RUES1 derivation and culture

Materials and Methods

Derivation, culture and embryoid body formation were peformed as previously described (Thomson et al., 1998). Blastocysts frozen at day 6 post fertilization were donated with informed consent from embryos in excess of clinical need according to institutional guidelines. Identifying information was removed before receipt of the vials and blastocysts were thawed by stepwise removal of cryoprotectant. Blastocysts were washed two times in recovery medium and incubated for two hours before immunosurgery to allow for blastocoel expansion and morphological grading. Recovery medium consisted of 10% Plasmanate, 1X non-essential amino acids, 1X essential amino acids, 1X GlutaMAX in M16 medium (Specialty Media). The blastocysts were treated with 2mg/ml pronase to remove the zona pellucida and then incubated in a 1:10 dilution of anti-human placental alkaline phosphatase antibody (DAKO). The embryos were washed three times in recovery medium and incubated in a 1:10 dilution of guinea pig complement (Sigma) and monitored for trophectoderm lysis. Lysed trophectoderm was removed by pipetting through a pulled pasteur pipette and isolated ICMs were washed 2X in HUESM medium. HUESM consisted of DMEM supplemented with 20% KSR, 1X non-essential amino acids, 1X essential amino acids, 1X GlutaMAX and 20ng/ml bFGF (Invitrogen). Human LIF was added (12ng/ml) during the initial outgrowth but was excluded from subsequent culture. ICMs were plated on a MEF feeder layer and outgrowths were micro-dissected and transferred to fresh feeder layers for three passages for expansion. Stable culture of RUES1 was maintained as previously described (Sato et al., 2004; Xu et al., 2001). Embryoid bodies were generated by incubation of cultures in Dispase until colonies detatched from the substrate. This was followed by culture of the aggregates in DMEM supplemented with 20%FCS, 1X penicillin-streptomycin, 1X GlutaMAX (all from Gibco) on non-tissue culture treated Petri dishes coated with a thin layer of agarose to prevent attachment.

Teratoma formation

To generate teratomas, 1-2X10^6 hESCs were injected into the rear leg muscle of SCID/Beige mice. Teratomas were allowed to develop for 6 weeks and were then excised and fixed in neutral buffered formalin and analyzed histologically by trained pathologist. Some teratomas were fixed, equilibrated in 30% sucrose and embedded for cryosectioning. Sections were processed immunohistochemically for markers of germ layers as described above.

Immunofluorescence

Undifferentiated hESCs plated on thermanox™ coverslips coated with MEFs or Matrigel, hESC derived EBs, teratomas and chimeric embryos were analyzed by immunofluorescence staining for markers of pluripotency and/or differentiation. Briefly, samples were fixed in 4% paraformaldehyde, washed in PBST and blocked in 5% donkey or goat serum. Samples were exposed to primary antibodies in blocking solution overnight at 4ºC, washed 3 times in PBST and exposed to fluorescent conjugated secondary antibodies. Primary antibodies included Oct3/4(Signal Transduction labs), SSEA4, Tra-1-60, and nestin (Chemicon), β-tubulinIII/Tuj1 (Sigma), Alpha-1-Fetoprotein (DAKO) HNF3β, Sox2, Oct3/4 (Santa Cruz), MuscleMHC/MF20 (Developmental Studies Hybridoma Bank), Neurofilament Heavy Chain, Phospho HistoneH3, Desmin (Abcam), and Cdx2 (BioGenex). Alexa conjugated secondary antibodies, SytoxGreen and SytoxOrange nuclear counterstains were purchased from Molecular Probes. Endogenous Alkaline Phosphatase was assayed using manufacturers instructions (Vector Labs). All imaging was performed using a Zeiss Pascal confocal microscope.

Blastocyst injections and embryonic outgrowth culture

RUES1 hESCs were manually dissected into 10-15 cells clumps using finely drawn glass Pasteur pipets and injected into embryonic day 3.5 mouse blastocysts flushed from the uterine horns of Swiss Webster mice. hESC clumps were drawn into custom pulled transfer pipets with a 25 μm bore (Eppendorf™) and injected into the blastocoel cavity of mouse embryos. hESC injected blastocysts were either fixed 24 hours post injection, or

cultured on Matrigel™ coated tissue culture plastic in culture medium containing 15% fetal bovine serum for 6 days. Resultant embryonic outgrowths were fixed and processed immunohistochemically as described above.

