ml pyruvate, and 50 mg/ml ITS + premix (Becton Dickinson; 6.25 µg/ml insulin, 6.25 µg/mL transferring, 6.25 ng/ml selenious acid, 1.25 mg/ml BSA and 5.35 mg/ml linoleic acid) (5)) to assess the differentiation capacity of established MSC lineages. Media were changed every other day for 3 weeks. Cells were then fixed with 4% paraformaldehyde (PFA) for 10 minutes and stained with Alizarin Red S, Oil Red O or Toluidine blue Sodium Borate accordingly.

All light microscopy figures in **Figure 1** were taken with phase contrast microscopy (Ph1), 10 x magnifications.

Induction of lung epithelial phenotypic differentiation of CB-MSCs

For 8 of 12 CB samples, passage 2-4 CB-MSCs were cultured in CB basal medium, mouse tracheal epithelium cells (MTEC) medium (DMEM-Ham's F-12 50:50, 15 mM HEPES, 3.6 mM sodium bicarbonate, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone with 2% NuSerum (Becton-Dickinson) and freshly added 0.01 µM retinoic acid) (6), small airway growth medium (SAGM) (Small airway basal media SABM (Cambrex) plus 0.5 mg/ml bovine serum albumin (Sigma), 5 µg/ml Insulin (Sigma), 10 µg/ml Transferrin (Sigma), 30 µg/ml Bovine Pituitary extract (Sigma), 0.5 µg/ml Epinephrine (Sigma), 6.5 ng/ml Tri-iodothyronin (Sigma), 0.5 µg/ml Hydrocortisone (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.5 ng/ml Human EGF (Sigma) and freshly added 0.1 ng/ml retinoic acid (Sigma)) (7), CB basal medium supplement with KGF at 50 ng/mL or retinoic acid 10 µg/mL for 1,

2 or 4 weeks. Cells were harvested and stored in TRIzol® (Invitrogen) at -80°C for PCR or fixed with 4% paraformaldehyde (PFA) and store in phosphate buffer saline (PBS) at 4°C for immunofluorescent staining.

Analysis of lung epithelial markers expression by RT-PCR

At each time point (1 week, 2 weeks or 4 weeks), total RNA was isolated from 5 of 8 samples of CB-MSCs with TRIzol® and purified using an RNeasy kit (Qiagen). First-strand cDNA was synthesized with random primers (Invitrogen Life Technologies) and Superscript II reverse transcriptase (Invitrogen Life Technologies) and Glyceraldehyde-3-phosphate dehydrogenase (GADPH), Clara cell secretory protein (CCSP), Cystic fibrosis transmembrane conductance regulator (CFTR), Aquaporin 5 (AQP-5), Thyroid Transcription Factor-1 (TTF-1), Surfactant Protein-C (SPC), a-smooth muscle actin and collagen, type I, alpha 1 (Col1A1) were amplified by PCR (Initial denatured with 94°C x 2 mins, then, 40 cycles of 94°C x 30 sec, 55°C x 30 sec, 72°C x 30 sec, final extension with 72°C x 5 mins) with Taq polymerase (Primer sequences are listed in **Table E1**).

All mRNA samples were subjected to PCR reaction to amplified ß-actin prior to reverse transcriptase reaction (no RT). Representative positive control samples (human lung (HL) and human lung fibroblast (HLF)) are shown in Figure 4A and 4C.

Analysis of lung epithelial markers expression by quantitative PCR

Quantitative PCR was performed on 40 ng of cDNA (5 samples at all time points) with Assays on Demand predesigned CCSP, CFTR and, hypoxanthine phosphoribosyltransferase (HPRT) primer/probe sets (Applied Biosystems), using TaqMan Universal PCR Master Mix and Applied Biosystems 7900HT Sequence Detection System. The level of CCSP or CFTR expression was normalized to HPRT housekeeping gene levels and relative CCSP or CFTR mRNA levels were determined according to the comparative cycle threshold method (Applied Biosystems 7900HT Sequence Detection System, User Bulletin No. 2; Applied Biosystems). Briefly, the threshold cycle (C_T) was determined for CCSP, CFTR and HPRT in each sample. The $?C_T$ was calculated for each sample by subtracting the C_T of HPRT from the C_T of CCSP or CFTR. The baseline $?C_T$ was assigned to human lung. The $??C_T$ values were calculated by subtracting the $?C_T$ of the baseline from the $?C_T$ of the experimental samples. The $??C_T$ values were transformed into absolute values using the equation $2^{-??CT}$ (Relative Quantification or RQ).

Analysis of lung epithelial marker expression by immunofluorescent staining

CB-MSCs cultured on collagen-coated glass coverslips were fixed with 4% PFA and stored in PBS at 4^{o}C. Cells were permeabilized with 0.5% DAKO Tween® 20 in PBS for 10 minutes at room temperature follow by series of washing with PBS. Samples were then blocked with 5% normal donkey serum, 0.5% BSA in PBS for

1 hour at room temp. Primary antibodies (goat anti-rat CCSP (courtesy of Barry Stripp, University of Pittsburgh) at 1:250 dilution and mouse anti-human CFTR (clone M3A7 and L12B4 (Chemicon)) at 1:1000 dilution) antibodies were applied and incubated overnight in the dark at 4^{o}C in diluent buffer (0.5% BSA, 0.1% DAKO Tween® 20 in PBS). Following series of washing with PBS, secondary antibodies (donkey anti-goat Alexa 488 at 1:250 dilution and goat anti-mouse Alexa 568 at 1:1,000 dilution (Invitrogen)) were applied and incubated for 2 hours at room temperature in the dark. Following series of washing, DAPI (Roche, IN) at 1:10,000 dilution in PBS were applied and incubated for 5 minutes at room temperature. Coverslips were then washed and mounted to slides for further analysis with Zeiss confocal microscopy.

