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Human Embryonic Stem Cells: A Model System for Delineating the Molecular Basis of Human Embryogenesis and Aging-related Diseases

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1. Introduction

The recent development of techniques to culture human embryonic stem cells (hESC) has allowed the study of reproductive (pregnancy) hormones involved in the growth and development of the early embryo. Until the advent of hESC culture techniques, no model system existed that could readily assess the requirement for pregnancy hormones in the growth and development of the human embryo. Hormonal manipulation of developing embryos *in utero* was technically cumbersome and complicated by competing *in vivo* maternal hormonal signals. This chapter describes recent experimental studies, utilizing hESC, aimed at identifying physiologically relevant signals that promote cell division, differentiation and apoptosis during early embryogenesis and summarizes our current knowledge of how reproductive hormones direct growth and development during embryogenesis. It also describes the potential for using hESC, embryoid bodies (EBs) and neuroectodermal rosettes to gain insights into how reproductive endocrine dyscrasia associated with menopause/andropause drives aberrant cell cycle signalling mechanisms leading to age-related diseases including neurodegeneration and associated cognitive decline.

2. Human embryogenesis

2.1 Hormonal regulation of pregnancy

Embryogenesis is a complex coordinated series of molecular and cellular changes that takes place within a well-defined internal environment. Human embryogenesis is orchestrated by a complex array of endocrine signals that commences with conception, is followed by the growth and development of the zygote into a blastocyst, its implantation into the endometrium, and the subsequent growth and development of the blastocyst into the neonate. Following conception, the developing embryo (zygote/morula/blastocyst) has 7-14 days in which to produce sufficient human chorionic gonadotropin (hCG), and subsequently progesterone (P_4 ; from both embryonic and corpus luteal sources), to allow implantation and halt degradation and discharging of the endometrium (menstruation) (Gupta, et al. 2007). The upregulation of hCG and P_4 not only allows for the maintenance of

the endometrium, blastocyst attachment and syncytiotrophoblast proliferation into the endometrium (Larson et al. 2003; Licht et al. 2001; Pepe and Albrecht 1995), but also prevents ovulation and prepares the immune, metabolic and psychological systems of the mother for pregnancy.

P₄ production is an *absolute* requirement for the maintenance of pregnancy (Larson et al. 2003). Indeed, administration of RU-486 (mifepristone), an anti-P₄ and anti-glucocorticosteroid agent to humans is used for the medical termination of pregnancies of up to 49 days gestation (up to 63 days gestation in Britain and Sweden), and in combination with prostaglandin E1, for termination of pregnancies between 13 and 24 weeks gestation (Fiala and Gemzel-Danielsson 2006). Inhibition of P₄ signaling using RU-486, a P₄ receptor (PR) competitive antagonist, results in endometrial decidual degeneration, trophoblast detachment and decreased syncytiotrophoblast production of hCG which in turn decreases P₄ production by the corpus luteum. In addition, RU-486 induces cervical softening and dilatation, release of endogenous prostaglandins and an increase in the sensitivity of the myometrium to the contractile effects of prostaglandins leading to the expulsion of the embryo/fetus (Gemzell-Danielsson et al. 2006).

Despite our understanding of the endocrinology of pregnancy, the lack of an appropriate model system limited experimentally our ability to answer fundamental questions such as what endocrine/paracrine/juxtacrine/autocrine factors 1) regulate embryonic cell division, 2) regulate cell migration, 3) specify differentiation into particular lineages, and 4) regulate apoptosis, during early embryogenesis.

2.2 Human embryonic stem cells: A model system for understanding the cellular and molecular mechanisms regulating early human embryogenesis

Thompson and colleagues first isolated pluripotent hESC lines from surplus embryos donated by individuals undergoing infertility treatment (Thomson et al. 1998). Inner mass cells isolated from these embryos were allowed to develop to the blastocyst stage and then passaged in defined media (to maintain pluripotency) to increase cell numbers. Five diploid cell lines (H1, H7, H9, H13 and H14) were obtained from 14 blastocysts. These cells are Oct-4, SSEA1, SSEA-3, SSEA-4, TRA 1-60, TRA 1-81 and alkaline phosphatase positive.

hESC derived from the inner cell mass of the blastocyst can be differentiated into EBs which resemble the early post-implantation embryo (blastocyst containing all 3 germ layers) (O'Shea 1999). hESC also can be differentiated into columnar neuroectodermal cells and mimics *in vivo* neuroectodermal development in terms of timing and morphology (Li and Zhang 2006). *In vitro*, hESC differentiate into primitive neuroectodermal (or neural precursor) cells at around day 10 and then neuroectodermal cells that exhibit neural tube-like rosettes in 14–17 days of differentiation in a chemically defined neural induction media (Fig. 1; Gallego et al., 2010; Zhang et al. 2001). These structures are predominantly composed of neuroectodermal cells akin to those that form the neural tube and are neural precursor cells/neural stem cells that can be further differentiated into different neural lineages. Considering hESC are equivalent to a 5–6 days embryo, development of the neuroectoderm *in vitro* takes about 18–20 days, the time window when the neural tube forms in a human embryo (Muller and O'Rahilly 1985; Zhang 2003). The ability to manipulate the hormonal milieu of cultured hESC, embryoid bodies or neuroectodermal rosettes during this time period, either positively or negatively, allows for the identification of signaling pathways involved in cell division, differentiation and apoptosis during embryogenesis.

