Trophoblastic hormones direct early human embryogenesis

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Division of the human zygote leads to the formation of the blastocyst containing human embryonic stem cells (hESC) that develop into the embryo. Little is known about the *physiological* signals that direct hESC division and differentiation during early embryogenesis. Although a number of growth factors, including the pregnancy-associated hormone human chorionic gonadotropin (hCG), are secreted by trophoblasts[1-3] that lie adjacent to the embryoblast in the blastocyst, it is not known whether these growth factors directly signal the epiblast. Here we show that hCG promotes the division of embryoblast-derived inner mass cells (hESC), and their differentiation during blastulation and neurulation. Inhibition of LH/hCG receptor (LHCGR) signaling with P-antisense oligonucleotides suppresses hESC proliferation. Similarly, hESC proliferation could be blocked using an antibody against the extracellular activation site of LHCGR, an effect that was reversed by treatment with hCG. hCG treatment rapidly upregulated steroidogenic acute regulatory protein-mediated cholesterol transport and the synthesis of progesterone (P_4), a neurogenic steroid[4,5]. P_4 treatment of hESC colonies induced neurulation as demonstrated by the

expression of nestin and the formation of columnar neuroectodermal cells and their organization into neural tube-like rosettes. Suppression of P_4 signaling by withdrawing P_4 or treating with the P_4 receptor antagonist RU-486 inhibited the differentiation of hESC colonies into embryoid bodies (blastulation) and rosettes (neurulation). These results explain why hESC default towards a neural stem cell fate in culture. Collectively, our findings implicate trophoblastic hCG secretion and signaling via LHCGR on the adjacent embryoblast in the induction of hESC proliferation, and their differentiation into a blastocyst and neurula. This paracrine/juxtacrine signaling of extraembryonic tissues is the commencement of trophic support by trophoblastic (placental tissues) in the growth and development of the human embryo.

Zygotic division into a blastocyst establishes the extra-embryonic tissues (trophoblast layer or outer cell mass) and hypoblast (extraembryonic endoderm) that support the embryonic epiblast (inner cell mass) early in embryogenesis. Trophoblasts secrete an array of hormones[1-3,6] including hCG during the migration of the blastocyst through the fallopian tube and its implantation into the endometrium. The dramatic elevation in the production of hCG by trophoblasts at this early embryonic stage (from 5 to ≥1000 mIU/mL in the maternal serum)[6,7] signals both the corpus lutea and trophoblast[2] to synthesize and secrete P_4[8,9] which is required for the maintenance of the endometrium, blastocyst attachment and synctiotrophoblast proliferation into the endometrium[10].

Given the close spatial localization of the developing trophoblast layer to the epiblast, it is conceivable that trophoblast-associated hormones directly signal the growth and development of the epiblast. Evidence supporting this concept includes our recent observations that, 1) hCG markedly increases hESC expression of the adhesion and neuritogenic protein amyloid-β precursor protein[11], and 2) P_4 signaling is necessary for human blastulation[3]. To examine the functionality of trophoblastic signaling to the epiblast, we examined if the LHCGR was expressed by pluripotent hESC. Full-length mature LHCGR (92-kDa)[6] was detected in hESC and expression was not altered upon differentiation

into embryoid bodies (EBs), which resemble early post-implantation embryos (blastocyst)[12], or into neuroectodermal rosettes, which consist of >90% columnar neural precursor cells (NPC) and are the *in vitro* equivalent of a rudimentary neural tube[13] (Fig. 1a). Decreased Oct-3/4 expression together with brightfield analysis indicated lineage commitment and loss of pluripotency during the differentiation of hESC colonies into rosettes. RT-PCR of RNA extracted from pluripotent hESC confirmed the presence of LHCGR message (data not shown). The comparable level of LHCGR expression between the different cell lineages is suggestive of a basal requirement for LH/hCG signaling during these early stages of embryogenesis.

hCG is mitogenic towards epithelial and endothelial cells of the endometrium and is a marker of carcinogenesis[14]. Treatment of hESC with a physiologically relevant concentration of hCG (500 mIU/mL) in *growth factor-free* TESR1 culture media resulted in a 3.3-fold increase in cell proliferation after 6 d (Fig. 1b), a response that did not vary with hCG concentration (5-50,000 mIU/mL; data not shown). Surprisingly, a similar increase (3.7-fold) in hESC proliferation was observed in growth factor-free TESR1 culture media, suggesting the autocrine production of hCG/LH or other mitogenic factors by hESC. RT-PCR amplification of RNA extracted from pluripotent hESC using sequence specific primers confirmed the presence of both hCGβ isoform V and LHβ message (Fig. 1c). While full length mature 30-kDa LH protein (α-GSU + LHβ subunits) and other variants of LH (47-kDa and 60-kDa)[15] could be detected by immunoblot analysis (Fig. 1d), hCG was not detectable (data not shown) as previously reported[2], suggesting differential translational control of the expression of these gonadotropins.

To examine the requirement for hCG/LH signaling in the proliferation of hESC *in vitro*, we treated hESC with P-antisense oligonucleotides against LHCGR. P-antisense oligonucleotides significantly decreased hESC proliferation (48%) compared to sense oligonucleotide treated hESC (Fig. 2a). To confirm that hESC production of LH or trophoblastic production of hCG promotes hESC proliferation, we treated hESC with increasing concentrations of an antibody against amino acids 15-38 of the extracellular activation site of LHCGR (Fig. 2b)[16]. This antibody significantly reduced hESC

proliferation in a dose-dependent fashion compared to 6 d untreated controls (Fig. 2c). Addition of hCG to hESC treated with this blocking antibody reversed this effect, confirming the specificity of the antibody for the receptor and of hCG signaling for hESC proliferation. The high binding affinities (Kd $\approx$ 0.4-5.5 x 10^{-10} M) of hCG/LH for the human receptor[17,18] indicates that the autocrine production of even low concentrations of these gonadotropins by hESC (or trophoblasts) is sufficient to signal hESC proliferation. A low level of hCG/LH expression by hESC/trophoblasts also is consistent with the low binding capacity (~2.2 fmol/mg tissue) of hCG for LHCGR[17]. In this respect, knockdown of Oct4 expression in hESC induces hCG and Gcm1 expression[19] and indicates any small differentiation of hESC in our cultures may provide sufficient hCG for hESC proliferation. Together, these results suggest the presence of a hCG/LH-dependent mechanism that signals embryonic growth.

