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USE OF A MURINE EMBRYONIC STEM CELL LINE THAT IS SENSITIVE TO HIGH GLUCOSE ENVIRONMENT TO MODEL NEURAL TUBE DEVELOPMENT IN DIABETIC PREGNANCY

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Abstract

Background—Neural tube defects (NTDs) are significantly increased by maternal diabetes. Embryonic stem cells (ESC) that can differentiate into neuroepithelium and can sense supraphysiological glucose concentrations would be very valuable to simulate the effects of maternal diabetes on molecular and cellular processes during neural tube formation.

Methods—LG-ESC, a recently established ESC line that expresses the glucose transporter, Scl2a2, and is sensitive to elevated glucose concentrations, were grown for up to 8 days in a 3-dimensional culture to form neural cysts (NC). We tested whether high glucose media inhibits expression of *Pax3*, a gene that is required for neural tube closure and whose expression is inhibited in embryos of diabetic mice, and inhibits formation of NC.

Results—*Pax3* expression was detected after 4 days of culture and increased with time. *Pax3* expression was inhibited by high glucose media, but not if cells had been cultured in low glucose media for the first 4 days of culture. *Pax7*, which is also expressed in dorsal neural tube, was not detected. *Pax6*, which is expressed in the ventral neural tube, was detected only after 8 days of culture, but was not inhibited by high glucose. High glucose media did not inhibit formation of NC.

Conclusions—LG-ESC can be used as a model of embryonic exposure to a diabetic environment during neural tube development. While high glucose exposure inhibits expression of a gene required for neural tube closure, it may not inhibit all of the processes involved in formation of a neural tube-like structure.

Keywords

Embryonic stem cells; Scl2a2; Glut2; hyperglycemia; neural tube defect; pregestational diabetic pregnancy; Pax3

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INTRODUCTION

Maternal diabetes, either type 1 or type 2, existing prior to conception, significantly increases risk for a variety of congenital malformations; neural tube defects (NTDs), including spina bifida, anencephaly, craniorachischisis, and encephalocele, are among the most common that occur (Correa and others, 2008; Zabihi and Loeken, 2010). The malformations arise very early during embryogenesis, as the neural tube is one of the first organs to form (beginning approximately two weeks after fertilization in humans, 8 days in the mouse) (Kaufman, 1992; Mills, 1982; Moore and Persaud, 1993; Phelan and others, 1997).

Rodent models of diabetic pregnancy have been widely used to study the mechanisms by which the NTDs occur (Gareskog and others, 2007; Phelan and others, 1997; Reece and Eriksson, 1996; Yang and others, 2008; Zabihi and Loeken, 2010). Our laboratory developed a mouse model and has demonstrated that exposure of embryos to elevated glucose levels (250 mg/dL, 14 mmol/L) is responsible for inducing NTDs (Fine and others, 1999). Several biochemical and signal transduction pathway disturbances have been found to be associated with diabetic pregnancy-induced malformations (Gareskog and others, 2007; Reece and Eriksson, 1996; Yang and others, 2008), but how they actually cause NTDs is not clear. We have found that insufficient expression of *Pax3*, a gene that is essential for neural tube closure, in response to maternal hyperglycemia, can lead to NTDs (Phelan and others, 1997). *Pax3* stimulates p53 degradation; thus, when *Pax3* levels are insufficient, p53 protein levels increase, thereby stimulating neuroepithelial apoptosis (Pani and others, 2002; Wang and others, 2011). The apoptosis is initially limited to the edges of the neural folds (Phelan and others, 1997), indicating that apoptosis of the migrating neuroepithelium causes the closure of the neural tube to abort.

While animal models can be very instructive of the regulation of neural tube development and etiology of NTDs, an *in vitro* model that would allow particular cell lineages to be studied in the absence of other tissues, and in which the environment can be perturbed in a controlled fashion, would be very valuable. Embryonic stem cells (ESC) that can be induced to form neuroepithelium offer such a model. ESC can be grown in quantity, enabling molecular and biochemical experiments that would be difficult or impossible using embryos. Additionally, understanding the control of neuroectodermal differentiation using ESC may be informative for utilization of ESC (or induced pluripotent stem cells) to repair NTDs or for spinal regenerative therapy.