Transfection of CB-MSCs with recombinant lentivirus

CB-MSCs were plated at 1,000 cells/cm^2 in 12 well tissue culture dishes (Corning). At 6 hours following the initial plating, recombinant lentivirus encoding either yellow fluorescent protein (YFP) or CFTR, were added at MOI ranging from 1-100. Cells were incubated with virus particle for 24 hours, washed with PBS for 5 minutes thrice, fresh CB basal medium were added and incubated for additional 48 hours prior to analysis. For CB-MSCs transduced with lentivirus-CFTR, cells were fixed with 4% PFA for 10 minutes and washed with PBS. Cells were stained with mouse anti-human CFTR as described above. Fluorescence was visualized with Olympus IX70 Inverted Microscope. Phase contrast microscopy technique was used for cell morphology assessments. Figure 6(a)

and (e) were taken with 40x magnification, Ph2 ring while figure 6(c) was taken with 40x magnification, Ph1 ring.

In vivo administration of CB-MSCs to NOD-SCID mice

CB-MSCs were harvested with 0.025% Trypsin in 0.033% EDTA and resuspended at 10^7 cells/ml. At 24 hours following sub-lethal irradiation (1.4 Gy in Bioradiator), each experimental mouse received 200 µL of cells suspension ($2x10^6$ cells/mouse) via tail vein injection. Control animals received equal volume of sterile PBS. Mice were subsequently euthanized at 1 day, 2 weeks, 1 or 3 months after transplantation. Bronchoalveolar lavage and lungs were harvested. The right lungs were frozen with liquid nitrogen and stored at -80^{o}C for RNA and DNA analysis. The left lung was gravity fixed with 4% PFA for 1 hour on ice and store in PBS for further analysis.

Analysis for the presence of human cells in mouse lung

Genomic DNA was extracted from frozen right lung (15 mg) using DNA extraction kit (Stratagene, CA) per manufacturer's instructions. 300 ng of total DNA obtained was amplified with human Alu sequences **(Supplemental data Table E2)** (8). Standard curves were generated using serial dilution of human genomic DNA (5 pg to 50 ng) into control mouse lung genomic DNA with total of 300 ng DNA per reaction (8).

Immunofluorescent staining for human cells in mouse lung

Paraffin-embedded left lungs were analyzed for presence of human cells by immunostaining for human ß2-microglobulin and either a-human/mouse pancytokeratin or a-human CFTR. De-paraffinized and rehydated 5 µm mounted lung sections were fixed in methanol for 10 minutes at room temperature. The sections were then immersed in 10 mM sodium citrate, pH 6 for 12 minutes at 96^{o}C for antigen retrieval. Sections were then washed in 2XSSC/0.05% DAKO Tween® 20 for 5 minutes at room temperature and blocked with blocking buffer (5% goat serum, 0.25% DAKO Tween® in PBS) for 1 hour at room temperature. Primary antibodies, polyclonal rabbit anti-human ß2 microglobulin at 1:400 dilution and monoclonal mouse anti-human pan-cytokeratin or mouse anti-human CFTR (clone M3A7 and L12B4) at 1:100 dilution in blocking buffer were applied to sections and incubated overnight at 4^{o}C. Control human lung and mouse lung slides were stained with Rabbit IgG antibody (DAKO) at 1:400 dilution. After a series of PBS washes, secondary antibodies (goat anti-rabbit Alexa 647 and goat anti-mouse Alexa 568 (Invitrogen)) were applied, at 1:400 dilution, in blocking buffer and incubated for 2 hours at room temperature in the dark. Following series of washing, DAPI (Roche, IN) at 1:10,000 dilution were applied and incubated for 5 minutes at room temperature. Sections were then washed and covered for further analysis with Zeiss confocal microscopy. Human lung section was utilized as a positive control and mouse lung was utilized as a negative control for human ß2-microglobulin antibody **(see Figures E1 and E2)**. Further, cytospin preparations of CB-MSCs injected to NOD-SCID mice aliquot were

analyzed for presence of pancytokeratin **(see Figure E3)**. Data was analyzed from 5 random high power fields (40x magnification) per section on 8 sections per mouse by counting 1) total cells (number of nucleus), 2) total cells stained positive with cytokeratin (CK +), 3) total cells stained positive with ß2-microglobulin (ß2+) and 4) cells dual stained with cytokeratin and ß2-microglobulin (ß2+ CK+).

References:

1. Sueblinvong V, L., R., Poynter, M.E., Bernstein, I.M., Weiss, D.J. . 2006. Development of Airway Epithelium from Cord Blood Stem Cells. *Proceedings of the American Thoracic Society* 3:A25.
2. Ridings, J., Weedon, H., Ioannou, C., Flego, L., Macardle, P. J., and Zola, H. 1996. Purification of cord blood lymphocytes. *J Immunol Methods* 195(1-2):43-8.
3. Peister, A., Mellad, J. A., Larson, B. L., Hall, B. M., Gibson, L. F., and Prockop, D. J. 2004. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. *Blood* 103(5):1662-8.
4. Phinney, D. G., Kopen, G., Isaacson, R. L., and Prockop, D. J. 1999. Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. *J Cell Biochem* 72(4):570-85.
5. Sakaguchi, Y., Sekiya, I., Yagishita, K., Ichinose, S., Shinomiya, K., and Muneta, T. 2004. Suspended cells from trabecular bone by collagenase digestion become virtually identical to mesenchymal stem cells obtained from marrow aspirates. *Blood* 104(9):2728-35.
6. You, Y., Richer, E. J., Huang, T., and Brody, S. L. 2002. Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. *Am J Physiol Lung Cell Mol Physiol* 283(6):L1315-21.