hCG functions to increase trophoblast and corpus luteal P_4 production[8,9]. To examine if hCG induces steroidogenesis in hESC as in steroidogenic tissues, we examined the expression of steroid acute regulatory protein (StAR), a key rate-limiting step in the production of sex steroids in reproductive tissues. hESC were found to express StAR mRNA and protein (37-kDa, 30-kDa and 20-kDa variants; Fig. 3a). Since truncation of the 37-kDa to the 30/32-kDa variants of StAR is indicative of increased cholesterol transport across the mitochondrial membrane for steroidogenesis[20], we treated hESC with hCG and measured P_4 secretion into the media. hCG (500 mIU/mL) treatment increased P_4 secretion into the media 15-fold (Fig. 3b). To understand the regulation of sex steroid synthesis by hESC, we treated hESC with increasing concentrations of gonadotropins (Fig. 3c). LHCGR expression was suppressed by treatment with increasing hCG and LH concentrations (Fig. 3c). Similarly, to determine whether gonadotropins regulate StAR expression and processing in hESC, as they do in the gonads[21], hESC were treated with increasing concentrations of hCG. The expression of the 37-, 30- and 20-kDa variants of StAR were dose-dependently suppressed by 6 d of treatment with increasing hCG concentration (Fig. 3d). Importantly, P_4 treatment decreased truncation of the 37-kDa StAR variant (increasing the 37:30-kDa ratio 83%; Fig 3e), indicating mechanisms at the level of both StAR

expression and processing exist to regulate hESC steroidogenesis. Increases in the expression of the mature variant (37-kDa) and decreases in the expression of the truncated (30- and 20-kDa) variants of StAR (Fig. 3a) with differentiation of hESC into EBs and rosettes is consistent with a decreased requirement for steroidogenesis in these more differentiated cell lineages. Together, these results indicate negative feedback pathways exist for the regulation of hCG/LH signaling and cholesterol uptake for the synthesis of sex steroids in hESC and differentiating lineages.

Increasing concentrations of hCG suppress the pluripotent marker Oct-3/4 (Fig. 3c), suggesting hCG, or steroid production initiated by hCG signaling, can direct lineage commitment. To test the effects of sex steroids on hESC proliferation and differentiation, we treated hESC with E_2, P_4, and E_2 + P_4 and observed a significant decrease in cell proliferation by 29%, 16% and 23%, respectively, compared to untreated control (Fig. 4a). These results were consistent with the slight decrease in cell proliferation observed following hCG treatment (Fig. 1b) that induced significant P_4 secretion (Fig. 3d). A screen of germline markers indicated that P_4, and to a lesser extent E_2, increase the expression of nestin, an early marker of NPC formation, in hESC (Fig. 4b). Interestingly, E_2 'priming' has been shown to be required for induction of P_4 receptor (PR) expression in other tissues[22]. Thus, the increase in nestin expression with E_2 treatment may reflect increased PR expression together with endogenous P_4 signaling, and explain the current requirement for serum priming of hESC colonies in the preparation of neuroectodermal rosettes. Previous studies have demonstrated the importance of P_4 and related steroids as neurotrophic agents that promote adult neurogenesis, neuronal survival and neuroprotection[4,5,23].

The induction of nestin expression by P_4 reveals a pivotal function for this pregnancy hormone during ectoderm formation. To confirm the requirement for P_4 signaling in epiblast development, hESC colonies just prior to entering the EB stage were treated with or without the PR antagonist RU-486[24]. When compared to controls, colonies treated with RU-486 failed to form cystic structures (cavitation) and instead formed solid irregular spheres (Fig. 4c) that did not express nestin (data not shown). We next examined the requirement for P_4 signaling during neurulation. hESC colonies grown into a pre-EB

stage were treated with either P_4, RU-486, P_4 + RU-486, or neither. In the presence of P_4, control rosettes displayed a minimum of three rosette structures inside of the cavity (Fig. 4c). Compared to controls, pre-EB's treated without P_4 or with RU-486 retained a spherical shape but failed to form rosettes with columnar neuroectodermal cells after 17 days in culture (Fig. 4c). Morphological changes were more severe in the absence of P_4 than with RU-486. That neuroectoderm failed to form was confirmed by the absence of nestin expression in RU-486 treated compared to P_4 treated pre-EBs (Fig. 4d). These results indicate the obligatory role of P_4 signaling in gastrulation and neurulation during early embryogenesis.

hCG/LH signaling via the LHCGR increases hESC proliferation, but also P_4 synthesis that decreases cell proliferation and promotes differentiation. At what point these functions bifurcate, and what other factors regulate hCG-induced proliferation versus hCG-induced P_4 mediated differentiation (e.g. BMP signaling) remain to be determined. Interestingly, hESCs default towards a primitive neural stem cell fate if maintained for any length of time in culture[25]. Since hESC express gonadotropins (Fig. 1c), and hCG signaling promotes P_4 production (Fig. 3d) which induces lineage commitment towards a neuroectodermal phenotype (Figs. 4b, c), we tested whether hCG might act to differentiate hESC toward a neuronal lineage. hCG treatment induced nestin expression (205-kDa variant) in hESC (Fig. 4e), indicating endogenous gonadotropin production by hESC (Fig. 1) or trophoblastic cells[2] may be sufficient for NPC formation, thereby explaining the *extrinsic* hormonal signals regulating the 'default pathway' of hESC differentiation into neuronal lineages[25].

