Bone marrow CCSP-expressing cells increase epithelium repair after ablation of Clara cells and lung stem cells

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SUPPLEMENTARY DATA

SUPPLEMENTARY TABLES

Table S1. CCSP^{+} BMC gene expression in culture

GENE	CCSP^{-} BMC	CCSP^{+} BMC	NORMAL TISSUE	Reaction Efficiency
CCSP	ND	0.1±0.052*	1 ±0.26^{L}	0.9843
Cyp2f2	0.007±0.0107*	ND	1 ± 0.09^{L}	1
Pon1	0.272±0.1*	0.036±0.01*	1 ± 0.15^{L}	0.9734
Fmo3	ND	ND	1 ±0.04^{L}	1
Aox3	ND	ND	1 ±0.12^{L}	1
Cldn10	1.17±0.14	3.54±0.12*	1 ± 0.20^{L}	0.958
Sftpc	ND	ND	1 ± 0.014^{L}	1
Aqp-5	0.007±0.012*	0.009±0.017*	1±0.084^{L}	0.9393
Foxj1	ND	ND	1 ±0.20^{L}	1
Krt14	0.36±0.01*	0.70±0.21*	1 ± 0.12^{L}	1
Krt18	ND	ND	1 ± 0.13^{L}	0.917
Krt5	ND	0.39±0.17*	1 ± 0.08^{L}	0.986
CFTR	0.45±0.32*	1.004±0.1	1 ± 0.26^{L}	1
ENaC	ND	0.047±0.006*	1 ± 0.058^{L}	1
Cubilin	0.25±0.19*	ND	1 ± 0.1^{K}	0.976
Megalin	ND	ND	1 ±0.08^{K}	1
18S	1.0032±0.017	1.009±0.08	1 ±0.084^{L}	1

CCSP= Clara cell secretory protein, Cyp2f2= Cytochrome P450, family 2, subfamily f, polypeptide 2, Pon1= Paraoxonase 1, Fmo3= Flavin containing monooxygenase 3, Aox3= Aldehyde oxidase 3, Cldn10= Claudin 10, Sftpc= Surfactant protein C, Aqp-5= Aquaporin 5, Krt14= Keratin 14, Krt18= Keratin 18, Krt5= Keratin 5, CFTR = Cystic fibrosis transmembrane conductance regulator; ENaC = Epithelial sodium channel, L= normal lung, K= normal kidney. ND= not detected. Data expressed as fold increase ± SEM. *$p<0.05$ when compared relative expression of normal tissue. n=4 mice per group.

Table S2. EXPRESSION OF EPITHELIAL PROTEINS BY DONOR CELLS IN LUNG

Epithelial Proteins	$CCSP^+$ BMC	$CCSP^-$ BMC	p Value
CCSP	79.53 ± 2.65%	3.08 ± 1.78%	< 0.001
PanKr	74.14 ± 2.29%	3.09 ± 1.79%	< 0.001
CFTR	60.61 ± 7.01%	9.49 ± 3.00%	0.002
ENaC	7.49 ± 2.17%	0.00%	0.041

BMC = Bone marrow cells; CCSP = Clara cell secretory protein; PanKr = pan cytokeratin; CFTR = Cystic fibrosis transmembrane conductance regulator; ENaC = Epithelial sodium channel. . The number of donor cells positive for both CMTMR and the epithelial cell-specific antigen was divided by the number of total $CMTMR^+$ cells and multiplied by 100 to give a percentage value. Data shown are means ± SEM. n=4 mice per group. Samples from mice administered with donor cells after 10 days of ganciclovir.

Table S3. Primers for real time PCR

Gene	Symbol	Forward primer	Reverse primer
Clara cell secretory protein	CCSP	ACATCACCCCACATCTACAGACACCAA	TGAGGAGGGCCTCAAGGACTTGAA
Cytochrome P450, family 2, subfamily f, polypeptide 2	Cyp2f2	GCATACCCCGTCTTTTTCAA	TCATCGTCATAGTCGAAGCG
Paraoxonase 1	Pon1	TCCTCCCGGCTCAGAGGTGC	TGCGTGCAGCTGGCTTGTCA
Aldehyde oxidase 3	Aox3	TCGGTCTCCTGCAGAGCACAGC	AGGCGCCACAGTCACCTCCT
Flavin containing monooxygenase 3	Fmo3	TCCCTGGGTGCCACCATCCC	CAGCCATAGGAGATTGGGCTTTGCA
Claudin 10	Cldn10	TCTGCAGTACCAACACCTTCA	ATGTTAAAGCCAGCCCATGA
Mus musculus keratin 14	Krt14	AGCGGCAAGAGTGAGATTTCT	CCTCCAGGTTATTCTCCAGGG
Mus musculus keratin 18	Krt18	GACATCCGCGCCCAGTAT	TCGGCAGACTTGGTGGTG
Mus musculus keratin 5	Krt5	GTCATGGCGATGACCTTCGA	CTTGACGTTGTCAATTTCAGATCT
Surfactant protein C	Sftpc	GCAAAGAGGTCCTGATGGAG	GCAGTAGGTTCCTGGAGCTG
Aquaporin 5	Aqp5	CAGCAACAACACAACACCA	TGCGGCGGGAGTCCGTGGA
Cubilin	Cubilin	AGTCAGTCCTGGCTCCTTGA	GGTGATGTGGAAGCCTTTGT
Megalin	Megalin	GGGACCGATGAGTCCCCGCT	GGGTCACAAGCGCACCGGAA
Forkhead box J1	Foxj1	GCCGGCACATCAACTGCCCT	CAGTCCTGCAGGTCAGCGGC
Cystic fibrosis transmembrane conductance regulator homolog (Mus musculus)	CFTR	GTGGCTGACACTTTGCTTGC	GAATCCCACCTGCTTTCAGC
Sodium channel, nonvoltage-gated 1 alpha (Mus musculus)	ENaC	TGCAGTGTGACCAACTACAAG	TCTCGAAGATCCAATCCTGGG
18S ribosomal RNA	18S	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
Sex determining region Y	Sry	GGGATGCAGGTGGAAAAGC	GTGACACTTTAGCCCTCCGAT