7. Ali, N. N., Edgar, A. J., Samadikuchaksaraei, A., Timson, C. M., Romanska, H. M., Polak, J. M., and Bishop, A. E. 2002. Derivation of type II alveolar epithelial cells from murine embryonic stem cells. *Tissue Eng* 8(4):541-50.

8. McBride, C., Gaupp, D., and Phinney, D. G. 2003. Quantifying levels of transplanted murine and human mesenchymal stem cells in vivo by real-time PCR. *Cytotherapy* 5(1):7-18.

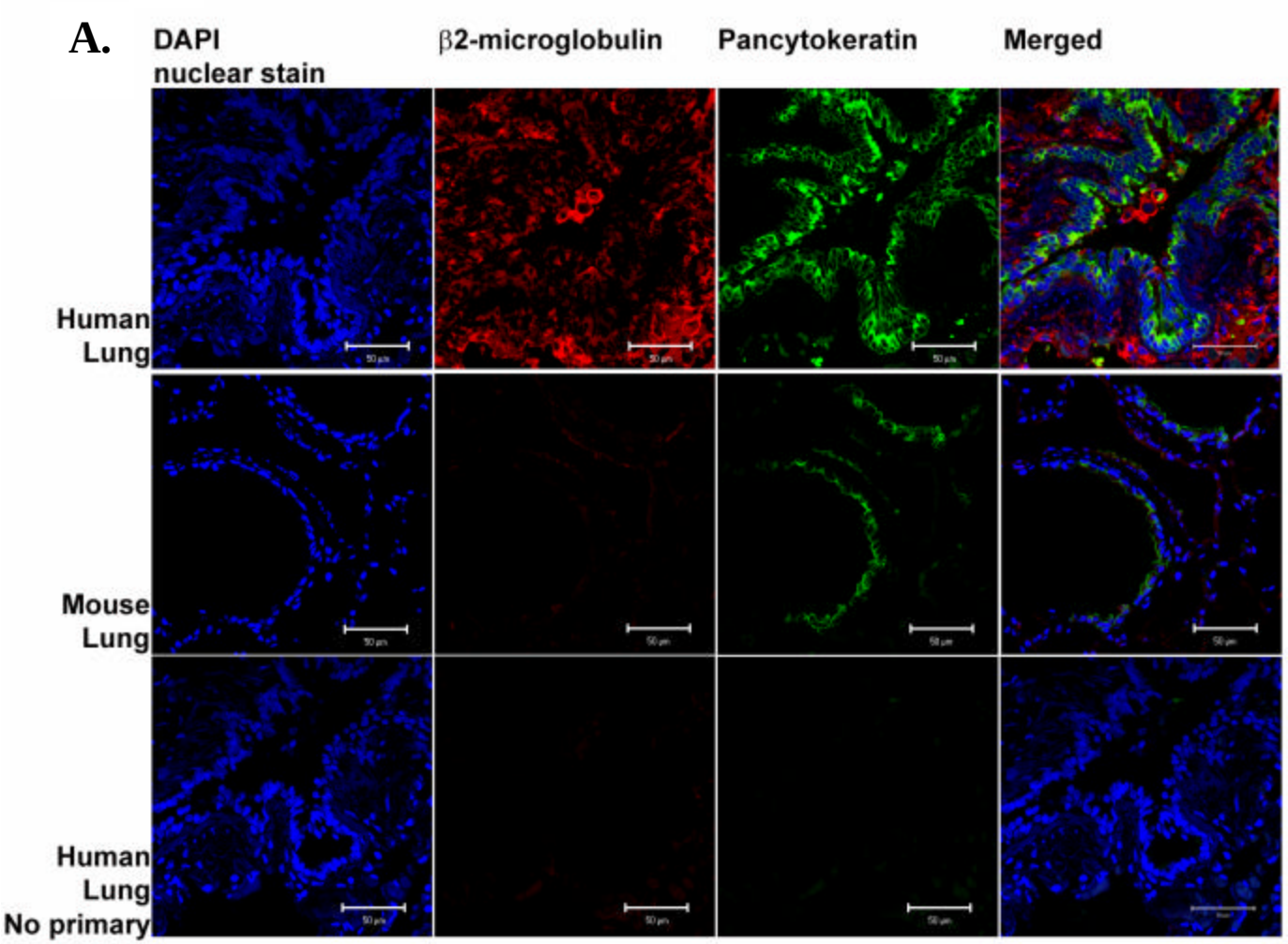
Table E1: Human primers sequences

Gene	Primer Sequences (5'-3')	Product size (bp)
GADPH	F: CCC TTC ATT GAC CTC AAC TAC AT R:ACG ATA CCA AAG TTG TCA TGG AT	407
CFTR 1-4	F: CAG CTG GAC CAG ACC AAT TT R: TTA TCC GGG TCA TAG GAA GC	160
CCSP	F: CTT TCA GCG TGT CAT CGA AA R: TTG AAG AGA GCA AGG CTG GT	232
SP-C	F: TGG TCC TCA TCG TCG TGG TGA TTG R: CCT GCA GAG AGC ATT CCA TCT GGA AG	327
TTF-1	F: CCTGTCCCACCTGAACTCC R: CGGCCAGGTTGTTAAGAAAA	197
AQP5	F: CAT CTT CGC CTC CAC TGA CT R: CCC TAC CCA GAA AAC CCA GT	193
α-smooth muscle actin	F: AGGTAACGAGTCAGAGCTTTGGC R: CTCTCTGTCCACCTTCCAGCAG	199
Col1A1	F: GCCTCCCAGAACATCACCTA R: GTG CAG CCA TCG ACA GTG	164