Until recently, a limitation of using existing ESC lines to study effects of a diabetic environment on neuroepithelial development was that they are not responsive to elevated glucose concentrations (simulating diabetic hyperglycemia). Preimplantation embryos (from which ESC are derived), as well as postimplantation embryos during formation of the neural tube, express *Scl2a2*, as well as *Scl2a1* and *Scl2a3* (Aghayan and others, 1992; Hogan and others, 1991; Pantaleon and others, 1997). *Scl2a2* encodes the high K_M Glut2 ($K_M \sim 16$ mmol/L) glucose transporter that is expressed by pancreatic β -cells and the liver and transports postprandial glucose into these tissues (Uldry and Thorens, 2004). Because the K_M of Glut2 is much higher than normal circulating glucose concentrations (~ 5 mmol/L), it

is probably not a major glucose transporter for the embryo in nondiabetic pregnancies but may be expressed to transport other solute(s). Rather, Glut1 and Glut3, encoded by *Slc2a1* and *Slc2a3*, respectively, with K_M 's of approximately 5 mmol/L, are the major embryo glucose transporters at physiological glucose concentrations. However, during episodes of maternal hyperglycemia, Glut2 transports glucose into embryo cells more effectively than Glut1 and Glut3 (Li and others, 2007). Indeed, *Slc2a2*^{-/-} embryos failed to develop NTDs in response to maternal hyperglycemia (Li and others, 2007), demonstrating that Glut2-mediated glucose transport during maternal hyperglycemia is responsible for susceptibility of the embryo to the adverse effects of maternal diabetes. Culture of blastocysts in high glucose media (450 mg/dL; 25 mmol/L), as is conventionally done during establishment and propagation of ESC, induces oxidative stress, just as does hyperglycemic exposure of the embryo *in vivo* (Chang and others, 2003; Reece and Eriksson, 1996; Viana and others, 1996; Wang and others, 2006). Therefore, as we recently reported, ESC lines that have been able to survive in high glucose media appear to have down-regulated expression of Glut2 (Jung and others, 2013). However, an ESC line that we established in low glucose media (100 mg/dL; 5.5 mmol/L), called, LG-ESC, retains expression of a functional Glut2 glucose transporter (Jung and others, 2013). Culture in high glucose media induces oxidative stress in LG-ESC (Jung and others, 2013). When induced to differentiate to neuroepithelium, high glucose media inhibits expression of *Pax3*, and the inhibitory effects of high glucose media are suppressed by antioxidants (Jung and others, 2013). Thus, LG-ESC can be used as a “development in a dish” model to study metabolic and molecular effects of a diabetic environment on neuroepithelial development.

In previous studies, ESC-derived neuroepithelial cells were selected from embryoid bodies (EBs) by culture in media containing insulin, fibronectin, selenium, and transferrin (Jung and others, 2013; Lee and others, 2000). While these cells express genes characteristic of neuronal precursors and have the potential to form functional neurons (Lee and others, 2000), they grow on a flat tissue culture surface and lack the 3-dimensional structure of the neural tube. Additionally, progenitors of each of the 3 germ layers arise in EBs, and so, it is difficult to study the early molecular events that induce neuroepithelial cell fate and the induction of *Pax3* expression. Furthermore, because ESC are developmentally comparable to day 4.5 epiblast (Boroviak and others, 2014) and neuroepithelium arises about 4 days later (Kaufman, 1992), study of neuroepithelial development from EB cultures does not replicate the time frame of this process *in vivo*. A method for 3-dimensional culture of ESC to replicate the embryonic patterning of the neural tube was recently reported (Meinhardt and others, 2014). Hollow spheres, or “neural cysts” of polarized neuroepithelium were formed from single cells. The neural cysts exhibit a gene expression pattern characteristic of the anterior region of the dorsal neural tube over a time course similar to formation of the neural tube from cells of the epiblast *in vivo*. Because the anterior dorsal neural tube is the most frequent the site of NTDs caused by diabetic pregnancy in mouse embryos (Phelan and others, 1997), we investigated whether *Pax3* expression and formation of neural cysts are inhibited by 3-dimensional culture of LG-ESC in high glucose media.

