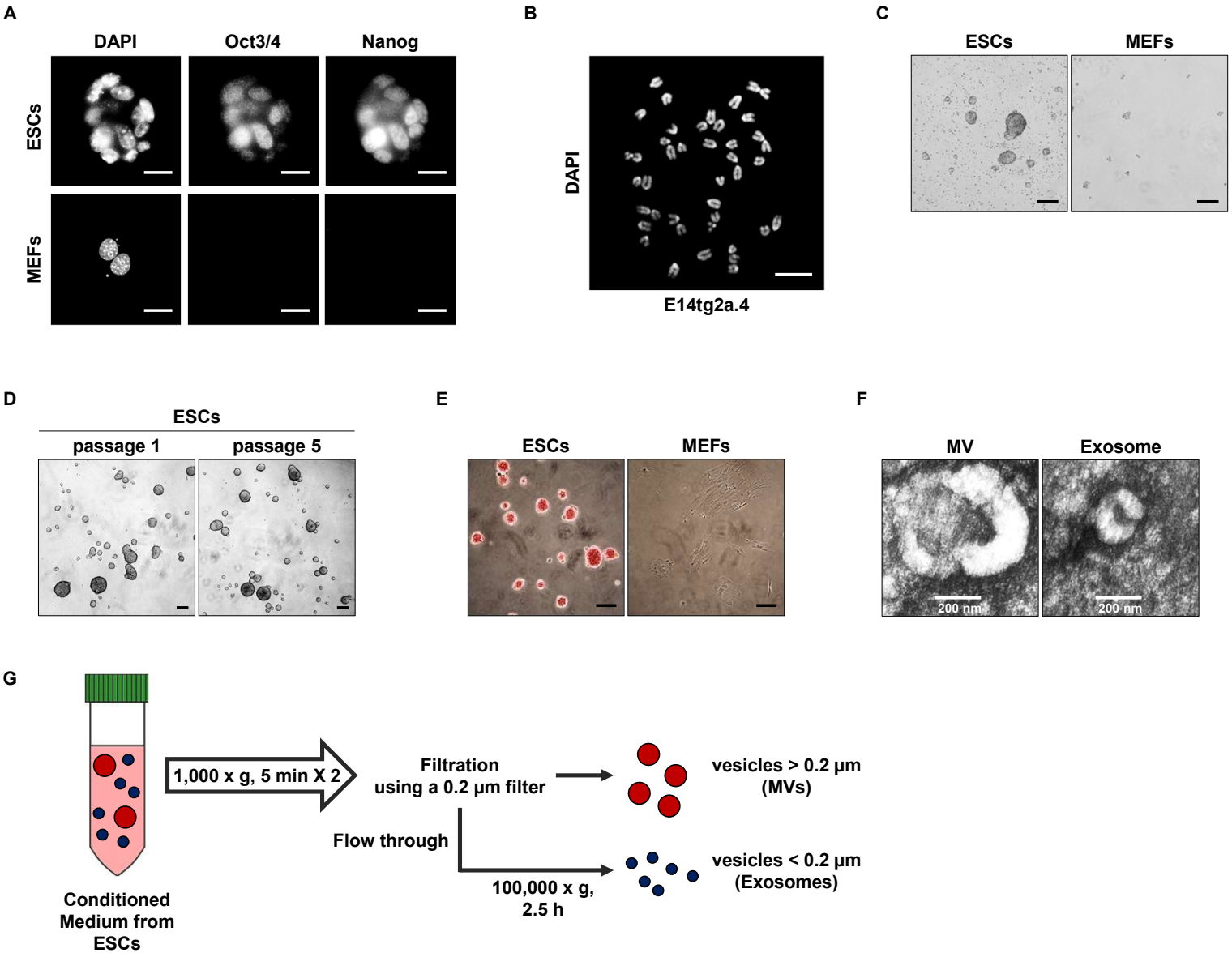
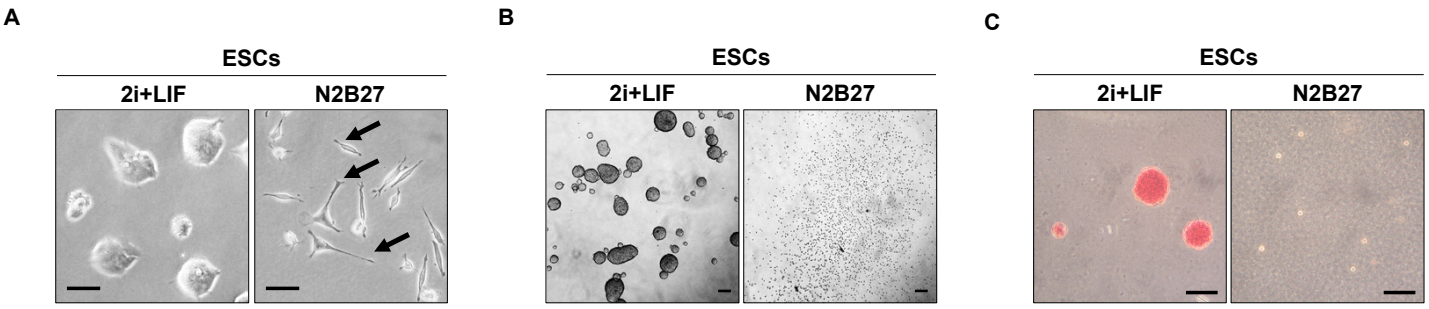
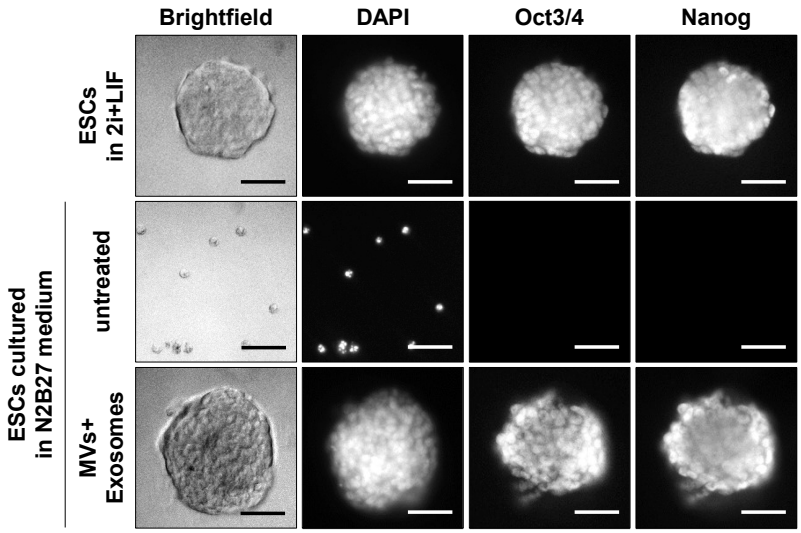


