STEM CELLS AND DEVELOPMENT XX:1–4 (2008)
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DOI: 10.1089/scd.2008.0190

# Opioid and Progesterone Signaling is Obligatory for Early Human Embryogenesis

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The growth factors that drive the division and differentiation of stem cells during early human embryogenesis are unknown. The secretion of endorphins, progesterone ($P_4$), human chorionic gonadotropin, 17$\beta$-estradiol, and gonadotropin-releasing hormone ~~[1]~~ by trophoblasts that lie adjacent to the embryoblast in the blastocyst suggests that these pregnancy-associated factors may directly signal the growth and development of the embryoblast. To test this hypothesis, we treated embryoblast-derived human embryonic stem cells (hESCs) with ICI 174,864, a $\delta$-opioid receptor antagonist, and RU-486 (mifepristone), a $P_4$ receptor ~~(PR)~~ competitive antagonist. Both antagonists potently inhibited the differentiation of hESC into embryoid bodies, an in vitro [illegible]cture akin to the blastocyst containing all three germ layers. Furthermore, these agents prevented the differentiation of hESC aggregates into columnar neuroectodermal cells and their organization into neural tube-like rosettes as determined morphologically. Immunoblot analyses confirmed the obligatory role of these hormones; both antagonists inhibited nestin expression, an early marker of neural precursor cells normally detected during rosette formation. Conversely, addition of $P_4$ to hESC aggregates induced nestin expression and the formation of neuroectodermal rosettes. These results demonstrate that trophoblast-associated hormones induce blastulation and [illegible]rulation during early embryogenesis.

## Introduction

Trophoblastic production of endorphins [1] suggests important, yet undefined, roles for this hormone during embryonic growth and development. Trophoblasts also have been reported to secrete an array of other pregnancy-associated hormones including progesterone ($P_4$), human chorionic gonadotropin (hCG), 17$\beta$-estradiol, and gonadotropin-releasing hormone (GnRH) [1]. Given the close spatial localization of trophoblasts to the embryoblast, it is conceivable that these pregnancy-associated factors may directly signal the growth and development of the embryoblast. Evidence supporting this notion includes the presence of placental opioid-enhancing factor in amniotic fluid and placenta~~, and that the ingestion of placenta potentiates $\delta$- and $\kappa$-opioid antinociception~~ [2]. Likewise, trophoblastic and corpa luteal production of hCG/$P_4$ is markedly elevated postconception and is obligatory for the maintenance of pregnancy [3]. This pilot study was therefore undertaken to determine if ~~blocking~~ opioid receptor and $P_4$ receptor (PR) signaling would affect early embryonic growth and development. Our results indicate that both opioid and progesterone signaling are vital for normal blastulation and neurulation.

## Methods ~~and Materials~~

### *Human embryonic stem cell culture*

Pluripotent H9 human embryonic stem cells (hESC; passage 29–33 XX karyotype; also known as WA09) obtained from WiCell Research Institute (Madison, WI, USA) were cultured on irradiated mouse embryonic fibroblast (MEF) feeder cells (Bio~~-~~vintage, CA, USA) plated on gelatin-coated wells and grown in Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with 20% Knockout™ Serum Replacement (KOSR), 1% nonessential amino acids (NEAAs), 1 mM L-glutamine, 4 ng/ml basic fibroblast growth factor (bFGF) (all from Invitrogen, Carlsbad, CA, USA) and 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA).

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hESC cultured in TESR1 media (lacking Li) ~~were~~ treated with $P_4$ [2 µM; stock $P_4$ was solubilized in dimethyl sulfoxide (DMSO) and then diluted into media] or control (the equivalent volume of DMSO) for 9 days and cells collected for the measurement of nestin expression by immunoblot analysis as previously described [4,5].