RUES1 aggregation with mouse blastomere embryos

Embryonic day 2.5 mouse embryos were flushed from the oviduct of superovulated CBA/B6 mice and treated with acid tyrodes to remove their zona pellucidae. In conical bottomed wells of a 96-well plate, one embryo was placed with a dispase-dissociated hESC clump of ~10-15 cells and the plate was centrifuged briefly in order to combine them. Embryos were allowed to recover for 48 hours, when they were fixed and processed immunocytochemically as described above. Under these conditions, mESCs aggregated in parallel showed contribution to host ICM after 48 hours.

Chimeric blastocyst implantation

RUES1 injected blastocysts were allowed to recover for 6 hours post-injection and injected into the uterine horns of pseudopregnant foster mice as previously described. Five days following injection, implanted embryos were recovered from the uterus of foster mothers and examined for hESC contribution.

Results

Derivation and characterization of RUES1

We isolated an hESC line on mouse embryonic fibroblasts by immunosurgery from 10 thawed blastocysts that had been frozen at day six of in vitro development after in vitro fertilization (Cowan et al., 2004; Thomson et al., 1998, Figure 1A, and B). Upon plating of the ICMs isolated from blastocysts, one expanded with continued culture and gave rise to colonies with tightly packed cells with a high nuclear to cytoplasm ratio (Figure 1C). These could be maintained on MEFs (Figure 1E) by manual dissection (Mitalipova et al., 2005; Oh et al., 2005) or transferred to and maintained on Matrigel coated plates in MEF conditioned medium (Xu et al., 2001) (Figure 1F) for more than 38 passages. This line was named RUES1 (for Rockefeller University Embryonic Stem-cell line 1). Karyotype analysis revealed that the line was male [46, XY] and most cells had a normal karyotype (26/30) after 6 passages (Supplemental Figure 1). Time-lapse video-microscopy established that the RUES1 cell cycle is about 24 hours (data not shown). This is equivalent to the rate of most hESCs, reported to be about 24 to 36 hours, with a range of 12 hours to 72 hours (Cowan et al., 2004).

RUES1 expressed previously described molecular markers of pluripotency (Thomson et al., 1998; Brivanlou et al., 2003). The markers Oct-3/4 (POU5F1, Figure 1D, and G), SSEA4 (Figure 1E, and H), TRA-1-60 (Figure 1F), and alkaline phosphatase (Figure 1I) were readily detected. By real-time RT-PCR, expression of Oct-3/4, Nanog, and Cripto-1 (Figure 1J) were also detected. We have recently reported the identification of a set of genes that are consistently enriched in undifferentiated hESCs across several independent microarray studies (Suarez-Farinas et al., 2005). We verified the enrichment of 91 of these markers in RUES1 hESCs by real-time RT-PCR (Supplemental Table 1). Together, these data demonstrate that RUES1 is similar to previously reported cell lines in origin, growth properties, and marker expression.

Differentiation of RUES1 into derivatives of all three embryonic germ layers

RUES1 also formed complex and cystic embryoid bodies when aggregated and cultured in suspension in vitro (Figure 2A, and B). Embryoid bodies could be maintained in suspension culture for at least 5 months. After prolonged in vitro culture or after reattachment to adhesive substrates, embryoid bodies generated multiple cell types indicative of the three embryonic germ layers (Figure 2C-F). Neural cell types were evident in outgrowths from the EBs (data not shown). Immunostaining for Nestin and Neurofilament Heavy Chain (NFH) confirmed the presence of ectoderm derivatives (Figure 2C) in these cultures. During culture, beating cardiac myocytes were observed, indicating the presence of functional mesoderm differentiation (arrow in Figure 2B). Staining for Desmin confirmed the presence of mesoderm (Figure 2D). Staining for HNF3β demonstrated the presence of endoderm derivatives (Figure 2E). We also found an early marker of trophectoderm, Cdx2 (Strumpf et al., 2005), in EB cultures (Figure 2F). These data indicate that markers of germ layer derivatives can be induced in vitro, however many cell types do not terminally differentiate in vitro under these conditions.

