Derivation and Characterization of RUES2

MATERIALS AND METHODS

RUES2 derivation and culture

Blastocysts used in this derivation were frozen 6 days post fertilization and were donated with informed consent and in excess of clinical need following institutional guidelines. Identifying information was removed before receipt of the vials. Blastocysts were thawed by stepwise removal of cryoprotectant, washed twice in recovery medium and incubated for 2 hours in G2v3 (VitroLife, Inc.) to allow for blastocoel expansion. Recovery medium consisted of 10% Plasmanate, 1X non-essential amino acids, 1X essential amino acids and 1X GlutaMAX in M16 medium (Specialty Media). They were then washed twice in HUESM medium and plated intact on a mitomycin-c inactivated MEF feeder layer (CF-1, Chemicon). HUESM consisted of DMEM supplemented with 20% KSR, 1X non-essential amino acids, 1X essential amino acids, 1X GlutaMAX, 1X B27 Supplement (without Vitamin A) and 20ng/ml bFGF (all from Invitrogen). The resulting outgrowth was micro-dissected and transferred to fresh feeder layers or to Matrigel (BD Biosciences) in MEF conditioned medium (CM) for expansion. CM was obtained by growing MEF with HUESM medium collected every 24 hours, and further supplemented with 20ng/ml bFGF prior to use. Stable cultures of RUES2 were maintained as previously described (James et al 2006). EBs were generated by incubation of cultures in dispase until colonies detached from the substrate. This was followed by culture of the aggregates in HUESM in low attachment 96-well plates (Evergreen). EBs were then transferred from individual wells to cell Matrigel coated Matek slides in the presence of HUESM to allow for attachment and further analysis.

Karyotype analysis

hESC lines were independently karyotyped after 6 passages by G-banding (Sloan-Kettering Cytogenetics core).

Teratoma formation

To generate teratomas, 1-2x10^6 hESCs were injected subcutaneously or into the rear leg

muscle of SCID/Beige mice. After 6 to 8 weeks, teratomas were excised and fixed in neutral buffered formalin and histologically analyzed by trained pathologist. In addition, some were fixed, equilibrated in 30% sucrose and embedded for cryo-sectioning. Sections were analyzed for the presence of germ layers markers as described below.

Real time RT-PCR

RNA from RUES2 hESCs grown on matrigel in the presence of CM or under differentiating conditions (non CM for 30 days) was isolated using RNA-Bee (Tel-Test Inc. Friendswood, TX). DNase treatment was performed using the DNA-free kit (Ambion) according to manufacturer's instructions and cDNA was generated with a SuperScript III cDNA First Strand Synthesis kit (Invitrogen) according to manfacturer's instructions. Real-time PCR reactions were performed using gene specific primer sets (table1) with LightCycler 480 SYBR Green I Master Kit (Roche) under universal cycling conditions. Data for genes of interest were normalized by a geometric average of expression of internal control genes TBP, ATP50, B2M and HPRT1 as described (Vandesompele et al 2002).

Immunofluorescence

Samples were fixed in 4% paraformaldehyde (PFA), washed in phosphate buffered saline solution and blocked in 0.1% triton-X and 3% donkey or goat serum in PBS. Samples were exposed to primary antibodies in blocking solution overnight at 4ºC, washed 3 times in PBS with 0.1% tween-20 at room temperature and exposed to fluorescent conjugated secondary antibodies (dilution 1:750). Primary antibodies included Oct3/4 (Signal Transduction labs), SSEA4, Tra-1-60, and MAP2ab (Chemicon), β-tubulinIII/Tuj 1 (Sigma), Alpha-1-Fetoprotein, PLAP (DAKO), GATA6 (Santa Cruz), MuscleMHC/MF20 (Developmental Studies Hybridoma Bank), Neurofilament Heavy Chain (SMI32, Sternberger), Desmin, HGCbeta (Abcam), Cdx2 (BioGenex), Nanog, Sox2 and Sox17 (R&D Systems). Alexa conjugated secondary antibodies and SytoxOrange nuclear counterstain were purchased from Molecular Probes. All imaging was performed on a Zeiss Pascal confocal microscope.