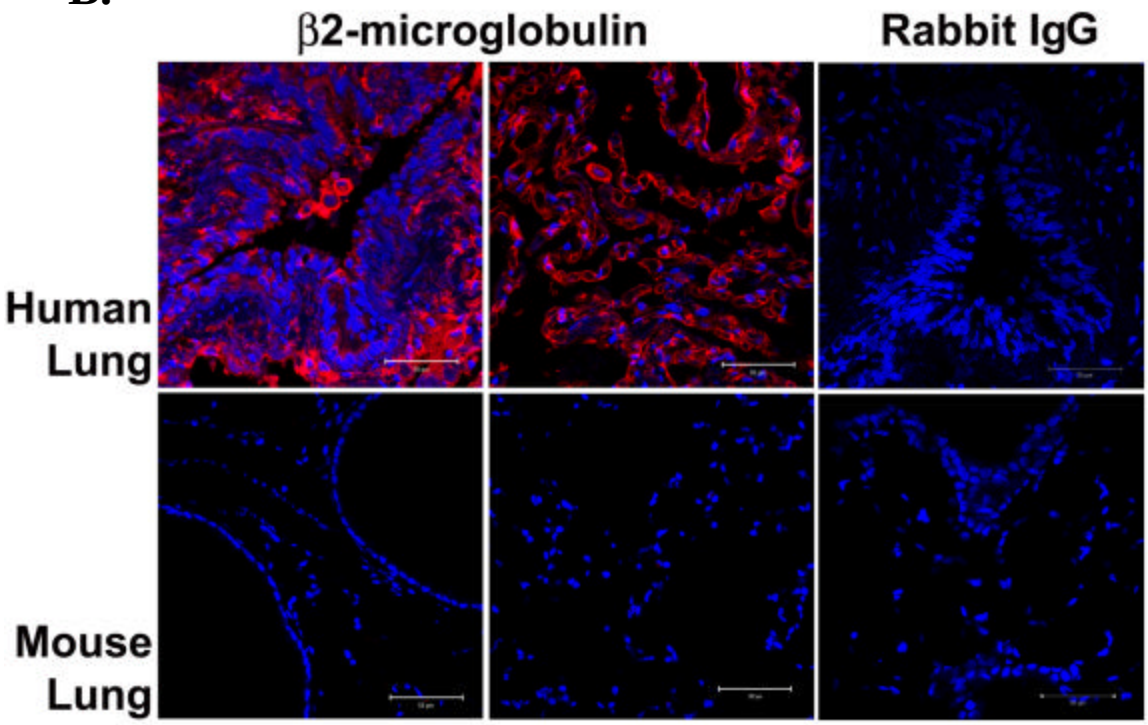
Table E2: Human Alu Sequences for quantitative PCR

Alu primers	F: CAT GGT GAA ACC CCG TCT CTA R: GCC TCA GCC TCC CGA GTA G
Alu Taqman probe	5’ FAM-ATT AGC CGG GCG TGG TGG CG-TAMRA 3’

Figure E1: Immunofluorescent staining of human ß2 microglobulin and Pancytokeratin or CFTR on control tissues. (A) Control human and mouse lung sections stained with polyclonal rabbit anti-human ß2-microglobulin (1:400) and monoclonal mouse anti-human pancytokeratin (1:100), (B) Control human and mouse lung sections stained with rabbit immunoglobulin G (IgG) (1:400), (C) Control human and mouse lung sections stained with polyclonal rabbit anti-human ß2-microglobulin (1:400) and monoclonal mouse anti-human CFTR (clone M3A7 and L12B4 at 1:100 dilution.



B.


β2-microglobulin
Rabbit IgG
Human Lung
Mouse Lung

C.