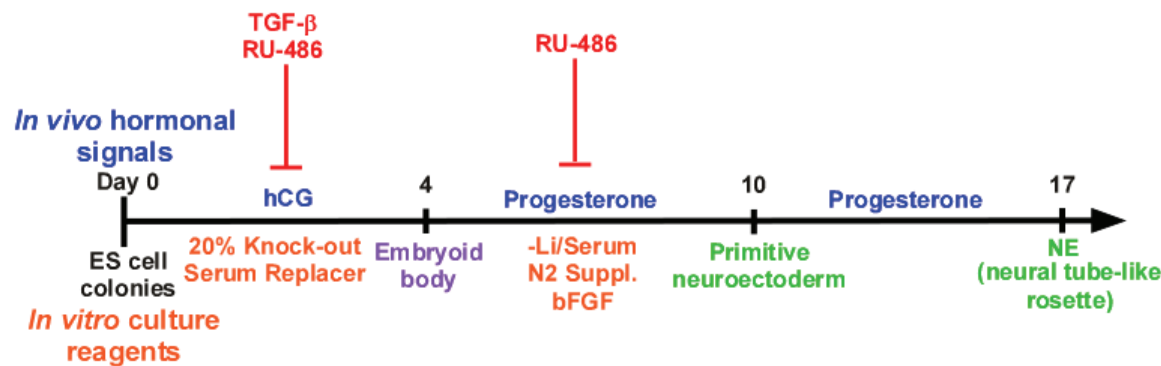


Fig. 1. Time course for the induction of embryoid bodies and neuroectodermal rosettes by hCG and P₄. hESC can be differentiated into primitive blastocyst-like structures (embryoid bodies (EBs) that contain the 3 germ layers) and then into primitive neuroectodermal cells (NE; or neural precursor cells) at ~day 10 and then NE that exhibit neural tube-like rosettes in 14–17 days of differentiation (Okabe et al. 1996; Zhang et al. 2001). Above Line: Physiological hormones secreted by trophoblasts demonstrated to induce cell division (hCG) during EB formation and cell differentiation (P₄) during neuroectodermal rosette formation. Below Line: Current unspecified medias used to generate EBs and neuroectodermal rosettes. These processes are negatively regulated by TGF-β signaling and by blocking signaling via PR

2.3 Trophoblastic hormone secretion during early embryogenesis

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 (Gilbert 2003). Trophoblasts secrete an array of hormones (Cemerikic et al. 1994; DiPirro and Kristal 2004; Gallego et al. 2009, 2010; Pidoux et al. 2007; Zhuang and Li 1991) including P₄, endorphins, hCG, 17β-estradiol (E₂) and gonadotropin-releasing hormone (GnRH) (Fig. 2). The dramatic elevation in the production of hCG by the trophoblastic layer of the blastocyst during the early embryonic stage (from 5 to ≥1000 mIU/mL in the maternal serum; Braunstein 1976; Pidoux et al. 2007) signals both the corpus lutea and trophoblast (Golos, et al. 2006) to synthesize and secrete P₄ (Bukovsky et al. 1995; Carr et al. 1982; Casper and Yen 1979; Duncan et al. 1996; Richardson and Masson 1985; Strauss et al. 2000). Trophoblastic secretion of these hormones appears to occur during the migration of the blastocyst through the fallopian tube and its implantation into the endometrium and subsequently from the placental tissues during later stages of embryogenesis. This is most clearly demonstrated by the elevation in maternal hCG during the early embryonic period.

The secretion of P₄, endorphins, hCG, E₂ and GnRH by trophoblasts that lie adjacent to the embryoblast in the blastocyst suggests that these hormones 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 (DiPirro and Kristal 2004). Likewise, trophoblastic and corpa luteal production of hCG/P₄ is markedly elevated post-conception and is obligatory for the maintenance of pregnancy (Larson et al. 2003). An autocrine/paracrine role for hCG secreted from invasive extravillous cytotrophoblasts (Hands Schuh et al. 2007) in the induction of neoangiogenesis during endometrial vascularization has previously been proposed (Licht et al. 2001). hCG signaling

via full-length LH/hCG receptors (LHCGR) on trophoblasts has been shown to modulate differentiation of the trophoblasts for subsequent villus projection and placentation. Given the close spatial localization of trophoblasts to the embryoblast, and the availability of hESC that can be cultured continuously, i.e. embryoblast-derived stem cells, it has become possible to explore trophoblastic hormone function in the development of the embryo.