Transgenic cell lines

Lentiviral transduction was used to generate hESCs that stably express green fluorescent protein (EGFP) and Neomycin resistance gene under the control of Rex1 promoter. We first transfected HEK 293 cells with a lentiviral vector. Supernatants from these cells containing infectious particles were collected and used to infect RUES2 at 5 X 10^5 infectious units/ml. After manually selecting for regions of strong GFP expression through two passages, homogenous, GFP-expressing RUES2 colonies were obtained (data not shown). GFP expression remained stable in these cells for more than 10 passages.

Compound Screening

Undifferentiated RUES2 Rex1-GFP-Neo cells were plated at the density of 100 cells/well on matrigel coated 96-well plates and maintained in CM for three days. Single cells were obtained by treatment with the ROCK inhibitor as described (Watanabe et al 2007). Cells were then exposed to chemical compounds at a final concentration of 10µg/ml in non CM (except for controls, as indicated). Compounds were obtained from a commercial library purchased from Prestwick Chemicals (Illkrich, France). Medium was changed everyday for 5 days with fresh compounds. EGFP expression was monitored during the time of the screen before cells were exposed to neomycin (200µg/ml) for 8 days. Viability assay was performed using resazurin to resofurin conversion (CellTiter-Blue Cell Viability Assay, Promega). Pink indicates live cells whereas blue indicates dead cells.

RESULTS

Derivation and characterization of RUES2, a novel hESC line

Work from several laboratories with current NIH approved lines suggests that these lines may change phenotype during adaptation to culture (Baker et al 2007). In order to minimize these effects, we undertook to derive a new hESC line and perform a screen on low passages. Human blastocysts at day 6 of development, originally destined to destruction, were thawed following standard protocols (James et al 2006; Cowan et al 2004; Thomson et al 1998) as described in the materials and methods section. Examples of two blastocysts with a small but distinguishable inner cell mass (ICM), composed of tightly packed cells are shown in Fig. 1A and D. They resulted in primary outgrowths (Fig.1B and E), further expanded by manual dissection to give rise to several colonies with a high nuclear to cytoplasmic ratio for over 40 passages (Fig. 1C, F). For both lines cell cycle length was 24 hours (data not shown), a growth rate equivalent to the reported rate for other hESCs (including our first derived RUES1) (James et al 2006; Cowan et al 2004) and karyotype analysis was normal (Figure S1 and S2). The first was a female (46, XX) we named RUES2, with which we decided to follow our molecular characterization. The second was a male (46, XY) we named RUES3 and will be described elsewhere.

When cultured under undifferentiating conditions, RUES2 expressed the conventional stemness markers Nanog, Oct3/4 and SSEA4 (Fig. 1G, H and I, respectively). In addition, expression level analysis of 10 genes by RT-PCR (5 enriched in undifferentiated hECS and 5 induced during differentiation; Sato et al 2003; Suarez-Farinas et al 2005) perfectly correlates with the state of differentiation of RUES2 (Fig. 2).

We next analyzed whether RUES2 was able to contribute to all three embryonic germ layers, ectoderm, mesoderm and endoderm, a hallmark of *bona fide* ESC lines. Fig. 3 shows that this is the case *in vitro* after the EBs formation assay. 24-day-old EBs stained positive for markers of the three germ layers, Neurofilament Heavy Chain for ectoderm, Muscle Actin for mesoderm and Sox17 for endoderm derivatives. In addition, the pluripotent character of RUES2 was analyzed *in vivo* by teratoma formation assay in NOD-SCID mice. Histology (Fig. 4 A-C) and immunofluorescence (Fig. 4 D-O) analyses

revealed that RUES2 was able to give rise to all three germ layers as well as trophectoderm.

Taken together, these results demonstrate that RUES2 fulfills all the molecular, cellular and embryological criteria of a hESC line.