These results suggest that trophoblastic hCG production adjacent to the embryoblast is required not only for trophoblast steroidogenesis and attachment to the uterine wall, but also for signaling normal growth and development of the epiblast. While the structural importance of P_4 and alloprogesterone has previously been recognized by its early synthesis (by at least day 13) within the developing rat central nervous system[26], our results demonstrate an early (within the first 7 days) and absolute requirement for P_4 during blastulation and neurulation as indicated by the findings that, 1) RU-486 prevents normal

cavitation of hESC colonies and columnar neuroectodermal rosette formation, 2) RU-486 prevents nestin expression, 3) P_4 induces nestin expression in hESC, 4) P_4 withdrawal from pre-EBs inhibits neuroectodermal rosette formation, and 5) hCG upregulates StAR processing for cholesterol transport and P_4 synthesis and secretion. Thus, these results suggest that paracrine/juxtacrine signaling of hCG for mobilization of cholesterol for P_4 production by the epiblast/synctiotrophoblast following conception is essential for blastulation and neurulation. Conversely, suppression of P_4 signaling at this time (e.g. with RU-486)[27] will block these time-sensitive developmental processes. The requirement of P_4 during cavitation processes indicates the structural influence of these molecular pathways on the developing embryo within the first 7 days, but also on the formation of the neural tube at around day 17-19, which will influence future neural connectivity. Thus, there exists a critical molecular signaling link between trophoblastic (and/or maternal) hormone production and early embryonic growth and development.

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Acknowledgments

Dr. Porayette was the recipient of a research fellowship from the American Foundation for Aging Research. Ms. Kaltcheva was the recipient of a Hilldale Research Scholarship. The authors thank the WiCell Research Institute for providing hESC lines and technical support.

Author Contributions

M.J.G. and P.P cultured, maintained, differentiated and treated hESC, EBs and rosettes, performed cell proliferation, immunoblot and morphological analyses, analyzed data and contributed to writing of the paper; M.M.K. cultured, maintained, differentiated and treated hESC, EBs and rosettes and analyzed data; S.V.M. was involved in study design, immunoblot analyses and data analysis; R.L.B. was involved in study design; C.S.A. conceived the study, designed experiments, analyzed data and wrote the paper. M.J.G. and P.P. contributed equally to the study. All authors discussed the results and commented on the paper.

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FIGURE LEGENDS

Figure 1. hESC, EBs and rosettes express LH/hCG ligands and receptor. (a) Pluripotent H9 hESC (passage 22–32; XX karyotype; also known as WA09, a National Institutes of Health registered line) were used to generate embryoid bodies (EB) and neural precursor cells (NPC) as previously described[3,11,28]and cell lysates were analyzed by immunoblot with **(i)** an affinity purified anti-human LH/hCG receptor polyclonal antibody generated against the N-terminal 15-38 amino acids (New England Peptides, GA, USA); **(ii)** a monoclonal antibody against the human POU5F1 transcription factor Oct-3/4 (C-10; against 1-134 amino acids of human Oct-4; Santa Cruz Biotechnologies, CA), and **(iii)** monoclonal antibodies against human C-11 β-actin (Santa Cruz, CA, USA) and human V-18 GAPDH (Santa Cruz, CA, USA)[11,3]. **(b)** hESC were cultured in growth factor free TESR1 media ± hCG (500mIU/mL) for 6 d and cell proliferation measured using the trypan blue assay. Results are expressed as mean ± SEM, n = 3 (*$p<0.05$, **$p<0.005$ compared to 6 d Control). **(c)** Total RNA isolated from hESC was amplified by RT-PCR using sequence specific primers for LHβ and isoform V of hCGβ. The expected LHβ band of 93 bp spanning exon 2 and 3 and 90 bp hCGβ of isoform V are shown. The sequence of the amplified cDNA matched the genomic sequence (minus the intronic sequences) demonstrating that the amplified cDNA was from LHβ and hCGβ mRNA templates. A molecular weight ladder is shown in the center. **(d)** Protein from cell lysates of hESC were analyzed by immunoblot using a polyclonal antibody generated against the entire β-subunit of the LH glycoprotein (Dr. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA, USA) as previously described[15].

Figure 2. Endogenously produced hCG signals via LH/hCG receptors for hESC proliferation. (a) hESC were treated with lipofectamine (control), lipofectamine + LHCGR sense oligonucleotides or lipofectamine + LHCGR P-antisense oligonucleotides for 6 days and the cells then counted using the trypan blue method. For experiments using oligomers with phosphorothioate bonds (antisense-P;

