INDUCED DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS TOWARD MOTOR NEURONS

by

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ABSTRACT

The understanding of mammalian cellular differentiation and cell fate specification are progressing intensively using an in vitro system, comprised of embryonic stem cells. Research on the cell fate specification in the central nervous system (CNS) is of enormous interest given the therapeutic potential in neuronal repair strategies. The main focus of this study was to derive motor neurons from human embryonic stem cells (hESC). For this main purpose, first study involved derivation and proliferation of neuroepithelial stem cells (NEP) which are the earliest multipotent neural stem cells from hESC. Embryonic stem cells cultured in serum-deprived defined medium developed a distinct canal structure which could be isolated either by dissociation or physical feeder separation. Dissociated cells formed colonies comprised of cells characterized as NEP in MEDII medium (HepG2 cell conditioned medium) dependent manner. However, cells isolated by feeder separation maintained adherence and developed enriched NEP like cells independent of exposure to MEDII. Further characterization indicates that these cells have a phenotype profile and differentiation potential of NEP. To proliferate NEP, ideal cell culture conditions were established and cells have been proliferated successfully in this condition for over six months, maintaining stable karyotype and without loss of their multipotent neural

stem cell characteristics. After successful derivation and proliferation of NEP, studies were conducted to differentiate them into specific type of neurons. To differentiate NEP into motor neurons, specific morphogens that have been demonstrated as important in development were introduced to short and long term cultured NEPs. First, freshly isolated (< one month, early) and propagated (> three months, late) cultures of NEP were characterized and both populations were exposed to inductive signals for the stimulation into motor neuron. Increased motor neuron gene expression was shown in both early and late NEP by retinoic acid and additional effect of sonic hedgehog was observed in early NEP. Finally, a spinal motor neuron phenotype was demonstrated in early and late NEPs. The acquired efficient neural induction, long term culture of NEP and subsequently derived motor neuron would serve as a great in vitro model to understand developmental cues and to overcome diseases related to motor neurons.

INDEX WORDS: Human embryonic stem cells, Neuroepithelial stem cells, motor neuron, induced differentiation, Sonic hedgehog, Retinoic acid, Fibroblast growth factor

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Chapter 1

Introduction

Embryonic stem cells are cell lines isolated from the blastocyst stage embryo. Pluripotency and their self renewal capacity make them a good in vitro model for developmental study, drug screening and regenerative medicine. With the exciting achievement in human embryonic stem cell (hESC) establishment in 1998, as well as adult stem cell research, new attention has been focused on clinical applications of stem cell therapy. Although adult stem cells have been shown to be more versatile in differentiation than has been thought, ESC can still be a cell source for therapy with their immortality and pluripotency. For the best use of these cells, the biology of ESC and their molecular and cellular mechanisms for directed differentiation need to be elucidated.

MOUSE EMBRYONIC STEM CELLS

Prior to derivation of ESC, studies on pluripotency and cell differentiation had been carried out using embryonic carcinoma cells (ECC) which originate from teratocarcinomas. This malignant tumor can be produced spontaneously and contain various tissues including undifferentiated populations. Embryonic Carcinoma cells are established from these undifferentiated tumor cells and studies done with ECC contributed to derivation, in vitro culture and in vitro differentiation of ESC.

The first ESC were established from the mouse in 1981 [1]. Embryonic stem cells are derived from the inner cell mass of the pre-implantation embryo. The inner cell mass is the forebear of the epiblast which has the potential to develop into all cell types in the body. Once the trophoblast is removed from the blast either by immunosurgery or by mechnical disruption, the isolated inner cell mass is plated on a supporting cell layer. To culture ESC, co-culture with a feeder layer was considered essential, but it was shown that diffusible factors from conditioned medium could inhibit differentiation of ESC [2] and the single cytokine Leukemia inhibitory factor (LIF) was found to maintain ESC in an undifferentiated status without feeder support [3]. LIF was secreted from mouse feeder layers and signal processed through a receptor complex containing the signal transducer of gp130 [4]. Among the down stream effectors of this signal pathway, STAT3 has been shown to be sufficient to support ESC self renewal [5]. However, it appears human derived ESC may use alternative pathways considering that LIF can not maintain undifferentiated status by itself. Transplantation of ESC resulted in bizarre tumorous growth known as a teratoma. In addition, ESC share stem cell characteristics with ECC. They are similar in morphology, growth behavior and also have similar phenotype marker expressions. They can self renew and expand in culture, and a single cell can differentiate into multiple cell types and contribute to embryogenesis following introduction into another embryo. However, ESC are expected to maintain stable diploid karyotype, which is critical both for cell therapy and for germ line transmission. One of the important uses of mouse embryonic stem cells (mESC) is for genetic modification. Embryonic stem cells enable precise controlled genetic modification and serve as good cellular vectors which can be incorporated into developing embryos. Through germ line transmission, transformed animals can be obtained. In addition, ESC are a versatile source of cells for tissue regeneration with their differentiation potential. It has been shown that