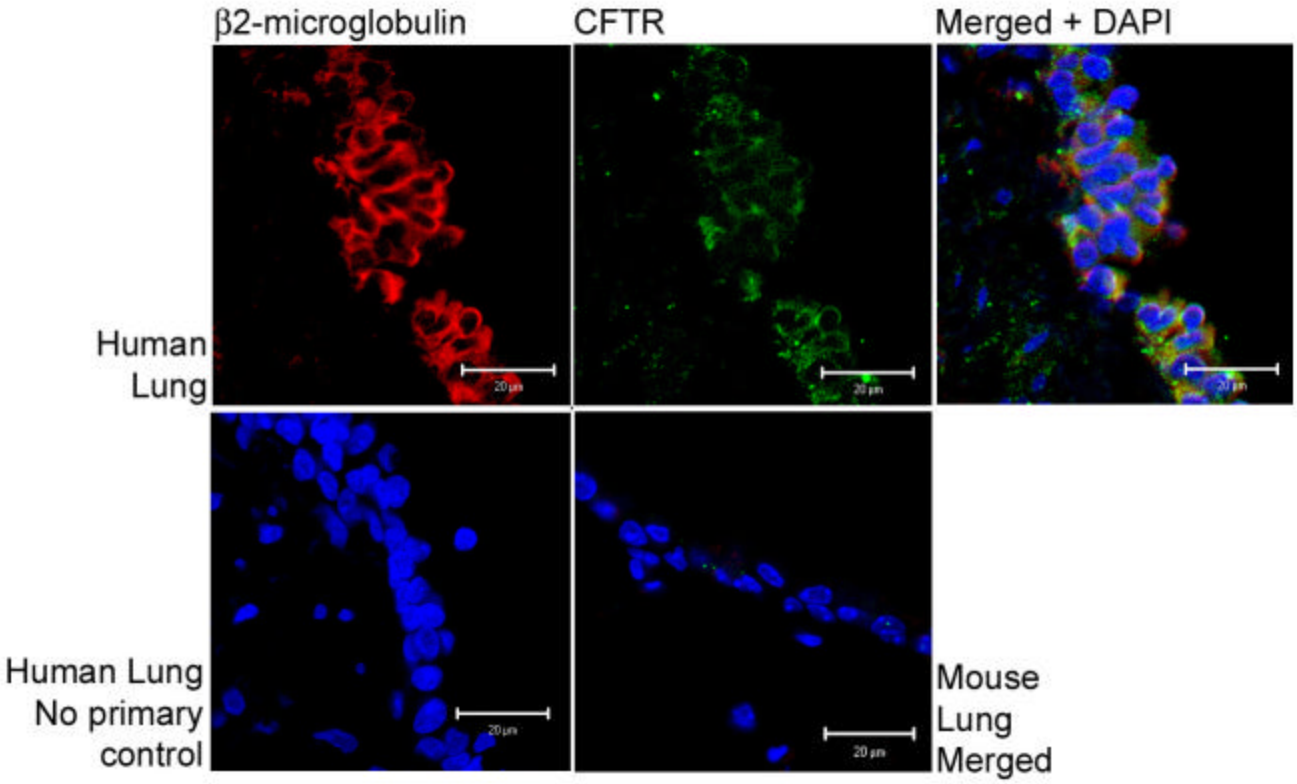
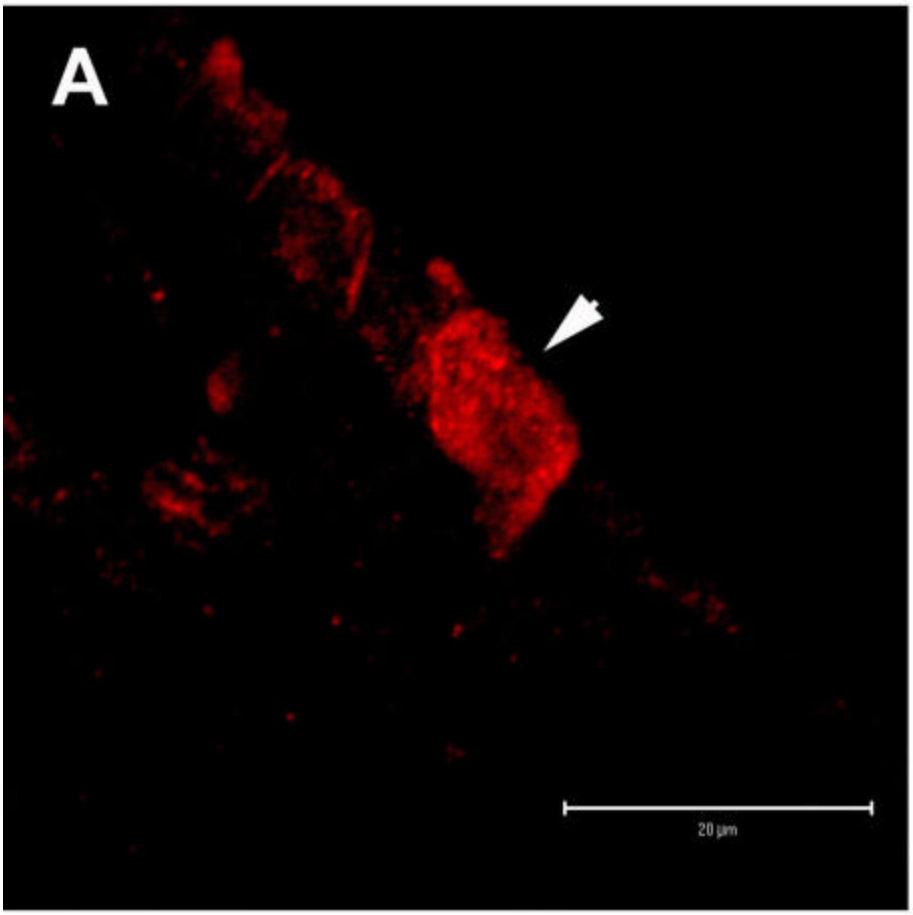
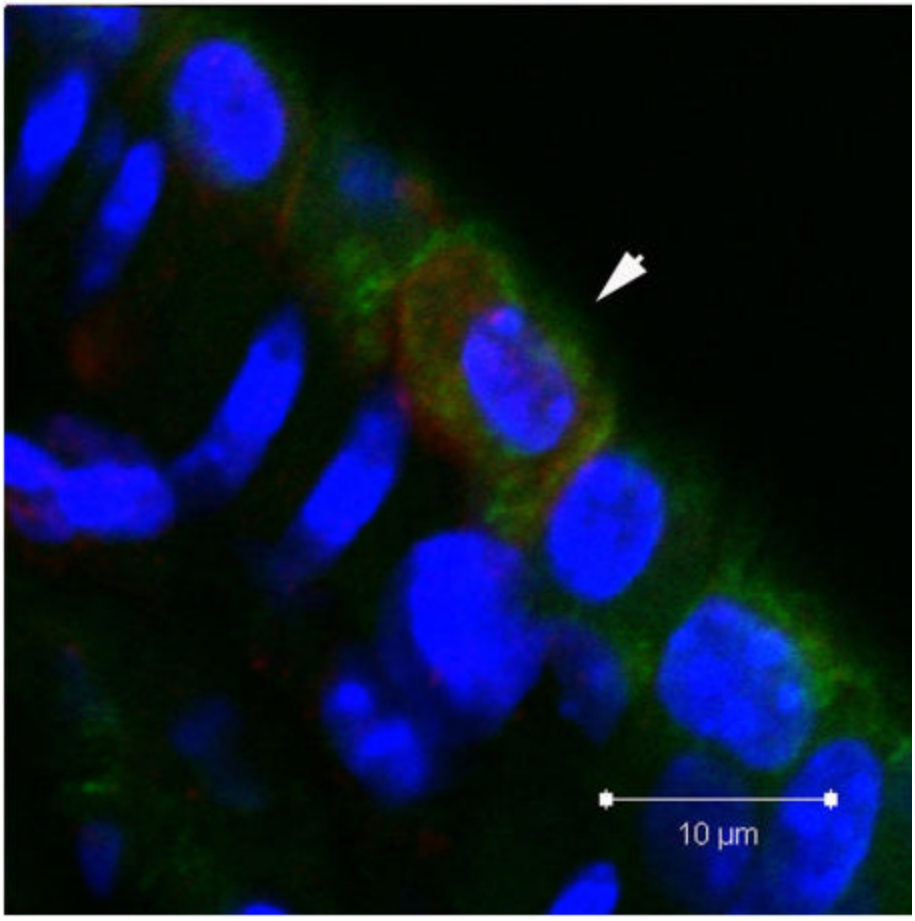