Table S4. Immunohistochemistry, immunocytochemistry and FISH

Antibodies and probe	Blocking	Antibodies	AlexaFluor-conjugated antibody	Isotype controls
CCSP	Normal Goat Serum [a1].	Anti-Clara Cell Secretory Protein (rabbit antiserum) [a2]. 1:1000 dilution	AlexaFluor® 488- goat anti-rabbit IgG [a3]. 1:200 dilution	Normal rabbit IgG [a4]. 1:1000 dilution
Ki-67	Normal Rabbit Serum [a3].	Rat anti-mouse Ki-67 [a5]. 1:50 dilution Biotinylated Rabbit Anti-Rat [a5]. 1:200 dilution	Streptavidin, AlexaFluor® 488 conjugate[a3]. 1:200 dilution	Purified Rat IgG[a6]. 1:50 dilution
β-tubulin IV	Normal Goat Serum [a1].	Mouse anti-β-Tubulin IV [a7]. 1:50 dilution	AlexaFluor® 488 goat anti-mouse IgG [a3]. 1:200 dilution	Purified Mouse IgG [a3]. 1:50 dilution
Cytokeratins	Normal Goat Serum [a1].	Mouse anti-pan-cytokeratin AE1/AE3 [a8].	AlexaFluor® 488 goat anti-mouse IgG [a3]. 1:200 dilution	Purified Mouse IgG [a3]. 1:50 dilution
CFTR	Normal Goat Serum [a1].	Mouse anti CFTR[a9] 3:37 dilution	AlexaFluor® 488 goat anti-mouse IgG [a3]. 1:200 dilution	Purified Mouse IgG [a3]. 3:37 dilution
ENaC	Normal Goat Serum [a1].	Rabbit anti-ENaC [a10] 1:100 dilution	AlexaFluor® 488- goat anti-rabbit IgG [a3]. 1:200 dilution	Normal rabbit IgG [a4]. 1:100 dilution
Mouse WCP FITC Chromosome Y probe[a11] and Cytokeratin	Normal Goat Serum [a1].	Mouse anti-pan-cytokeratin AE1/AE3 [a8].	AlexaFluor® 488 goat anti-mouse IgG [a3]. 1:200 dilution	Purified Mouse IgG [a3]. 1:50 dilution
CCSP	Normal Goat Serum [a1].	Anti-Clara Cell Secretory Protein (rabbit antiserum) [a2]. 1:1000 dilution	Goat anti Rabbit IgG Biotinylated [a12] 1:200 dilution	Normal rabbit IgG [a4]. 1:1000 dilution
MHC II	Normal Goat Serum [a1].	Anti-Mouse MHC Class II (I-a/I-E) [a13] 10 μg/ml dilution	AlexaFluor® 488 goat anti-rat IgG [a3]. 1:200 dilution	Purified Rat IgG[a6]. 10 μg/ml dilution

[a1.] Vector Laboratories Inc, Burlingame, CA. [a2.] Upstate Laboratories, Tremecula, CA. [a3.] Invitrogen, Eugene, OR. [a4.] *Imgenex* Corp, San diego, CA. [a5.] Dako, Mississauga, Canada. [a6.] Invitrogene, Carlsbad, CA. [a7.] Sigma. [a8.] Abcam, Cambridge, MA. [a9]R & D, Minneapolis, MN USA. [a10]Chemicon, Temecula, California. [a11]*Cambio*, *Cedarlane* Laboratories, Hornby, Ontario. [a12]VECTASTAIN Elite ABC kit Goat anti Rabbit IgG Biotinylated, Vector Laboratories Inc, Burlingame, CA. [a13] eBiosciences, San Diego , CA.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Characterization of the CCSP$^+$ BMC population in FVBn mice. Representative flow histograms of BMC freshly isolated (a) or 7 days after culture (b) show a population of CCSP$^+$ BMC (red) that in fresh bone marrow contained 1.74 ± 0.16% CCSP$^+$ cells, which expanded in culture to 22.42 ± 1.66% compared to isotype staining (green). Data shown are means ± SEM. To test the possibility that the CCSP protein was present in the BMC by adsorption the quantitative RT-PCR gene expression for cubilin (c) and megalin (d) was assessed on FACS-sorted CCSP$^+$ BMC (+) and CCSP$^-$ BMC (-). The dissociation curves shown cubilin expression only in CCSP$^-$ BMC and no expression of megalin by any of both groups compared to the gene expression in normal Kidney (K) where both CCSP receptors are expressed. (e) FACS-sorted CCSP$^+$ BMC showed expression of CCSP while the CCSP$^-$ BMC did not express the gene; both groups were compared to a normal lung (L) sample that also showed amplification for CCSP. Immunocytochemistry using 3 different antibodies for CCSP: anti-CCSP upstate antibody (f and j), anti-CC10S20 Santa Cruz antibody (g and k) and anti-CC10T18 Santa Cruz antibody (h and l), showed that most sorted CCSP$^+$ BMC had a positive staining for all the antibodies after sorting (f-h), and retained this CCSP expression for at least three days after sorting (j-l). In contrast the CCSP$^-$ BMC didn't show expression of CCSP on fresh or 3 days cultured sorted cells (i and m). n=4 samples per group.