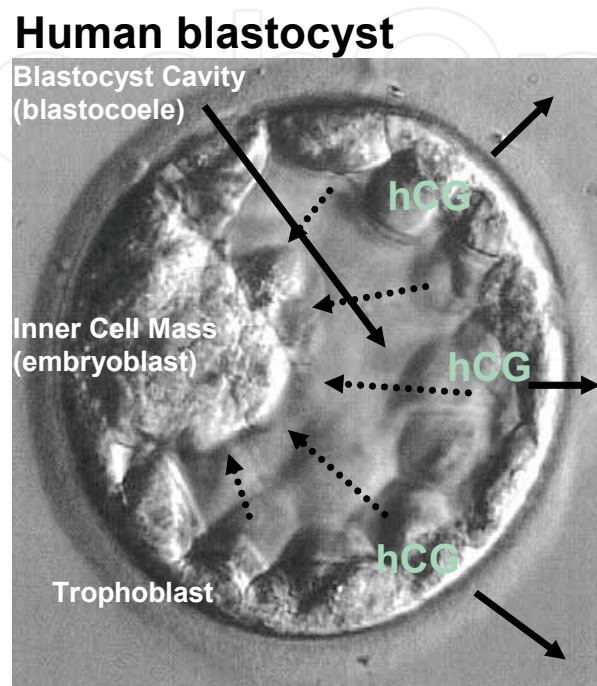


Fig. 2. Trophoblastic hCG secretion from the human blastocyst. Illustration of hCG autocrine (dotted line) and paracrine/endocrine (full line) secretion from the trophoblastic layer of a human blastocyst. Modified from: Muckle, C, Feinberg, E, Glob. libr. women's med., (ISSN: 1756-2228) 2008; DOI 10.3843/GLOWM.10002

3. Trophoblastic hormones and early human embryogenesis

3.1 Regulation of blastulation by human chorionic gonadotropin, progesterone and opioids

The first evidence for a function of any trophoblastic hormone in the regulation of human embryogenesis was ironically demonstrated by the finding that hCG induces the expression of the adhesion and neuritogenic protein amyloid- β precursor protein (A β PP; Porayette et al. 2007), a protein normally associated with the neurodegenerative pathology of Alzheimer's disease (AD; Hardy and Selkoe 2002). A β PP expression was detected at the transcriptional and translational levels (Porayette et al. 2007, 2009). These results indicated a critical molecular signaling link between the hormonal environment of pregnancy and the expression of A β PP in hESC that was suggestive of an important function for this protein during early human embryogenesis prior to the formation of neural precursor cells (Porayette et al. 2007).

That hCG could induce changes in epiblast protein expression was subsequently supported by the finding that hESCs express mRNA and protein for the full-length mature LHCGR (Gallego et al. 2008, 2010). That LHCGR is expressed on hESC implicates hCG as an

important signaling molecule in the growth and development of the embryo. LHCGR expression did not alter upon differentiation into EBs (structures that resemble early post-implantation embryos containing all three germ layers) (O'Shea 1999), or into neuroectodermal rosettes, which consist of >90% columnar neural precursor cells (NPC) and are the *in vitro* equivalent of a rudimentary neural tube (Gallego et al. 2008, 2010; Li and Zhang 2006). The comparable level of LHCGR expression between the different cell lineages was suggestive of 1) the existence of a tight regulatory system for the maintenance of hCG signaling during embryonic stem cell division and differentiation, and 2) a basal requirement for LH/hCG signaling during this early stage of embryogenesis. Indeed, hCG signaling via its hESC receptor was found to be essential for the proliferation of hESC (**Fig. 1**); inhibition of LHCGR signaling with P-antisense oligonucleotides suppressed hESC proliferation, as did a specific blocking antibody against the extracellular activation site of LHCGR, an effect that was reversed by treatment with hCG (Gallego et al. 2008, 2010). These data are supported by the known proliferative properties of (hyperglycosylated) hCG, which has been demonstrated to act as an autocrine factor on extravillous invasive cytotrophoblast cells to initiate and control invasion as occurs at implantation of pregnancy and the establishment of hemochorial placentation, and malignancy as occurs in invasive hydatidiform mole and choriocarcinoma (Cole 2009).