Supplemental Figure 1

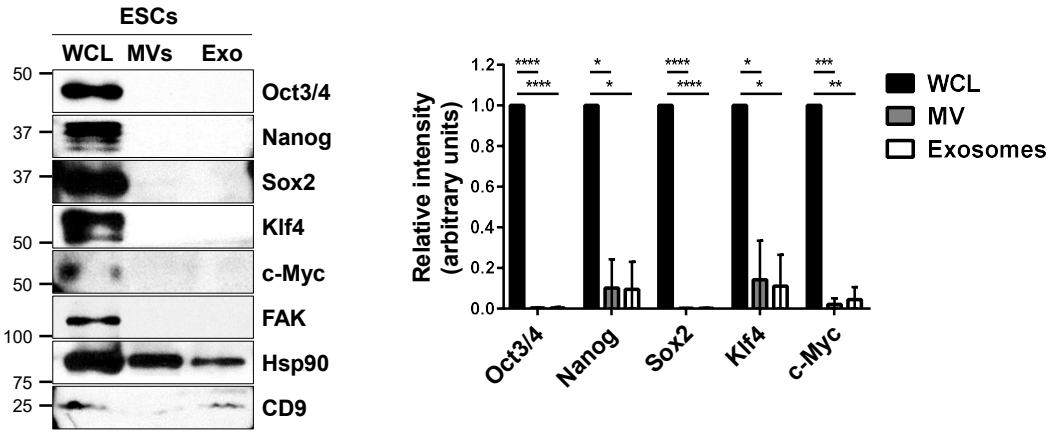


Supplemental Figure 2



Supplemental Figure 3

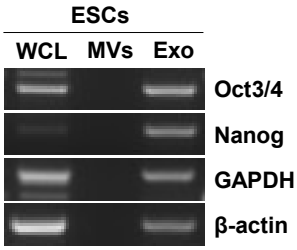
A



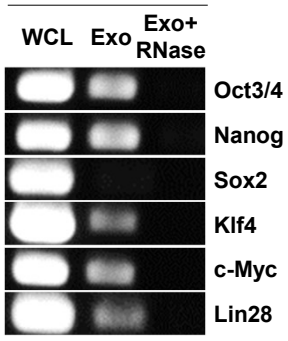
B

Sample	RNA concentration (ng/μl)
ESCs	1084.53±181.46
MV preparation	7.13±4.55
Exosome preparation	46.30±34.80