To further demonstrate the differentiation potential of RUES1 we generated teratomas in SCID-beige mice and analyzed for tissue derivatives of the three embryonic germ layers (Figure 2G-N and Supplemental Figure 2). Several teratomas were analyzed including a single teratoma from which we could identify representatives of ectoderm, mesoderm, and endoderm by histology (Supplemental Figure 2). We immunostained a separate teratoma for germ layer markers to verify these results and identified neuroepithelium that stained positively for Nestin, NFH, and TuJ1 (Figure 2G-I), mesodermal tissue that stained positively for Desmin and Muscle MHC (Figure 2J, K) and endoderm tissue that stained positively for AFP and HNF3β (Figure 2L, M). We also identified trophectoderm, as marked by Cdx2 (Figure 2N). Taken together the results presented above establish that RUES1 is a bona fide new hESC line meeting the current criteria of prolonged undifferentiated proliferation while maintaining the ability to differentiate into trophectoderm and germ layer derivatives.

hESCs incorporate and differentiate in mouse blastocyst outgrowths

Although the functional qualities of mESCs and hESCs are very much the same, mouse and human ESCs show significant differences. For example, cell cycle length and the signaling factors that mediate self-renewal have been shown to be different between the two cell types (James et al., 2005; Sato et al., 2004; Xu et al., 2002a). The fact that hESCs are grown on top of MEFs in culture experiments clearly demonstrates that embryonic cell types from the two species can coexist. But factors secreted by MEFs are important for the maintenance of self renewal in hESCs, so it is possible that paracrine signaling between mouse cells and hESCs within mosaic embryos could affect the differentiation process of one or both cell types. In order to assess the ability of hESCs to proliferate, integrate and differentiate in mouse embryos, we injected e3.5 blastocysts with small RUES1 colonies (~10-15 cells) and cultured the embryos in vitro for six days (Figure 3). These experiments described below were designed to minimize hESC input into host embryos, in accordance with policies in place at the Rockefeller University, and are also in line with guidelines recommended by the National Academy of Sciences. Figure 3A and B shows the injection protocol.

As RUES1 hESCs do not tolerate trypsin-passaging, two independent means of RUES1 dissociation, trypsin and micro-dissection into cell clumps, were compared for their ability to integrate into host blastocysts. In each case a total of 10 to 15 cells, either as individual cells or in clumps, were microinjected into the blastocoel cavity of a mouse blastocyst. When trypsin dissociated cells were compared to cell clumps, micro-dissected colonies showed the best quality and quantity of contribution (data not shown). Other available cell lines have shown poor recovery from trypsin-passaging in tissue culture (Amit et al., 2000), and this harsh enzymatic treatment may account for poor contribution of trypsin dissociated RUES1 hESCs to mouse blastocysts. But another cell line HUES#6 (Cowan et al., 2004) (generously provided by Doug Melton), which is routinely trypsin-passaged in cell culture, also showed poor contribution (data not shown). For this reason, the embryonic chimeras in these experiments were generated by injection of manually dissociated clumps.

In order to determine whether human cells would proliferate and mix with the mouse host, injected blastocysts were cultured on Matrigel™ for 6 days in vitro (Figure 3C-I). Embryonic outgrowths showed a complex and disorganized three-dimensional structure with human cells present in significant numbers (>500 nuclei). Cells were actively proliferating, as evidenced by the co-localization of Phospho-HistoneH3 (Gurley et al., 1978) (green in Figure 3G), and human nuclear antigen (red in Figure 3G). This evidence established that hESCs could proliferate and intermingle with their mouse embryonic counterparts in cultured blastocyst outgrowths.