they can be differentiated into endoderm [6, 7], mesoderm [8, 9] and ectoderm [10]. Among various differentiation studies, neural induction will be the focus of this dissertation.

HUMAN EMBRYONIC STEM CELLS DERIVATION AND PROPAGATION

As in the mouse, efforts were undertaken to derive a counterpart ESC population in the primate. In 1995, Thomson and colleagues reported isolation of pluripotent cells from non-human primates [11] which ultimately led to establishment of ESC from human blastocysts in 1998 [12]. Established ESC from primates shared many characteristics with their mouse counterpart. Like mESC, hESC originate from the ICM/epiblast, have unlimited self renewal capacity, high telomerase activity and can generate all types of tissue in vitro and in teratoma.

There are distinct differences between mouse and human ESC. First, the profile of phenotype marker expression is not the same as summarized in Table 1.1. Second, LIF cannot inhibit differentiation of hESC without other support [13]. Ginis et al, examined the expression of LIF receptor(LIFR) and gp130 in hESC [14]. Expression was easily detected in mouse D3 cell line for both molecules. However, both LIFR and gp130 expression varied among hESC lines. They concluded that the variable expression and detected inhibitors suggest this pathway is not active in hESC self renewal. Third, clonal proliferation was not efficient yet in hESC. One of the routine methods used to passage hESC has been mechanical dissection of the formed colony [13, 15] and re-plating on to new substrate. However, this method restricted large scale propagation. Amit et al, have demonstrated that it was possible to clonally derive hESC that retained pluripotency [16]. But the cloning efficiency was less than 1% and routine passage was made using collagenase which separates hESC from the feeder layer as clumps. Also, in the

human, the confirmation of hESC contribution to embryogenesis was not an approved procedure or ethically acceptable. Therefore teratoma formation was used as alternative criteria.

REGENERATIVE MEDICINE

Human embryonic stem cells are expected to contribute to numerous research fields such as early development, functional genomics, discovery of novel genes involved in tissue regeneration, in vitro models for drug discovery and replacement therapy. However, for successful use as a source material for regenerative medicine, several things need to be resolved. First, a sufficient number of desired cell types need to be obtained and purified without undesired cell type contamination. Also, cells need to be delivered to target location without causing an immune response. Elimination of animal materials such as mouse feeder layers will avoid potential zoonotic transmission to patients.

To maintain the undifferentiated status of hESC, mitotically inactivated mouse fibroblast cells have been used as the supporting feeder layer. Attempts were made to replace this feeder layer. Xu et al, reported that hESC could be supported without contact with the feeder layer. They still used conditioned medium from the feeder layer but tried to define the culture system. They exchanged serum replacement for serum and used matrigel or laminin as a substrate [17]. Recently, Amit et al, have demonstrated that combinations of growth factors can replace the feeder layer [18]. They supplemented medium with 15% serum replacement, Transforming growth factor β (TGF β), LIF, basic Fibroblast growth factor (bFGF) and used fibronectin matrix. Also, in 2002, attempts were made to establish a human origin feeder system. The established human feeder layer could support prolonged undifferentiated hESC growth of an existing cell line [19]. The other approach was to induce differentiation of hESC toward target tissue, thus

eliminating both the need for feeders and unwanted chaotic differentiation like in teratomas or in spontaneous differentiation of hESC.

