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Amyloid-β precursor protein expression and modulation in human embryonic stem cells: A novel role for human chorionic gonadotropin

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Abstract

The amyloid-β precursor protein (AβPP) is a ubiquitously expressed adhesion and neuritogenic protein whose processing has previously been shown to be regulated by reproductive hormones including the gonadotropin luteinizing hormone (LH) in human neuroblastoma cells. We report for the first time the expression of AβPP in human embryonic stem (hES) cells at the mRNA and protein levels. Using N- and C-terminal antibodies against AβPP, we detected both the mature and immature forms of AβPP as well as truncated variants (~53 kDa, 47 kDa, and 29 kDa) by immunoblot analyses. Expression of AβPP is regulated by both the stemness of the cells and pregnancy-associated hormones. Addition of human chorionic gonadotropin, the fetal equivalent of LH that is dramatically elevated during pregnancy, markedly increased the expression of all AβPP forms. These results indicate a critical molecular signaling link between the hormonal environment of pregnancy and the expression of AβPP in hES cells that is suggestive of an important function for this protein during early human embryogenesis prior to the formation of neural precursor cells.

Keywords: Amyloid-β precursor protein; Human embryonic stem cell; Embryogenesis; Human chorionic gonadotropin; Pluripotency; Protein processing; Pregnancy hormone; Oct-3/4; GAPDH; Beta-actin

The amyloid-β precursor protein (AβPP) is a single pass transmembrane protein with a large extracellular domain (~88% of total protein mass) and a small cytoplasmic tail [1–4]. These characteristics together with numerous other functional studies indicate that AβPP functions as a receptor and/or an adhesion molecule to other cells or to extracellular matrix components [5–7]. AβPP also has structural similarity with growth factors [8]. Importantly, the high expression of AβPP in radial glia, which guide future neurons to their correct positions in the embryonic cortex, suggests that the adhesion of neurons to glia cells promoted by AβPP plays an important role in brain development [9]. As is also the case for other contact receptors, it has been reported that the adhesion promoting activity of AβPP in migratory cells is accompanied by a neuritogenic activity in stationary, immature neurons *in vitro* and *in vivo* ([10–12], reviewed in [13]).

Given the above function of AβPP as an adhesion and neuritogenic molecule, it is not surprising that AβPP has been detected immunochemically in the human fetal brain as early as 17 weeks of gestation [14,15]. Likewise, *APP* mRNA has been detected by Northern blot in both 19-week normal as well as 19-week Down Syndrome affected human fetal brain [2]. In the mouse, *APP* mRNA including the splice variants 771, 770, and 695 have been found to be

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expressed during early embryogenesis in the oocyte, pre-implantation embryo, and post-implantation embryo through to the late embryonic stages [16]. *APP* transcripts also have been found in mid-gastrulation zebrafish embryos [17].

Thus far, no studies have examined the expression of AβPP prior to the formation of neural precursor cells. In this study, we chose human embryonic stem (hES) cells as a model of early embryonic development to examine AβPP expression and the influence of hormones on AβPP expression. We find that hES cells express AβPP and that both the stemness of the cells and the pregnancy-associated hormone human chorionic gonadotropin (hCG) alter its expression. These results indicate that this molecule is functional during a developmental period where proliferation, specification, and differentiation are particularly important.

Materials and methods

Propagation of human embryonic stem cells. Pluripotent H9 hES cells (passage 22–32; XX karyotype; also known as WA09, a National Institutes of Health registered line) obtained from WiCell Research Institute (WI, USA) were grown on Matrigel™ (BD Biosciences, CA, USA) in the presence of mTeSR1 media (WiCell Research Institute [18]). Every 4–5 d, the cells were passaged by enzymatically lifting colonies with a sterile solution of dispase (Invitrogen, CA, USA; 1 mg/ml in DMEM-F12) and then dissecting the colonies into multiple small pieces prior to transfer onto freshly plated Matrigel™ in mTeSR1 media (2.5 ml per well). mTeSR1 media was replaced with fresh media every day.

Hormonal treatment of human embryonic stem cells. Pluripotent H9 hES cells were plated in 6-well plates coated with Matrigel™ in 2.5 ml of mTeSR1 media per well. After overnight culture, these cells were treated every day with hCG (0–50,000 mIU/ml; Ray Biotech Inc., GA, USA) in mTeSR1 media for 6 d. Cells were then collected in Dulbecco's phosphate-buffered saline (DPBS) and stored at −80 °C for immunoblot analyses.

