Online data supplement to:

Hyperoxia Injury in the Developing Lung is Mediated by Mesenchymal Expression of Wnt5A

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METHODS

All 37 °C incubations took place in a cell culture incubator with 5% CO_2.

Epithelial cell and fibroblast isolation from human fetal lung:

Enriched epithelial cell samples were isolated from these lung tissues using a technique that generally yields >85% epithelial cells with the remaining cells consisting of fibroblasts (~10-15%) and endothelial cells (< 1%)(1, 2). Second trimester fetal lungs were processed according to previously published protocols(1). Specifically, lung tissue was chopped into 1 mm cubes, and plated onto 60 mm dishes with Waymouth's medium (serum-free). After incubation overnight, medium was removed and collagenase solution was added, 4 ml/gm of tissue. Collagenase solution is prepared with collagenase 1A (Sigma) 10 mg/4 ml PBS plus 50□l DNase (2 mg/ml MEM stock) (Sigma) and filtered through syringe filter. Tissue was incubated in collagenase solution from 1 hr at room temperature (RT). After incubation, the cell suspension was vigorously pipetted up and down and then filtered through 4 layers of sterile gauze. The solution was centrifuged at 1200 RPM, 3 minutes at room temperature. The supernatant was discarded and the cell pellet was resuspended in 0.125% dispase solution (2 ml dispase stock 50 U/ml (Sigma) in 13 ml PBS with 160 μl DNase (2 mg/ml)), incubated at 30 min at RT with gentle stirring. After incubation, cells were pipetted vigorously and then filtered through a 40 mm sterile filter and centrifuged at 1200 RPM for 3 minutes at RT. The pellet was resuspended in Waymouth's medium with 10% fetal calf serum (FCS) (36 ml/gram tissue). This suspension was plated onto 100 mm culture dishes (6 ml per dish) and placed into 37 °C incubator for 30 minutes. After 30 minutes, the nonadherent cells were transferred to a fresh 100 mm dish and the adherent cells were changed to fibroblast media (DMEM with 10% FCS plus 1% non-essential amino acids and 1% Glutamax). These early

adherent cells are fibroblasts, and they were grown out for two days and passaged. They were confirmed to be fibroblasts by morphology and positive vimentin staining. Fibroblasts between passage 5 and 15 were used for experiments. The nonadherent cells were left in 37 °C incubator for 30 minutes, and transferred again to a fresh 100 mm dish (a total of 3 adherence steps). All non-adherent cells were collected in Waymouth's medium with 10% FCS centrifuged at 1200 RPM for 3-4 min at RT. They were resuspended in Waymouth's medium with FCS and counted. These epithelial cells were either used directly for experiments or frozen as stocks in Waymouth's with freezing medium (DMEM medium with 10% DMSO, 20% FCS, 2 mM glutamine, 100U/ml penicillin, 100 μg/ml streptomycin).

MLE-15 Cell Culture:

MLE-15 cells were obtained from Dr. Jeffrey Whitsett (Cincinnati Children's Hospital, Cincinnati, OH)(3). MLE-15 cells were cultured in HITES medium (Dulbecco's medium: Ham's F12, 50:50 mix, 0.005mg/ml insulin, 0,01mg/ml transferrin, 30nM of sodium selenite, 10nM of hydrocortisone, 10nM of β-estradiol, 10mM HEPES, 2mM L-glutamine) supplemented with 10% fetal bovine serum. Absence of mycoplasma in cultures was verified with the LookOut Mycoplasma PCR detection kit (Sigma) according to manufacturer's instructions.

Hyperoxia exposure:

3D organotypic cultures of human fetal primary AT2 cells and fibroblasts were placed into HeraCell 150i incubators (Thermo Fisher) set to have an ambient oxygen concentration of 70% O_2 (5% CO_2) and were cultured in hyperoxia for 48hrs. They were compared with 3D organotypic co-cultures made at the same time cultured under normoxic controls (5% oxygen,

which produces a pO_2 of 60-70 mmHg in growth media and mimics physiologic oxygen tension(4)).

3D-organotypic co-culture processing:

At the conclusion of the experiments, co-cultures were cut in half. One half was fixed in 4% paraformaldehyde for 30 minutes, processed by paraffin embedding and sectioned on edge. The remaining half was processed for RNA and protein isolation.