Figure S2. Ablation of CCSP$^+$ cells in CCtk mice treated with ganciclovir. Immunohistochemical analysis of CCSP expression in lung tissue from wild type (FVBn) or CCtk transgenic animals treated with saline or ganciclovir 4.5 mg/day for 10 days is shown. Antigen-antibody complexes were detected with 3,3'-diaminobenzidine and tissue was counterstained with hematoxylin. CCSP$^+$ cells (brown staining) in lungs from FVBn (a) and CCtk (c) mice treated with saline or FVBn mice treated with ganciclovir (b) showed a normal distribution of CCSP$^+$ cells in the airways while CCtk mice treated with ganciclovir (d) showed a dramatic depletion of CCSP$^+$ cells. Green insets: higher-magnification image of boxed region. Black insets: representative isotype staining controls. n=4 mice per group.

Figure S3. Transtracheal administration of $CCSP^+$ BMC at 5 days of ganciclovir treatment has a beneficial effect. (a) Female CCtk mice were administered with ganciclovir (GCV) 4.5 mg/day for 10 days. Transtracheal administration of 1.3 million FACS-sorted, CMTMR-labeled $CCSP^+$ or $CCSP^-$ BMC from male wild type mice was performed at day 5 of ganciclovir treatment. Lung analysis was performed 7 days after cell delivery. (b) Quantitative RT-PCR for the Sry gene shown that mice treated with $CCSP^+$ BMC at day 5 had 9.1 times more male cells in the lung compared to mice treated with $CCSP^-$ BMC (*$p<0.01$), while no amplification of male DNA was detected (ND) in lungs from mice treated with saline. There was no difference in $CCSP^+$ BMC retention comparing day 5 or 10 of ganciclovir as the day of cell delivery. (c) Administration of $CCSP^+$ BMC after 5 days of ganciclovir treatment increased survival while none of the mice treated with $CCSP^-$ BMC survived for 10 days (*$p<0.05$). There was no difference in survival between groups administered $CCSP^+$ BMC at either day 5 or day 10 of ganciclovir treatment. (d) Lung sections from mice treated with $CCSP^-$ BMC showed 0.11 ± 0.08% of donor cells identified by $CMTMR^+$ $DAPI^+$ staining (red cytoplasm with blue nucleus; percentage based on 1 $DAPI^+$ $CMTMR^+$ cell per 100 $DAPI^+$ cells) while (e) mice treated with $CCSP^+$ BMC showed 2.01 ± 0.43% of donor cells (*$p=0.013$). Insets show high-power magnification areas with donor cells. Data shown are means ± SEM. n=8 mice per group.

Figure S4. CCtk mice treated with CCSP$^+$ BMC have preserved epithelium in small airways. Mice treated with CCSP$^-$ BMC (a) have a poorly preserved epithelium in small airways when compared to (b) mice treated with CCSP$^+$ BMC. Green insets: higher-magnification image of boxed region. Confocal microscopic analysis of the ciliated cell marker β-tubulin IV (c-f) shown that donor cells (CMTMR$^+$; red fluorescence) do not express β-tubulin (green fluorescence) but are surrounded by host β-tubulin$^+$ squamous cells in the CCSP$^+$ BMC group. The basal regions of these β-tubulin$^+$ squamous cells is extended covering the bronchiolar surface (d and f). There were areas were donor and host cells expressed CCSP (g-j; green fluorescence) in the small airways of CCSP$^+$ BMC group. Insets are representative isotype staining controls. Arrows point to donor cells and arrowheads indicate host positive cells. Scale bar represents 10 μm. n=4 mice per group. Samples from mice administered with cells after 10 days of ganciclovir.

Figure S5. CCSP$^+$ BMC express CFTR and ENaC, while CCSP$^-$ cells express only CFTR. Immunocytochemistry was carried out on isolated bone marrow cells for CFTR (a-d) and ENaC (e-h). Isolated CCSP$^-$ cells stained positive for CFTR (a,b), but were negative for ENaC (e,f). Approximately 30% of CCSP$^+$ cells were weakly positive for CFTR (c,d) and 15% were positive for ENaC (g,h). Isotype controls for CFTR (i) and ENAC (j) are also shown.

Figure S6. Corroboration of donor origin for CMTMR^{+} cells. To evaluate whether the CMTMR (red fluorescence) label could be acquired by phagocytosis or efferocytosis the expression of the MHC II protein was analyzed by immunohistochemistry showing that only 1.2 ± 0.87% of the CMTMR^{+} donor cells were positive for MHC II for the CCSP^{+} BMC group (a-c). As donors and recipients were sex-mismatched it was possible to track the donor cells by confocal microscopy analysis for lung sections stained for Y-chromosome FISH and then subjected to immunohistochemistry for the pan-cytokeratin epithelial cell marker (d-f). Finally the proliferation of the donor BMC by the expression of the cell proliferation marker Ki67 shown no significant differences in proliferative index of donor CCSP^{+} BMC (1.8±0.69%; g-i) and CCSP^{-} BMC (0%; p=0.08). Arrows point to donor cells and arrowheads indicate host positive cells. Scale bar represents 10 μm. n=4 mice per group. Samples from mice administered with cells after 10 days of ganciclovir.