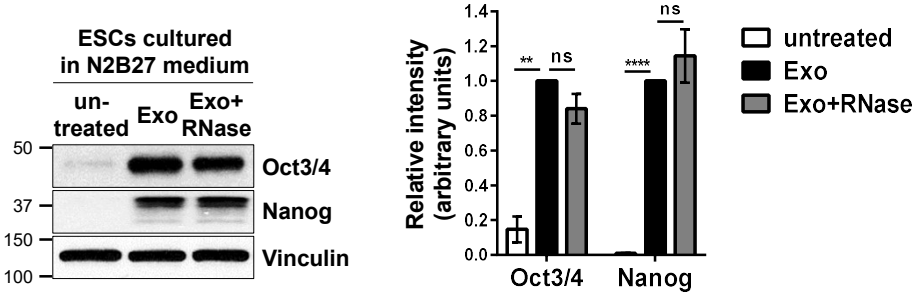
C

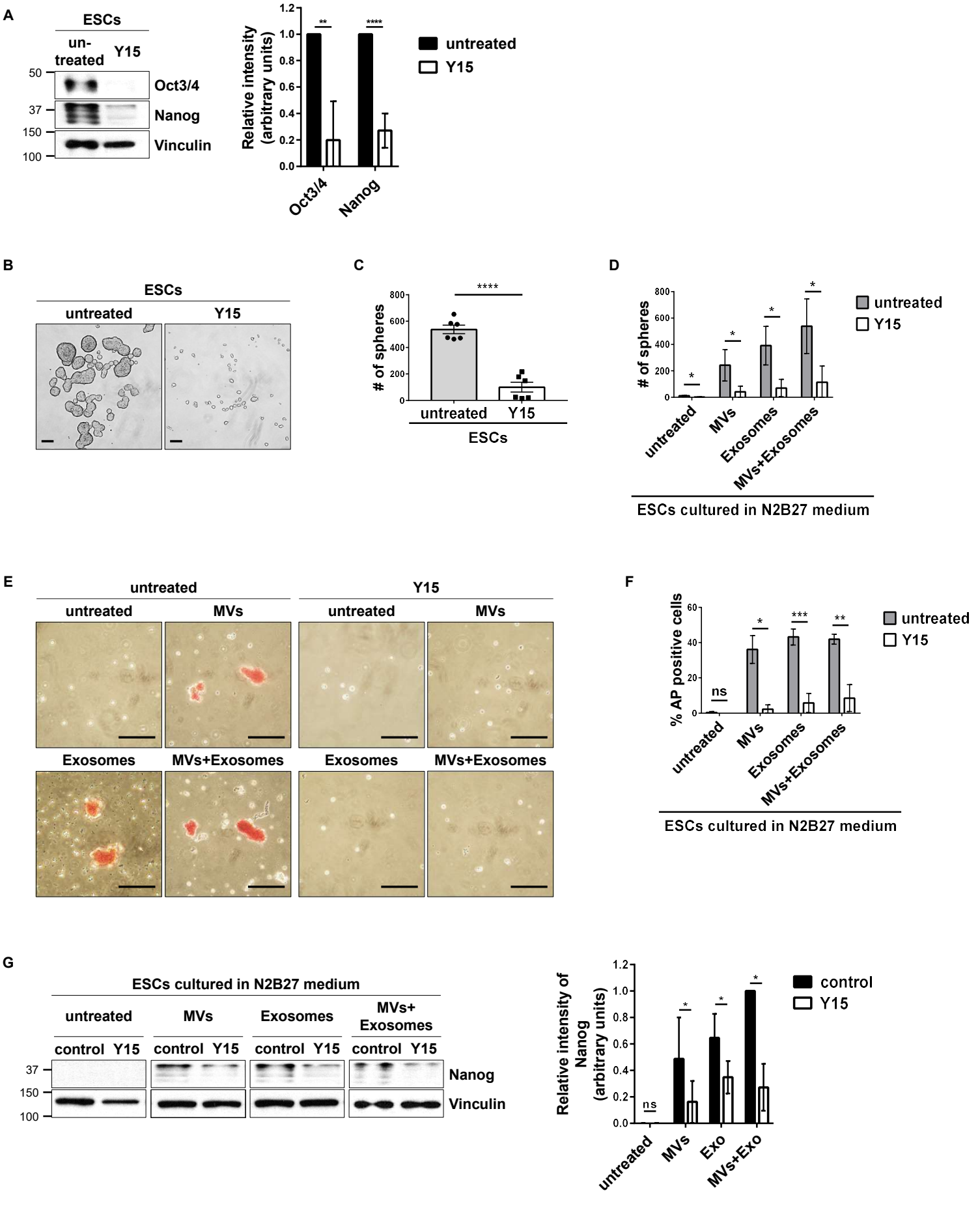


D

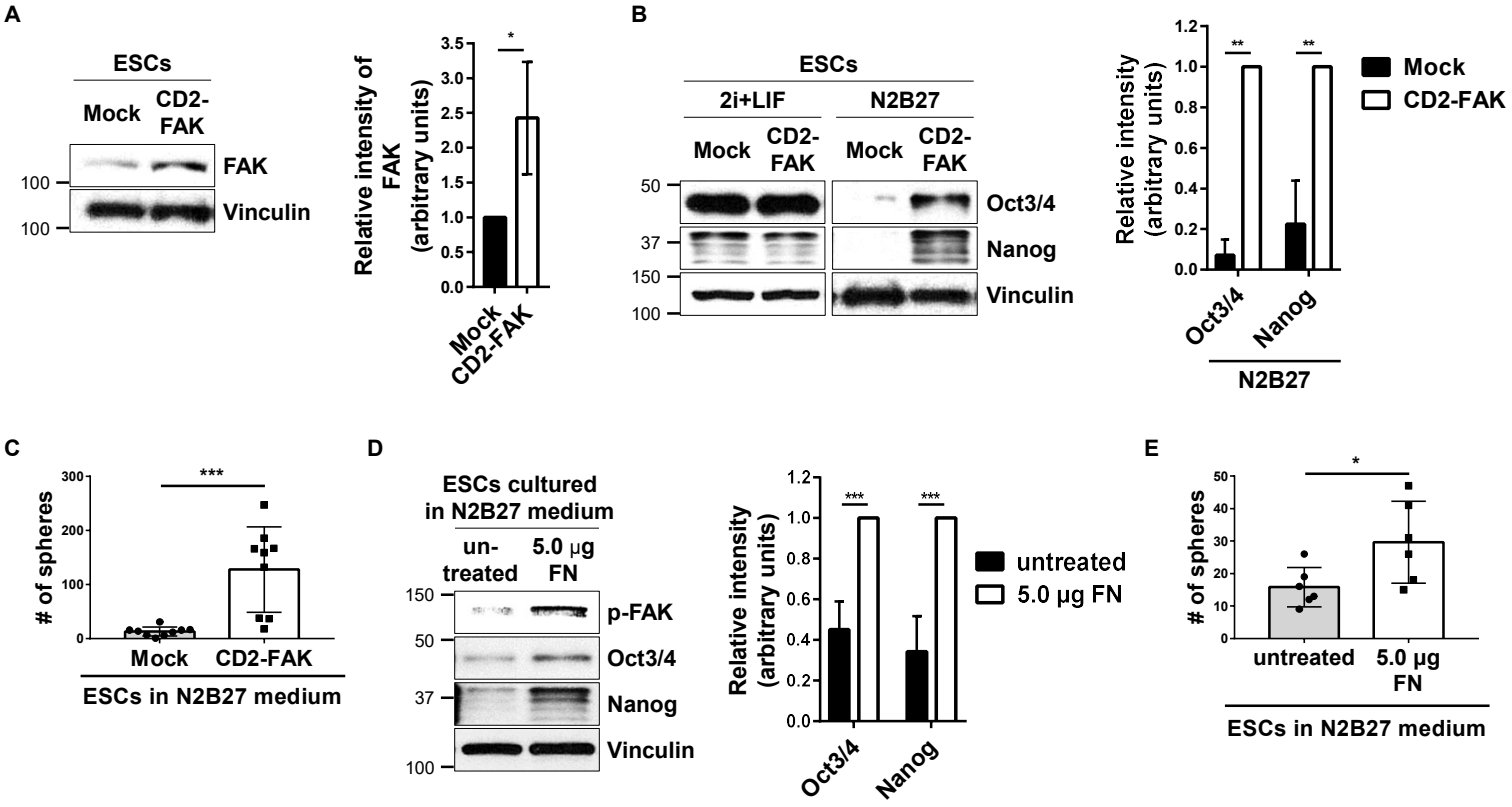


E

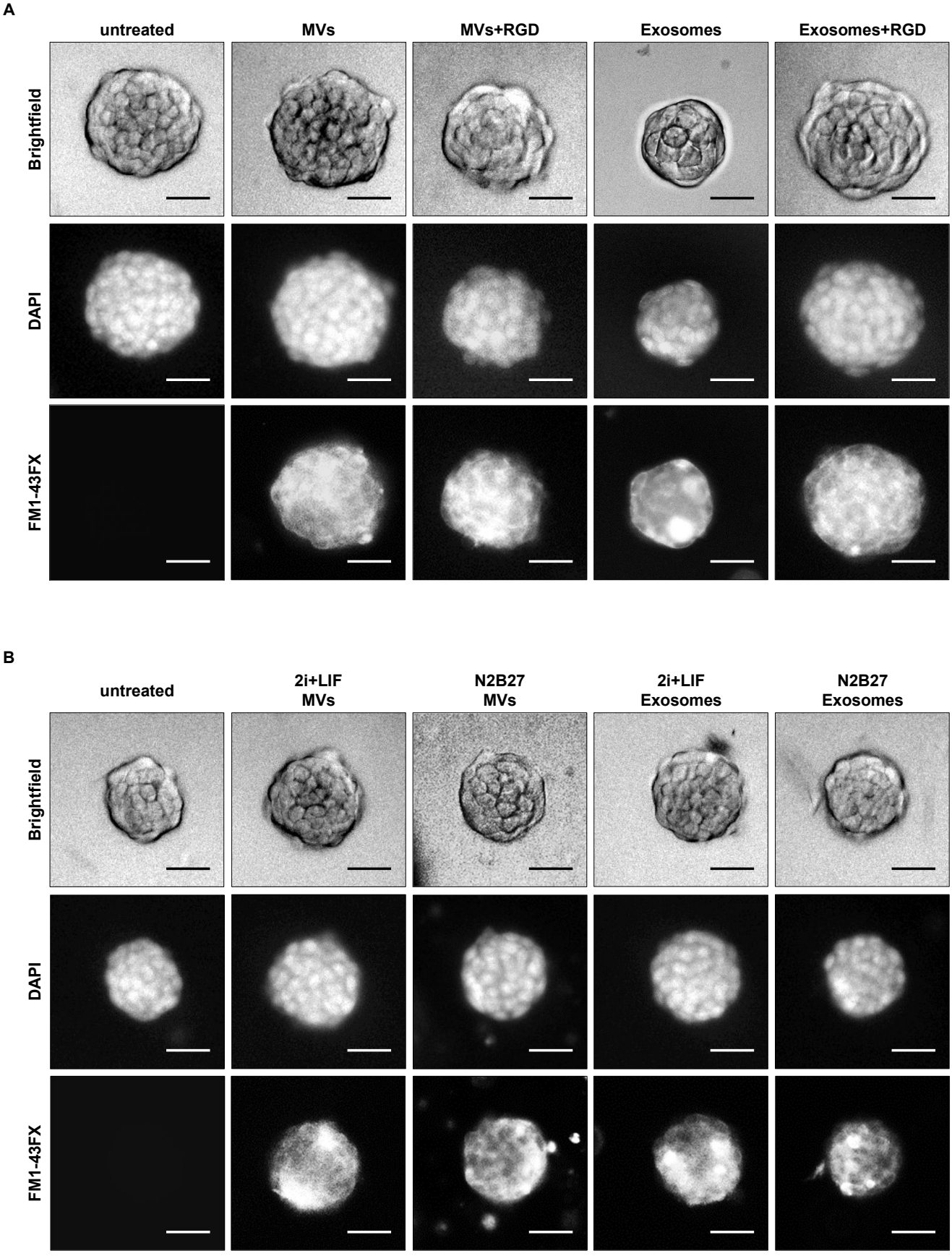




Supplemental Figure 5



Supplemental Figure 6



Supplemental Figure 7

Supplemental Figure Legends

Supplemental Figure 1. Characterization of ESCs, Related to Figure 1.

(A) Fluorescent microscopy images of ESCs and MEFs stained for Oct3/4 and Nanog. The cells were also stained with DAPI to label the nuclei. Scale bar, 20 μm

(B) Fluorescent microscopy image of an ESC chromosome spread stained with DAPI. Scale bar, 10 μm.

(C) Images of sphere formation assays performed on ESCs and MEFs. Scale bar, 100 μm.

(D) Images of sphere formation assays performed on ESCs that had been passaged once (passage 1) or 5 times (passage 5). Scale bar, 100 μm.