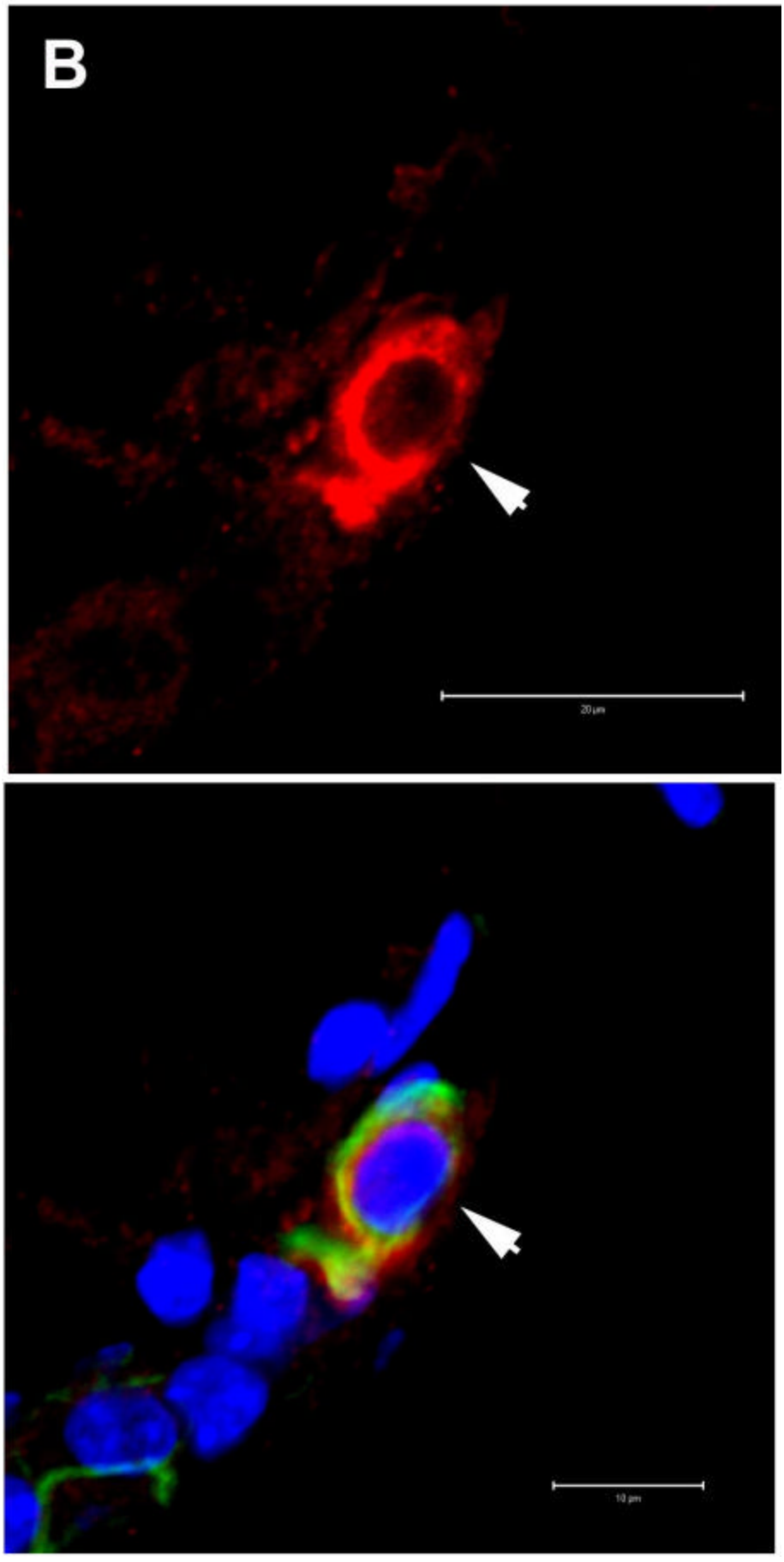
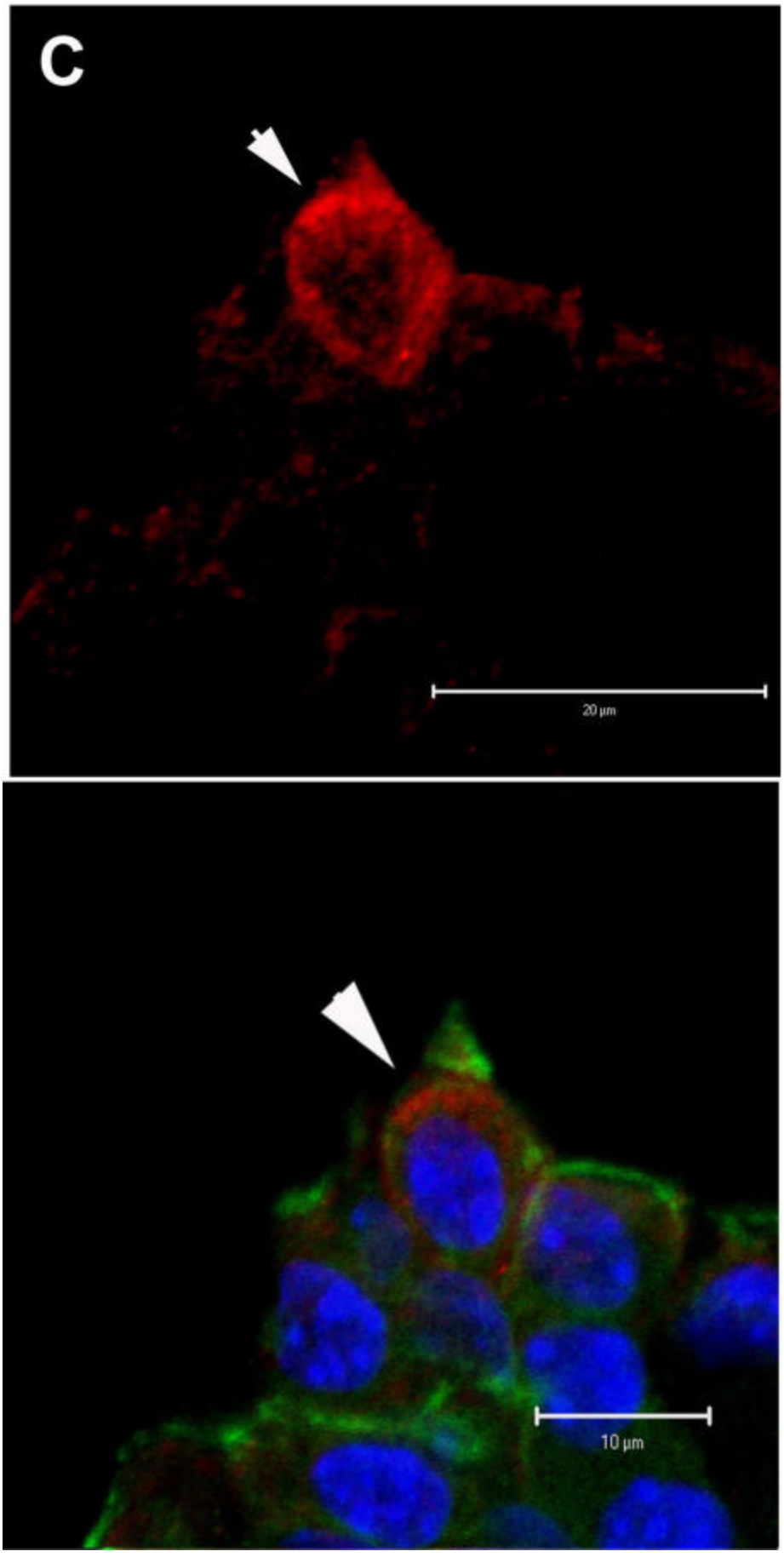