Figure S7. Expression of MHCII. Immunocytochemistry was carried out on sorted bone marrow cells for MHC II. Isolated CCSP^{-} cells showed no expression of MHCII (a,b), while a few sorted CCSP^{+} cells were found to faintly express MHCII (c,d). Isotype control is also shown (e).

Fig.S1

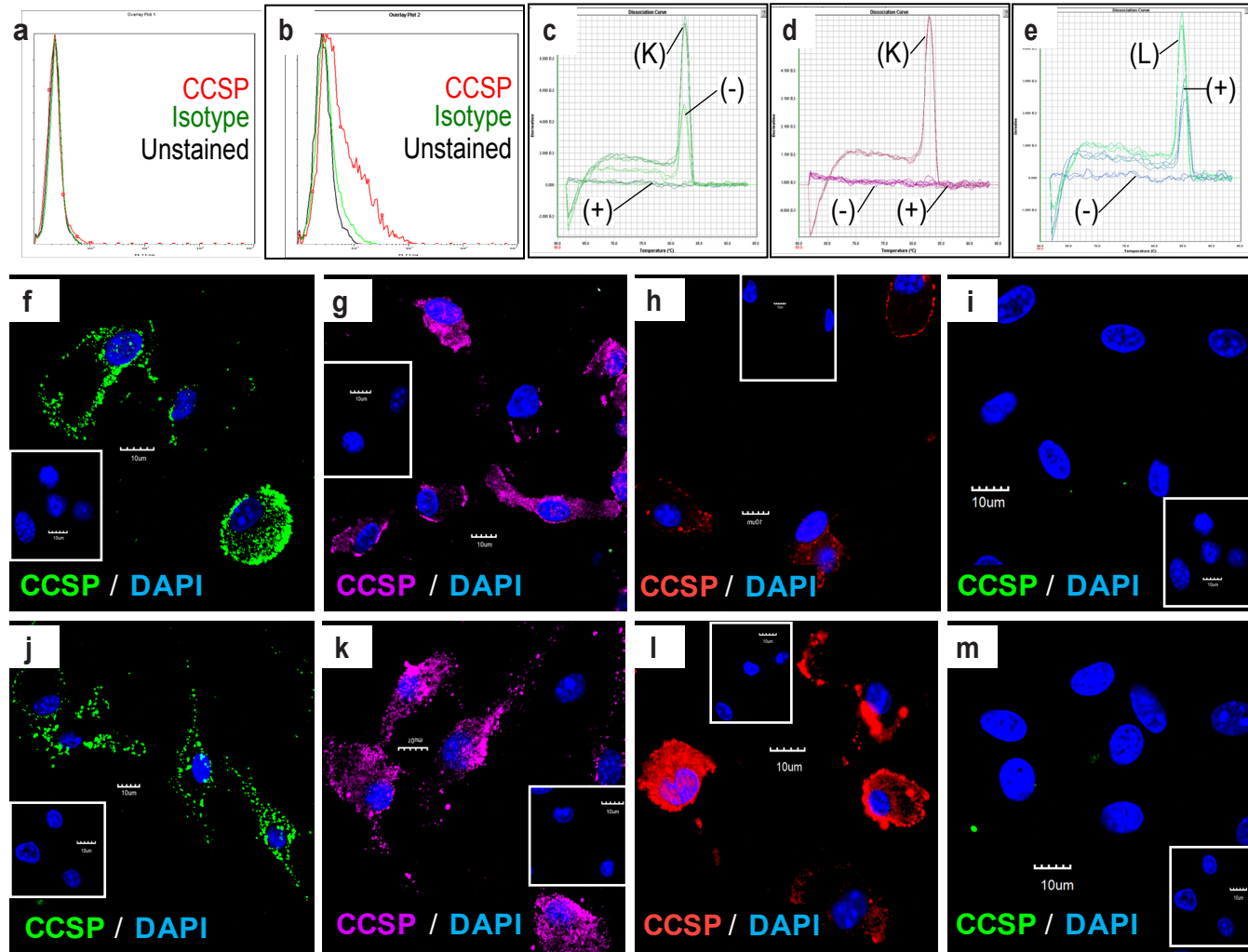
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Fig.S2