Immunofluorescence

Immunofluorescence (IF) was performed as described previously(5, 6) using primary antibodies specific to α-SMA (MilliporeSigma, A2547), vimentin (Bioss, Woburn, MA USA, bs-0756r), pro- and mature SP-B (surfactant protein B) (Abcam, Cambridge, MA USA, ab408726), pro-SP-C (surfactant protein C) (Abcam ab90716), p-β-catenin^{Y489} (DSHB), a-SMA (), Vimentin ()and Ki67(Abcam ab16667). Primary antibody binding was visualized using Alexa 488-, Alexa-594, and Alexa 647-conjugated secondary antibodies (Thermo Fisher). Each section was counterstained with DAPI (Vector Laboratories, Burlingame, CA USA) and mounted with ProLong Gold (Thermo Fisher). Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay (Sigma Aldrich) was used to measure apoptotic cells.

Confocal microscopy

The Nikon TiE inverted fluorescence confocal microscope was outfitted with a Yokogawa X1 spinning disk head and Andor DU-897 EM-CCD. Lasers utilized for

excitation included 405, 488, 561, and 647 nm, and emission filters were 455/50, 525/36, 641/75, and 700/74 (peak/bandwidth), respectively.

Confocal Image Analysis

In order to enumerate the number of RNAscope puncta, a custom automated script was written as a macro inside NIS-Elements and applied in batch so as to treat all images equally. For this script, seeing that the *number* of puncta was the desired output (and not intensity), focus stacking (extended depth of focus) was applied to generate a single focused plane from axial stacks of images. Subsequently, nuclei were bounded by simple thresholding and conversion to regions of interest to restrict RNAscope analysis/enumeration to only nuclear borders. Finally, spot detection was utilized to detect individual puncta, which were subjected to both contrast and size restrictions. The macro then exported field measurement of all datasets for final statistical analysis in Prism Graphpad Version 7. It should be noted that the parameters of the macro (segmentation, contrast, and size restrictions), were quality controlled by selecting images at random and assessing the macro output against manual counting methods to ensure accuracy. This method kept dataset to dataset variability to a minimum due to universal application of an automated script, thus making the ensemble measurement robust, but also ensured that the output was in keeping with a trained eye's assessment. The script is provided below:

```
ClearBinary();

//Focus Stacking on Z Stack and Extract as Single Plane

_EDF_CreateFocusedImageDef(0);
```

```
ActivateDocument("85 RNA Scope Wnt5a ACTA2 AXIN2-1006-Focused Images");

ND_CreateViewProjection();

//Threshold DAPI on EDF'd and Extracted Dataset

DefineThresholdMCHOperation(1);

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DefineThresholdRestrictionSizeOnChannel(0,0,0.000000,484.000000);

DefineThresholdRestrictionCircularityOnChannel(0,0,0.000000,1.000000);

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Threshold();

//Spot Detection for RNAScope

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(647)");

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ViewComponents("0010");

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BinLayerStore("SpotDetection (649)", "SpotDetection (649)");

ViewComponents("1111");

//Send Data to Excel
```

Export("AutoMeasResults",3);

RNA isolation and real-time qPCR

Total RNA was extracted from 3D organotypic co-cultures or 2D cell cultures using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, including DNase treatment of the column. TaqMan Reverse Transcription Reagents (Thermo Fisher) were used to make cDNA libraries. Real-time quantitative PCR (qPCR) was performed on an Applied Biosystems StepOne-Plus Real Time PCR System using TaqMan PCR Fast Advanced Master Mix (Applied Biosystems, Thermo Fisher) (40 cycles, per manufacturer's instructions). The following TaqMan probes were used for human genes:*COL1A1*, *ACTA2, ELN, FOXM1, MYB, MCM2, MCM3, WNT5A, AXIN2* . The following TaqMan probes were used for mouse genes:*Axin2, Wnt5a.* Data obtained from Thermo Fisher support no cross-reactivity between human and murine AXIN2/Axin2 probes or between the WNT5A/Wnt5a probes. Expression was normalized to expression of housekeeping gene 18S with TaqMan probe 4319413E. The Wnt pathway array was purchased from ThermoFisher (cat. No 441875).

RNA *in situ* hybridization:

RNAScope probes (with catalog numbers from ACD Bio) to the following human genes were used: *WNT5A* (604921)*, SFTPC* (452561)*, S100A4* (422071), *AXIN2*(400421). Probes to the following mouse genes were used: *Wnt5a* (316791)*, Axin2* (400331)*, S100a4* (412971)*, Sftpc* (314101).