METHODS

Culture of LG-ESC

Derivation of Low Glucose Embryonic Stem Cells (LG-ESC) from FVB blastocysts was previously described (Jung and others, 2013). LG-ESC were maintained as monolayer cultures on gelatin-coated dishes in ESC media containing low glucose (100 mg/dL; 5.5 mmol/L) Dulbecco's Modified Eagle's Media (DMEM) (Life Technologies, Grand Island, NY) and in a 37°C incubator in an atmosphere of 5% CO₂: 5% O₂ (balance room air) as described (Jung and others, 2013). The glucose concentration of 1-2 µl of media was checked with a blood glucose monitor (ACCU-CHECK Advantage, Roche Diagnostics, Indianapolis, IN) twice/day and the actual glucose concentration of the media was determined using a curve that had been generated using dilutions of media standards with known glucose concentrations. Volumes of a 50% glucose solution that were calculated to maintain the glucose concentration at 100 mg/dl were then added to media. Cells were detached from culture dishes with trypsin (Life Technologies) and passaged at a concentration of 5.0×10^4 cells/ mL every four days.

For gene expression assays, undifferentiated (UD) LG-ESC, EBs, or neuronal precursors were harvested after 4 days of culture in low glucose (100 mg/dl) or high glucose (315 mg/dl; 17.5 mmol/L) ESC media. High glucose ESC media was made by adding 430 µl 50% glucose per 100 ml low glucose ESC media. EBs were formed from UD ESC as described (Jung and others, 2013; Wu and others, 2012). Briefly, cells were removed from culture dishes by trypsinization and were transferred into nonadherent bacterial-grade culture dishes containing the same media as used for culture of UD LG-ESC, except lacking leukocyte inhibitory factor (Millipore, Billerica, MA), but containing 0.5 µmol/L retinoic acid (Sigma-Aldrich, St. Louis, MO). Neuronal precursors were selected from EBs as described (Lee and others, 2000; Wu and others, 2012) except using low glucose media as described (Jung and others, 2013). All cultures were performed in triplicate.

Induction of Neural Cysts

LG-ESC were induced to form neural cysts using methods developed by E. Tanaka and colleagues (Meinhardt and others, 2014), except using either low or high glucose media. Briefly, LG-ESC were dissociated to single cells with Accutase (Life Technologies). Reactions were stopped with Low Glucose Neural Cyst media (LG-NC media), which was made as described (Meinhardt and others, 2014) except that N-2 media were made using low glucose DMEM/Ham's F-12 (final glucose concentration = 140 mg/dL; 7.8 mmol/L). The cells were centrifuged at $193 \times g$ for five minutes. The cell pellet was resuspended in LG-NC media and the cell concentration was adjusted to 2×10^6 cells/mL. Ten µL of the cells were dispensed into pre-chilled 1.5 mL tubes on ice. Using pre-chilled pipet tips, 150 µL of Matrigel (Corning Discovery Labware, Bedford, MA) (kept on ice) were added to one tube of cells, mixed, and immediately dispensed onto one 35 mm culture dish. The cell-Matrigel suspension was allowed to solidify at room temperature for approximately ten minutes. Fifty µL of additional Matrigel were overlaid on each cell-Matrigel suspension. After an additional 10 minutes, 3 mL of LG-NC media were added to the culture dish. The cells were incubated as above. The glucose concentration in the media was maintained at 140 mg/dL

on subsequent days of culture by testing media glucose concentrations and adding 50% glucose as described above. Where indicated, some cultures were grown in High Glucose Neural Cyst media (HG-NC media), which was made by adding 350 μ L of 50% glucose solution per 100 mL LG-NC media to bring the glucose concentration to 315 mg/dL. The glucose concentration was tested on subsequent days of culture and maintained at 315 mg/dl as described above. All cultures were performed in triplicate.