Mouse embryonic fibroblasts have commonly been used to maintain the undifferentiated state of hESCs, so it is possible that the mouse embryonic environment may impede the differentiation of hESCs. In order to address this, we examined whether human cells within the outgrowths expressed markers of the differentiated state. Human cells derived from all three germ layers were detected (Figure 4E-J). Furthermore, we concluded that all the human cells were differentiated, as no RUES1 nuclei were positive for the pluripotency marker Oct3/4. In fact, a cluster of Oct3/4 positive mouse cells that was retained in one outgrowth provided a valuable internal control for the absence of Oct3/4 in human cells (Figure 4A-D).

Figure Legends

Figure 1. Derivation and pluripotent marker expression of RUES1 hESCs.
RUES1 was derived from a frozen 6 day old blastocyst (A). The ICM after isolation by immunosurgery is shown (B). This ICM attached to the MEF feeder layer an produced a primary outgrowth of small ICM-like cells with a high nuclear to cytoplasmic ratio (C). Colonies on MEFs (D-F, I) and on Matrigel (G,H) were analyzed for the pluripotency markers Oct-3/4 (D,G), SSEA4 (E,H), TRA-1-60 (F) by immunofluoresence. Colonies on MEFs were also positive for Alkaline phosphatase (I) by cytochemistry. Real-time RT-PCR analysis of pluripotency markers (J). Shown are amplification plots of relative fluorescence vs. cycle number for Oct-3/4, Nanog, and Cripto-1. β-2-microglobulin as an

amplification control. The no-RT controls are indicated for each primer. Red lines indicate the threshold cycle of amplification used to determine the level of expression.

Figure 2. Germ layer differentiation of RUES1 hESCs in embryoid bodies and teratomas.

RUES1 generated complex aggregates after 14 days of in vitro differentiation in suspension (A). These subsequently formed complex embryoid bodies during 2 months of culture (B). The arrow in B indicates an area of contracting cardiac muscle after 2 months of culture, indicating mesoderm differentiation. When plated on adhesive substrates, the EBs generated multiple differentiated cell types, including neural tissue (C). The neural cell types can be propagated in vitro and stain for markers of Ectoderm: nestin (C, blue) and Neurofilament Heavy Chain (C, red). Mesoderm, marked by Desmin (D, red), and Endoderm, marked by HNF3β (E, red) as well as Trophectoderm, marked by Cdx2 (F, red), can also be found in EBs. RUES1 hESCs at passage 11 were also injected intramuscularly into SCID/beige mice and allowed to develop for 6 weeks to generate teratomas. Germ layer markers were verified by immunofluoresence on cryosections of the teratoma (G-N). Examples of Ectoderm: Nestin (G, red), Tuj1 (H, red) and NFH (I, red), Mesoderm: Desmin (J, red) and Muscle MHC (K, red), and Endoderm: AFP (L, red) and HNF3β (M, red) are shown. In addition, Trophectoderm: Cdx2 (N, red) is present. SytoxGreen nuclear conterstain is shown in green. Scale bars are shown at 50 μM.

Figure 3. hESCs survive, proliferate and incorporate into cultured mosaic embryos.

hESCs were dissociated enzymatically by trypsin or manually by micro-dissection and injected into the blastocoel of e3.5 mouse embryos. The injection scheme is shown in panels A and B. hESC-Injected embryos were cultured in vitro on matrigel coated tissue culture plastic (C) for 6 days. Resultant outgrowths showed complex three dimensional structure and human cells were present in significant numbers (D-I). Panel G shows human cells near the end of mitosis; Phoso-HistoneH3, which is phosphorylated in the context of mitosis, is shown in green. The inset in panel F is magnified as a single optical slice in panels H and I, which show intermingling of human cells with the host. Human

nuclei are represented in red in E-I. SytoxGreen nuclear counterstain is shown in blue. Scale bars – C, D, F, H, I – 50 μM; G – 20 μM.

Figure 4. hESCs differentiate into three primary germ layer derivatives within mosaic outgrowths.

hESCs were injected into e3.5 mouse blastocysts and cultured for 6 days on Matrigel. Resulting outgrowths were fixed and processed immunohisto-chemically using antibodies specific for Oct3/4 (Green in B and D), neurofilament heavy chain (Green in F), desmin (Green in H) and HNF3β (Green in J). Human nuclei are stained by an antibody to human nuclear antigen in red and SytoxGreen nuclear counterstain in blue. Panels C and D represent a magnified view of the inset in B. Scale bars - A-D, I and J – 50 μM; E - H – 10 μM).