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure E1: Supplementary data from assays for proliferation and apoptosis: TUNEL staining shows rare apoptotic cells in 3D organotypic co-cultures, with TUNEL+ nuclei in green and surfactant protein-C (SPC) shown in red. A) 3D organotypic co-cultures exposed to hyperoxia and normoxia had rare (<1%) cells that were TUNEL positive; scale bar =10μm. B) Positive control for TUNEL staining, with known dead cells cultured (green) on a cell-free matrix, a previously described cytotoxic stimulus(7); scale bar =10μm C) The increase in Ki67+ cells (green) seen in hyperoxia-exposed PCLS relative to normoxia-exposed PCLS is unchanged by addition of recombinant Wnt-5A or neutralizing antibody. PCLS are stained with SPC (red), a marker of AT2 cells; scale bar =10μm. D) PCLS exposed to hyperoxia and normoxia had rare (<1%) cells that were TUNEL positive (green) and this was unchanged in culture with either recombinant Wnt5A, anti-Wnt5A neutralizing antibody; PCLS also staining with SPC (red) with scale bar =10μm. N=4 mice per group.

Supplemental Figure E2: With hyperoxia exposure, 3D organotypic co-cultures comprised of primary fetal lung mesenchymal cells and type 2 alveolar epithelial cells have no significant difference in the expression of FGF18, a gene associated with airway branching, NS = not significant, p=0.385. N=4.

Supplemental Figure E3: A) Exposure of 3D organotypic co-cultures to normoxia or hyperoxia with the addition of recombinant Wnt5A had no significant differences in the expression of *ELN, MCM3,* or *MYB.* The previously observed differences in expression between hyperoxia and normoxia alone were reproduced, $**p<0.01$ by two-way ANOVA with Tukey correction for multiple comparisons. B) Exposure of 3D organotypic co-cultures to normoxia or hyperoxia with

the addition of neutralizing antibody had no significant differences in the expression of *ELN, MCM3,* or *MYB.* The previously observed differences in expression between hyperoxia and normoxia alone were reproduced, $**p<0.01$ by two-way ANOVA with Tukey correction for multiple comparisons.

Supplemental Figure E4: Table E1: Multiplex array of inflammatory analytes from hyperoxia-exposed precision cut lung slices. Cytokine multiplex analysis on conditioned media from PCLS from P4 lungs cultured in normoxia and hyperoxia. (n =4 mice/ group). $*=p < 0.05$ by Welch's t-test with correction multiple comparisons using the Holm-Sidak method.

Supplemental Figure E5: A) RNA *in situ* hybridization (ISH) of PCLS exposed to normoxia or hyperoxia, with or without treatment with BAY11-7082, an inhibitor or NFκB signaling, for fibroblast marker *S100a4* (white), *Sftpc,* and *Wnt5a* (red), scale bar = 10μm. B) Multiplex RNA ISH comparing expression of *Wnt5A* (red) in pups exposed to normoxia or hyperoxia until PN14 and treated with BAY11-7082 on PN12-13 or vehicle control. *Sftpc* expression is in green, *S100a4* expression is in white, with scale bar=10μm. N= 4 mice/group.