Morphological Analyses

Phase-contrast images of cultures were captured using an inverted stage microscope with attached DP70 camera using cellSens Standard software 1.7.1 (Olympus, Center Valley, PA).

RT-PCR Assays

At the end of culture, media were removed and culture dishes were washed three times with ice cold PBS. The Matrigel was depolymerized to release cells using Cell Recovery Solution (Corning) as instructed by the manufacturer. RNA was prepared from all cultures using Ultraspec RNA Total Isolation (BIOTECHX, Houston, TX). RNA was resuspended in 100 μ L of low TE buffer and 5 μ L were reverse transcribed in quadruplicate using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). The reverse transcription reaction was diluted 5-fold and 2 μ L were amplified by real time PCR using TaqMan PCR Master Mix (Life Technologies). Primers and VIC-labeled probe to detect *rRNA* (Life Technologies #43189E) was used as the normalization control as previously described (Chang and others, 2003). *Pax3* primers and FAM-labeled probe were as previously published (Chang and others, 2003). Primers and FAM-labeled probes for *Pax6* (Mm00443081_m1), *Pax7* (Mm01354484_m1), *Sox1* (Mm00486299_s1), *smooth muscle actin* (*Sma*; Mm01546133_m1), and *α -fetoprotein* (*Afp*; Mm00431715_m1) were obtained from Life Technologies. A FAM-labeled *Nestin* probe (CAGCCTCCAGCAGAGTCCTGTATG) was prepared by Life Technologies, and *Nestin* primers (F: GAAGGTGGGCAGCAACTGGCA; R: CCAGGTGTCTGCAAGCGAGAG) were synthesized by Integrated DNA Technologies (Coralville, IA).

Statistical Analyses

RT-PCR results were analyzed by One-way ANOVA followed by Neuman-Keuls post test using GraphPad Prism software v. 4.0 (La Jolla, CA). $P < 0.05$ was determined to be statistically significant.

RESULTS

Effects of Glucose on Cyst Morphology by LG-ESC

To Investigate whether LG-ESC can form neural “cysts” in 3-dimensional culture similar to those recently reported (Meinhardt and others, 2014), and whether high glucose media can inhibit this process, LG-ESC were cultured in Matrigel suspensions with media (LG-NC or HG-NC) containing glucose concentrations that correspond to nondiabetic or diabetic blood glucose concentrations, respectively, as described in Methods, for up to 8 days. Because high glucose could induce oxidative stress and reduce survival of LG-ESC that express a

functional Glut2 transporter, some cultures were grown for the first 4 days in LG-NC media, followed by growth in HG-NC media for the last 4 days. Cultures were examined after 2, 4, 6, and 8 days of culture for morphology. Although the cells had been dispersed to single cells prior to 3-dimensional culture, within 2 days, the cells had divided to form clusters (Fig. 1 A, B). During 4-8 days of culture, the clusters formed spherical “cysts” (Fig. 1 C-J). Although we did not examine the cysts to determine whether they contained a lumen surrounded by an epithelium with apical-basal polarity as reported by Meinhardt, et al. (Meinhardt and others, 2014), their morphology is distinct from EBs (not shown). There were no notable differences in the ability to form cysts in any of the culture conditions. However, in general, the cysts that were cultured for all or part of the time in HG-NC media appeared larger (i.e., more cells per cyst) after 6 or 8 days of culture than cysts cultured the entire time in LG-NC.