Immunoblotting. Cells were collected in DPBS and homogenized in lysis buffer (20 mmol/L Tris–HCl, pH 7.6, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% sodium dodecyl sulfate (SDS)), and protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mmol/L phenylmethylsulfonyl fluoride; Roche Diagnostics, IN, USA) and then sonicated indirectly at 30 Hz for 30 s with intermittent cooling. Following protein assay (Bicinchoninic Acid Protein Assay Kit; Pierce Biotechnology, Inc., Rockford, IL), equal amounts of protein were loaded onto 10–20% tricine gels (Invitrogen) for SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, CA, USA), fixed with 4% glutaraldehyde in TBS-Tween 20 (TBST), blocked with milk (10%, w/v) for 4 h and then probed with primary antibody overnight at 4 °C. The blot was then incubated with the corresponding horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature, washed again and developed with ECL plus reagent (Amersham ECL™ Advance Western Blotting Detection Kit, GE Healthcare, UK) as per the manufacturer's instructions. The chemiluminescent signal was captured on autoradiograph (Eastman Kodak, NY, USA). The membrane was then stripped using a stripping buffer (2% SDS, 60 mM Tris, pH 6.8, and 1% 2-mercaptoethanol) and probed with the next primary antibody of interest. Images were captured, scanned, and the intensity of the autoradiograph signals (including a blank region) was determined using the NIH Image J software (http://rsb.info.nih.gov/nih-image/). Control and treatment values were corrected for blank values, normalized to their respective control band intensity and the results then expressed as a fold-change over control levels. The following antibodies (with dilution ratio) were used throughout this study: 22C11, a monoclonal antibody against the N-terminal 66–81 aa of the human amyloid-β precursor protein (Chemicon International, CA, USA; 1:1000); 4G8, a monoclonal antibody against aa 17–24 of human amyloid-β (Signet Laboratories, MA, USA; 1:1000); anti-human Oct-3/4 monoclonal antibody (1:1000); anti-human β-actin (C-11) polyclonal antibody (1:1000), anti-human GAPDH (V-18) polyclonal antibody (1:1000), horseradish peroxidase-linked goat anti-mouse antibody (1:6000) and horseradish peroxidase-linked donkey anti-goat antibody (1:6000; all from Santa Cruz Biotechnology).

RT-PCR. Total RNA was isolated from cultured hES cells using the RNeasy Mini Kit (Qiagen, CA, USA) according to the manufacturer's instructions. *APP* cDNA was synthesized and amplified using the SuperScript III One-Step RT-PCR system (Invitrogen). Both cDNA synthesis and PCR amplification were carried out using gene-specific primers: *APP* forward 5′ CCGCGCAGAACAGAAGGACAGAC 3′, reverse 5′ AGGGCGGGCATCAACAGGCTCAA 3′ (Integrated DNA Technologies, IA, USA). PCR amplification of *APP* was performed for 35 cycles of 95 °C 30 s, 60 °C 45 s, 72 °C 60 s, with a final extension time of 5 min. The PCR product was stained with GelStar® Nucleic Acid Stain (Cambrex Bio Science, ME, USA), run on 2.5% Metaphor agarose gel (Cambrex Bio Science) and imaged using the EC^3 Imaging System (UVP Bioimaging Systems, CA, USA).

Results

To determine whether AβPP is expressed during early human embryonic development, we first assessed H9 hES cells for the expression of *APP* mRNA. Amplification of cDNA using sequence specific primers indicated a product of the expected length (439 bp) in pluripotent hES cells (Fig. 1), indicating that *APP* mRNA is transcribed in hES cells. To determine if *APP* mRNA was translated, we performed an immunoblot analysis of cellular protein using an antibody to the N-terminus of AβPP. Like the M17 neuroblastoma cell line (Fig. 2) [19], hES cells expressed both the mature (AβPPm) and immature (AβPPim) forms of full-length AβPP (Fig. 2), although the

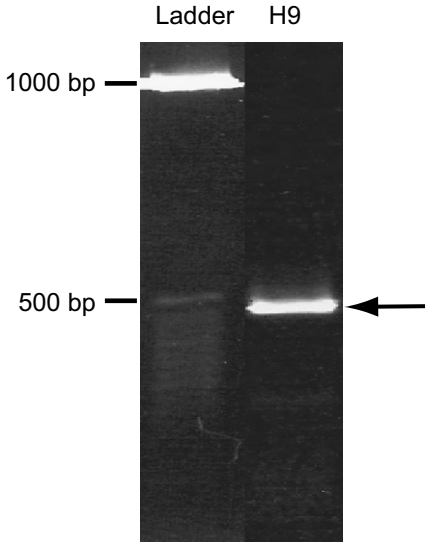

Fig. 1. *APP* mRNA is expressed by pluripotent hES cells. Total RNA was extracted from pluripotent H9 hES cells and amplified via RT-PCR using sequence specific primers (see Materials and methods). The expected 439 bp cDNA was observed (arrow). A molecular weight ladder is shown on the left.

