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Expression and knockdown of cellular prion protein (PrP^C) in differentiating mouse embryonic stem cells

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ABSTRACT

The mammalian cellular prion protein (PrP^C) is a highly conserved glycoprotein that may undergo conversion into a conformationally altered isoform (scrapie prion protein or PrP^{Sc}), widely believed to be the pathogenic agent of transmissible spongiform encephalopathies (TSEs). Although much is known about pathogenic PrP conversion and its role in TSEs, the normal function of PrP^C is poorly understood. Given the abundant expression of PrP^C in the developing mammalian CNS and the spatial association with differentiated stages of neurogenesis, recently it has been proposed that PrP^C participates in neural cell differentiation. In the present study, we investigated the role of PrP^C in neural development during early embryogenesis. In bovine fetuses, PrP^C was differentially expressed in the neuroepithelium, showing higher levels at the intermediate and marginal layers where more differentiated states of neurogenesis were located. We utilized differentiating mouse embryonic stem (ES) cells to test whether PrP^C contributed to the process of neural differentiation during early embryogenesis. PrP^C showed increasing levels of expression starting on Day 9 until Day 18 of ES cell differentiation. PrP^C expression was negatively correlated with pluripotency marker Oct-4 confirming that ES cells had indeed differentiated. Induction of ES cells differentiation by retinoic acid (RA) resulted in up-regulation of PrP^C at Day 20 and nestin at Day 12. PrP^C expression was knocked down in PrP-targeted siRNA ES cells between Days 12 and 20. PrP^C knockdown in ES cells resulted in nestin reduction at Days 16 and 20. Analysis of bovine fetuses suggests the participation of PrP^C in neural cell differentiation during early embryogenesis. The positive association between PrP^C and nestin expression provide evidence for the contribution of PrP^C to ES cell differentiation into neural progenitor cells.

1. Introduction

The mammalian cellular prion protein (PrP^C) is a highly conserved glycoprotein localized in membrane lipid rafts and anchored to the cell surface by glycophosphatidylinositol (GPI) (McKinley et al., 1991). It is present in many cell types, and is particularly abundant in neurons (Taraboulos et al., 1992). Under certain conditions PrP^C may undergo conversion into a conformationally altered isoform (scrapie prion protein or PrP^{Sc}) widely believed to be the pathogenic agent in prion diseases or transmissible spongiform encephalopathies (TSEs) (Caughey et al., 1991; Pan et al., 1993). Although much is known about the effect of PrP^{Sc} in prion disease, the normal function of PrP^C is poorly understood. PrP^C binds copper ions, can function as a Cu/Zn superoxide dismutase and has been shown to protect cells against oxidative stress (Vasallo and Herms, 2003). Alternatively, PrP^C may act as an antiapoptotic agent by blocking some of the internal or environmental factors that initiate apoptosis (Bounhar et al., 2001; Roucou et al., 2005). Despite these putative roles, mice null for PrP^C display no consistent phenotype apart from complete resistance to TSE infection (Bueler et al., 1992, 1993).

Recently, several authors have proposed that PrP^C participates in transmembrane signaling processes associated with hematopoietic stem cell replication and neuronal differentiation (Mouillet-Richard et al., 2000; Zhang et al., 2006; Steele et al., 2006). Abundant expression of PrP^C has been detected during mouse embryogenesis in association with the developing nervous system (Manson et al., 1992; Miele et al., 2003; Tremblay et al., 2007). In the developing mouse brain, undifferentiated neural progenitor cells in the mitotically active ventricular zone do not express PrP^C. In contrast, post-mitotic neurons express high levels

Abbreviations: PrP^C, cellular prion protein; PrP^{Sc}, scrapie prion protein; Prnp, prion gene; TSE, transmissible spongiform encephalopathy

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of PrP^C after their last mitosis in the neuroepithelium as migrate towards the marginal layers and differentiate (Steele et al., 2006; Tremblay et al., 2007). Thus, PrP^C may be expressed exclusively in differentiated neurons. Studies in vitro have showed that expression of PrP^C is positively correlated with differentiation of multipotent neural precursors into mature neurons (Steele et al., 2006). In addition, treatment of embryonic hippocampal neurons with recombinant PrP^C enhances neurite outgrowth and survival (Kanaani et al., 2005).

Given the abundant expression of PrP^C in the developing mammalian CNS and the spatial association with differentiated stages of neurogenesis in the neuroepithelium, we investigated the role of PrP^C in neural development during early bovine embryogenesis (gestation Days 27 and 39; total gestation interval = 283 days). The spatial localization of PrP^C in the nervous system of early bovine fetuses was first analyzed. We examined whether PrP^C shared a common location with nestin, a marker of neuronal progenitor cells and MAP-2, a mature neuron marker. PrP^C was differentially expressed in the neuroepithelium, showing higher levels at the intermediate and marginal layers which are occupied by more differentiated neuronal cells. Expression of PrP^C in the nervous system at these early developmental stages suggested that PrP^C might play a role in nervous system development. However, PrP^C levels do not lend themselves to convenient experimental manipulation in the bovine system. Therefore, we chose to examine the effect of siRNA-mediated knockdown of PrP^C in differentiating mouse embryonic stem (ES) cells. We found evidence for a significant contribution of PrP^C in the process of neural cell differentiation.