Effects of Glucose on *Pax3* Expression by LG-ESC During Cyst Formation

To investigate whether *Pax3* expression by LG-ESC is induced during 3-dimensional culture, and whether it would be inhibited by high glucose, RNA was recovered after 2, 4, 6, or 8 days of 3-dimensional culture from LG-ESC. Insufficient amounts of total RNA were recovered for detection of *Pax3* mRNA after 2 days of culture (suggesting that some cells did not survive the first 2 days of culture), but *Pax3* mRNA was increased compared to the start of culture after 4 days. There was no significant difference between the expression of *Pax3* in cultures that had been grown in LG-NC or HG-NC media on day 4 (Fig. 2 A). *Pax3* expression increased after 6 and 8 days of culture, but was significantly reduced in the cultures grown the entire time in HG-NC media compared to the cultures grown for all or part of the time in LG-NC. There was no significant difference in *Pax3* expression on day 6 in cultures that had been cultured the entire time in LG-NC and those that were cultured for 4 days in LG-NC and then 2 days in HG-NC, and there was significantly more *Pax3* expression by the cells that had been cultured for 4 days in LG-NC and then 4 days in HG-NC compared to cells that had been cultured the entire time in LG-NC.

Pax7, which is expressed in a similar spatial and temporal pattern as *Pax3* (Mansouri and others, 1996) in embryos and observed by Meinhardt et al. in neural cysts (Meinhardt and others, 2014), was not detected in any of the cultures at any time point (data not shown). However, *Pax6*, which is expressed in the ventral neural tube and was not detected by Meinhardt et al. in neural cysts (Meinhardt and others, 2014), was expressed at barely detectable levels on days 0-6 and slightly increased on day 8 (Fig. 2 B). As in the embryo (Wei and Loeken, 2014), there was no effect of glucose exposure on *Pax6* expression.

To further investigate whether neural cysts formed from LG-ESC are neuroepithelial and are distinct from EBs, additional neuroectodermal markers, *Nestin* and *Sox1*, along with the endodermal marker, *α -fetoprotein* (*Afp*), and the mesodermal marker, *smooth muscle actin* (*Sma*), were assayed by RT-PCR. As shown in Fig. 3 A, *Nestin* mRNA was increased on days 4, 6, and 8 of culture compared to day 0. There was no difference in *Nestin* expression between neural cysts cultured in LG-NC and HG-NC media, although there was a nonsignificant trend toward decreased *Nestin* expression on day 8 in the high glucose cultures. *Sox1* expression increased after 6 and 8 days of culture, but only by the neural

cysts cultured in LG-NC (Fig. 3 B). Neither the endodermal marker, *Afp*, nor the mesodermal marker, *Sma*, were detected in any of the neural cyst cultures (not shown). We also assayed expression of *Pax3*, *Pax7*, and *Pax6*, as well as *Nestin*, *Sox1*, *Afp*, and *Sma* by undifferentiated (UD) LG-ESC and EBs that had been grown in ESC media containing low or high glucose media. Gene expression was expressed relative to neuronal precursors after 4 days of selection from EBs in low glucose media. *Pax3* was expressed by EBs grown in either low or high glucose media, and while less *Pax3* was expressed by EBs grown in high glucose media, the difference was not significant (Fig. 4 A). Similar to neural cysts, *Pax7* was either not detected or barely reached detection by any cultures (not shown). *Pax6* was expressed by EBs, and there was no difference between high and low glucose media (Fig. 4 B). The neuroectodermal markers, *Nestin* and *Sox1*, were expressed by EBs, and, similar to neural cysts, high glucose media inhibited expression of *Sox1* (Fig. 4 C, D). However, unlike neural cysts, the endodermal and mesodermal markers, *Afp* and *Sma*, were expressed by EBs (Fig. 4 E, F). There was no significant effect of glucose on *Afp* or *Sma* expression.

DISCUSSION

Many classical procedures to induce lineage-specific differentiation of murine ESC involve formation of EBs from pluripotent ESC by growing in nonadherent culture dishes in the absence of LIF (Turksen, 2002). When transferring monolayer ESC to embryoid body cultures, the cells are generally not separated into individual cells, so clusters of cells give rise to “balls” of proliferating cells (EBs). The proportion of cells in the EBs that gives rise to specific germ layers can be influenced by EB size, the microenvironment, and whether growth factors or agents that regulate signal transduction pathways are added. However, EB cultures are generally heterogeneous and lack the tissue architecture that arises during normal embryogenesis. Commonly, there are one or more steps to the differentiation procedure, in which cells of the unintended lineages are selected against, while the intended lineage proliferates and continues to differentiate. However, these processes can take days to weeks, much longer than the corresponding differentiation in the embryo.