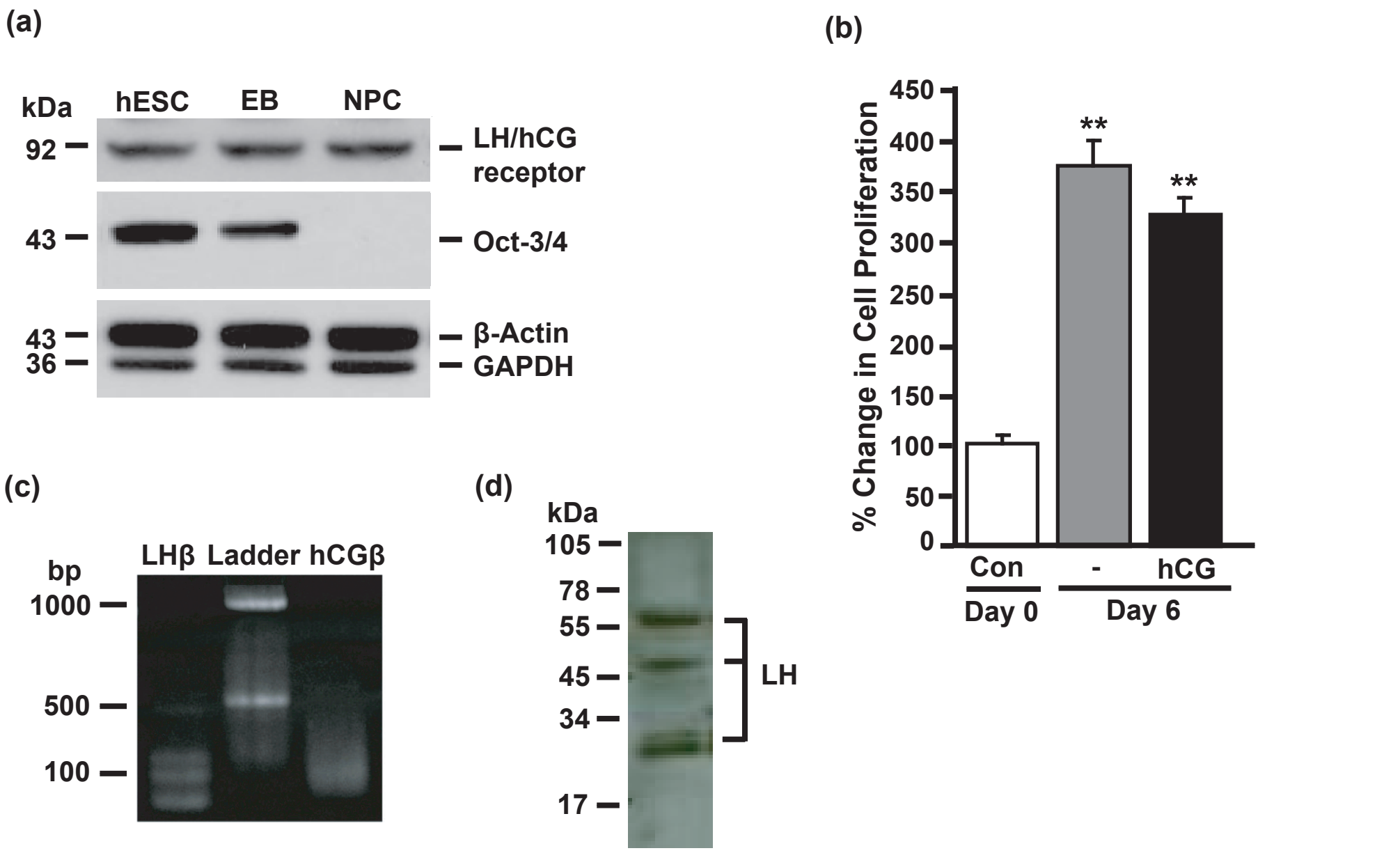
Integrated DNA Technology, Coralville, IA), oligomers were added to media (240 µl) that had been preincubated with lipofectamine (4 ng/µl; Invitrogen Corporation, Carlsbad, CA) for 5 min. at room temperature. This mixture was then incubated at room temperature for 20 min. prior to addition to cells. Antisense-P was used at a final concentration of 0.4 µM. LHCGR antisense-P: 5'-TCCAGTTCAGAGTCCCATT TC-3', 21nt, 49% G/C; Sense 5'-GAAATGG GACTCTGAACTGGA-3. Results are expressed as mean ± SEM, n = 4; significant differences between groups are indicated by different letters, $P < 0.05$). **(b)** Schematic of the LH/hCG receptor activation site and binding site of rabbit polyclonal antibody against amino acids 15-38 of the extracellular binding domain of LH/hCG receptor (New England Peptides, GA, USA). **(c)** hESC grown in 6-well plates coated with Matrigel™ in mTeSR1 media were treated for 6 d with, i) hCG (500 mIU/mL; lane A; Ray Biotech Inc., GA, USA), ii) increasing concentrations of the affinity purified rabbit polyclonal antibody against amino acids 15-38 of the extracellular binding domain of LH/hCG receptor (1:1000, 1:200, 1:100; lanes B, C, D, respectively), and iii) in combination (lanes E, F, G). Cell number was counted. Results are expressed as mean ± SEM, n = 3 (*$p<0.05$, **$p<0.005$ compared to d 6 control).

Figure 3. hCG induces P_4 synthesis and secretion from hESC. (a) Total RNA isolated from hESC was amplified by RT-PCR using 2 pairs of sequence specific primers for StAR. The expected 404bp and 408bp cDNA fragments were detected. The sequence of the amplified cDNA matched the expected lengths of the StAR gene. Equal amounts of protein from cell lysates of hESC, EBs and rosettes were analyzed by immunoblot with an anti-human StAR polyclonal antibody (Dr. Strauss, University of Pennsylvania[29]). Three bands were identified at 37-, 30-, and 20-kDa. **(b)** hESC were treated with hCG (500 mIU/mL) in TESR1 media each day for 6 d, the media collected and pooled each day (15 mL total), lyophilized and resuspended in 2 mL of TESR1 media for ELISA of P_4 (Cayman Chemical Company, MI, USA). Results are expressed as µg P_4/µg cellular protein (mean ± SEM, n = 3, *t*-test, *$p<0.001$). **(c)** Equal amounts of protein from cell lysates of hESC grown in mTeSR1 media and

treated with LH (5, 10, and 100 mIU/mL; National Peptide Hormone Program, Harbor-UCLA, USA) or hCG (50, 500, and 5,000 mIU/mL; Ray Biotech Inc., GA, USA) for 6 d were analyzed by immunoblot with antibodies against LH/hCG receptor and Oct-3/4 expression as described in Fig. 1. **(d)** hESC cultured for 6 d in mTeSR1 media were treated with hCG (0, 5, 50, 500, 5,000, 50,000 mIU/mL) and equal amounts of protein from cell lysates analyzed by immunoblot for StAR as described above. **(e)** hESC were treated with and without P_4 (2 μM) and/or RU-486 (20 μM; Sigma Laboratories, St. Louis, MO) for 5 d. The colonies were collected and equal amounts of protein from cell lysates were analyzed by immunoblot for StAR, β-actin and GAPDH as described earlier.