(E) Images of AP activity assays performed on ESCs and MEFs. Cells positive for AP activity are red. Scale bar, 100 μm.

(F) Electron microscopy images of MVs and exosomes present in the conditioned medium collected from cultures of ESCs. Scale bar, 200 nm.

(G) Approach used for isolating MVs and exosomes from conditioned medium. See STAR Methods for details.

Supplemental Figure 2. ESCs lose their stem cell phenotypes when cultured in N2B27 medium, Related to Figure 2.

(A) Images of ESCs maintained in 2i+LIF or N2B27 medium. Many of the cells cultured in N2B27 medium had protrusions, denoted with arrows. Scale bar, 100 μm.

(B) Images of sphere formation assays performed on ESCs cultured in 2i+LIF or N2B27 medium. Scale bar, 100 μm.

(C) Images of AP activity assays performed on ESCs cultured in 2i+LIF or N2B27 medium. Cells positive for AP activity are red. Scale bar, 100 μm.

Supplemental Figure 3. EVs from ESCs help maintain the expression of pluripotency markers, Related to Figure 3.

Brightfield and fluorescent microscopy images of ESCs cultured in 2i+LIF medium, or N2B27 medium supplemented without (untreated) or with MVs and exosomes from pluripotent ESCs. The cells were stained for Oct3/4 and Nanog, and DAPI was used to label nuclei. Scale bar, 50 μm.

Supplemental Figure 4. The transfer of core stemness proteins, or the RNA transcripts encoding these proteins, is not responsible for the ability of ESC EVs to promote stemness, Related to Figure 5.

(A) ESCs (whole cell lysate; WCL), MVs, and Exosomes (Exo) were immunoblotted for markers of pluripotency (i.e. Oct3/4, Nanog, Sox2, Klf4, and c-Myc), the cytosolic protein FAK, the general EV marker Hsp90, and the exosome-specific marker CD9.*

(B) The amount of RNA detected in ESCs, and associated with the MVs and exosomes that these cells produce, was quantified using a NanoDrop Spectrophotometer. RNA isolation and quantification were performed three independent times, and the data are presented as mean ± SD.

(C) Reverse transcriptase (RT)-PCR was performed on the RNA isolated from ESCs (whole cell lysate; WCL), and on the MVs and exosomes (Exo) these cells produce, using primer sets that specifically amplify Oct3/4, Nanog, GAPDH, and β-actin.

(D) RT-PCR was performed on the RNA isolated from ESCs (whole cell lysate; WCL), intact exosomes (Exo), and exosomes incubated with RNase A (Exo+RNase), using primer sets that specifically amplify markers of pluripotency (i.e. Oct3/4, Nanog, Sox2, Klf4, c-Myc, and Lin28).