Engineering RUES2 for HTS of compounds maintaining pluripotency

From the parental RUES2 line, we next engineered a dual system enabling HTS to identify new drugs interfering with stemness or differentiation. Our aim was to create a system combining the possibility to distinguish undifferentiated from differentiated cells both in real time during the screening process, and at the end of the screen by a quick readout of the cells's stemmnes status, a feature making our strategy amenable to robotization and HTS. This double aim was achieved by stably integrating in RUES2 genome a multicistronic construct that co-expresses specifically in undifferentiated stem cells (under the control of Rex1 promoter) the reporter gene encoding the green fluorescent protein (EGFP) together with a selectable marker (the neomycin phosphotransferase gene, Neo). EGFP expression is crucial for the dynamic *in vivo* analysis, whereas the selectable marker allows the quick readout of cells' status at the end of the screen, after neomycin selection and a colorimetric viability assay. When the cells are maintained undifferentiated, they express EGFP and are resistant to neomycin treatment, whereas the expression of these two genes is lost under differentiating conditions (Figure 5 and 6). The new-engineered line, further referred to as RUES2 Rex1-EGFP-Neo, retains all the characteristics of the parental line (data not shown).
In order to validate RUES2 Rex1-EGFP-Neo line as a screening tool, we performed a series of positive and negative controls. CM maintains cells undifferentiated, whereas non CM pushes the cells towards differentiation. Fig. 5 shows that cells maintained in CM alone, in CM+DMSO, in the presence of non CM+BIO (a small molecule we previously showed to be sufficient to maintain cells undifferentiated in non CM; Sato et al 2004) or cells constitutively expressing the construct, remain fluorescent after several days and show a pink color after the viability assay indicating the presence of live cells after neomycin treatment. In contrast, Rex1-EGFP-Neo RUES2 cells maintained in non CM alone, non CM+DMSO, non CM+an inactive BIO analog, MeBIO or the parental

cell RUES2 line do not or weakly express EGFP and show a blue color after the viability assay (dead cells). These results suggested that the cell line we engineered responded as expected and could be used for larger scale screens.

Screening for pluripotency maintaining compounds using RUES2 Rex1-EGFP-Neo

We then undertook a pilot screen with the Rex1-EGFP-Neo RUES2 cell line, using manual plating of single cell suspensions in 96-well dishes, as described in the material and methods section. For this pilot experiment, we focused on pluripotency maintaining compounds and therefore, cells were exposed to compounds in non CM, a medium that pushed them towards differentiation. Out of 80 molecules tested, 7 showed a significant maintenance of pluripotency markers after 5 days in non CM, as observed by both the persistence of EGFP expression and the resistance to neomycin selection (Fig 5) (compounds C5, C11, G3, G6, H2, H7 and H8). In order to confirm the activity of our hits by an independent method, the expression level of the pluripotency marker Oct-4 was monitored by immunofluorescence on the parental RUES2. Results shown in Fig. 6 show that 6 out of 7 primary hits are able to maintain a high level of Oct-4 expression after 5 days in non CM, compared to controls in non CM alone. These results were also observed on two other hESC lines, H1 and HUES6 (data not shown). For 5 of them (C5, G6, H2, H7 and H8), the effect was dose-dependent. Interestingly, compound G3 was able to maintain high levels of Oct-4 expression at low concentration (2 μM), whereas this activity was lost at higher concentrations (20 and 50μM), suggesting that G3 may be hitting a pathway acting as a morphogen, e.g., inducing different fates at different concentrations.

References

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FIGURE LEGENDS

Fig.1: RUES2 Derivation and molecular marker characterization.

(A) Human embryo at day 6 used for derivation (blastocyst stage) (B) Outgrowth morphology of on MEFs. (C) hESCs colonies on Matrigel in MEF CM. (D-F) Immunohistochemistry on RUES2 line for molecular markers of pluripotency, including (D) Nanog (green), (E) Oct-3/4 (red), and (F) SSEA4 (green).

Fig.2: Dynamic Molecular marker of undifferentiated or differentiated RUES2.

CM = RUES2 undifferentiated; noCM = RUES2 differentiated for 24 days. On upper row, all pluripotency markers declined upon differentiation: A) Nanog, B) Pim2, C) Pou5F1, D) ZNF74, and E) Sox2. On lower row, all differentiation markers increased upon differentiation: F) BHLHB2, G) FOSL2, H) NR2F2, I) EPAS1, and J) Sox9.