In addition to its cell cycle signaling activity, signaling of hCG via the hESC receptor rapidly upregulated steroidogenic acute regulatory protein (StAR)-mediated cholesterol transport and the synthesis of P_4 , a neurogenic steroid (**Fig. 3**; Brewer et al. 1993; Wang et al. 2005). StAR, a key rate-limiting step in the production of sex steroids in reproductive tissues, was detected in hESC at both mRNA and protein (37-kDa, 30-kDa and 20-kDa variants) levels. hCG treatment dose-dependently suppressed the expression of these StAR variants, while P_4 treatment decreased the truncation of the 37-kDa to the 30/32-kDa variants of StAR, indicative of decreased cholesterol transport across the mitochondrial membrane for steroidogenesis (Epstein and Orme-Johnson 1991; Krueger and Orme-Johnson 1983; Pon et al. 1986; Stocco 2001; Stocco and Chen 1991; Yamazaki et al. 2006). Importantly, hCG treatment markedly increased P_4 secretion 15-fold, indicating that embryoblast-derived hESC already possess the machinery to transport cholesterol and synthesize sex steroids. Together, these findings indicate negative feedback pathways exist for the regulation of hCG/LH signaling and mitochondrial cholesterol uptake for the synthesis of sex steroids in hESC and differentiating lineages.

hESC and EBs express P_4 receptor A (PR-A; Gallego et al. 2010; Hong et al. 2004; Sauter et al. 2005) implying P_4 signals hESC differentiation. The requirement for P_4 in the differentiation of hESC into EBs was confirmed by the finding that RU-486, a PR competitive antagonist (Fiala 2006), potently inhibited the differentiation of hESC into EBs (**Fig. 1**). RU-486 treated 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 EBs (Gallego et al. 2008, 2009, 2010).

Trophoblastic production of endorphins (Zhuang and Li 1991) also is crucial for embryogenesis (Gallego et al. 2009). Treatment of hESC colonies with the delta opioid receptor selective antagonist ICI 174,864 (Corbett et al. 1984; Paterson et al. 1984) inhibits the formation of the EB cystic structure, and instead forms non-spherical structures ~40% the size of normal spheroidal EBs. The mechanism by which opioid signaling promotes blastulation is unclear, however delta opioid antagonists may function to inhibit

embryogenesis by regulating hCG release (Cemerikic et al. 1992) required for P₄ production. Thus, the tight regulation of hCG signaling, and sex steroid and opioid synthesis and signaling, is required to coordinate hESC proliferation and differentiation during gastrulation.