Figure 4. P_4 promotes hESC blastulation and neurulation. (a) hESC were grown in lithium free TESR1 media in the presence of E_2 (10 nM), P_4 (2 μM) or E_2 (10 nM) + P_4 (2 μM) for 6 d and cell proliferation quantitated using the trypan blue assay. Results are expressed as mean ± SEM, n = 4 (*$p<0.05$, **$p<0.005$ compared to 6 d control). **(b)** Equal amounts of protein from cell lysates of hESC treated for 9 d as described above were analyzed by immunoblot using a monoclonal antibody against nestin (clone 10C2; Chemicon, CA, USA). **(c)** EB formation: hESC were allowed to form colonies by culturing for 4 d on an MEF feeder layer, then were enzymatically lifted and the colonies placed into EB media (containing serum) in the absence (control) or presence of RU-486 (20 μM) and rocked gently for an additional 10 d to allow EB formation. Structures were then assessed morphologically. Rosette formation: hESC were allowed to form colonies by culturing for 4 d on an MEF feeder layer, colonies were then enzymatically lifted and placed into EB media (containing serum) and rocked gently for an additional 4 d. Colonies were then placed in neural induction media with and without P_4 (2 μM) or RU-486 (20 μM) for an additional 11 days. At 19 d the structures were analyzed morphologically, the structures collected and equal amounts of protein from cell lysates were analyzed for nestin **(d)** by immunoblot analysis. The relative binding affinity of RU-486 for the PR is twice that of P_4[30], and is used at a dose of 200–600 mg for the termination of pregnancies (this equates to ~6–19 μM, equivalent to that

used in our study (20 μM)[24]. **(e)** hESC were grown in mTeSR1 media and treated with 500 mIU/mL hCG for 8 days. Cells were collected, protein content determined using the BCA assay, and equal amounts of protein run on SDS-PAGE and the immunoblot probed for human nestin.


(a)
kDa
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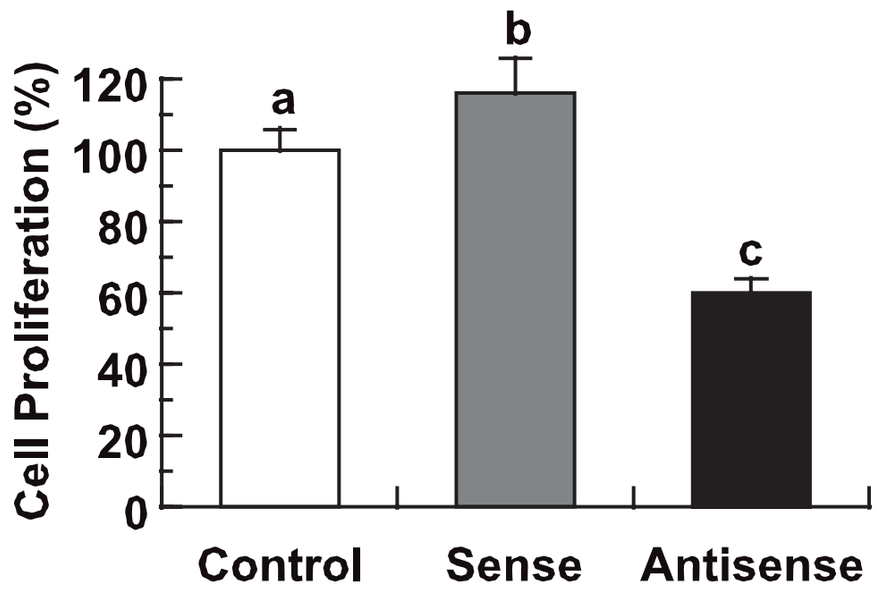
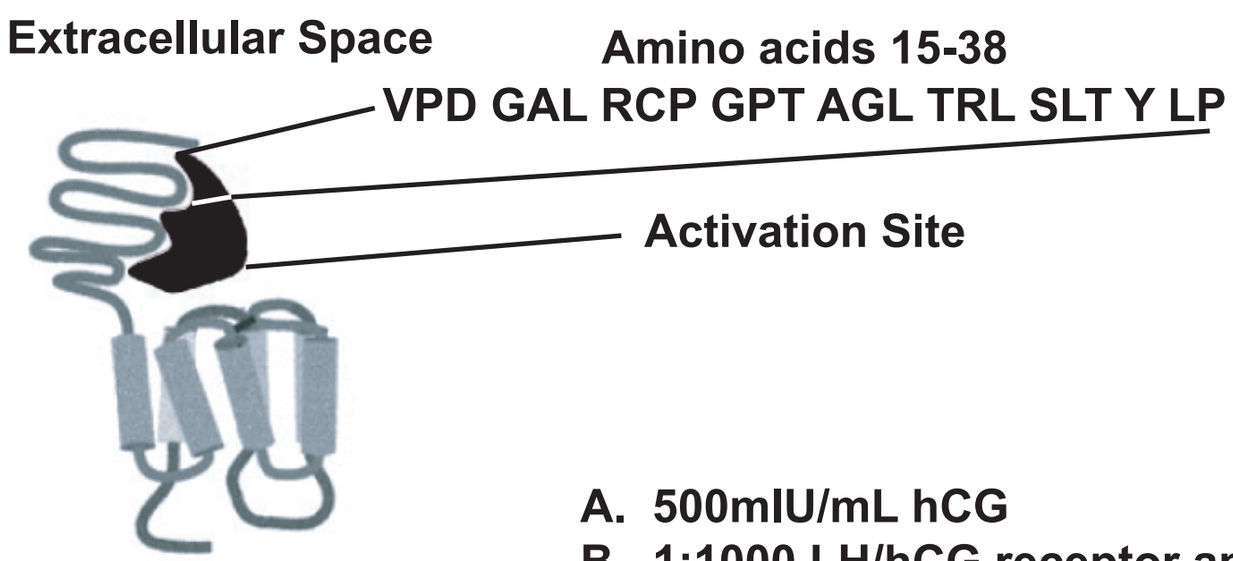
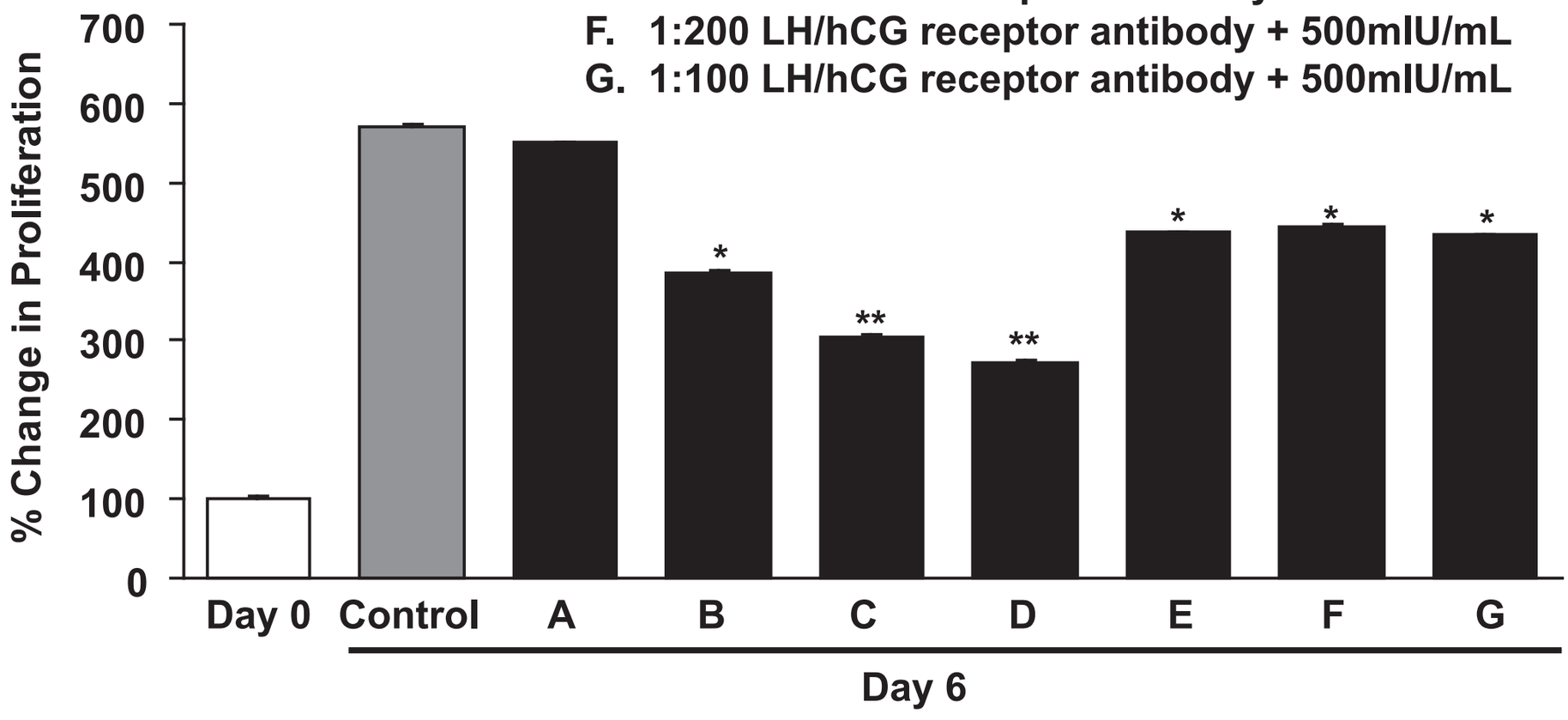