Fig.3: Characterization of RUES2 embryoid bodies.

Derivatives of all three germ layer detected by immunofluorescence on 24-day-old RUES2 embryoid bodies. A) Neurofilament Heavy Chain positive staining (Red) indicates ectoderm formation. B) Mesoderm formation as marked by muscle actin positive cells (Red). C) Sox17-positive cells (Red), demonstrating the presence of endoderm derivatives in RUES2 EBs. Nuclei in EBs were counterstained with Sytox Orange (White).

Figure 4: Differentiation potential of RUES2 in teratomas. Teratomas were analyzed by histology (A-C) and immunofluorescent staining of cryosections (D-O). A) Retinal pigmented epithelium, B) bone, C) and glandular tissue. Ectodermal differentiation was detected by staining sections with D) Map2, E) NFH, and F) cytokeratin. Mesodermal tissue was marked by G) Muscle Actin, H) Cardiac Actin and I) Desmin. Endodermal tissue was detected by staining for J) GATA6, K) AFP, L) IFABP, and M) HNF3beta. Trophectoderm formation was marked by O) HCG and P) PLAP.

Figure 5: Pilot Screen to identify new pluripotency maintaining molecules

A) EGFP expression patterns in RUES2 Rex1-EGFP-Neo transgenic line exposed to control conditions or compounds C5, C11, G3, G6, H2, H7 and H8.

B) 96-well plate at the end of the screen after neomycin selection and cell viability assay. Blue indicates dead cells (negative wells) and pink indicates live cells (positive hits). Compounds C5, C11, G3, G6, H2, H7 and H8 were identified as positive in this pilot screen.

Figure 6: Verification of hits' activity by immuno-histochemistry.

RUES2 parental cells were immuno-stained for Oct-4 (Red) and nuclei were counterstained with Sytox Orange (White, inset). Mean fluorescence was quantified and expressed as a percentage of mean Oct-4 expression in cultures maintained in CM.

A) Cells exposed to various concentrations of compounds (as indicated). Compound structures are shown on the right. B) Cells exposed to control conditions (as indicated in the text).