β2-microglobulin
CFTR
Merged + DAPI
Human Lung
Human Lung No primary control
Mouse Lung Merged

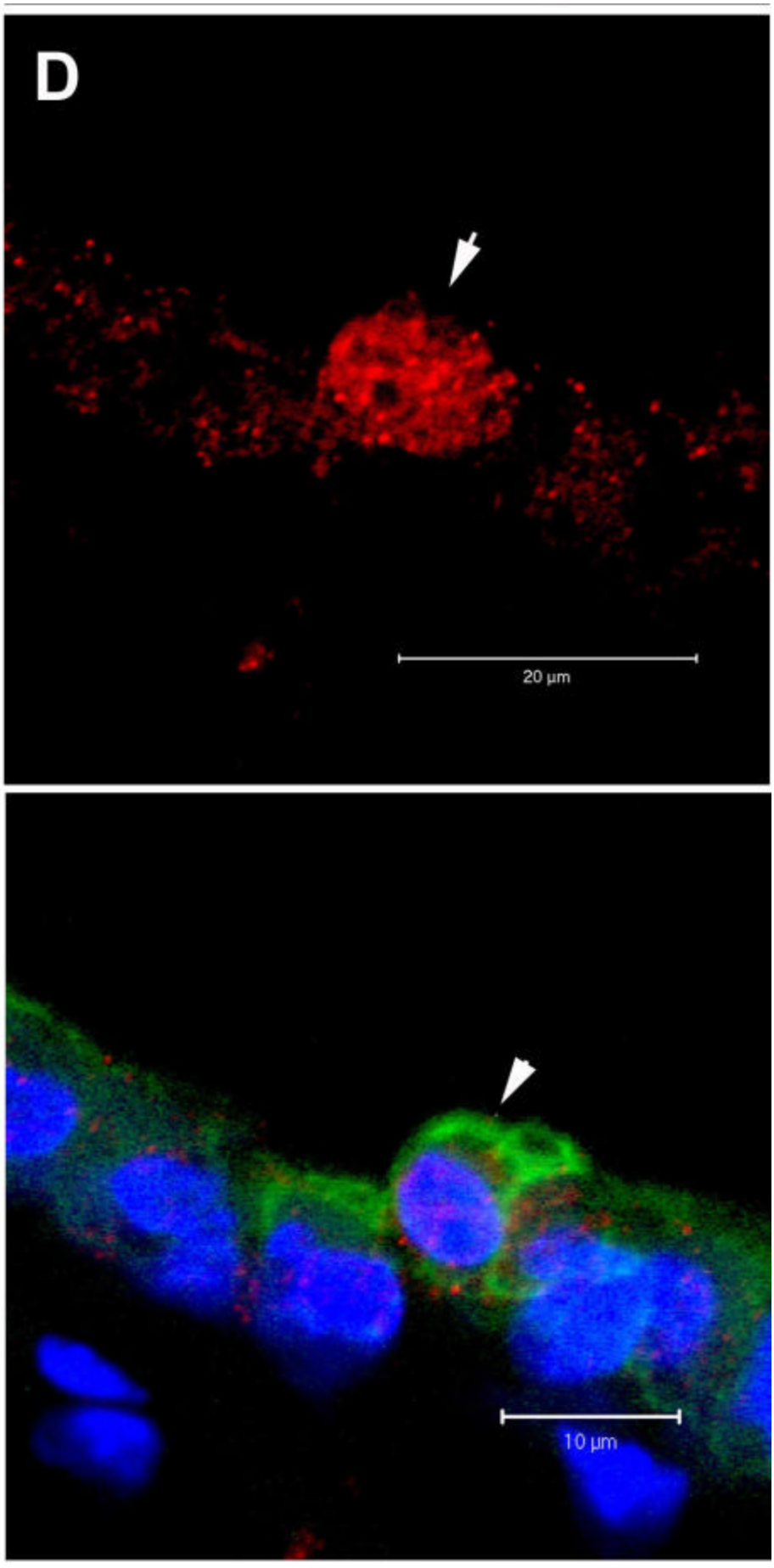
Figure E2: Human ß2-microglobulin positive cells can be detected in NOD-SCID mice airways following systemic administration of CB-MSCs. A small percentage of ß2-microglobulin positive cells stained positively for pancytokeratin or human CFTR (white arrows). (A-C) at 2 weeks, (D) at 1 month, and (E) at 3 months following CB-MSCs administration. Blue =DAPI nuclear stain, green = pancytokeratin, red = b2-microglobulin. Original magnification 200x.