(b)

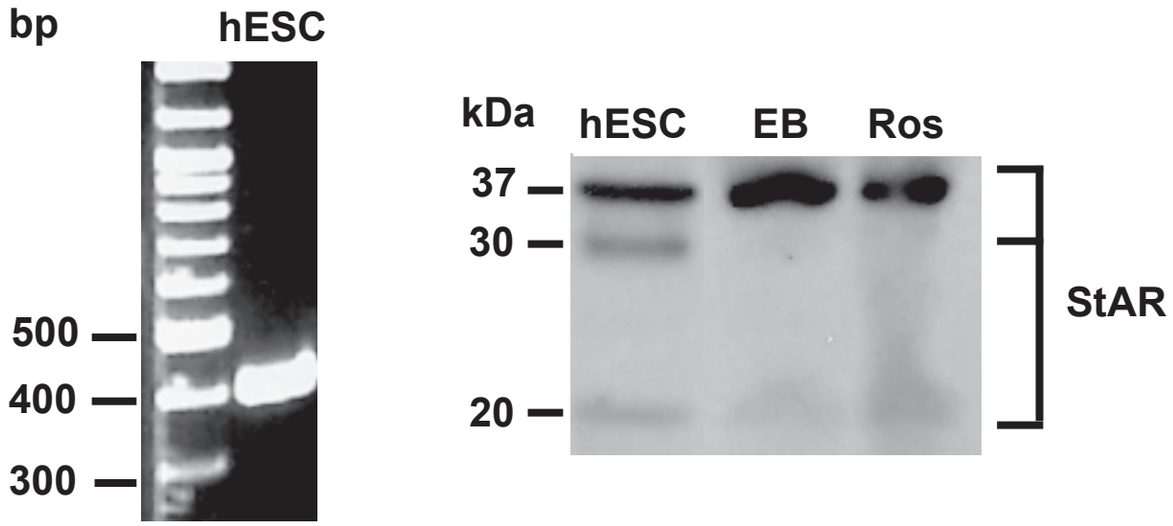


A. 500mIU/mL hCG
B. 1:1000 LH/hCG receptor antibody
C. 1:200 LH/hCG receptor antibody
D. 1:100 LH/hCG receptor antibody
E. 1:1000 LH/hCG receptor antibody + 500mIU/mL
F. 1:200 LH/hCG receptor antibody + 500mIU/mL
G. 1:100 LH/hCG receptor antibody + 500mIU/mL