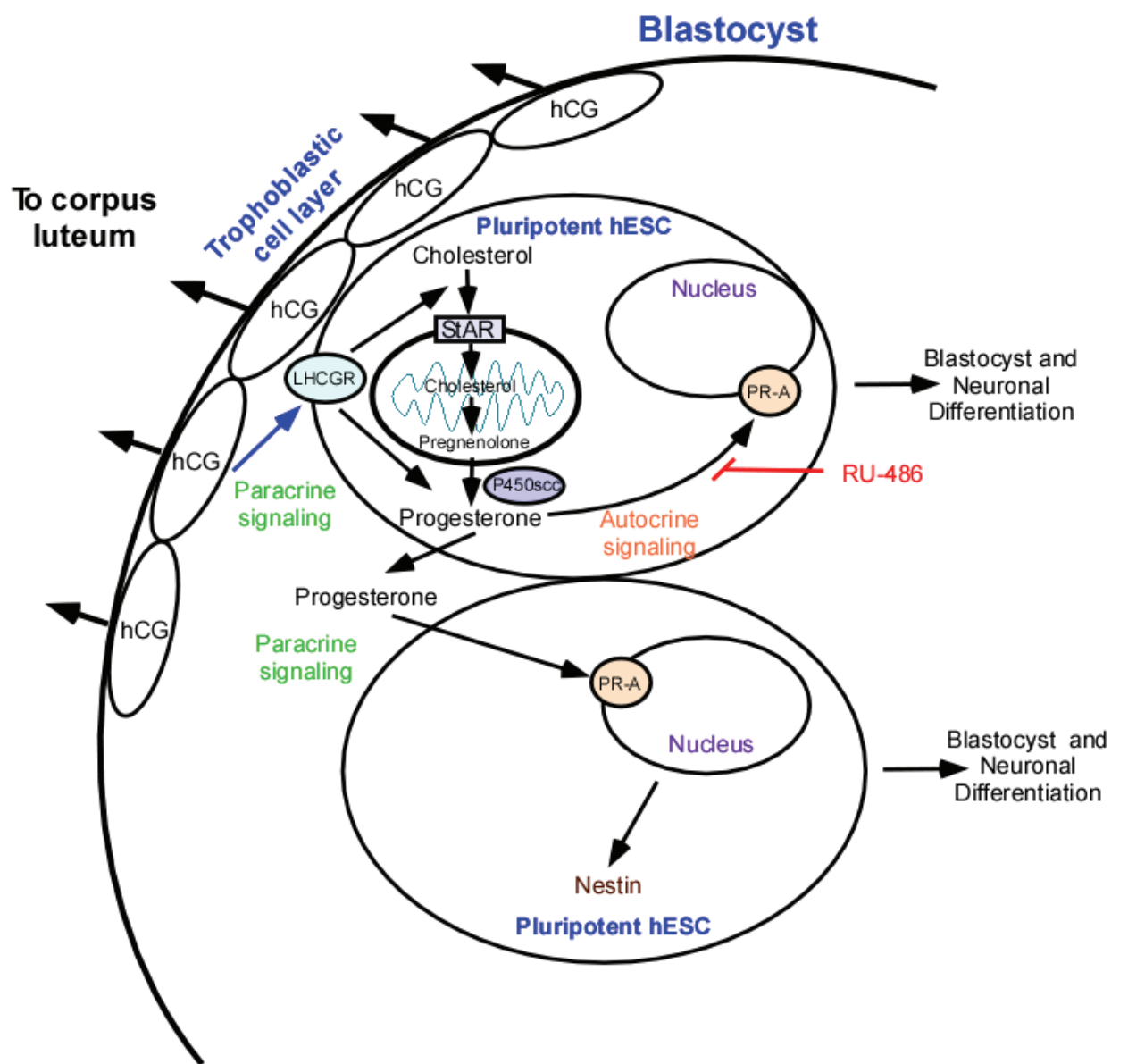


Fig. 3. Model of the autocrine and paracrine pathways regulating blastulation and neurulation. Putative autocrine and endocrine signalling pathways involved in cell proliferation, steroidogenesis and differentiation of the blastocyst and primitive neural tube. For details see Gallego et al., (2010)

3.2 Regulation of neurulation by human chorionic gonadotropin and progesterone
In addition to the obligatory signaling of P₄ for gastrulation, P₄ signaling also is required for the specification of NPCs from hESC (Fig. 1; Gallego et al. 2008, 2009, 2010). hCG treatment suppresses expression of the pluripotent marker Oct-3/4, suggesting hCG, or steroid production initiated by hCG signaling, could direct lineage commitment (Gallego et al. 2008,

2010). In the presence of P_4 , hESC colonies differentiate into spherical structures containing a minimum of three neuroectodermal rosettes inside of the cavity, while hESC colonies treated without P_4 or with RU-486 failed to form rosettes with columnar neuroectodermal cells after 17 days in culture (Gallego et al. 2008, 2009, 2010). Morphological changes were more severe in the absence of P_4 than with RU-486. P_4 , and to a lesser extent E_2 , were found to increase the expression of nestin, an early marker of NPC formation, in hESC. RU-486 completely suppressed nestin expression. Interestingly, ' E_2 priming' is required for induction of PR expression in other tissues (Atwood et al. 2000; Mylonas et al. 2007). 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.

Interestingly, hESCs default towards a primitive neural stem cell fate if maintained for any length of time in culture (Munoz-Sanjuan and Brivanlou 2002). Since hCG treatment induces nestin expression in hESC, endogenous gonadotropin production by hESC or trophoblastic cells (Golos et al. 2006) may be sufficient for NPC formation, thereby explaining the intrinsic hormonal signals regulating the 'default pathway' of hESC differentiation into neuronal lineages (Munoz-Sanjuan and Brivanlou 2002).

These results suggest that trophoblastic hCG production adjacent to the embryoblast is required not only for trophoblast steroidogenesis and attachment of the blastocyst to the uterine wall (Wahabi et al. 2007), but also for signaling normal proliferation and differentiation of the epiblast (Fig. 3). hCG-induced P_4 synthesis therefore has, in addition to its role in uterine decidualization for the implantation and maintenance of pregnancy, an obligatory role prior to the formation of neural precursor cells, as well as an inductive role in the directed differentiation and specification of the first neuronal cell types (organogenesis) and the formation of the neural tube. 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 (Pomata et al. 2000), these results demonstrate an early (within the first 7 days) and absolute requirement for P_4 during human blastulation and neurulation. In this respect, it has been shown that P_4 is necessary and sufficient (in Neurobasal media) for the maintenance and differentiation of primary hippocampal/cortical/striatal neurons *in vitro* (Brewer et al. 1993). That P_4 is the hormone regulating these key events is perhaps not surprising given its location high in the steroidogenic synthetic pathway; P_4 is the first steroid synthesized from pregnenolone, the precursor to all other steroids.

Opioid signaling also is required for neuroectodermal rosette formation since ICI 174,864 (Corbett et al. 1984; Paterson et al. 1984) inhibits normal neuroectodermal rosette formation and nestin expression (Gallego et al. 2009, 2010). 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 (Dufourny et al. 2005; Gu and Simerly 1994). Thus, delta opioid receptor signaling is required both for normal human blastulation and neurulation, but it remains to be determined if there is crosstalk between opioid signaling and the regulation of GnRH/gonadotropin secretion.

Previous studies have demonstrated the importance of P_4 and related steroids as neurotrophic agents that promote adult neurogenesis, neuronal survival and neuroprotection (Brewer et al. 1993; Ciriza et al. 2004; Cutler et al. 2006; Guo et al. 2006;

Mauch et al. 2001; Schumacher et al. 2003, 2004; VanLandingham et al. 2006; Wang et al. 2005). Clinical studies supporting the neurotrophic actions of P_4 administration are demonstrated by the decrease in mortality rate and improved outcome following acute traumatic brain injury in humans (Wright et al. 2007).