Figure E1

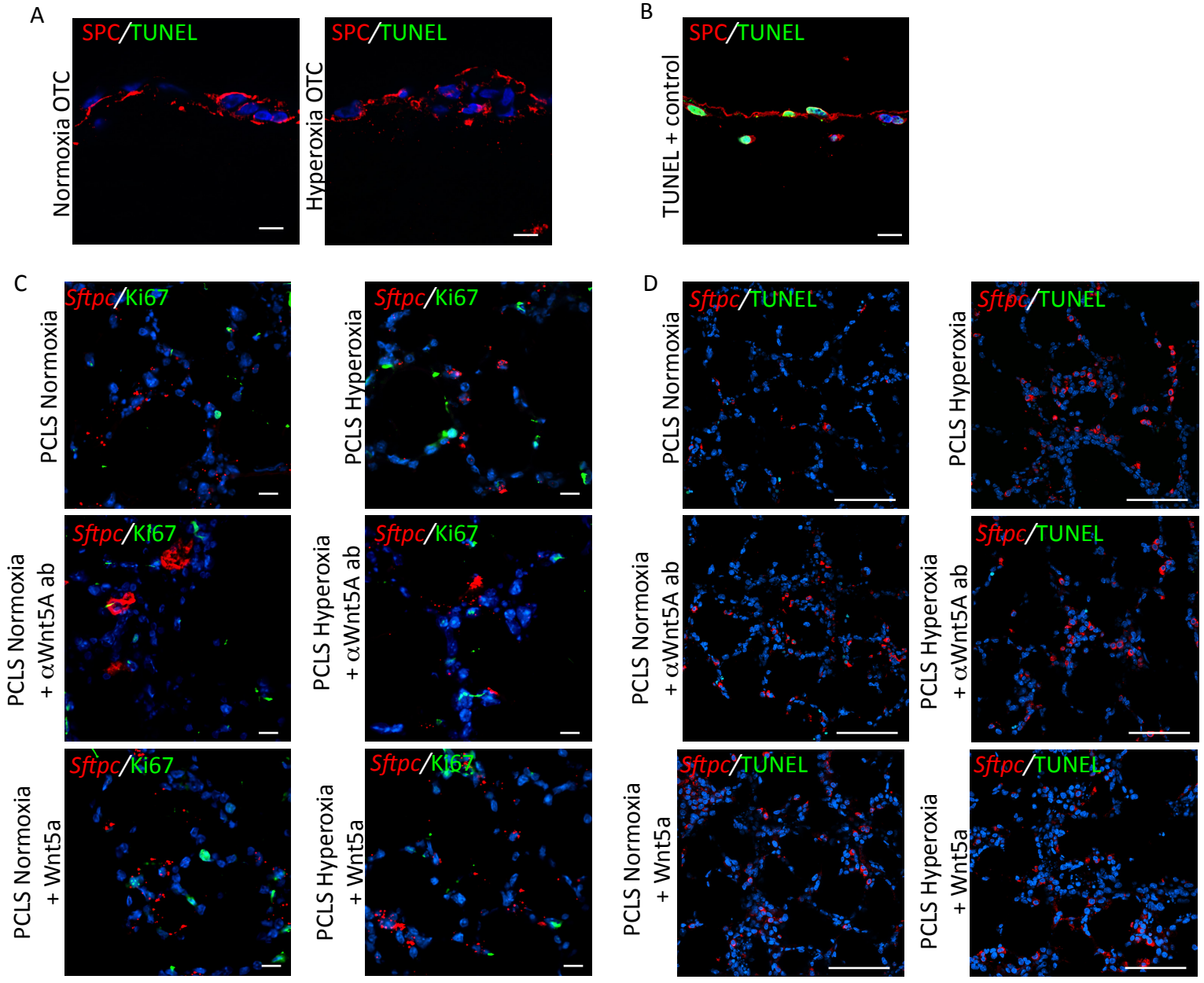

A
SPC/TUNEL
Normoxia OTC
SPC/TUNEL
Hyperoxia OTC
B
SPC/TUNEL
TUNEL + control
C
Sftpc/Ki67
PCLS Normoxia
Sftpc/Ki67
PCLS Hyperoxia
Sftpc/Ki67
PCLS Normoxia + αWnt5A ab
Sftpc/Ki67
PCLS Hyperoxia + αWnt5A ab
Sftpc/Ki67
PCLS Normoxia + Wnt5a
Sftpc/Ki67
PCLS Hyperoxia + Wnt5a
D
Sftpc/TUNEL
PCLS Normoxia
Sftpc/TUNEL
PCLS Hyperoxia
Sftpc/TUNEL
PCLS Normoxia + αWnt5A ab
Sftpc/TUNEL
PCLS Hyperoxia + αWnt5A ab
Sftpc/TUNEL
PCLS Normoxia + Wnt5a
Sftpc/TUNEL
PCLS Hyperoxia + Wnt5a