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Figure 1

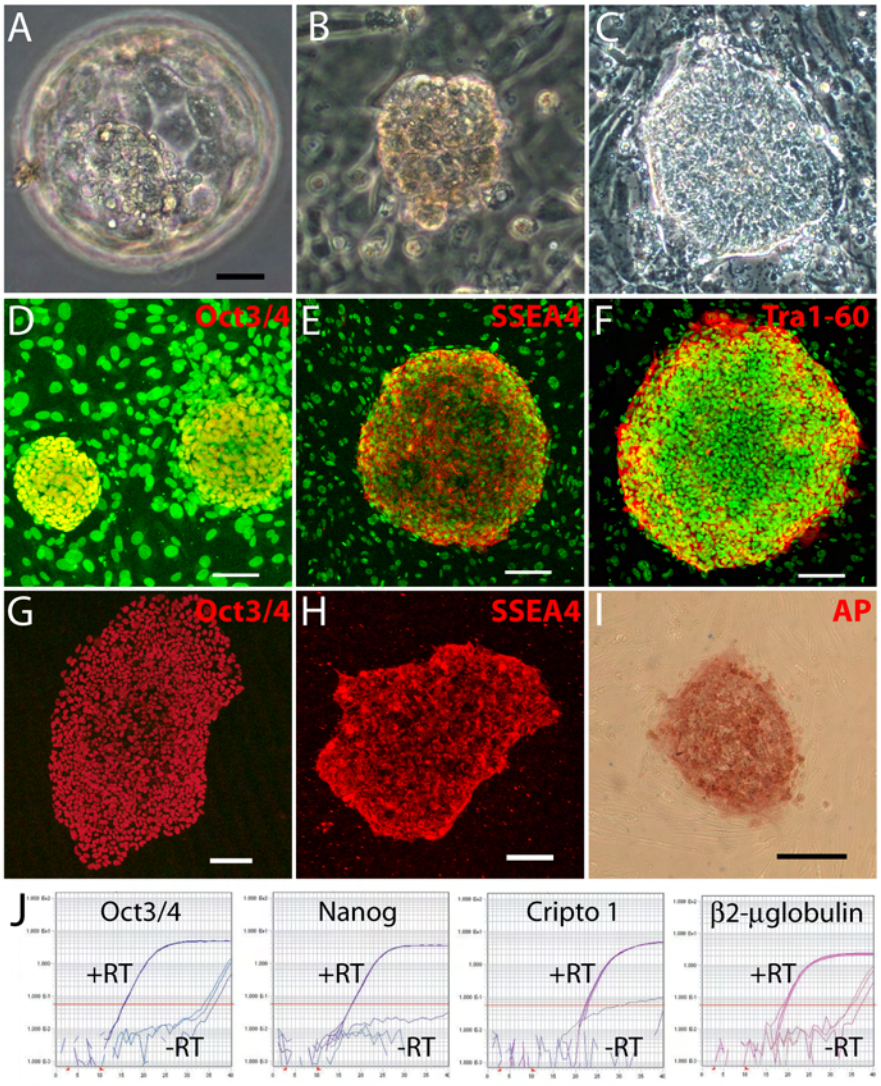

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Figure 2