Dependent upon the timing of administration during pregnancy, suppression of P_4 signaling with RU-486 (Fiala and Gemzel-Danielsson 2006) aside from intrauterine disruptive functions (decidual breakdown and trophoblast detachment) also will disrupt 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. The relative binding affinity of RU-486 for the PR is twice that of P_4 (Heikinheimo et al. 2003), and is used at a dose of 200-600 mg for the termination of pregnancies (Fiala 2006) (this equates to $\sim 6-19 \mu\text{M}$, equivalent to that used in Gallego et al. 2008, 2009, 2010). Thus, the abortifacient effects of RU-486 in blocking PR signaling also extend to blocking blastulation and neurulation and the normal growth and development of the embryo.

3.3 Regulation of organogenesis by human chorionic gonadotropin and progesterone

Aside from the induction of blastulation and neurulation early in embryogenesis, hCG/LH and P_4 signaling may play a role in the development of other tissues (LH is the adult hCG homolog with 83 % homology and binds the same receptor - LHCGR). LHCGR and PR have been identified on numerous reproductive and non-reproductive tissues (Ascoli et al. 2002; Bouchard 1999; Bukovsky et al. 2003; Mulac-Jericevic and Conneely 2004). With regard hCG/LH, the free glycoprotein α -subunit of gonadotropins has been shown to stimulate differentiation of prolactin cells in the pituitary (Avsian-Kretchmer and Hsueh 2004) and endometrial stromal cell decidualization in the placenta (Blithe et al. 1991). Although it has not been demonstrated if hCG/LH has a developmental function during organogenesis, hyperglycosylated hCG β has potent cell growth and invasion properties as observed in early pregnancy, gestational choriocarcinoma and testicular cancers (Cole 2009). Interestingly, in the adult brain, subcutaneous administration of LH has been shown to induce neurogenesis in the hippocampus of the adult mouse (Mak et al. 2007), while in sheep there is evidence that GnRH directly, or indirectly via LH, induces neurogenesis in the hippocampus (Hawken et al. 2009). hCG also is known to promote angiogenesis by inducing the up-regulation of vascular endothelial growth factor (Berndt et al. 2006; Licht et al. 2002; Zygmunt et al. 2002) and P_4 (Rogers et al. 2009).

The potential for P_4 to regulate organogenesis has been reported during puberty and adulthood, where P_4 is obligatory for the development of the tertiary ducts on the mammary gland, and the functional differentiation of the lobuloalveolar system from the lobular buds (Atwood et al. 2000). In the adult, P_4 and related metabolites have been demonstrated to regulate bone formation (Prior 1990), promote angiogenesis and arteriogenesis (Rogers et al. 2009), promote formation of the placenta and promote neurogenesis by increasing rat neuroprogenitor cell proliferation and human neural stem cell proliferation (Wang et al. 2005). While our knowledge of hCG/LH and P_4 during organogenesis is rudimentary at this point, the above evidence indicates these hormones likely play important functions in many tissues during organogenesis.

4. Utilizing human embryonic stem cells as a model system to understand the molecular basis of aging-related diseases

It is becoming clear that the hormonal mechanisms that regulate the coordinated division and differentiation of cells early in life become dysregulated later in life following menopause and during andropause (which starts at ~ 30 years of age; see Vadakkadath Meethal et al. 2005; Atwood and Bowen 2011), and that these dysregulated hormonal mechanisms drive aberrant re-entry of cells into the cell cycle and their abortive death leading to tissue compromise and ultimately disease (Atwood et al. 2005).

The reproductive endocrine dyscrasia associated with menopause and andropause are intimately associated with disease (Bowen and Atwood 2004; Atwood and Bowen, 2011). The decline in sex steroid production by the gonads following menopause and during andropause leads to a loss of hypothalamic feedback inhibition that stimulates GnRH and gonadotropin production (Carr 1998). In addition, the decrease in gonadal inhibin production at this time (Reichlin 1998) results in decreased activin receptor inhibition, and together with the increase in bioavailable activin (Gray et al. 2002) leads to a further increase in the secretion of GnRH and gonadotropins (MacConell et al. 1999; Schwall et al. 1988; Weiss et al. 1993). Thus, the lack of negative feedback from the ovary (P_4 , E_2 and inhibin) is responsible for the unopposed and marked elevations in the secretion of GnRH and gonadotropins with ovarian and testicular senescence (Atwood et al. 2005; Chakravarti et al. 1976; Neaves et al. 1984; Reame et al. 1996; Schmidt et al. 1996).

The concentration of brain sex steroids, including P_4 , is a mixture of peripherally derived sex steroids, converted peripheral steroids, and neuro-sex steroids. The contribution of peripheral sex steroids to total brain sex steroids is unknown, but post-reproductive declines in peripheral P_4 would be expected to impact brain P_4 concentrations. While elevations in GnRH and gonadotropin concentrations might promote brain neurosteroid production (see Vadakkadath Meethal et al. 2009), including P_4 , it is not known if this is sufficient to counter the loss of peripherally-derived sex steroids. The consequences of these hormonal alterations are discussed below in the context of AD.