Figure E2

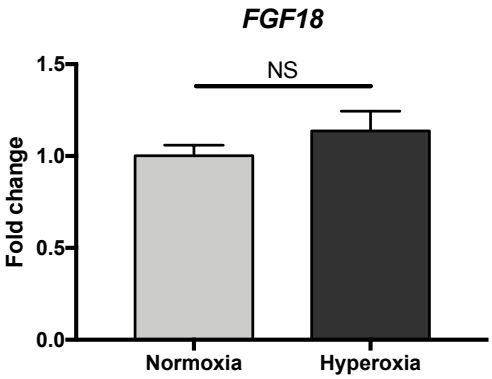
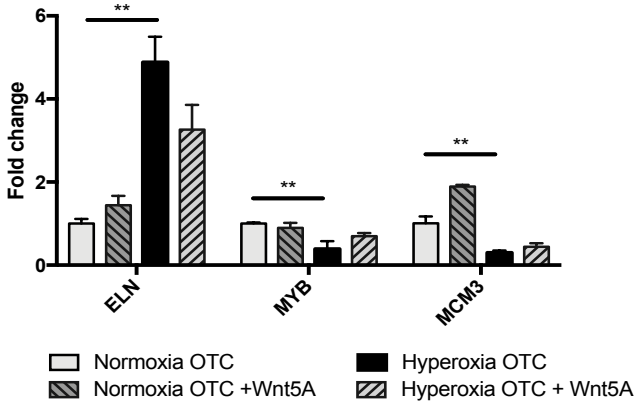


Figure E3

A


Fold change
6
4
2
0
**
**
**
ELN
MYB
MCM3
Normoxia OTC
Normoxia OTC +Wnt5A
Hyperoxia OTC
Hyperoxia OTC + Wnt5A

B

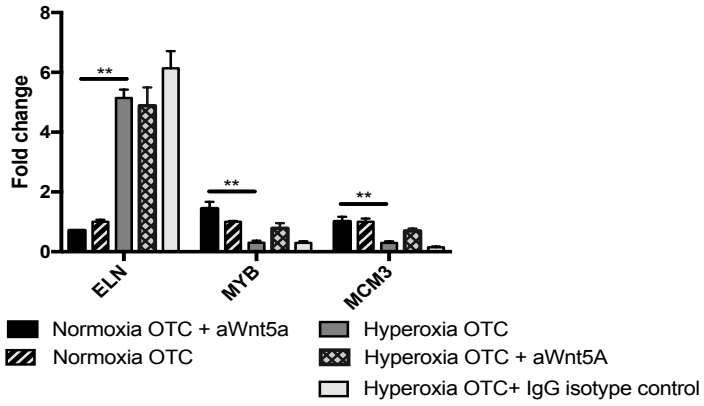
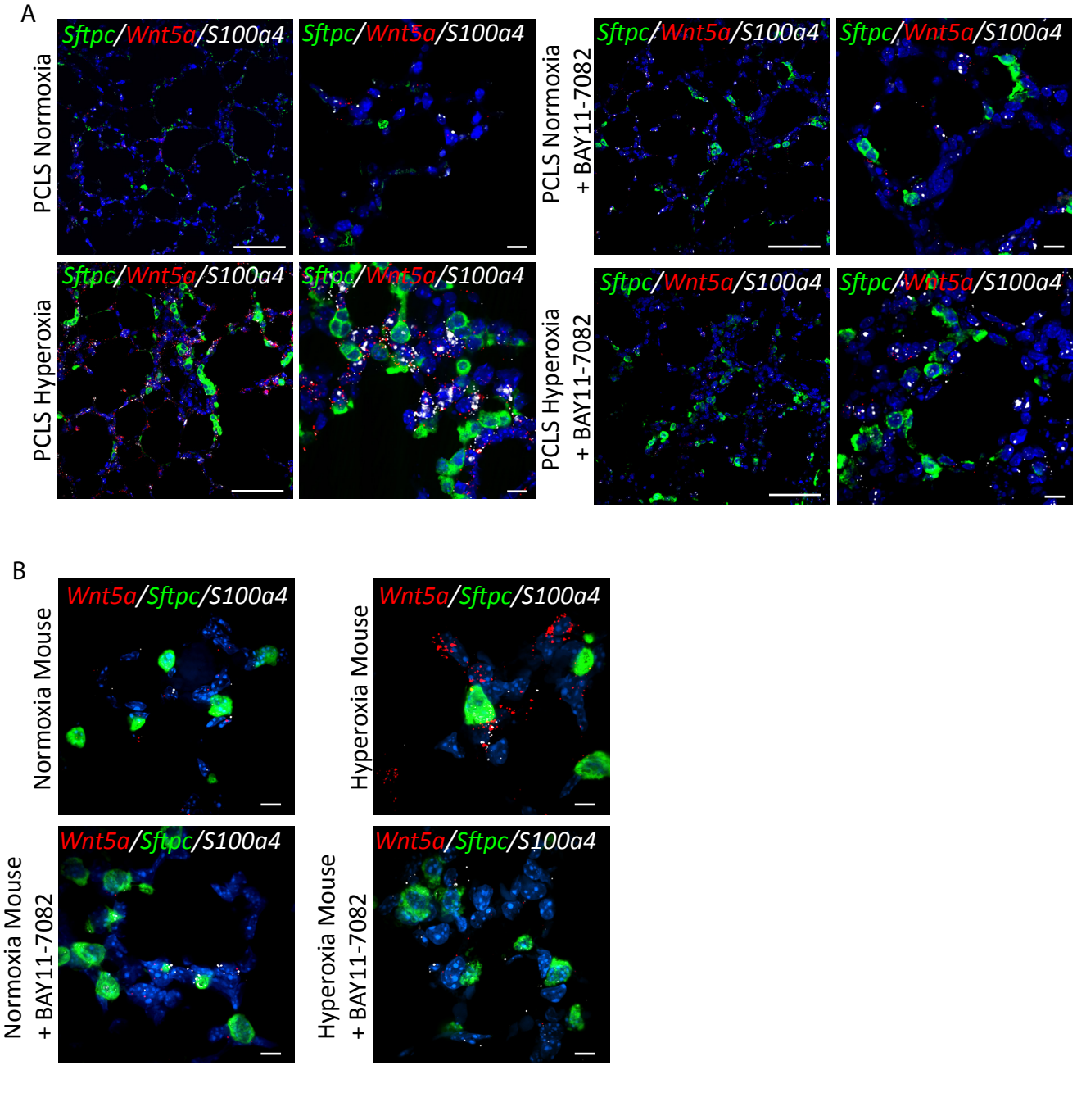