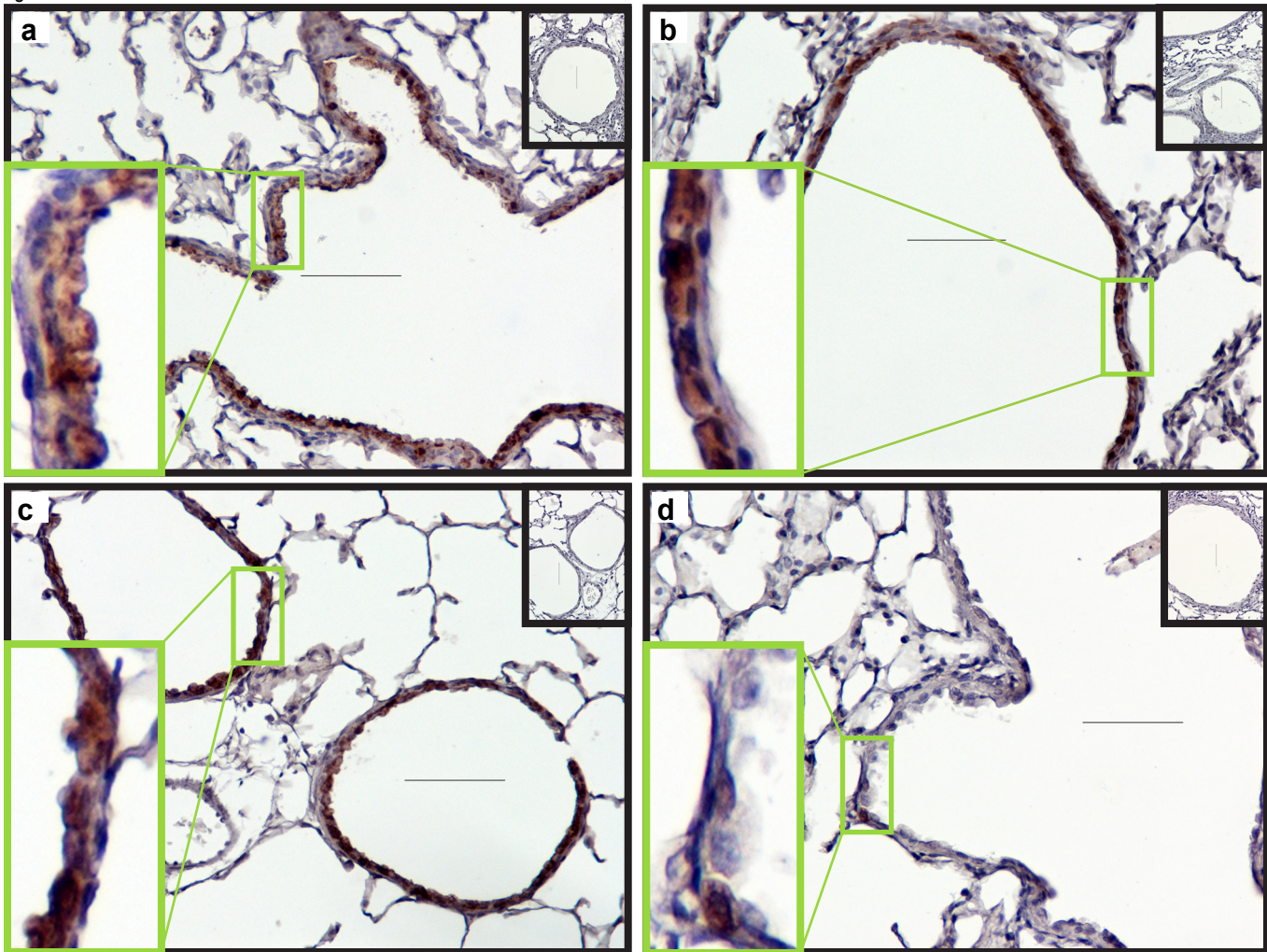
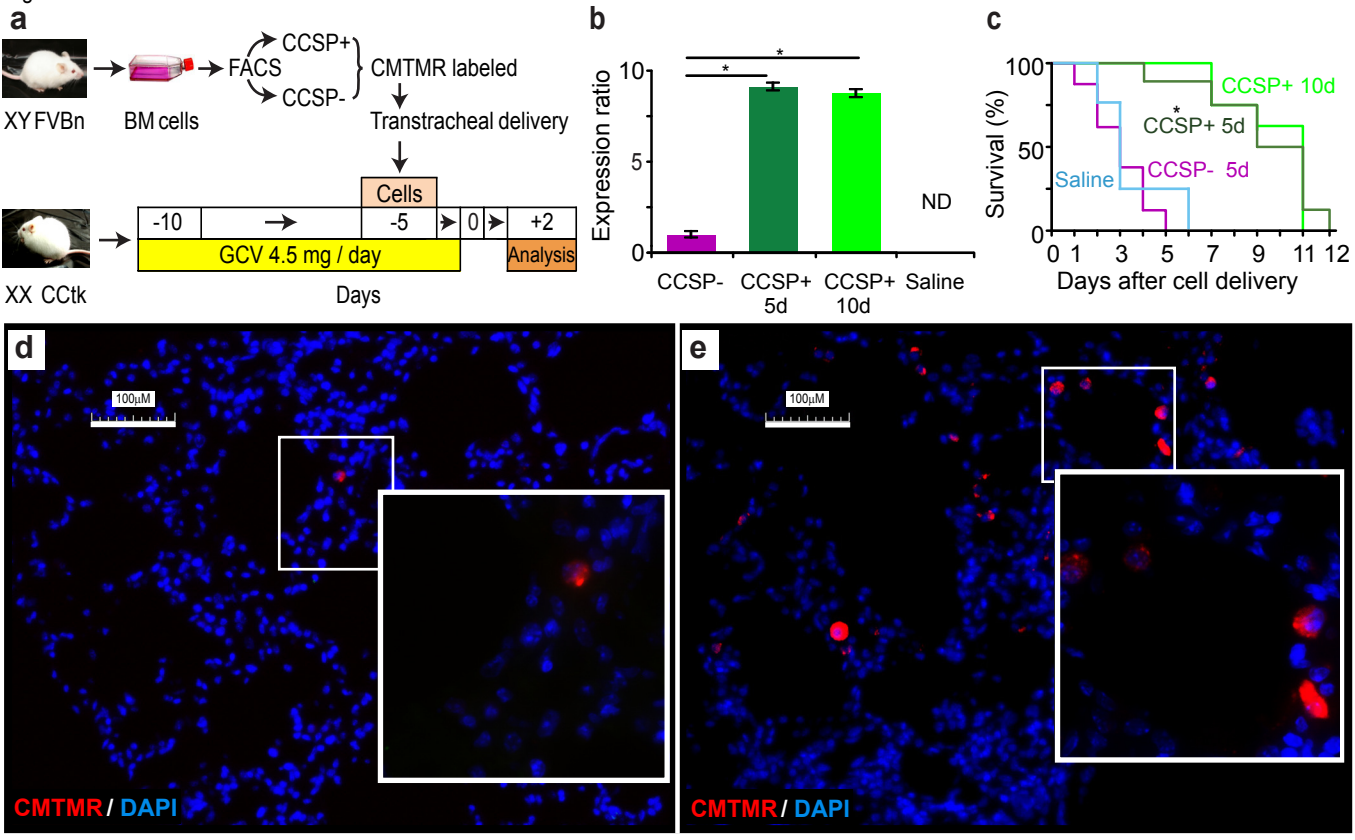