4.1 Alzheimer's disease

Dementia accounts for 3% of deaths in the USA (CDC National Vital Statistics Report, 2009) although by the age of 85 ~45% of the population has some form of dementia. AD accounts for ~70% of all dementia cases and is characterized neurologically by age-related progressive memory loss, impairments in behavior, language, and visuo-spatial skills (Atwood et al. 2005; Vadakkadath Meethal et al. 2005).

Unlike development, where mitogenic and differentiative signaling are both elevated, senescence is associated with elevated mitogenic (i.e. gonadotropins, GnRH) signaling but decreased differentiative signaling (i.e. sex steroids).

The age-related loss of P_4 is of particular importance given the differentiative properties of this steroid described above. Also of importance are the aging-related elevations in serum GnRH, FSH and LH, especially given the known proliferative properties of hCG/LH (Cole 2009; Gallego et al. 2008, 2010). In this regard, LH/hCG is known to have powerful mitogenic properties in certain reproductive tissues (Davies et al. 1999; Harris et al. 2002; Horiuchi et al. 2000; Sriraman et al. 2001; Webber and Sokoloff 1981), the brain (Mak et al., 2007; Hawken et al., 2009) and are frequently expressed by tumor cells (Krichevsky et al.

1995; Whitfield and Kourides 1985; Yokotani et al. 1997 and reviewed in Cole 2009). Thus, these multiple changes in hormonal signaling with menopause/andropause, i.e. increased mitogenic signaling but decreased differentiation signaling (*dyotic signaling*) might be expected to impact normal cell cycle dynamics. Indeed, accumulating evidence suggests there is a reactivation of the cell cycle with aging (see Bowen and Atwood 2004) as has been demonstrated for post-mitotic pyramidal neurons of the brains of aged individuals with AD (Herrup and Yang 2007; Raina et al. 2000). This data includes 1) the ectopic expression of cell cycle proteins in those regions of the brain affected by AD, but not in areas unaffected by AD pathology or in control brains, 2) chromosomal replication (endoreduplication) in differentiated AD neurons, demonstrating entry into S-phase of the cell cycle, 3) elevated cytoplasmic mitochondrial DNA and Cox-1 expression, suggestive of *de novo* mitochondrion synthesis, and 4) upregulated growth factor signal transduction pathways. Importantly, the spatio-temporal expression of sex hormone receptors throughout the brain is in those areas of AD neurodegeneration. Other parallels between embryonic neurogenesis and adult neurodegeneration include the expression of A β PP, secretases and tau, together with the processing of A β PP either towards the amyloidogenic or non-amyloidogenic pathways, and the phosphorylation of tau (Porayette et al. 2009) (Atwood and Porayette, unpublished data). Similarly, the fetal brain has been reported to display a number of biochemical similarities to the AD brain, namely the presence of A β and A β PP (Arai et al. 1997; Takashima et al. 1990), presenilin-1 expression (Berezovska et al. 1997) and hyperphosphorylated tau (Goedert et al. 1993). The phosphorylation of tau is a mitogenic-associated event that normally occurs during metaphase of neuronal division, and is observed during differentiation of neurons in the fetal brain (Goedert et al. 1993; Liu, et al. 2004).

This increased developmental protein expression in the AD brain suggests reactivation of the cell cycle in differentiated neurons of the AD brain (Herrup and Yang 2007) and explains the majority of the biochemical and pathological features associated with the disease (Atwood et al. 2005; Meethal et al. 2005). Two recent studies support this claim. Forced cell cycle activation in terminally differentiated neurons via conditional expression of the simian virus 40 large T antigen (oncogene) forms A β deposits and tau pathology in the mouse cortex (Park et al. 2007). Similarly, forced cell cycle activation in primary neurons is accompanied by tau phosphorylation (McShea et al. 2007). These data suggest that AD neuropathology is a result of an imbalance in cell cycle regulation in the adult brain.

4.2 Amyloid- β precursor protein and neurogenesis

That amyloidogenic pathways are involved in neurogenesis has recently been reported by a number of workers (Calafiore et al. 2006; Heo et al. 2007; Liu et al. 2004; Lopez-Toledano and Shelanski 2004). In this context, an increase in neurogenesis has been reported in young transgenic mice overexpressing human mutant A β PP (Jin et al. 2004; Lopez-Toledano and Shelanski 2007). Moreover, the overexpression of wild-type or FAD mutant A β PP, which promotes A β generation (Citron et al. 1997), also has been shown to promote the re-entry of primary neurons into the cell cycle, as demonstrated by the induction of DNA synthesis and cell cycle markers (McPhie et al. 2003). Not surprisingly, A β PP has structural similarity to growth factors (Trapp and Hauer 1994) and modulates several important neurotrophic functions, including neuritogenesis, synaptogenesis, and synaptic plasticity (Gralle and Ferreira 2007).