2. Material and methods

2.1. Bovine fetuses

Intact early gestation bovine fetuses were obtained from six clinically healthy cows at a local abattoir. Reproductive tracts were obtained at slaughter and gently palpated for the presence of a four-to six-week old fetus (bovine gestation interval = 283 days, or about 40 weeks). Though detailed descriptions of early bovine nervous system development have not been made, neural tube closure occurs on Day 22 and the formation of sulci and gyri commences around Day 60 (Winters et al., 1942). Thus, fetuses of four-to-six week's gestational age clearly represent early stages of neural development. After extraction from the uterus and placental membranes, crown-rump length of each fetus was measured in order to estimate fetal age as described in Winters et al. (1942). Fetuses were then fixed in 10% formalin.

2.2. Immunohistochemistry

Formalin-fixed fetuses were embedded in paraffin and sectioned at 5–7 μm using a microtome (Historange, LKB Bromma, Sweden). Tissue sections were mounted on adhesive coated slides (Newcomer supply; Middleton, Wisconsin) and incubated overnight at 37 °C. Mounted tissues were deparaffinized in xylene and rehydrated in serial alcohol solutions. Slides were subjected to an unmasking protocol by autoclaving at 120 °C for 5 min in an unmasking solution (Vector Laboratories, Burlingame, CA, USA). Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxidase diluted in 0.1 M PBS for 30 min. Slides were then rinsed two times in PBS and blocked in 2.5% horse serum for 15 min. Tissues were probed for PrP^C by incubating overnight at RT with 1° antibody (SAF-32, Cayman Chemical Company, Ann Arbor, MI) diluted 1:400 in 1.5% equine serum (Vector Labs.). Serial slides were probed similarly for nestin using a mouse monoclonal antibody (1:200; 2Q178, Santa Cruz Biotechnology, Santa Cruz Biotech, CA) and for MAP-2 using a mouse monoclonal (1:200; MT-01, Santa Cruz Biotech). After two washes in PBS, bound 1° antibody were detected using pan-specific 2° antibody conjugated to horseradish-peroxidase (Vector Labs.) by incubating for 10 min at RT. Immune complexes were visualized using 3, 3′-diaminobenzidine (DAB) substrate for 5 min or until the signal became visible. Probed sections were then counterstained with hematoxilyn and dehydrated in serial alcohol solutions. Sections were mounted with permount (Fisher Scientific, Hampton, NH) under coverslips. Bovine obex tissue was prepared and probed in parallel to serve as a positive control for PrP^C. The following procedural controls were performed on neighboring sections: (1) replacement of the 1° antibody with non-immune serum, (2) replacement of the 2° antibody with non-immune serum and (3) omission of both 1° and 2° antibodies, followed by incubation in DAB alone. Digital photos of tissue sections were obtained using bright microscopy (Olympus Vanox-T, Tokyo, Japan).

2.3. ES cell culture

Mouse ES cells (D3) and STO feeder cells (SNL) were obtained from ATCC (Manassas, VA, USA) and cultured according to Rudnicki and McBurney (1987). STO cells were seeded onto 0.1% gelatin-coated 100 mm diameter tissue culture dishes and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml gentamicin. When 80% confluent, STO cells were mitotically inactivated in 10 μl/ml mitomycin C for 1 h (Sigma Aldrich, St. Louis, MO). Approximately 1×10^6 ES cells were then seeded on feeders in DMEM supplemented with 1% non-essential aminoacids, 10^{-4} M β-mercaptoethanol, 1000 units/ml leukemia inhibitory factor (LIF; Millipore, MA), 15% ES cell-qualified FBS (Hyclone, Logan, UT) and 50 μg/ml gentamicin. Under these conditions, undifferentiated ES cells form typical compacted colonies attached to monolayer of STO cells. Medium was changed every other day and ES cells were detached using 0.25% trypsin and 1 mM EDTA in 0.1% PBS (trypsin/EDTA) and passaged 1:4 onto STO feeders every 3-days.

To initiate differentiation, ES cell were gently harvested when 80% confluent using trypsin/EDTA. Relatively intact ES cell colonies were resuspended in 10 ml ES cell medium in a conical tube separated from STO cells by gravity sedimentation for 10 min. The denser ES cell colonies formed a soft pellet, while most of the STO cells remained in suspension. After discarding the supernatant, the pellet was resuspended in ES cell medium and further separation of ES cells and STO cells was achieved by repeated differential plating after which most STO cells attached to the dish. At this point, the unattached ES cell colonies were reduced in trypsin/EDTA to a monocellular suspension by trituration. To initiate differentiation feeder-depleted ES cells were centrifuged at 200 g for 10 min and resuspended in differentiation medium (ES cell medium without LIF), seeded into non-adherent plastic bacteriological petri dishes and placed in the incubator. Under these conditions, ES cells aggregated within a few hours and formed embryoid body (EBs). After four days, EBs were disaggregated by trituration in trypsin/EDTA, resuspended in differentiation medium and seeded into plastic tissue culture dishes and cultured for an additional 17 days. Samples were obtained at three-day intervals for 21 days and analyzed for PrP^C and Oct-4 expression by western blot; PrP^C expression was also analyzed by real-time quantitative PCR.

To study the effect of retinoic acid (RA) on ES cell differentiation, ES cells were cultured according to the 4−/4+ protocol reported by Bain et al. (1995). Briefly, ES cells were allowed to