Therefore, a method to derive neuroepithelium directly from single ESC without first forming EBs contaminated by cells of other germ layers, and which cells follow a similar expansion and folding process as occurs during neural tube formation *in vivo*, offers some advances for studying the regulation of neuroepithelial development over classical selection from EBs. Further morphological analyses are necessary in order to determine whether our neural cysts were, indeed, hollow spheres with appropriate apical-basal cell polarity. However, our results indicate that a high glucose environment does not inhibit formation of a cyst-like structure. Further investigation is necessary to determine whether the same regulators of planar cell polarity that are important for neural tube closure (Wallingford, 2012) regulate the assembly as well as the polarity in neural cysts, and whether high glucose media interferes with these processes.

When using classical methods (Lee and others, 2000) to select *Pax3*-expressing neuronal precursors from EBs, we observed that *Pax3* expression begins in EBs, then increases during the 4 subsequent days of culture in selective media (Fig. 4). Additionally, expression of endodermal and mesodermal markers was still detected in neuronal precursors, but

endodermal and mesodermal markers were undetectable in neural cysts. Thus, the time course with which to study regulation of *Pax3* expression by neural cysts, as well as tissue homogeneity, more closely resemble the mouse embryo than study using neural progenitors after first inducing EBs. Interestingly, the onset and magnitude of *Pax3* expression after 4 days of culture was not inhibited by high glucose, but expression was less robust after 6 and 8 days by cells culture the entire time in HG-NC. This suggests that oxidative stress induced by high glucose during the first 4 days of culture does not block the initial processes that are needed to “turn on” *Pax3* expression, but suppresses some of the processes needed for full activation of *Pax3* transcriptional machinery. We can only speculate how this occurs. One possibility is that the hypomethylation that is necessary for differentiation-induced *Pax3* expression is inhibited in the high glucose cultures, as we have recently reported using differentiating ESC in which oxidative stress is induced with the mitochondrial inhibitor, antimycin A (Wei and Loeken, 2014). However, high glucose media did not inhibit *Pax3* expression if the neural cysts were cultured for the first 4 days in low glucose media. This suggests that, like in the embryo, there is a window of time preceding the onset of *Pax3* expression (day 7 in embryos) in which high glucose can suppress the full activation of *Pax3* transcription, but beyond this point, *Pax3* expression cannot be attenuated (Fine and others, 1999). This further illustrates the differences in the time course of induction of *Pax3* expression between neural cysts and neuroepithelium selected from EBs, as *Pax3* expression by LG-ESC cultured through the EB stage in low glucose media was inhibited by high glucose media during selection of neuronal precursors (Jung and others, 2013).

Pax7 was not detected in any of the cultures, although, in preliminary experiments, we sometimes detected *Pax7* expression. This may be because in the hindbrain, *Pax7* expression is limited to rhombomeres 1, 3, and 5, whereas *Pax3* is expressed along the length of the hindbrain, except at the most rostral level (Mansouri and others, 1996). Thus, while the neural cysts appear to express markers of the anterior dorsal neural tube by default (Meinhardt and others, 2014), the exact location along the neuraxis that they correspond to may vary. On the other hand, *Pax7* was barely detectable or undetectable in EB and EB-derived neuronal precursor cultures as well, so this may be a characteristic of LG-ESC or the sensitivity of the *Pax7* assay, rather than the differentiation method. *Pax6* was expressed at very low levels after 8 days of culture, suggesting that some cells may have adopted a more ventral phenotype. *Pax6* was also expressed by EBs and at lower levels by EB-derived neuronal precursors (perhaps because including retinoic acid in EB and neuronal precursor selection media appears to favor selection of *Pax3*- over *Pax7*-expressing cells, unpublished results). As in the embryo (Wei and Loeken, 2014), *Pax6* expression was not regulated by glucose concentration.