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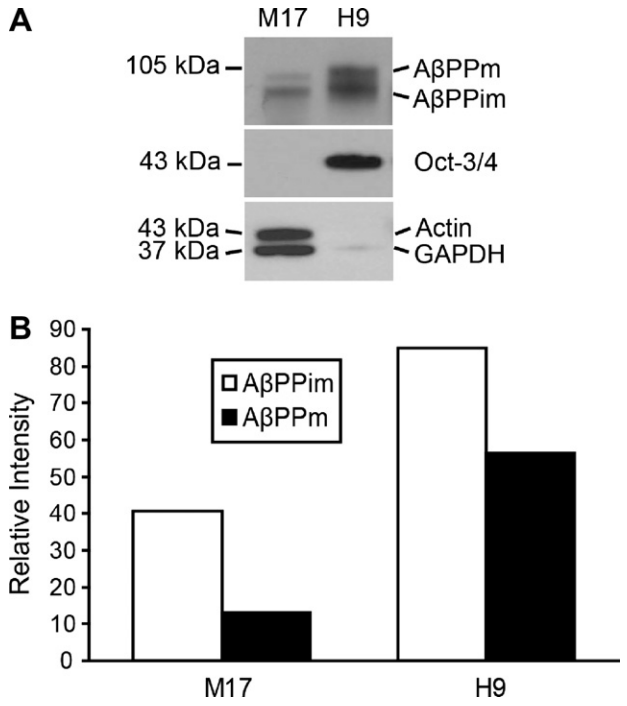

Fig. 2. AβPP expression in hES cells. (A) Equal amounts of cellular protein from pluripotent H9 hES cells and human M17 neuroblastoma cells (positive control) were analyzed by immunoblot for AβPP expression using the 22C11 monoclonal antibody against amino acids 66–81 of AβPP (top panel). The immunoblot in the top panel also was probed with the Oct-3/4 pluripotency marker (anti-human Oct-3/4 monoclonal antibody), a polyclonal GAPDH antibody and a polyclonal β-actin antibody. Molecular weight markers are shown on the left-hand side. Anti-human AβPP antibody recognized AβPPim and AβPPm in both M17 and hES cells; anti-Oct-3/4 antibody recognized a 43 kDa band in hES cells only. (B) The graph shows the relative intensities of the AβPPim and AβPPm bands in H9 hES cells compared to M17 neuroblastoma cells.

expression in the hES cells was 4- and 2-fold higher, respectively. Interestingly, the ratio of AβPPm/AβPPim in hES cells was higher (~67%) compared to M17 neuroblastoma cells (~32%), indicating that the post-translational machinery is active in hES cells and that more post-translational modification of AβPP occurs during development compared with a transformed neuroblastoma cell line.

To confirm that the hES cells were pluripotent, we probed the immunoblot for Oct-3/4, a 43-kDa POU transcription factor that is required to maintain stem cell self-renewal (i.e. pluripotency) and is a master regulator of pluripotency that controls lineage commitment [20]. This marker was absent in human M17 neuroblastoma cells as expected for a lineage-committed cell, but was clearly present in H9 hES cells. These results indicate (1) that hES cells express AβPP, (2) that the level of AβPP expression is dependent upon the stemness of the cell, and (3) that M17 neuroblastoma cells, although not considered differentiated, are lineage committed. Despite equal loading of protein onto the gel, we consistently observed very low expression of GAPDH (37 kDa) and β-actin (43 kDa) in hES cells compared to the M17 neuroblastoma cell line, indicating that their expression is different between cell types and that these proteins are not useful as loading controls at least between experimental conditions.