### *Embryoid body formation*

H9 hESC colonies were cultured for 4 days on an MEF feeder layer as described above. Intact colonies were enzymatically detached and cultured on an orbital shaker at 5% $CO_2$ in medium containing Iscove's modified Dulbecco's medium and 20% fetal bovine serum (embryoid body media; both from Invitrogen) with or without ICI 174,864 (0.1 mM; Tocris Bioscience, Ellisville, MO, USA; stock ICI 174,864 was solubilized in water) and/or RU-486 (20 µM; Sigma Laboratories, St. Louis, MO, USA; stock RU-486 was solubilized in EtOH and then diluted into media) for another 10 days (when embryoid body formation occurs under normal conditions—day 14). Controls were treated with the equivalent volume of water or EtOH. Structures were then examined morphologically and the area of the structures quantitated using ~~the~~ Image J Software (http://rsb.info.nih.gov/ij/).

### *Neuroectodermal cell formation*

~~The protocol below for the differentiation of~~ hESC into columnar neuroectodermal cells ~~is~~ described elsewhere [6] ~~and~~ mimics in vivo [illegible]roectodermal development in terms of timing and morp[illegible]ology ~~as previously described~~. In brief, hESC colonies were removed intact from MEF layer and were cultured in a special hESC growth medium (78.5% DMEM-F12, 20% KOSR, 1% NEAA, 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol) for 4 days with daily replacement of media to form hESC aggregates. ~~They~~ were then adhered to the culture surface where they form~~ed~~ monolayer colonies in a chemically defined neural induction medium [32.6% F-12, 65.2% DMEM, 1% N2 supplement, 1% NEAA, 10 ng/ml bFGF (all from Invitrogen), 0.2% of 1 mg/ml Heparin (Sigma-Aldrich)]. Under this culture condition, columnar neuroectodermal cells appear in the center of each colony and organize into neural tube-like rosettes after a total of 9–10 d of differentiation culture. The neural induction media was replaced every other day. The neuroectodermal cells in the rosettes were selectively isolated through differential enzymatic treatment using dispase (0.5 mg/ml in DMEM-F12) and incubated for 2 h in neural induction medium to allow the nonneural cells to differentially attach to the flask. ~~After this, t~~he floating cells (mostly aggregates of neuroectodermal cells) were transferred to new flasks where they rolled up to form round clusters. For treatments, cells were cultured in ~~a similar fashion~~ in the presence of RU-486 (20 µM) and/or ICI 174,864 (0.1 mM), and then examined morphologically and the cells collected for measurement of nestin expression by immunoblot analysis [4,5].

## AQ1 ults and Discussion

To investigate opioid signaling during early human embryogenesis, we utilized hESC derived from the inner cell mass of the blastocyst as a model of early embryogenesis [4,5]. Treatment of hESC colonies with the δ-opioid receptor–selective antagonist ICI 174,864 [7,8] for 10 days inhibited the formation of the embryoid body cystic structure, and instead formed nonspherical structures that were ~40% the size of normal spheroidal embryoid bodies (Fig. 1A and B). ICI 174,864 also inhibited normal neuroectodermal rosette formation from hESC, inducing a more dense morphology compared with control rosettes (Fig. 1A). To confirm that blocking opioid signaling inhibits hESC differentiation into neuroectodermal cells, the treated hESC were collected for immunoblot analysis and probed with a monoclonal antibody against human nestin, an early marker of neural precursor cell formation. Two bands representative of nestin (205 and 220 kDa) were detected in control rosettes, but neither band was detected in cell aggregates treated with ICI 174,864 (Fig. 1C). These results indicate that ICI 174,864, in addition to disrupting the normal development of hESC into embryoid bodies, also inhibits ~~neural tube~~ formation demonstrating that δ-opioid receptors are required for normal human blastulation and neurulation.

δ-Opioid antagonists may function to inhibit embryogenesis by regulating hCG release [9] required for $P_4$ production. Trophoblastic and corpa luteal production of hCG/$P_4$ is markedly elevated postconception and is obligatory for the maintenance of pregnancy [3]. Inhibition of $P_4$ signaling using the PR antagonist RU-486 is known to result in endometrial decidual degeneration, trophoblast detachment and decreased hCG production from the syncytiotrophoblast, and $P_4$ production from the corpus luteum [10]. In addition, RU-486 induces cervical softening and dilatation, release of endogenous prostaglandins and an increase in myometrial sensitivity to the contractile effects of prostaglandins leading to the expulsion of the embryo/fetus. Although these abortive actions of RU-486 are well demonstrated, the effect of suppressing $P_4$ production with RU-486 on the development of the early human embryo has not been studied.