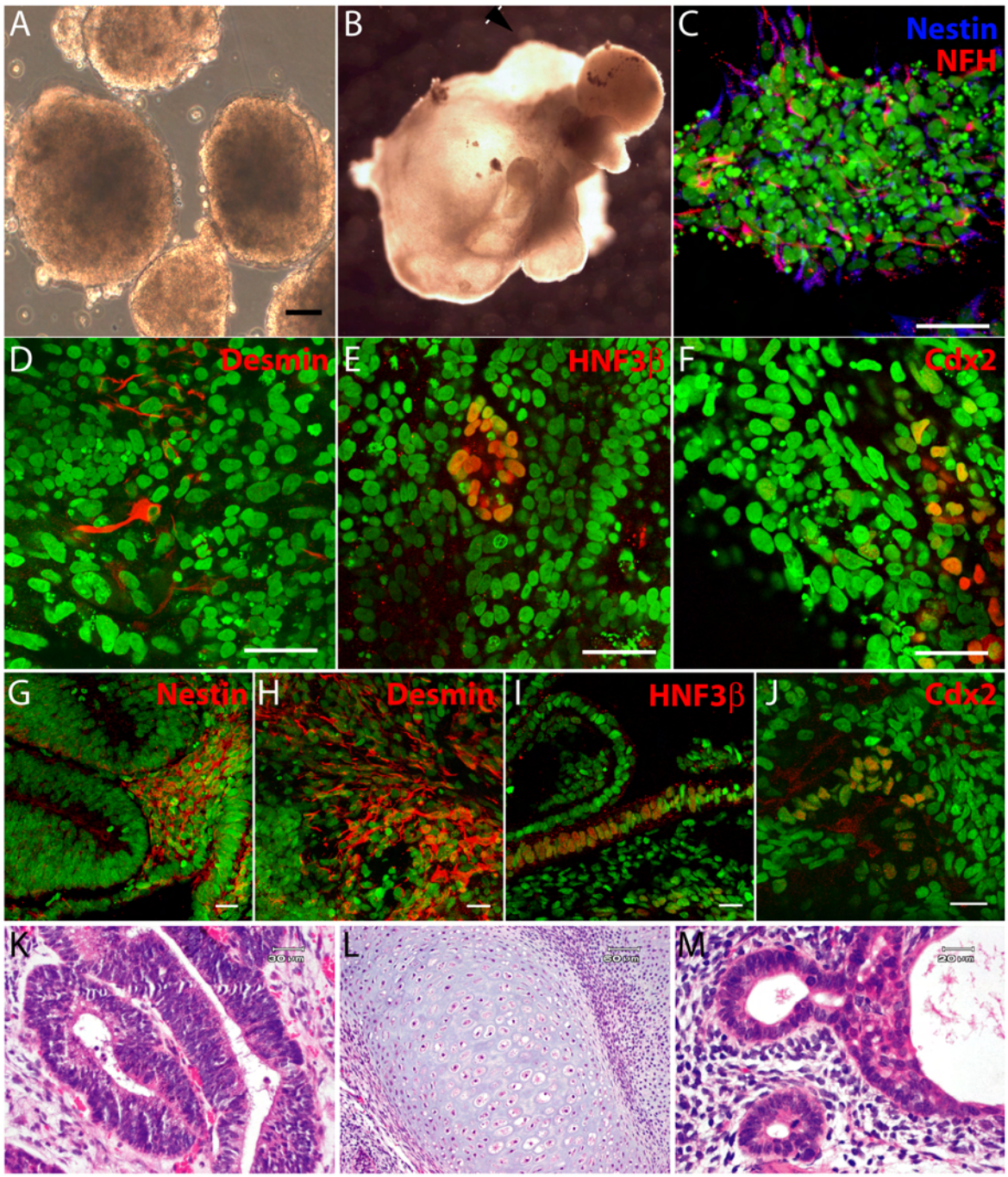

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Figure 3

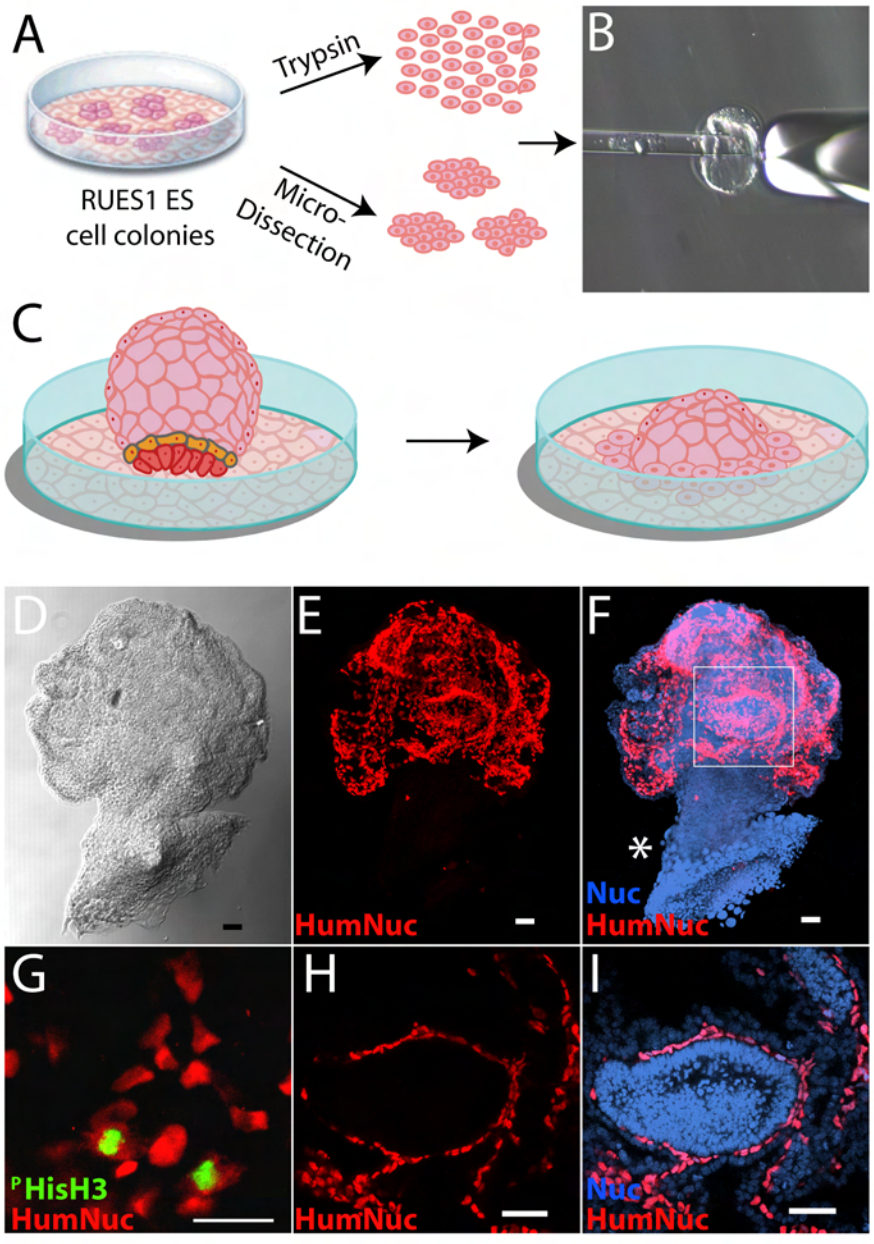