Given that hCG (which has 83% homology to LH and binds the same receptor-LH/hCG receptor) expression by the blastocyst is adjacent to the inner mass cells from which hES cells are derived, and that our previous published data indicate that LH promotes the processing of AβPP [19], we tested whether hCG alters AβPP expression in hES cells. Immunoblot analysis using two different monoclonal antibodies against the N- and C-terminus of AβPP (22C11 and 4G8, respectively; Fig. 3A and B) indicated an hCG induced dose-dependent increase in the expression of both the mature and immature forms of AβPP over a physiological range of hCG encountered by hES cells during embryogenesis. Both AβPPim and AβPPm forms increased in parallel by ~2.5- to 4-fold with treatment of hCG to 50,000 mIU/ml. In addition, hCG induced an increase in the expression of ~53 kDa (Fig. 3A and B), ~47 kDa (Fig. 3B), and ~29 kDa (Fig. 3B) cleavage products of AβPP [21]. These results indicate that AβPP is expressed and processed in pluripotent hES cells and that expression of full-length and processed AβPP forms is dependent upon hCG levels. The basal level of AβPP expression observed in the absence of added exogenous hCG may reflect autocrine hCG production by hES cells (data not shown).

Discussion

Our results demonstrate for the first time that AβPP is expressed both at the mRNA and protein level in hES cells suggesting that AβPP is functionally important as early as the blastocyst stage of human embryogenesis (Figs. 1–3). This finding is consistent with earlier findings that the *APP* gene is expressed in mouse oocytes and embryos [16,22] and that AβPP immunoreactivity is found in differentiating neurons of the mouse neural tube, motor neurons of the spinal cord and hind brain [23]. Although previous studies have indicated a role for AβPP in cell adhesion or as a receptor [5–7], in neuritogenesis [10–12,24] and synaptogenesis [25,26], our data indicate that AβPP likely has a separate function prior to the specification of neural precursor cells. Previous studies of AβPP, APLP1, and APLP2 knockout mice indicate that AβPP family members possess essential, but partially redundant, functions, and that they play important roles in normal brain development and early postnatal survival [27–29]. Triple-knockout mice for AβPP, APLP1, and APLP2 survive through embryonic development and die shortly after birth but show cortical dysplasia [28] with the presence of focal ectopic neuroblasts that had migrated through the basal lamina and pial membrane, a phenotype that resembles a human type II lissencephaly. In AβPP and APLP2 (nearest homologue) double knockout mice, motoneuron axons bypass their targets in muscle fibers and do not form synaptic terminals at the correct sites, leading to a dramatic increase in transmission failure on the day of birth [30] and to the death of these

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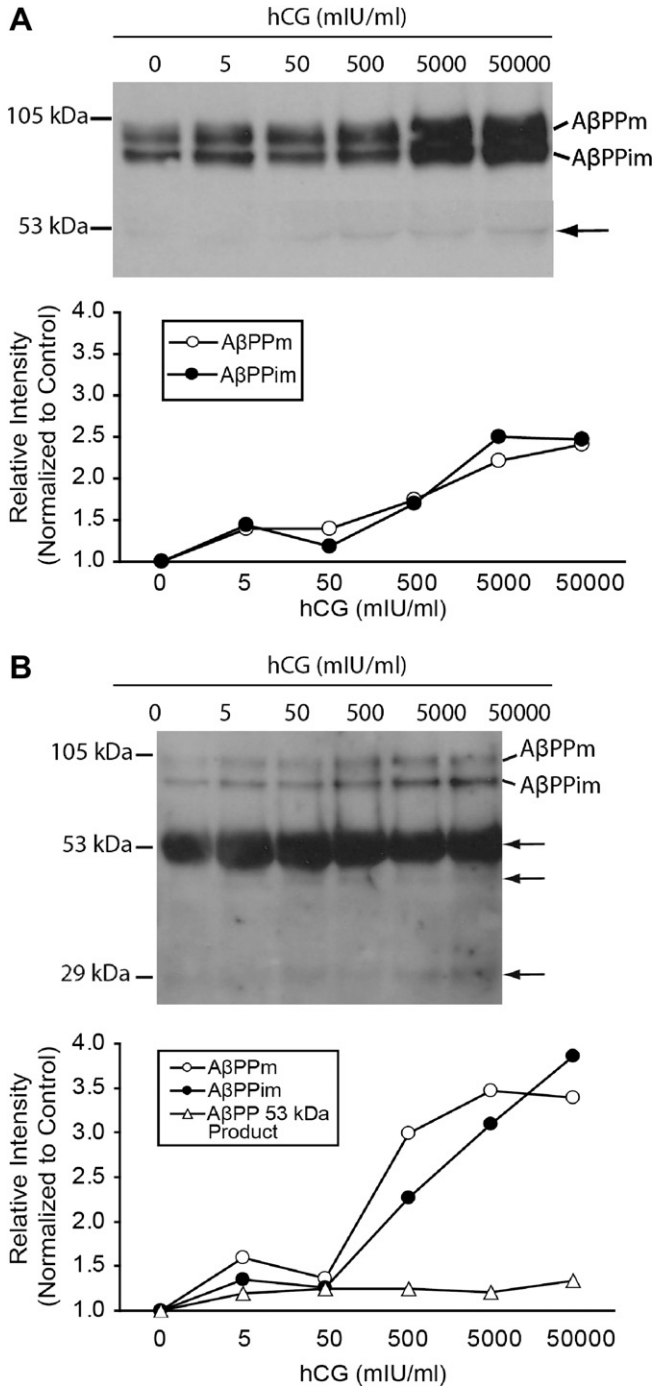

