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TECHNICAL ARTICLE

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Induction of a high population of neural stem cells with anterior neuroectoderm characters from epiblast-like P19 embryonic carcinoma cells

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Abstract The epiblast, derived from the inner cell mass (ICM), represents the final embryonic founder cell population of mouse embryo and can give rise to all germ layer lineages including the neuroectoderm. The generation of neural stem cells from epiblast-like cells is of great value for studying the mechanism of neural determination during gastrulation stages of embryonic development. Mouse embryonic carcinoma (EC) P19 cells are equivalent to the epiblast of early post-implantation blastocysts. In this study, we establish a feasible induction system that allows rapid and efficient derivation of a high percentage (~95%) of neural stem cells from P19 EC cell in N2B27 serum-free medium. The induced neural stem cells bear anterior neuroectoderm characters, and can be efficiently caudalized by retinoic acid (RA). These neural stem cells have multilineage potential to differentiate into neurons, astrocytes, and oligodendrocytes. Mechanistic analysis indicates that inhibition of the bone morphogenetic protein (BMP) pathway may be the main reason for N2B27-neural induction, and that fibroblast growth factor (FGF) signaling is also involved in this process. This method will provide an *in vitro* system to dissect the molecular mechanisms involved in neural induction of early mouse embryos.

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Introduction

Around the time of implantation of mouse embryo, the surface of the inner cell mass (ICM) differentiates into primitive endoderm, while the remainder of the ICM undergoes rapid proliferation, selective apoptosis, differentiation and reorganize to form the final embryonic founder cell population, the epiblast. The epiblast represents the direct origin of the entire embryo, including the germline and other extraembryonic tissues (Gardner and Beddington, 1988). The anterior visceral endoderm (AVE), which derives from the primitive endoderm, induces anterior characters in the underlying epiblast as it sweeps anteriorly. During gastrulation, the anterior mesendoderm (AME) derived from the anterior primitive streak moves anteriorward to displace the AVE and convert the competent anterior epiblast into anterior neuroectoderm (Thomas and Beddington, 1996; Beddington and Robertson, 1999; Lu et al., 2001). The central nervous system (CNS) derived from the neural plate is then patterned to acquire regional specifications along the anterior–posterior (A–P) and dorsal–ventral (D–V) axes. In the early phase of the A–P specification, the neural tube is subdivided into the forebrain, midbrain, hindbrain, and spinal cord. To date, knowledge of the genetic hierarchies governing these events is limited, due in part to the small size, complexity, and inaccessibility of the early post-implantation embryo, as well as the rapid pace of maturation.

The establishment of embryonic stem (ES) cell lines has provided many new experimental approaches to

study early mammalian development. ES cells are undifferentiated and pluripotent cells derived from the ICM of blastocysts (Evans and Kaufman, 1981; Martin, 1981). When cultured *in vitro* on feeder layers of embryonic fibroblasts, or in the presence of leukemia inhibitory factor, they maintain an undifferentiated phenotype. Upon induced differentiation *in vitro*, they form embryoid bodies that contain cells of all three germ layer lineages (Doetschman et al., 1985). Many protocols have been developed to induce ES cells to differentiate into the neuroectoderm lineage, and they provide a feasible platform to study the process from the ICM to the neuroectoderm. However, these ES cell neural induction systems do not recapitulate well the *in vivo* neural induction process, which is generally assumed to begin in the early gastrula stage (Lu et al., 2001; Stern, 2005). ICM must reorganize to form the epiblast before it can initiate gastrulation and be committed to the neuroectoderm. These ES cell neural induction protocols mixed the two transitions (i.e., from ICM to epiblast and from epiblast to neuroectoderm) together, and those signals that contribute to the epiblast formation may interfere with the detailed study of neural induction. Moreover, the ES cell-derived neural stem cells usually do not acquire the intrinsic A–P polarity as their counterparts do *in vivo*. Therefore, it is still necessary to establish an *in vitro* system to convert a high percent of epiblast-like cells into neural stem cells with the correct A–P axis identities for the study of the process of transformation from the epiblast to the neuroectoderm.