Based on the above observations that opioid signaling is important for embryogenesis and the relationship between opioid signaling and $P_4$ production [9], we tested the relevance of $P_4$ signaling in early embryogenesis using RU-486. Treatment of hESC colonies with RU-486, like ICI 174,864, prevented the development of embryoid bodies (Fig. 1A); hESC colonies failed to form normal cystic structures after 10 days in culture, and instead formed solid irregular spheres that were ~20% the size of normal spheroidal embryoid bodies (Fig. 1B). Since [illegible]o is a known neurotrophic factor in the adult brain **[11]**, we examined whether PR signaling ~~was~~ required for neurogenesis. RU-486 treatment of hESC colonies ~~for 17 days~~ blocked the normal differentiation of hESC into neuroectodermal rosettes cultured in neural induction media containing $P_4$ (Fig. 1A; RU-486 is a competitive receptor antagonist in the presence of $P_4$). This was confirmed by the suppression of nestin expression in RU-486 ~~treated~~ compared to $P_4$-treated hESC aggregates (Fig. 1C). These results indicate the obligatory role of PR signaling in normal embryoid body and neuroectodermal rosette formation during early embryogenesis. To test whether $P_4$ ~~was~~ directly required for neuroectodermal rosette formation, we treated hESC aggregates with $P_4$ and