Fold change
8
6
4
2
0
**
**
**
ELN
MYB
MCM3
Normoxia OTC + aWnt5a
Normoxia OTC
Hyperoxia OTC
Hyperoxia OTC + aWnt5A
Hyperoxia OTC+ IgG isotype control

Figure E4

Table E1

Analyte	Normoxia	Hyperoxia
	pg/ml (Std dev)	pg/ml (Std dev)
Eotaxin	<3.2 (0)	72.6 (47.81)
G-CSF	<3.2 (0)	7.1 (1.9)
GM-CSF	6.47 (2.5)	6.28 (0.5)
IFNγ	<3.2 (0)	<3.2 (0)
IL-1α	<3.2 (0)	<3.2 (0)
IL-1β	<3.2 (0)	<3.2 (0)
IL-2	<3.2 (0)	<3.2 (0)
IL-3	<3.2 (0)	<3.2 (0)
IL-4	<3.2 (0)	<3.2 (0)
IL-5	<3.2 (0)	10.72 (6.3)
IL-6	**<3.2 (0)**	**20.8 (4.6)***
IL-7	<3.2 (0)	<3.2 (0)
IL-9	4.96 (1)	10.45 (6.2)
IL-10	<3.2 (0)	<3.2 (0)
IL-12(p40)	<3.2 (0)	<3.2 (0)
IL-12(p70)	<3.2 (0)	3.87(0.7)
IL-13	<3.2 (0)	<3.2 (0)
IL-15	<3.2 (0)	<3.2 (0)
IL-17	<3.2 (0)	<3.2 (0)
IP-10	<3.2 (0)	49.5 (32.2)
LIF	**<3.2 (0)**	**21.54 (5.6)***
LIX	6.31 (0.6)	12.13 (5.9)
KC	**<3.2 (0)**	**74.89 (13.0)***
M-CSF	<3.2 (0)	<3.2 (0)
MIG	<3.2 (0)	<3.2 (0)
MIP-1α	4.36 (0.6)	13.24 (6.6)
MIP-1β	<3.2 (0)	16.92 (9.6)
MIP-2	10.47 (3.6)	16.93(8.6)
RANTES	<3.2 (0)	<3.2 (0)
VEGF	**<3.2 (0)**	**60.39 (12.2)***
TNFα	<3.2 (0)	<3.2 (0)

Figure E5


A
PCLS Normoxia
PCLS Hyperoxia
PCLS Normoxia + BAY11-7082
PCLS Hyperoxia + BAY11-7082
Sftpc/Wnt5a/S100a4
B
Normoxia Mouse
Hyperoxia Mouse
Normoxia Mouse + BAY11-7082
Hyperoxia Mouse + BAY11-7082
Wnt5a/Sftpc/S100a4