Fig.S3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-5
0
+2
GCV 4.5 mg / day
Analysis
Days
b
Expression ratio
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5
0
*
*
ND
CCSP-
CCSP+ 5d
CCSP+ 10d
Saline
c
Survival (%)
100
50
0
CCSP+ 10d
CCSP+ 5d
Saline
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Days after cell delivery
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100μM
CMTMR / DAPI
e
100μM
CMTMR / DAPI

Fig.S4

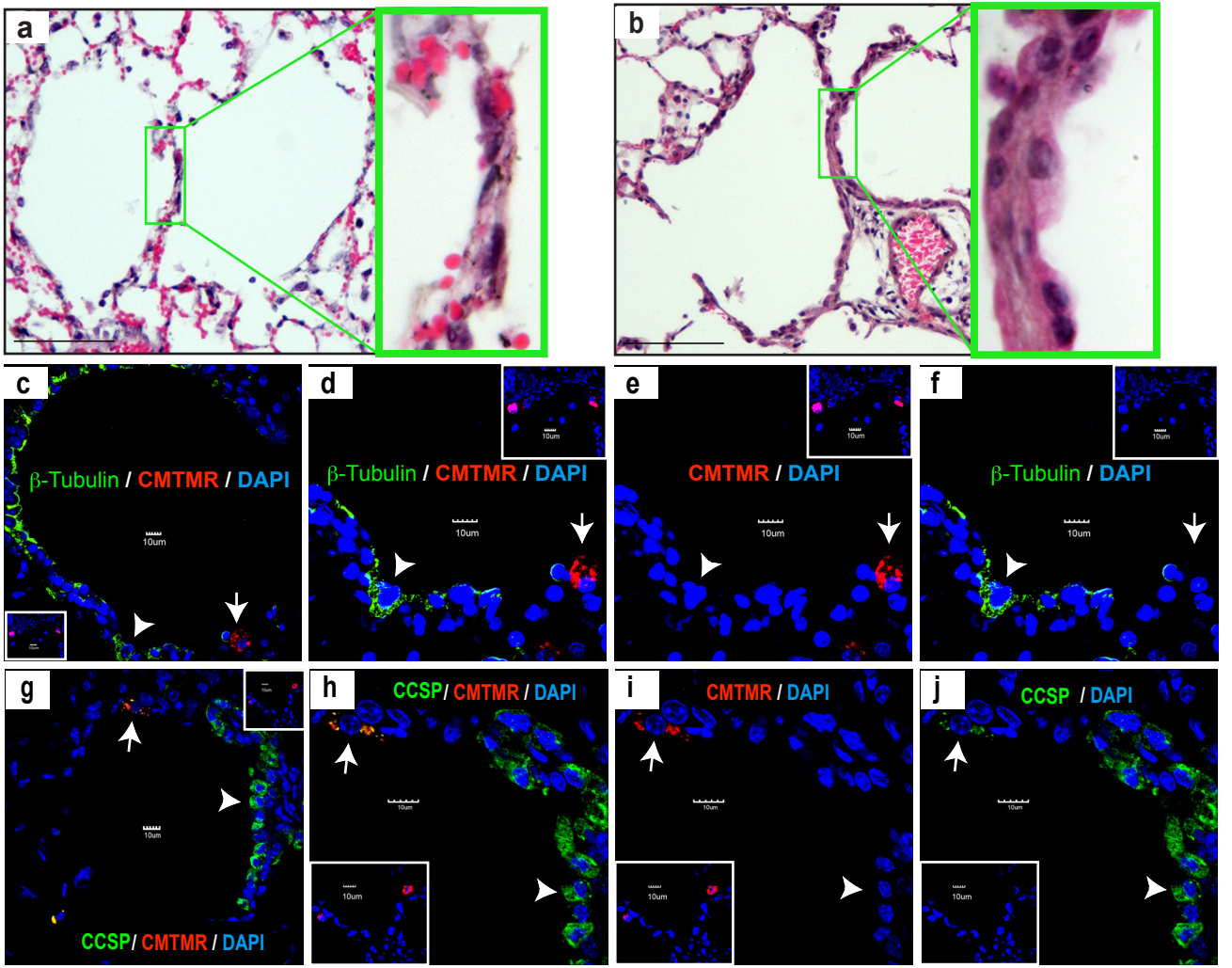
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Fig.S5