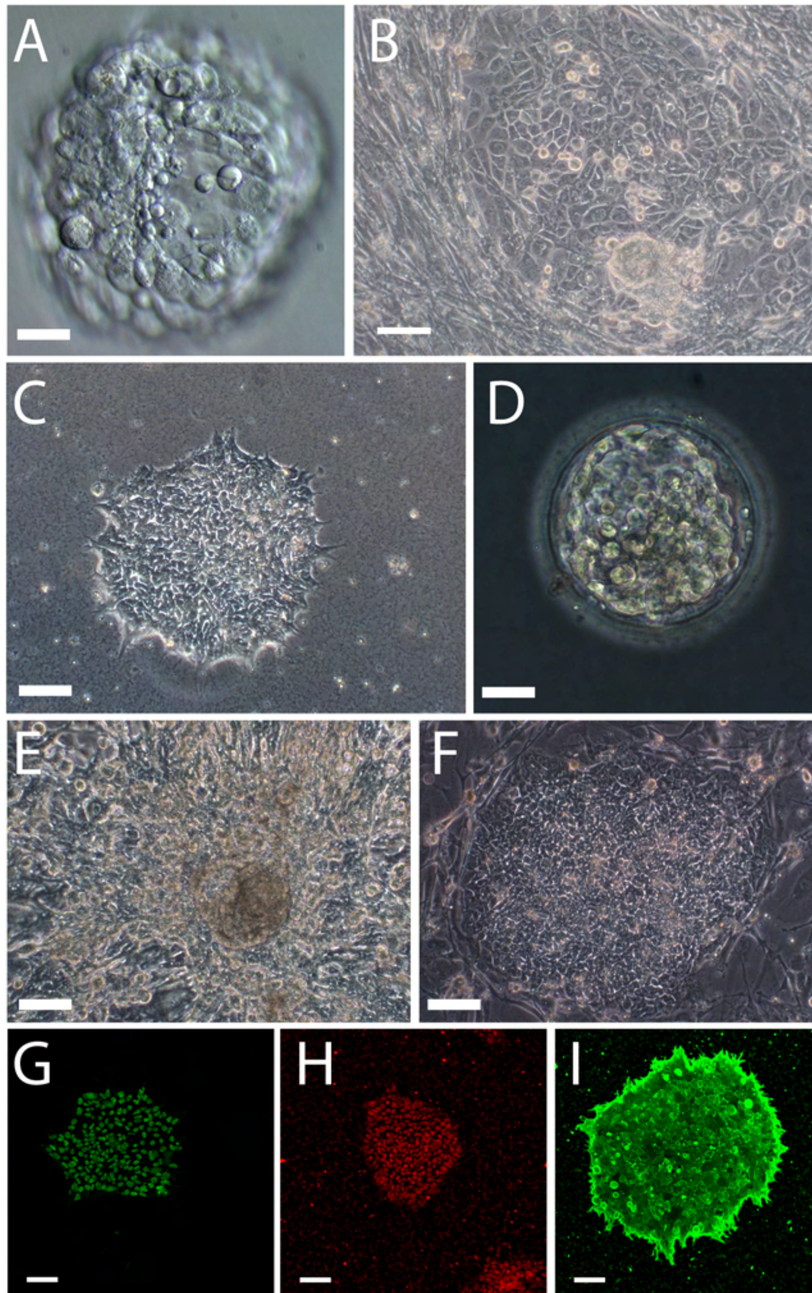


Figure 1