(E) ESCs maintained in N2B27 medium supplemented without (untreated) or with intact exosomes isolated from ESCs (Exo), or with exosomes incubated with RNase A (Exo+RNase), were immunoblotted for Oct3/4 and Nanog. The blot was re-probed for vinculin as the loading control.*

*The data shown in (A), (B), and (E) are presented as mean ± SD. All experiments were performed at least three independent times, and statistical significance was determined using Student's t-tests; ****; $p < 0.0001$, ***; $p < 0.001$, **; $p < 0.01$, *; $p < 0.05$, and ns; not significant.

Supplemental Figure 5. EVs from ESCs help maintain the pluripotency of recipient stem cells by activating FAK, Related to Figure 5.

(A) ESCs cultured in 2i+LIF medium supplemented without (untreated) or with 2.5 μM Y15 were immunoblotted for Oct3/4, Nanog, and vinculin as the loading control.*

(B) Images of sphere formation assays performed on ESCs maintained in 2i+LIF medium supplemented without (untreated) or with 2.5 μM Y15. Scale bar, 100 μm.

(C) Quantification of the assays shown in (B).*

(D) Quantification of sphere formation assays performed on ESCs cultured in N2B27 medium

supplemented with various combinations of MVs and exosomes produced by pluripotent ESCs, and treated without (untreated, shaded bars) or with 2.5 μM Y15 (clear bars).*

(E) Images of AP activity assays performed on ESCs cultured in N2B27 medium supplemented with different combinations of MVs and exosomes from pluripotent ESCs, and treated without (untreated) or with 2.5 μM Y15. Scale bar, 100 μm.

(F) Quantification of the assays shown in (E).*

(G) ESCs cultured in N2B27 medium supplemented with various combinations of MVs and exosomes from ESCs, and treated without (control) or with 3.5 μM Y15, were immunoblotted for Nanog and vinculin as the loading control.*

*The data shown in (A), (C), (D), (F), and (G) are presented as mean ± SD. All experiments were performed at least three independent times, and statistical significance was determined using Student's t-tests; ****; $p < 0.0001$, ***; $p < 0.001$, **; $p < 0.01$, *; $p < 0.05$, and ns; not significant.

Supplemental Figure 6. The ectopic expression of an activated form of FAK in ESCs promotes their pluripotency, Related to Figures 5 and 6.

(A) ESCs that had been either mock transfected (Mock), or transfected with pLV-neo-CD2-FAK (CD2-FAK), were immunoblotted for FAK and vinculin as the loading control.*

(B) The transfectants in (A) were cultured in 2i+LIF or N2B27 medium and then immunoblotted for Oct3/4, Nanog, and vinculin as the loading control.*

(C) Sphere formation assays were performed on the transfectants in (A).*

(D) ESCs cultured in N2B27 medium supplemented without or with 5.0 μg of purified fibronectin (FN) were immunoblotted for phosphorylated FAK (p-FAK), Oct3/4, Nanog, and vinculin as the loading control.*

(E) Sphere formation assays were performed on ESCs cultured in N2B27 medium supplemented without or with 5.0 μg of purified fibronectin (FN).*

*The data shown in (A-E) are presented as mean ± SD. All experiments were performed at least three independent times, and statistical significance was determined using Student's t-tests; ***; $p < 0.001$, **; $p < 0.01$, and *; $p < 0.05$.

Supplemental Figure 7. Fibronectin associated with the EVs from ESCs is not required for their ability to dock onto recipient cells, Related to Figure 6.

(A) MVs and exosomes from pluripotent ESCs that had been labeled with the fluorescent

membrane dye FM1-43FX were used to treat cultures of ESCs incubated without or with 25 μg/ml of the RGD peptide for 1 hour, at which point the cells were washed extensively, fixed, and stained with DAPI to label nuclei. Brightfield and fluorescent microscopy images of the assay are shown. Scale bar, 50 μm.

(B) MVs and exosomes from ESCs that had been cultured in either 2i+LIF medium (2i+LIF MVs and 2i+LIF Exosomes) or N2B27 medium (N2B27 MVs and N2B27 Exosomes) were labeled with FM1-43FX and then were used to treat ESCs. One hour later, the cells were washed extensively, fixed, and stained with DAPI to label nuclei. Brightfield and fluorescent microscopy images of the assay are shown. Scale bar, 50 μm.