A
20 µm


10 µm


B
20 µm
10 µm


C
20 µm
10 µm


D
20 µm
10 µm

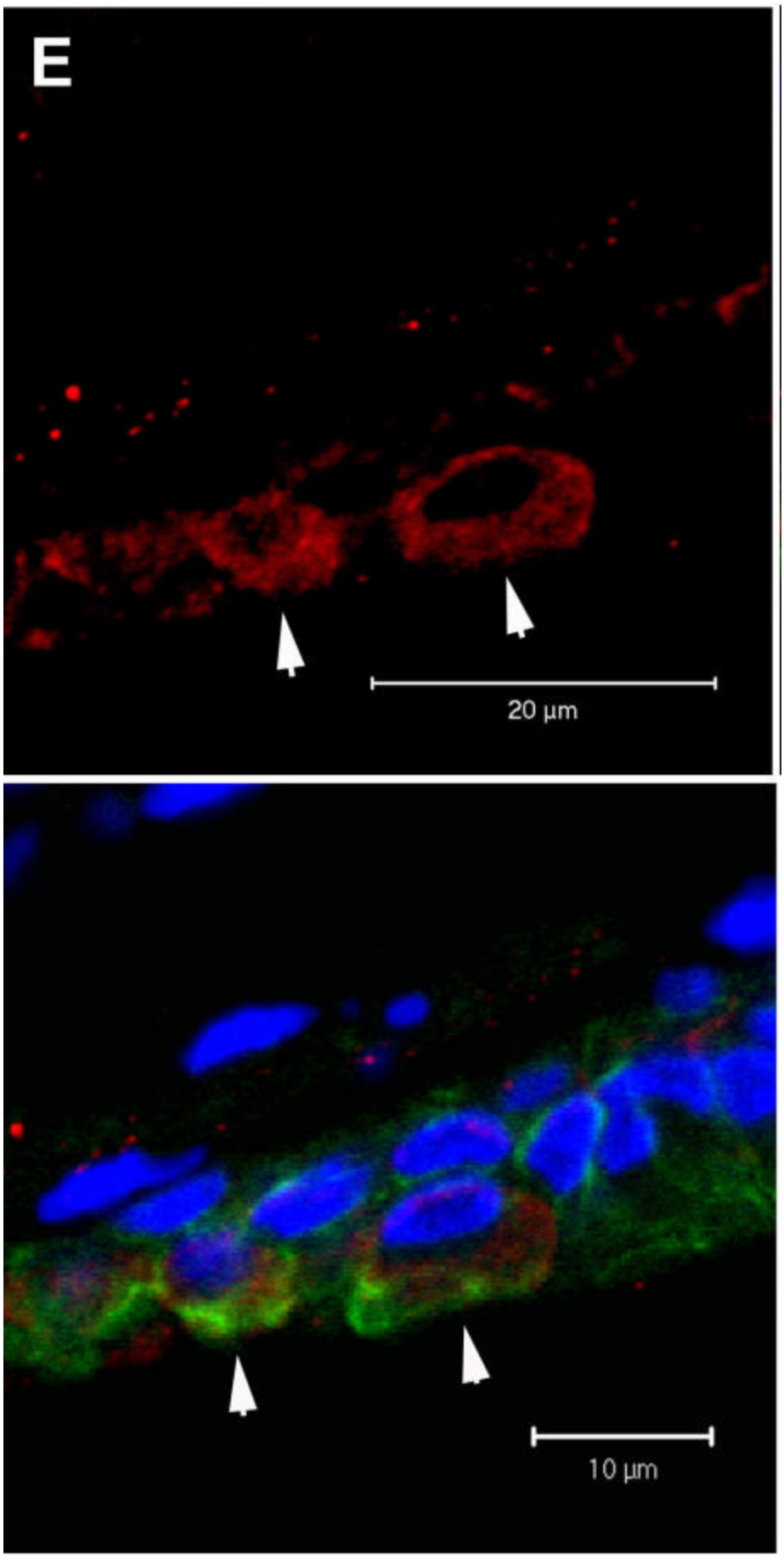

E
20 µm
10 µm

Figure E3: Immunofluorescent staining of pancytokeratin on CB-MNCs and CB-MSCs. These stains demonstrated CB-MNCs and CB-MSCs did not express cytokeratin *in vitro*. (A) Human Bronchial Epithelial (HBE) cells, (B) HBE cells, no primary antibody control, (C) CB-MNCs, and (D) CB-MSCs. Red = pancytokeratin, Blue = DAPI nuclear stain. Original magnification, 40X.