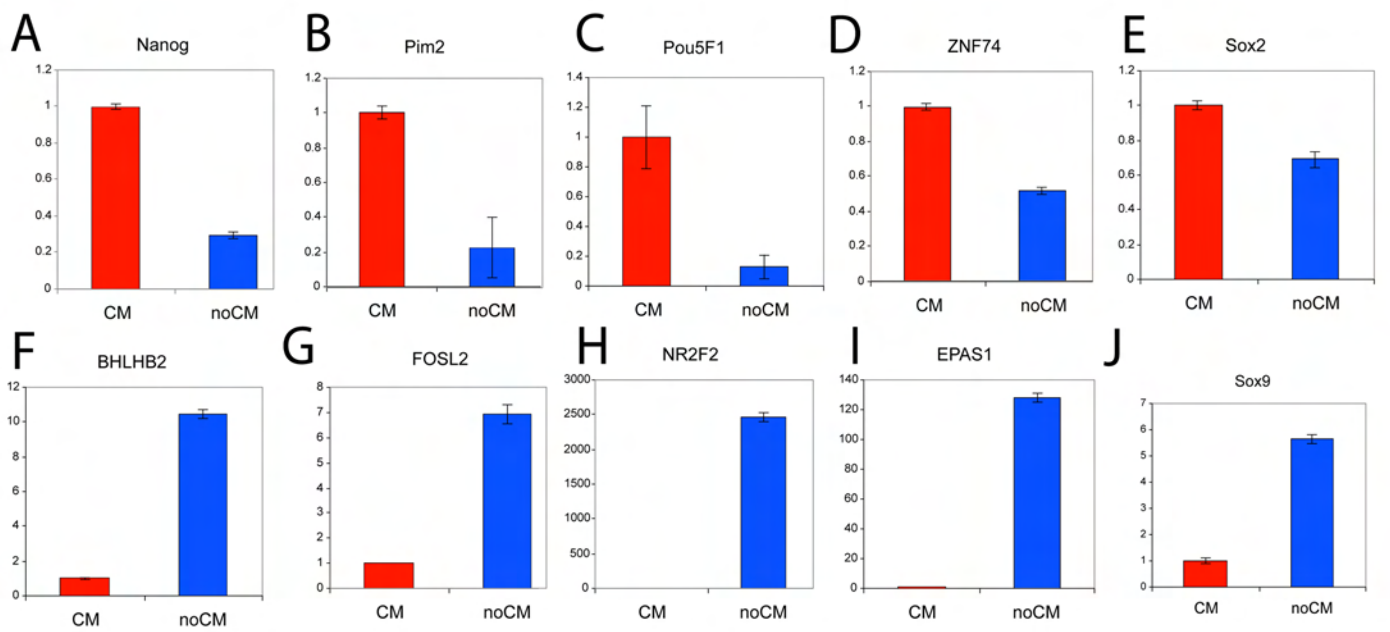
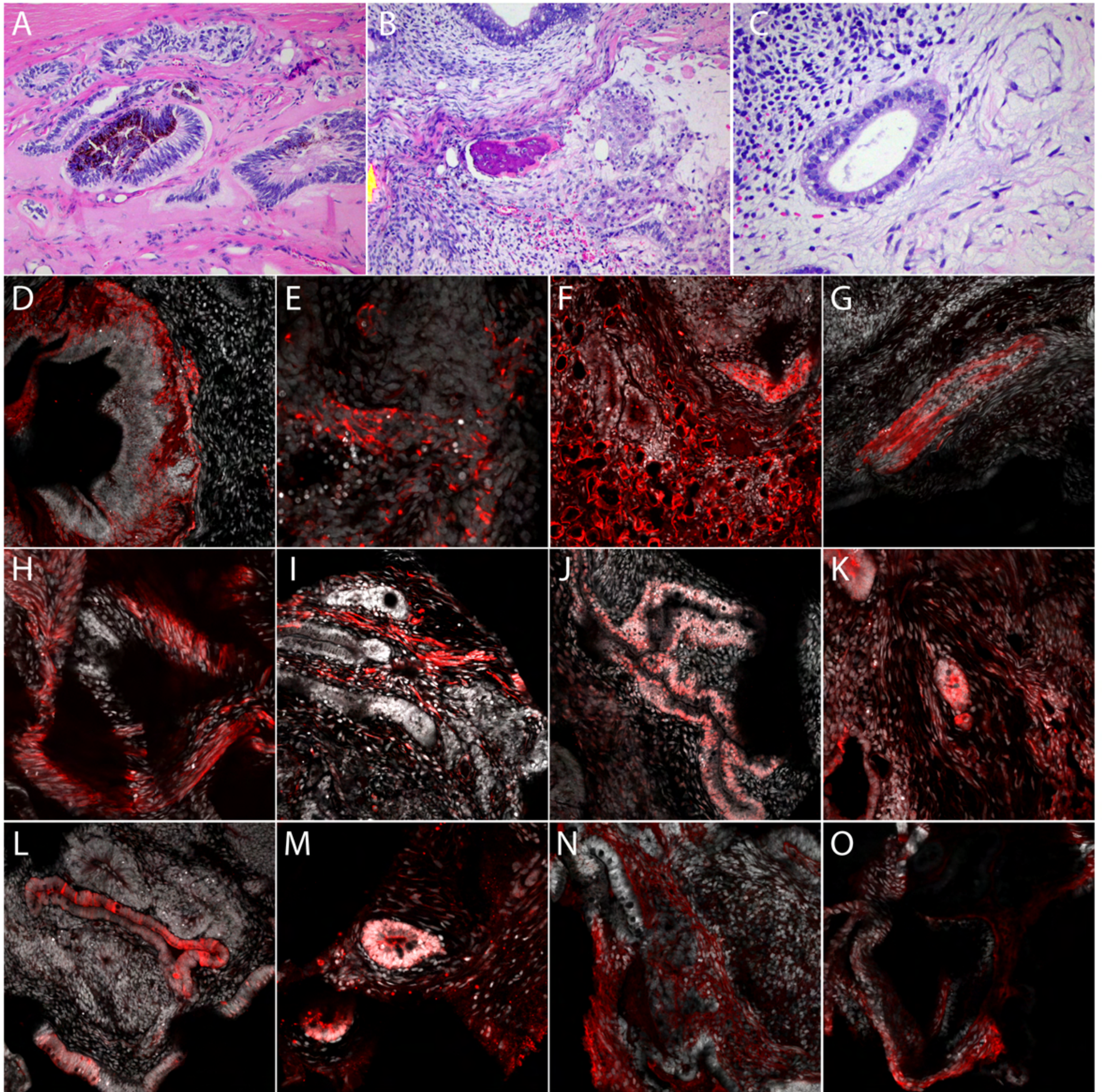