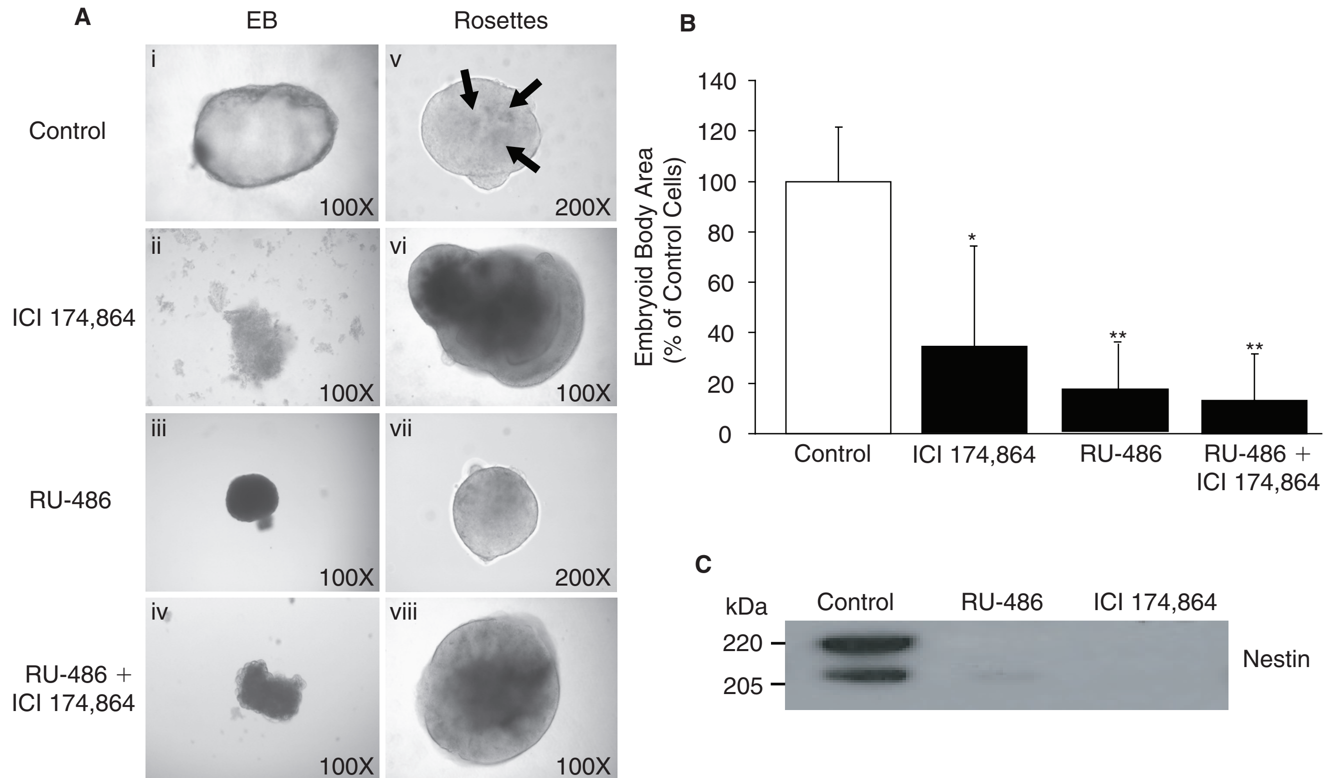


**FIG. 1.** RU-486 and ICI 174,864 prevent embryoid body and neuroectodermal rosette formation. (**A**) Embryoid body formation: H9 hESC colonies (day 4) were cultured in the presence of (1) embryoid body media containing (2) RU-486 (20 μM), (3) ICI 174,864 (0.1 mM) or (4) RU-486 (20 μM) + ICI 174,864 (0.1 mM). Colonies were followed for 10 days (when embryoid body formation occurs under normal conditions—day 14) and then examined morphologically. Rosette formation: H9 hESC colonies (day 4) were cultured in hESC growth media for 4 days before being placed in (5) neural induction media containing (6) RU-486 (20 μM), (7) ICI 174,864 (0.1 mM) or (8) RU-486 (20 μM) + ICI 174,864 (0.1 mM). Colonies were assessed morphologically at 17+ days (when rosette formation occurs). Control structures typically display a minimum of three rosettes within the neuroectodermal aggregate (arrows). Figures are representative examples of each treatment and have been converted to grayscale. (**B**) Embryoid body size was quantified using Image J Software. Results are expressed as mean ± SEM, $n = 6$ ($^*p < 0.05$, $^{**}p < 0.01$ compared to control). (**C**) Structures in (Av-viii) were collected and an equal amount of protein run on SDS-PAGE and the immunoblot probed with the 10C2 monoclonal antibody against human nestin (Chemicon, CA, USA). ~~For details of all methodology see Materials and Methods.~~

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measured nestin expression. $P_4$ induced the expression of the 205- and 220-kDa forms of nestin (Fig. 2) confirming the requirement for $P_4$ signaling in the formation of neuroectodermal rosettes. Together, these results suggest that the upregulation of trophoblastic $P_4$ production following conception [3] is required not only for trophoblast attachment, but also for the normal development of the embryo. These results also reveal for the first time the basis for why neural induction media induces neural precursor cell formation; the N2 supplement used in the neural induction media contains $P_4$.

The relative binding affinity of RU-486 for ~~the~~ PR is twice that of $P_4$ [12], 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; ref. 13). Thus, blocking PR signaling with RU-486 during early pregnancy will block blastulation and neurulation.

Previous data has implicated $P_4$ as acting in the arcuate nucleus and anteroventral periventricular nucleus through

beta-endorphin and dynorphin B neurons to affect preoptic area GnRH neurons and gonadotropin secretion [14,15]. Our results also suggest that there may be signaling crosstalk between $P_4$ and opioids during early embryogenesis in the

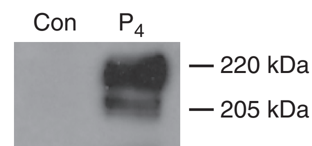


**FIG. 2.** P4 Induces hESC differentiation into neural precursor cells. H9 hESC colonies (day 4) were cultured in TESR1 media with ~~P4~~ (2 μM) or control (DMSO) for 9 days. Equal amounts of protein from cell lysates were analyzed by immunoblot using a monoclonal antibodies against nestin (clone 10C2; Chemicon). DMSO did not increase nestin expression (data not shown).

regulation of GnRH secretion, although the role of GnRH and gonadotropins during embryogenesis remains to be elucidated.

### *Conclusion*

These data indicate for the first time that pluripotent hESC have an absolute requirement for steroid and opioid signaling during blastulation and neurulation and suggest a critical molecular signaling link between trophoblastic and/or maternal hormone production and early embryonic growth and development. The abortifacient effects of RU-486 therefore also extend to blocking normal embryonic growth and development.

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Received for publication July 8, 2008; accepted after revision September 19, 2008.

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