Fig. 3. hCG increases AβPP expression. Pluripotent H9 hES cells were cultured overnight as described in Materials and methods prior to treatment with 0, 5, 50, 500, 5000, and 50,000 mIU/ml hCG in mTeSR1 media with daily replacement of media. After 6 d of treatment, equal amounts of cellular protein extracted from the collected cells underwent immunoblot analysis using (A) anti-human AβPP, N-terminus (22C11) monoclonal antibody, and (B) anti-human amyloid-β, 17–24 aa (4G8) monoclonal antibody. Arrows indicate the 53 kDa, 47 kDa, and 29 kDa bands. The lower graphs show the relative intensities of the bands for both antibodies normalized to the control. The relative band intensities increased with hCG concentration.

animals a few days later [27,28,30,31]. Although the knock-out of AβPP alone has a subtle effect on adult animals, 3 amino acid substitutions within the rodent Aβ sequence compared with the human sequence render the rodent Aβ peptide non-toxic [32,33]. The peptide also does not deposit in the rodent brain [34,35]. Given these key differences in function, and the vast pathological and functional differences between the rodent and human brains, it remains to be determined whether AβPP expression is more crucial during early human embryogenesis and neurogenesis.

Our results also demonstrate that hCG increases AβPP expression in hES cells. Considering the fact that hCG is maximally expressed during the embryonic period of human pregnancy to maintain the corpus luteal production of progesterone, it might also play a crucial role in inducing developmental proteins like AβPP that can affect early embryogenesis. In our earlier study, LH, the adult homologue of hCG that binds the same receptor (LH/hCG receptor), did not change the expression of full-length AβPP, but did change the processing of AβPP, in the lineage-committed M17 neuroblastoma cells [19]. These results indicate a key difference between the signaling of hCG and LH, and provide a clue as to how the developmental and senescent expression of these proteins could influence AβPP expression and processing required during these different stages of life. Indeed, the functional differences between the cellular signaling of LH and hCG, despite the fact that they bind the same receptor, has remained a puzzle. In addition, the expression of both AβPP mature and immature forms was higher in the hES cells compared to the lineage-committed M17 cells, suggesting that full-length AβPP may be more important during the stem cell stage.

AβPP cleavage products were observed in both pluripotent and hCG treated hES cells (Fig. 3). The ~53 kDa (22C11 antibody) and 53 kDa and 29 kDa bands (4G8 antibody) have been observed in PDAPP transgenic mice expressing human AβPP with the familial AD AβPP 717 Val → Phe mutation [21]. This similarity in the expression of AβPP and its cleavage products between degenerative and developmental stages of life suggests a developmental function of AβPP during the pathology of Alzheimer's disease (AD).

The finding that M17 neuroblastoma cells, considered to be 'undifferentiated' transformed cells, do not express the pluripotency marker Oct-3/4 indicates that the specification of cell lineage and the acquisition of functional characteristics, both considered to be differentiation characteristics, are two separate components of the 'differentiation' pathway.

These results indicate that AβPP is expressed and processed in pluripotent hES cells during early human embryogenesis and that the pregnancy hormone hCG modulates these processes. Further studies on this novel role of hCG in signaling via LH/hCG receptors to modulate the expression of AβPP (and other proteins) in hES cells could have far-reaching implications for understanding the role of AβPP in human embryogenesis and its critical molecular signaling link to the immediate hormonal environment of pregnancy.

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