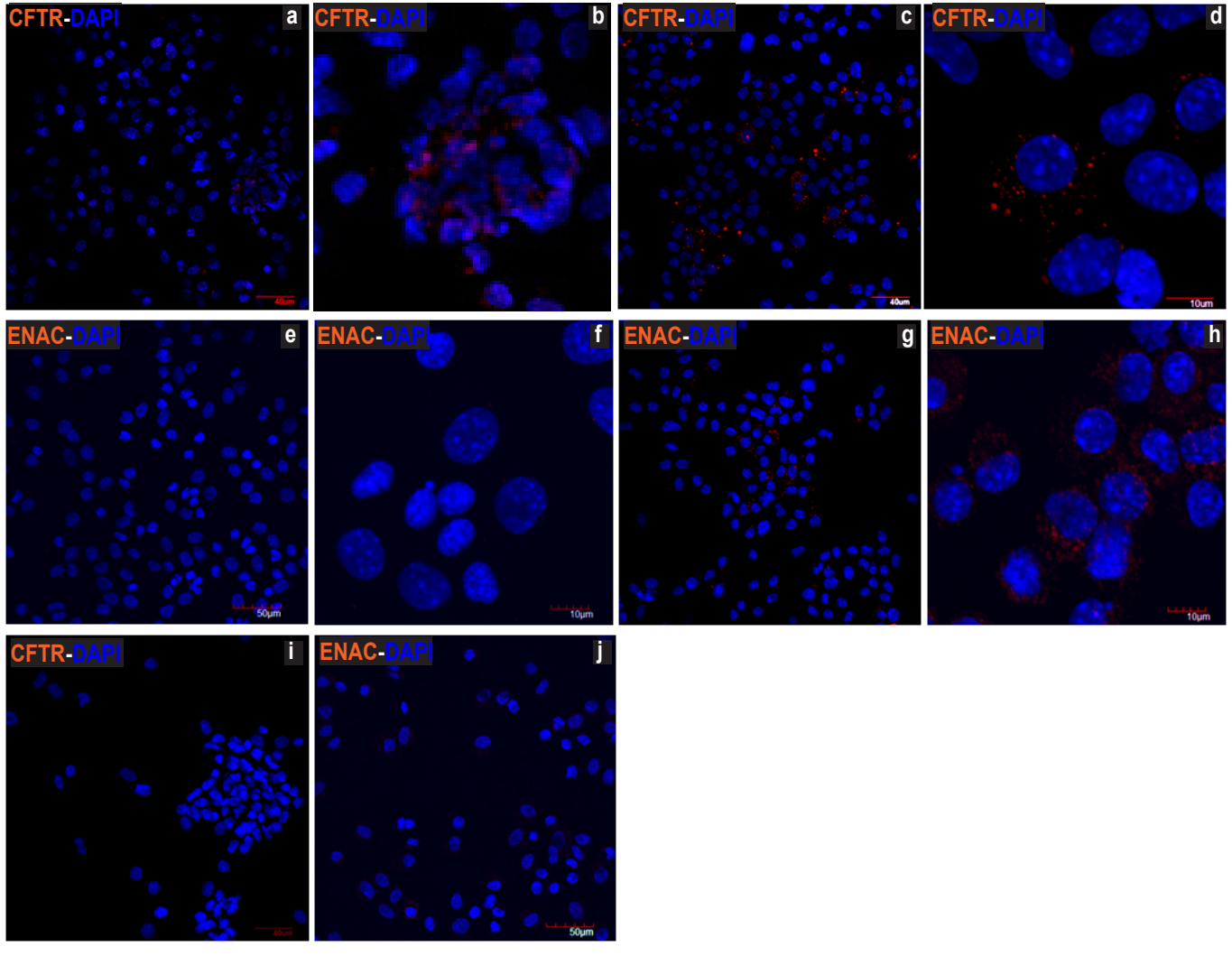
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Fig.S6