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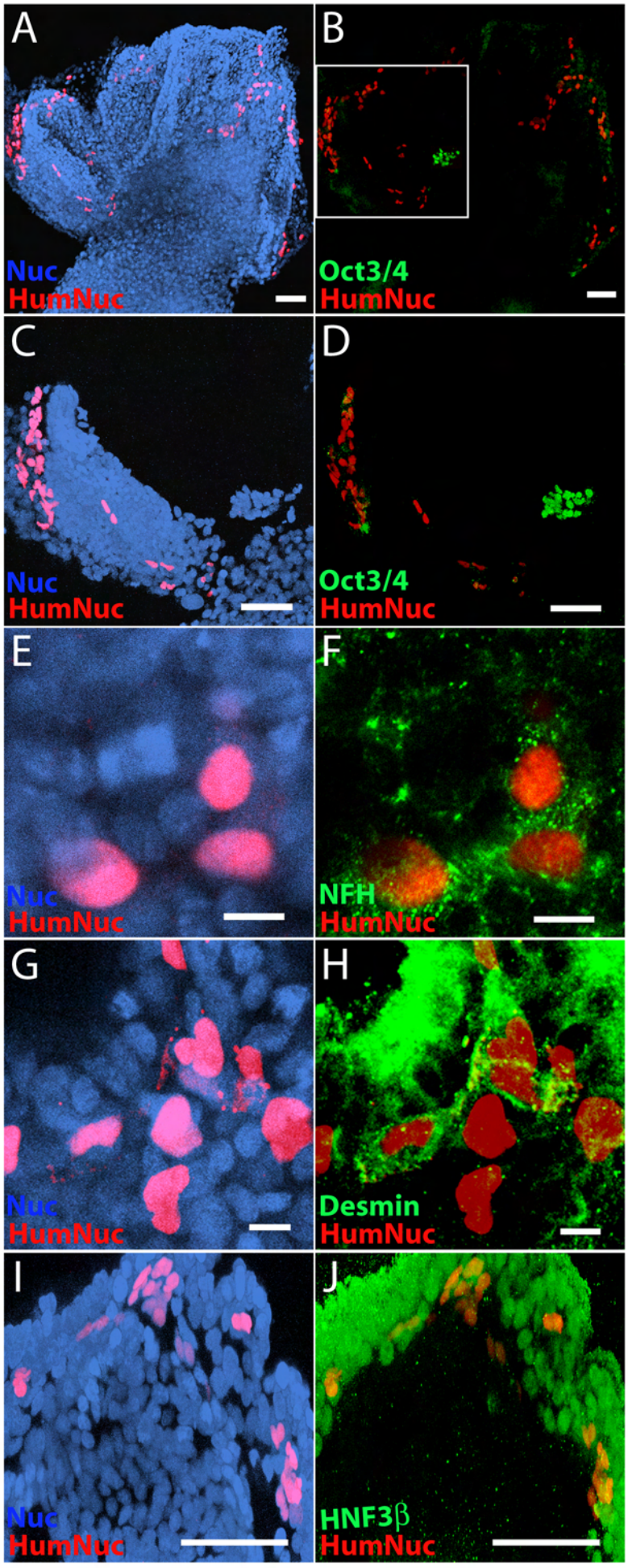
Figure 4