Mouse embryonic carcinoma (EC) P19 cell is a teratocarcinoma cell line derived from transplanted epiblast stage cells of mouse embryo (McBurney and Rogers, 1982; van der Heyden and Defize, 2003). P19 EC cells are pluripotent cells with the ability to differentiate into derivatives of three germ layers in response to different chemical inducers (McBurney, 1993). These cells are considered to be equivalent to epiblast cells of the early post-implantation blastocyst according to their developmental stages (Riego et al., 1995; Yeom et al., 1996) and to their cell-surface antigen expression and protein synthesis patterns (Evans and Kaufman, 1981). It has been widely used as a representative cell system to study events that occur during gastrulation stages of embryonic development (van der Heyden and Defize, 2003). Modern developmental biology has benefited significantly from studies on P19 cells to understand the mechanisms of neural and skeletal muscle differentiation (McBurney, 1993; Bain et al., 1994; Skerjanc, 1999).

P19 EC cells can be induced to differentiate into neurons and glial cells when aggregated in the presence of retinoic acid (RA) (Jones-Villeneuve et al., 1982, 1983). This method is commonly used for molecular analysis of neural induction and differentiation (Vojtek et al., 2003; Akiyama et al., 2004; Seo et al., 2005). However, there are several drawbacks to this RA-induced P19 cell neural differentiation. First, only a small percentage of neural stem cells can be induced from P19 cells; many different cell types exist in the induced cell population, and these non-neural cells may interfere with detailed analysis of neural differentiation (McBurney et al., 1982; Doetschman et al., 1985). Second, in early embryos, the inhibition of retinoid signaling does not affect neural plate/neural tube formation (Blumberg, 1997; Niederreither et al., 1999), suggesting that RA does not participate in the neural induction process *in vivo*. Thus, molecular mechanisms studied by RA-induced P19 cell neural determination do not necessarily recapitulate the related events *in vivo*. Third, RA is particularly required to pattern the neural plate along the A–P axis during CNS development (Blumberg, 1997; Niederreither et al., 1999). It induces caudalization of anterior neural tissues, and inhibits rostral neural tissue formation (Ruiz i Altaba and Jessell, 1991). Moreover, RA treatment also perturbs the neural patterning and neuronal identities of ES cell aggregates (Wichterle et al., 2002). Neural precursors produced by RA induction appear to be developmentally restricted, and can only generate a limited range of neural cell types (Renoncourt et al., 1998). Therefore, a system with a high population of neural stem cells and without RA treatment for P19 cell neural induction is needed.

In this study, we established an efficient neural induction method to induce P19 EC cells to differentiate into neural stem cells without RA treatment. These induced-neural stem cells bear anterior neuroectoderm characters and can differentiate into neurons, astrocytes, and oligodendrocytes. This neural induction system recapitulates well with the transition process from epiblast to neuroectoderm, and could be used to study the molecular mechanisms of this process in early mouse embryos.

Materials and methods

Cell culture and neural induction in N2B27 serum-free medium

P19C6, a subclone of the mouse EC P19 cell line, was used in this study, and the RA-induced P19 cell neural differentiation was performed as described previously (Gao et al., 2001; Tang et al., 2002).

For N2B27 induction (Fig. 2A), nearly confluent P19 cells were dissociated into single cells by Trypsin-EDTA (Gibco, Carlsbad, CA), and allowed to aggregate in the bacteriological petri dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ) at a seeding density of 1×10^5/ml in N2B27 medium (1:1 mix of DMEM/F12 supplemented with 25 μg/ml insulin, 100 μg/ml apo-transferrin, 6 ng/ml progesterone, 16 μg/ml putrescine, 30 nM sodium selenite, 50 μg/ml bovine serum albumin fraction V, and neurobasal medium supplemented with B27 without Vitamin A). All chemicals were from Sigma (St. Louis, MO), except the BSA and B27 media, which were from Gibco. After 4 days of culture in suspension, the cell aggregates were collected, rinsed once with phosphate-buffered