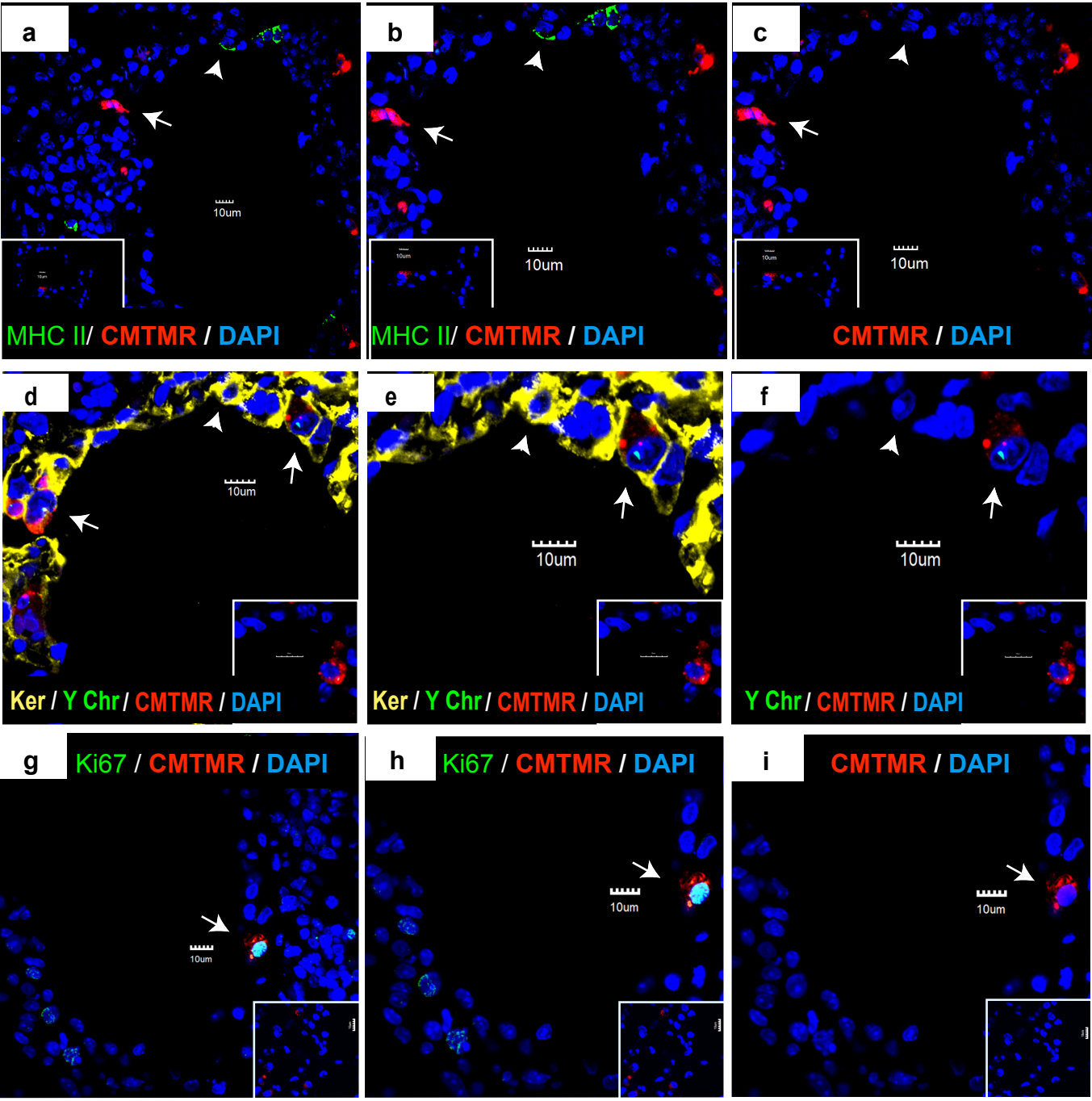
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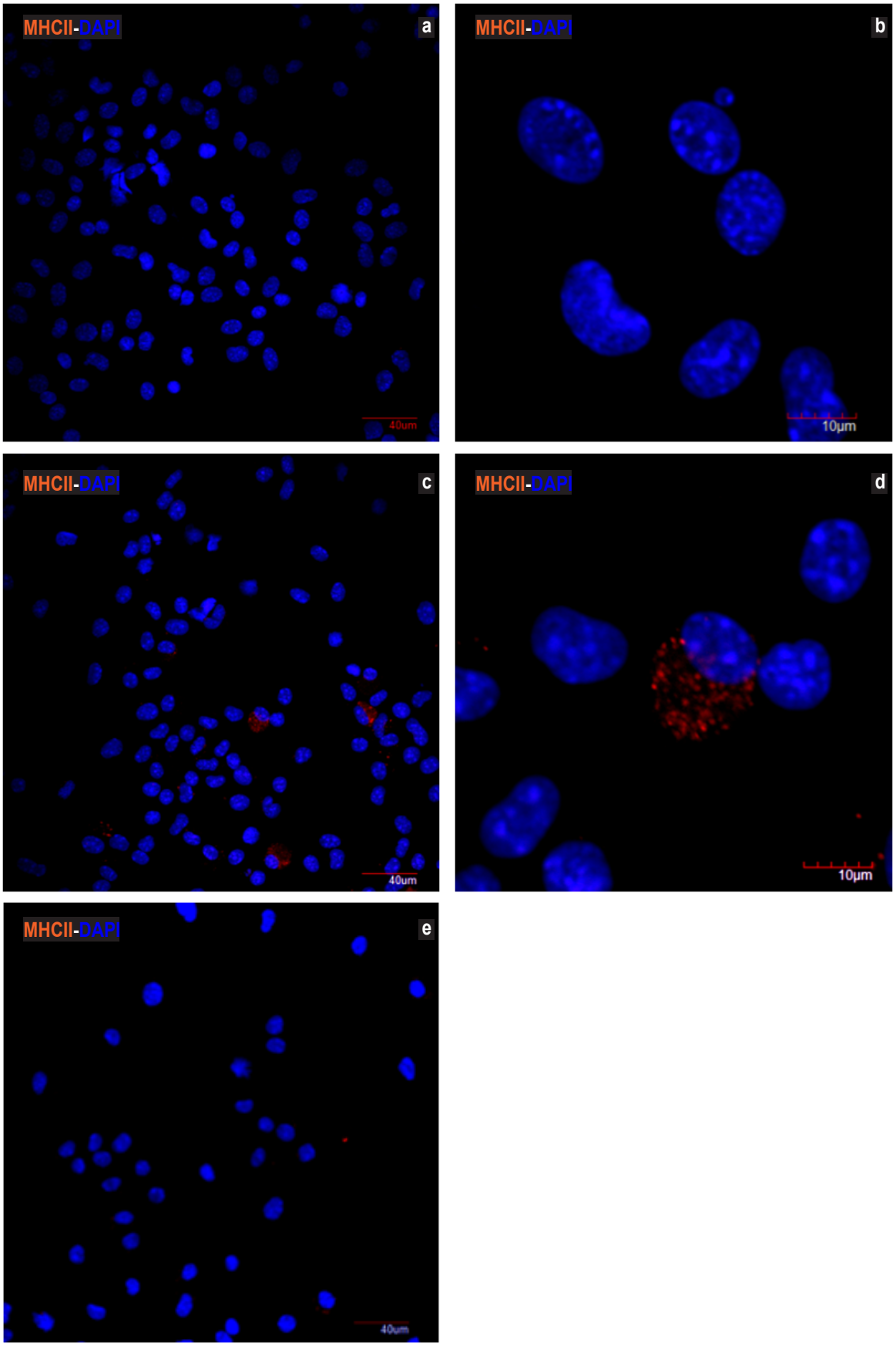
Fig.S7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