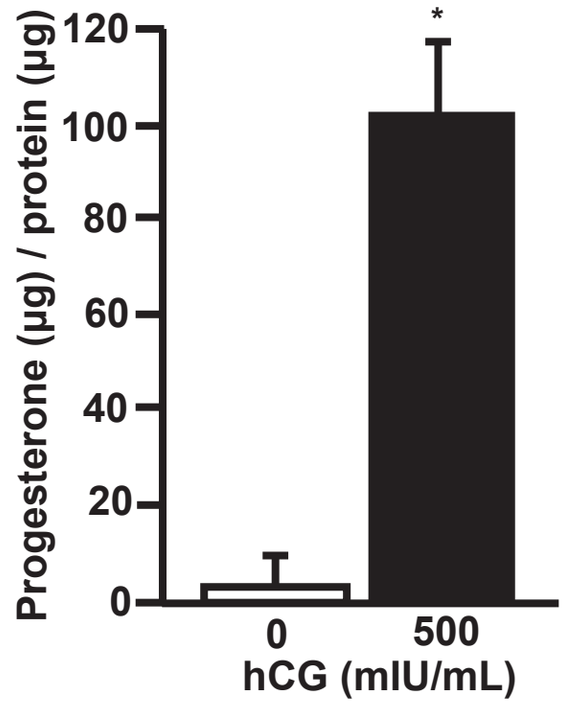
(c)



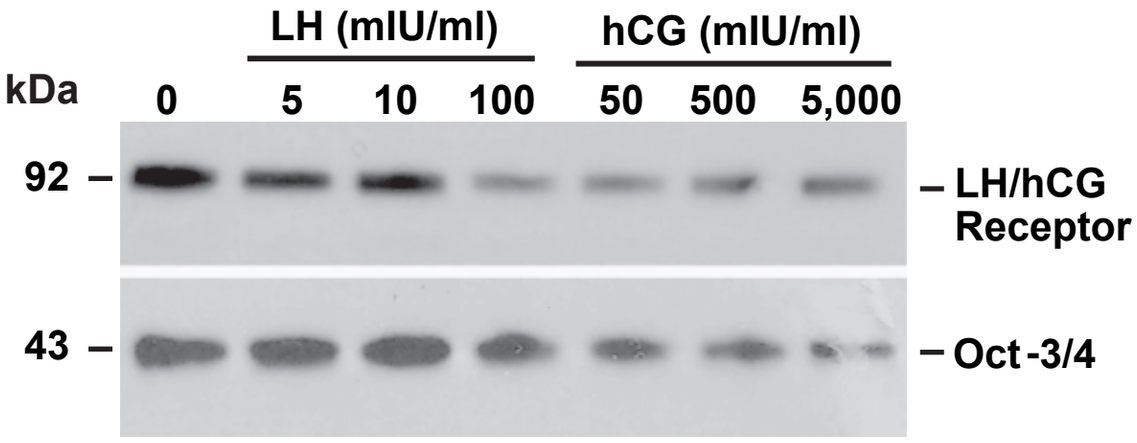
(a)


bp
hESC
500
400
300
kDa
hESC
EB
Ros
37
30
20
StAR

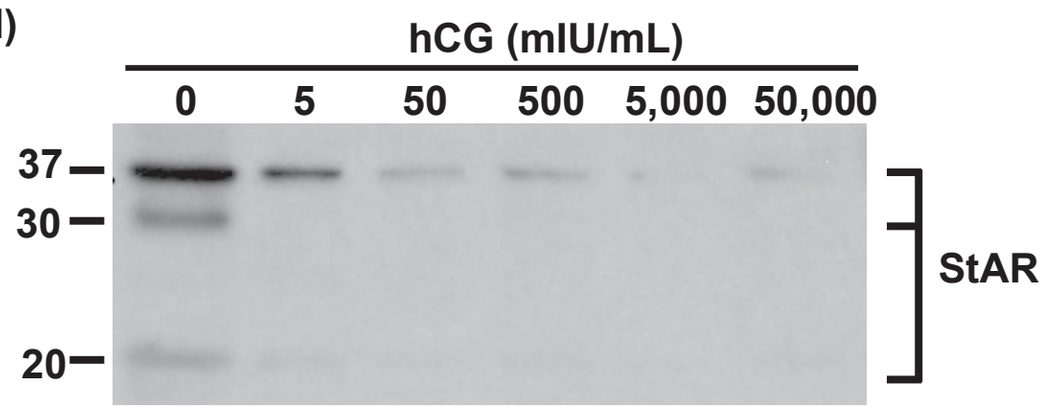
(b)


Progesterone (µg) / protein (µg)
120
100
80
60
40
20
0
*
0
500
hCG (mIU/mL)

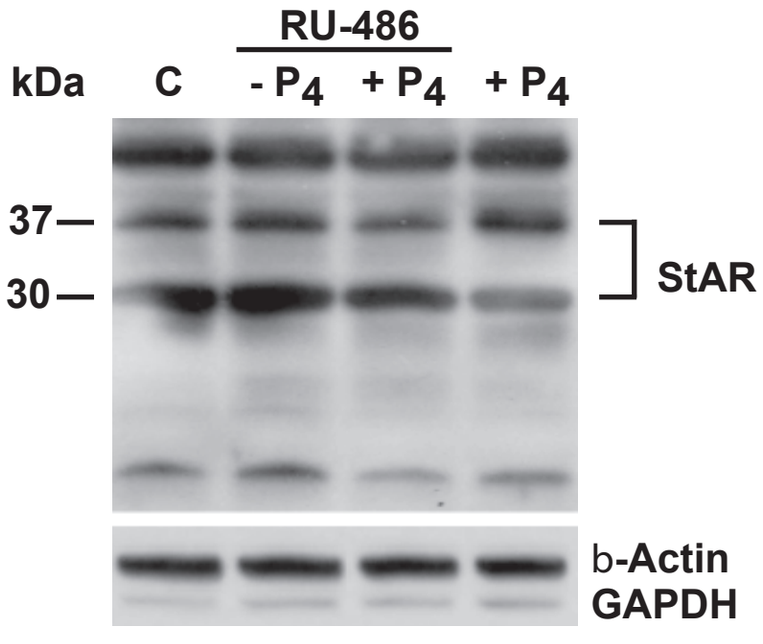
c)