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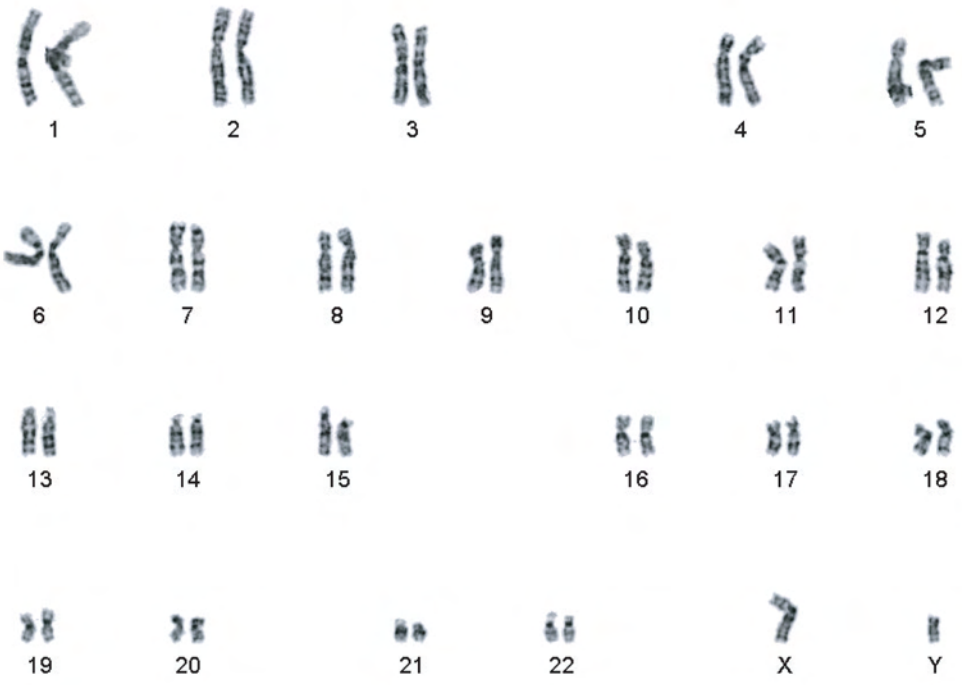
Supplemental Figure 1



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