4.3 Hormonal regulation of neurogenesis via modulation of amyloid- β precursor protein metabolism

In hESC, the differential processing of A β PP via secretase enzymes regulates the proliferation and differentiation of hESC; processing towards the amyloidogenic pathway is associated with cell proliferation, processing towards the non-amyloidogenic pathway is associated with cell specification and differentiation. Specifically, P₄ induces processing of A β PP towards the production of the soluble A β PP α in hESC (Porayette et al. 2009), which has known differentiative properties (Milward et al. 1992). Similarly, E₂ has been shown to stimulate the processing of A β PP by the nonamyloidogenic α -secretory pathway and reduces cellular A β production in both nonneuronal (Jaffe et al. 1994) and neuronal cultures (Greenfield et al. 2002; Manthey et al. 2001; Xu et al. 1998). Conversely, the loss of sex steroids and elevation in gonadotropins following ovariectomy has been shown to increase A β generation in non-transgenic animals (Petanceska et al. 2000). Importantly, we have demonstrated that LH promotes the processing of A β PP towards the amyloidogenic pathway *in vitro*, while suppression of serum LH in mice using GnRH agonists decreases the concentration of brain A β 1-40 and A β 1-42, the 2 major variants that deposit in the AD brain (Bowen et al. 2004). It is therefore plausible that the interaction of these hormonal pathways on the modulation of the processing of A β PP may regulate cell cycle events throughout life, with dyotic signaling by these hormones leading to the reactivation of the cell cycle in differentiated neurons of the AD brain (Herrup and Yang 2007). This aberrant, albeit unsuccessful, re-entry of neurons into the cell cycle leads to synapse contraction and neuron death (see Atwood et al. 2005; Herrup et al. 2004; Raina et al. 2000 for reviews). In addition to the loss of neurons following the reactivation of the cell cycle in differentiated neurons, it is possible that dyotic signaling prevents normal neurogenesis from resident neural stem cells, thereby preventing replacement of neurons. Further studies are required to determine whether post-reproductive levels of GnRH/gonadotropins are sufficient to induce neurosteroidogenesis in neural stem cells and neuronal cell types, and whether the level of post-reproductive neurosteroid synthesis dictates normal or dyotic signaling in these cell types.

5. Conclusion

The advent of the human embryonic stem cell era has allowed experimental determination of the physiological hormone requirements for early embryogenesis. In this respect, although progestagens are often considered primarily reproductive hormones with maternal influences, it is now clear that progestagens and hCG are essential for the growth and development of the embryo as well as the normal health of the brain throughout life. Paracrine/juxtacrine signaling of hCG (and opioids) for mobilization of cholesterol for P₄ production by the epiblast/syncytiotrophoblast following conception is obligatory for human blastulation and neurulation (Fig. 3). This paracrine/juxtacrine signaling by extraembryonic tissues is the commencement of trophic support by placental tissues in the growth and development of the human embryo. The identification of these hormones that regulate cell proliferation and differentiation of hESC *in vitro* will help direct the development of medias that most closely reflect their *in utero* environment for *in vitro* culture of these cells and their differentiation towards various cell lineages. Such medias could be used to further delineate the molecular basis of embryogenesis and organogenesis, as well as establish *ex utero* embryonic and fetal cultures.

The above discussion also indicates how hESC can provide insight into neurodegenerative disease (and likely many other aging-related diseases). While appropriate gonadotropin/ GnRH and P₄ signaling is necessary for normal growth and development during embryogenesis, fetal life and childhood, and for the maintenance of brain health during adult reproductive life, the unopposed elevations in GnRH/gonadotropins with the loss of sex steroids following menopause/andropause appears to lead to dysregulation of cell cycle events (Bowen and Atwood 2004).

It will be possible in the future to use hESC cells as a model for understanding how endocrine, paracrine and autocrine signals regulate cell cycle progression, from entry to exit, and how dysregulated signaling leads to entry but no exit from the cell cycle, i.e. leading to endoreduplication such as is apparent in the pyramidal neurons of the AD brain. Thus, hESC are a useful cell model system for examining questions related to early embryonic neurogenesis, adult neuroregeneration and neurodegeneration.

6. References

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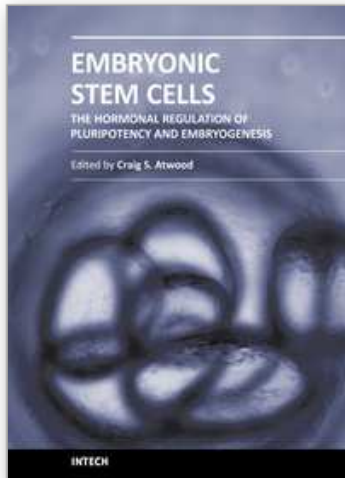
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