Figure 2



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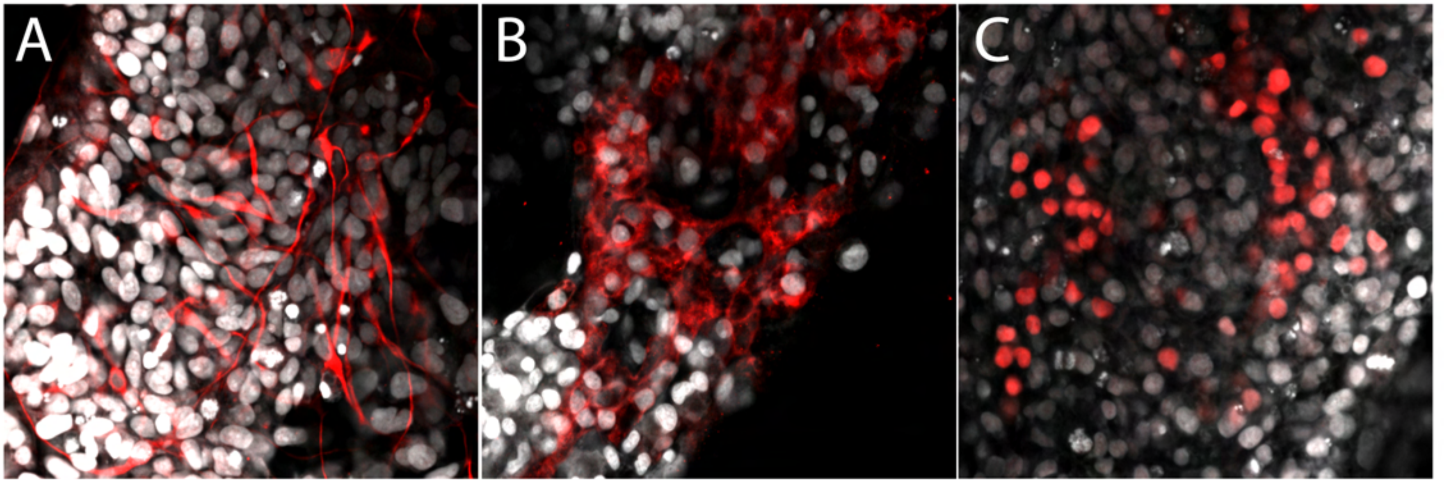