Expression of the neuroectodermal markers, *Sox1* and *Nestin*, but not endodermal or mesodermal markers, by neural cysts supports that they are distinct from EBs. The inhibition of *Sox1* by high glucose, both in EBs and neural cysts, was unexpected. Further investigation will be necessary to determine whether *Sox1* and *Pax3* are coordinately regulated by glucose metabolism.

In conclusion, direct induction of neuroepithelium in 3-dimensional culture from undifferentiated ESC allows the study of inhibition of *Pax3* expression using a glucose-

responsive ESC line to model effects of diabetic pregnancy on neural tube development. Although *Pax3* expression was inhibited by high glucose media, the formation of neural cysts was not. Thus, this system may be useful for study of processes during induction of neuroectoderm and establishment of a tube-like structure. However, because, at least in our hands, formation of neural cysts did not appear to exhibit convergent extension, an important process during primary neurulation (Pryor and others, 2012; Ybot-Gonzalez and others, 2007), LG-ESC-derived neural cysts may not replicate all of the physical and mechanical processes involved in neural tube closure *in vivo*. Whether such processes are disturbed by high glucose *in vivo* and contribute to NTDs in embryos, or whether inhibition of *Pax3* expression was not sufficient to induce apoptosis, and therefore, halt neural cyst formation, will require further investigation. There may be other limitations of the neural cyst model as well. For example, if cells other than neuroepithelial contribute to NTDs, it may not be possible to replicate the processes involved, due to the apparent homogeneity of the neural cysts cell type. Also, although ESC can be grown in large quantities, there is a limit to the size of individual Matrigel suspensions that can feasibly be cultured which may limit large-scale production of 3 dimensional cultures.

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Figure 1.

Morphology of LG-ESC cysts with time and grown in LG-NC and HG-NC media. Cultures were grown the entire time in LG-NC (A, C, E, H), or HG-NC (B, D, F, I), or the first 4 days in LG-NC, and the last 4 days in HG-NC (G, J). Panels H and J each show 2 individual cysts that are in close proximity to the other but are separate structures.

Figure 2.

A. *Pax3* expression by LG-ESC with time compared to 0 days of culture and grown in LG-NC and HG-NC media. Cultures were grown the entire time in LG-NC (Low), HG-NC (High), or the first 4 days in LG-NC, and the last 4 days in HG-NC (Low → High). **B.** *Pax6* expression by LG-ESC with time compared to 0 days of culture and grown in LG-NC and HG-NC media. Cultures were grown the entire time in LG-NC (Low), or HG-NC (High) media. Because no effects of initial culture in LG-NC followed by culture in HG-NC were observed on *Pax3* expression, only effects of entire culture in LG-NC or HG-NC media were assayed. *P<0.05 vs. other cultures on the same day.

Figure 3.

A. *Nestin* expression by LG-ESC with time compared to 0 days of culture and grown as in Fig. 2B. **B.** *Sox1* expression by LG-ESC with time compared to 0 days of culture and grown as in Fig. 2B. **P<0.01 vs. high glucose culture on the same day.

Figure 4.

A. *Pax3* expression by undifferentiated LG-ESC (UD ESC), embryoid bodies (EB), or neuronal precursors selected from embryoid bodies (NP). Undifferentiated ESC and embryoid bodies were grown in low (100 mg/dl) or high (315 mg/dl) media for comparison with gene expression patterns in high or low glucose media by neural cysts shown in Figs. 2 and 3. *Pax3/rRNA* by all cultures was expressed relative to that by neuronal precursors. **B.** *Pax6* expression by LG-ESC cultured as in A. **C.** *Nestin* expression by LG-ESC cultured as in A. **D.** *Sox1* expression by LG-ESC cultured as in A. **E.** *α-fetoprotein (AFP)* expression by LG-ESC cultured as in A. **F.** *Smooth muscle actin (Sma)* expression by LG-ESC cultured as in A. **P<0.01 vs. high glucose cultures at the same stage of development.