LH (mIU/ml)
hCG (mIU/ml)
kDa
0
5
10
100
50
500
5,000
92
– LH/hCG Receptor
43
– Oct-3/4

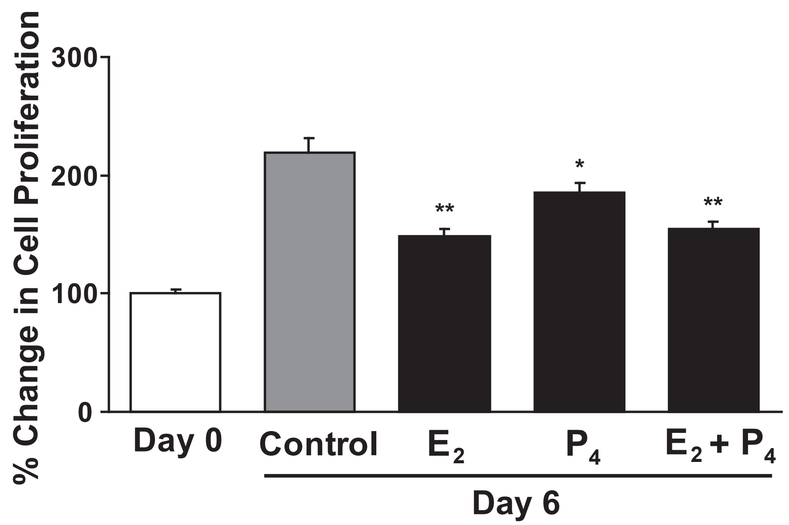
(d)


hCG (mIU/mL)
0
5
50
500
5,000
50,000
37
30
20
StAR

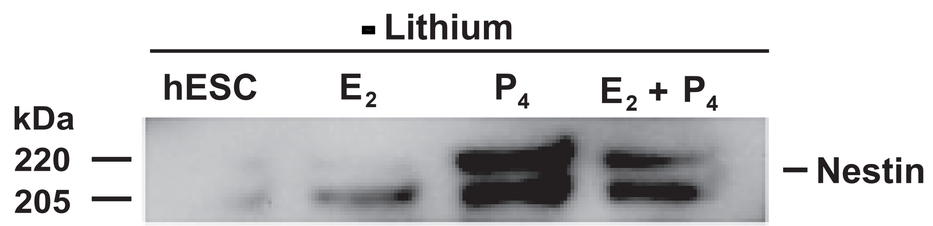
(e)


RU-486
kDa
C
- P4
+ P4
+ P4
37
30
StAR
b-Actin
GAPDH

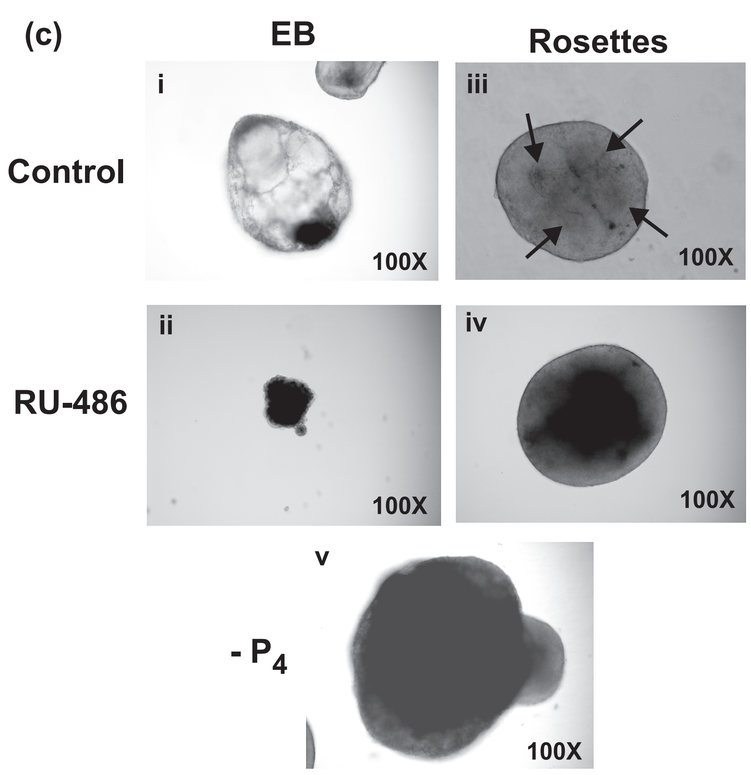
(a)


% Change in Cell Proliferation
300
200
100
0
**
*
**
Day 0
Control
E2
P4
E2 + P4
Day 6

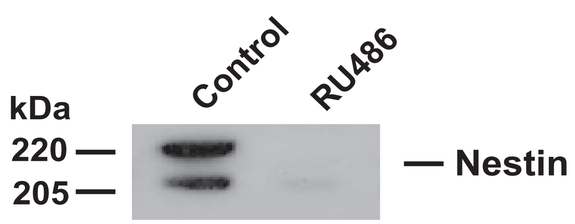
(b)


- Lithium
hESC
E2
P4
E2 + P4
kDa
220
205
Nestin

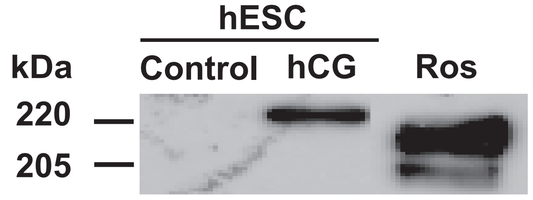
(c)


EB
Rosettes
Control
RU-486
- P4
i
ii
iii
iv
v
100X
100X
100X
100X
100X

(d)


Control
RU486
kDa
220
205
Nestin

(e)


hESC
kDa
Control
hCG
Ros
220
205