Figure 4

A

Controls

hits

No CM

BIO + No CM

H2

H7

CM

MeBIO + No CM

G3

H8

DMSO + CM

RUES2 + No CM

C5

C11

DMSO + No CM

PGK-EGFP-T-Neo + No CM

G6

B

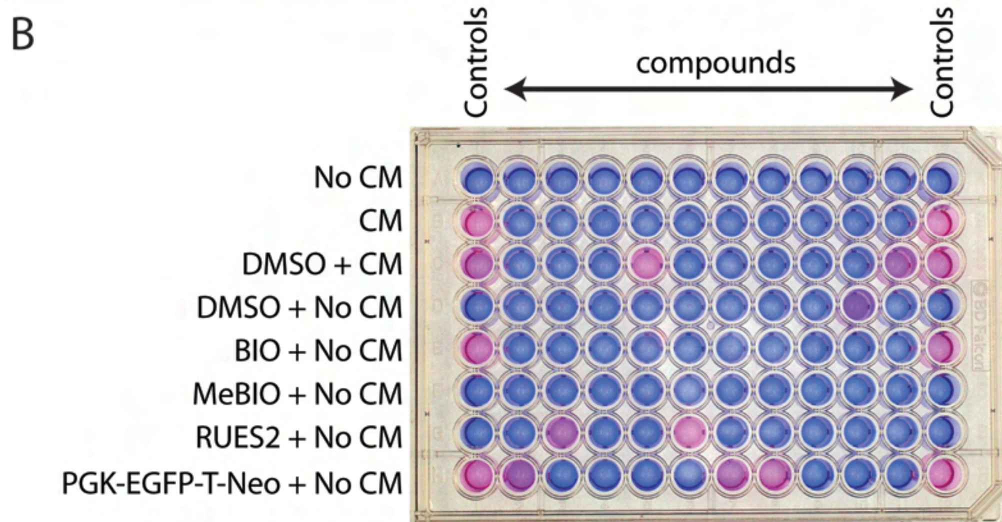

Figure 5

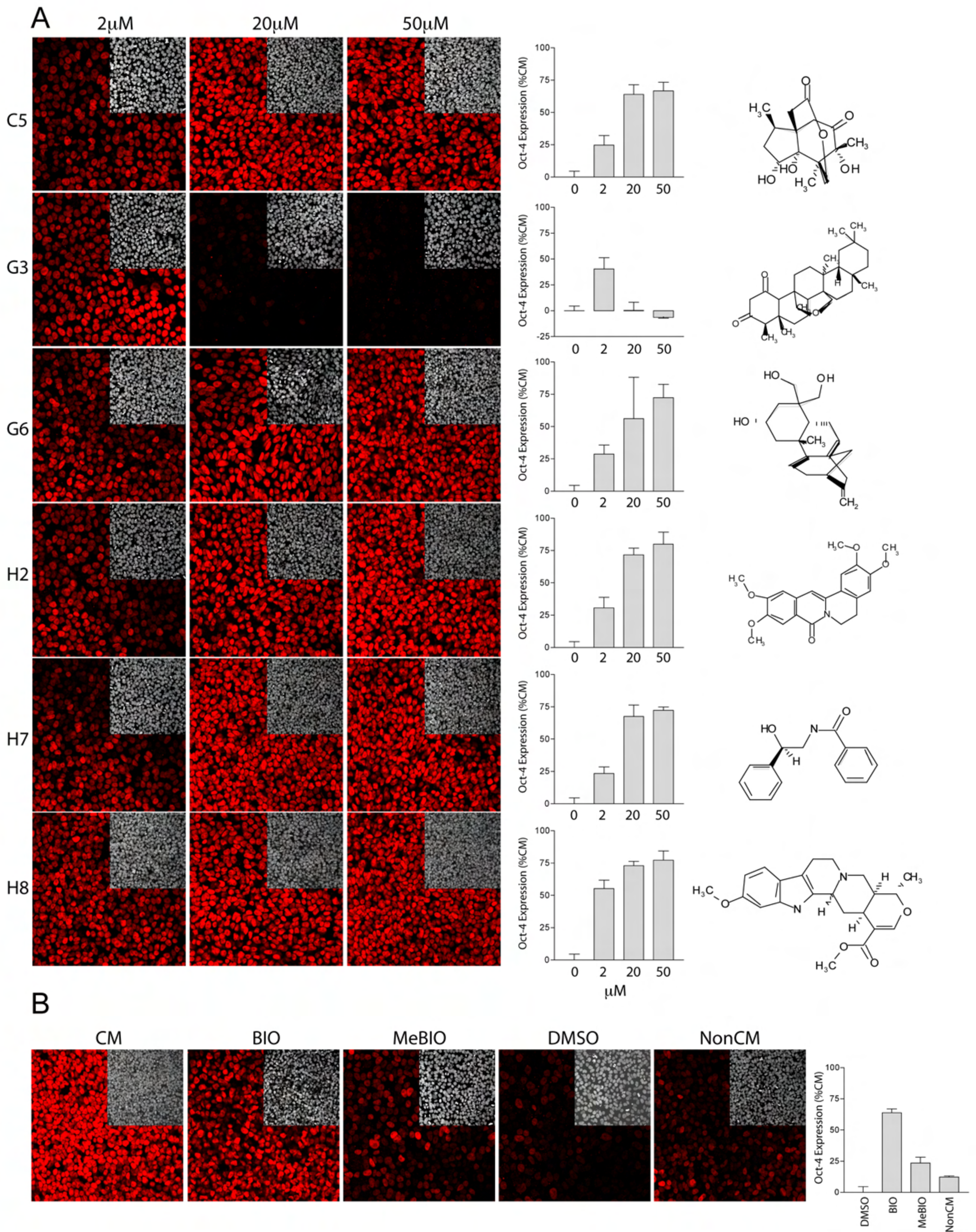

Figure 6