Table 1.1 Phenotype marker expression for mouse and human ESC

Phenotype marker	Mouse ESC	Human ESC	Reference
SSEA-1	+	-	[20]
SSEA-3	-	+	[20]
SSEA-4	-	+	[20]
TRA-1-60	-	+	[20]
TRA-1-81	-	+	[20]
GCTM-2	-	+	[21] [22]
TG343	NA	+	[21] [22]
TG30	NA	+	[21]
CD9	+	+	[23] [24]
Alkaline phosphatase	+	+	[21]
Oct 4	+	+	[14]
Nanog	+	+	[14]
Rex1	+	+	[14]
Sox2	+	+	[14]
TERT	+	+	[14]
Vimentin	-	+	[14]
β III tubulin	+	-	[14]

NEURAL INDUCTION IN DEVELOPMENT

The purpose of this dissertation is to induce hESC to differentiate into Neuroepithelial stem cells (NEP) and then force the NEP into the specific lineage of motor neuron. One of the plausible strategies for neural differentiation of ESC would be to recapitulate the processes in

development. In the next sections, major signaling pathways involved in developmental process for spinal motor neuron are specifically addressed. The mammalian central nervous system is developed from the neural tube. The early neural tube is composed of a single layer of pseudostratified columnar epithelium of NEP.

NEP are self renewing cells that can differentiate into neurons, oligodendrocytes, and astrocytes. According to Liu, who examined the immunohistochemistry pattern in mouse tissue sections, phenotype of NEPs can be summarized by several specific markers (table 1.2) [25]. Likewise, it has been shown that human NEP cells have a phenotype characterization similar to the mouse [26]. They express Nestin and Sox2 but do not express any other late stage neuronal nor glial lineage markers. These cells divide symmetrically or asymmetrically to give rise to all the cells which comprise the mammalian central nervous system, including various types of neurons and glial cells.

During early human development, NEP have been shown to form the neural tube during the third and fourth weeks of gestation [27]. To acquire this NEP induction from epiblast, several molecules are required. Major signaling pathways involved in this are bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and Wnts [28]. Bone morphogenetic protein signals block neural fate and promote epidermal fate and are excluded from prospective neural cells. Noggin, chordin and follistatin are known BMP inhibitors. Fibroblast growth factor has the dual role of repression of BMP expression and promotion of a neural pathway independent of the repression of BMP pathways. In this pathway, Wnts block one of FGF roles of BMP repression (Figure 1.1).

Table 1.2 Phenotype profile of Neuroepithelial stem cell
Liu et al. Glia 40:25-43 (2002) [25]

Antigens	NEP cells
Nestin	+
Sox2	+
Nkx2.2	+/-
A2B5/4D4	-
GFAP/CD44	-
RC1/S100/Vimentin	-
Sox10/NG2/PDGFRα	-
O4/GALC	-
PLP-DM20/CNP/MBP	-

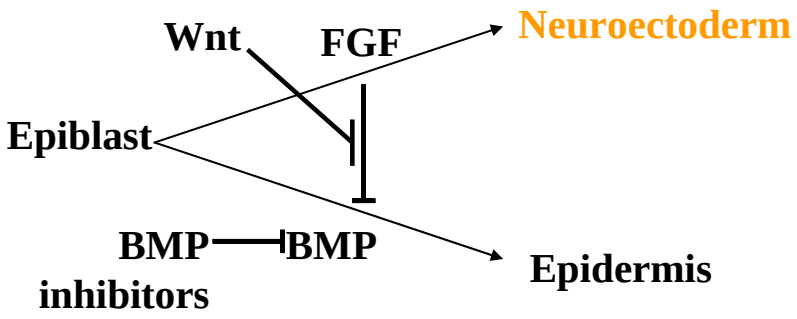

Figure 1.1 Regulatory pathways involved in neural induction of Epiblast

NEURAL PATTERNING IN DEVELOPMENT

Along with neural induction, cell fate determination occurs during and following neural tube closure. Among several molecules, Sonic hedgehog (Shh), Retinoic acid (RA) and Fibroblast growth factors (FGFs) have been well defined molecules involved in dorsal ventral and anterior posterior axis formation and motor neuron development as well.

THE ROLE OF SONIC HEDGEHOG

Dorsoventral axis formation involves the action of two opposing signaling pathways: Sonic hedgehog (Shh) ventrally from the notochord and later from the floor plate and bone morphogenic protein (BMP) dorsally from the boundary of neural and nonneural ectoderm and later from the roof plate. Notochord is the source of signals involved in the specification of the floor plate and secondarily to the formation of motor neurons and ventral interneurons and Shh is the major regulator of these signals [29]. Loss and gain of function studies of Shh have suggested that it is both necessary and sufficient to induce the floor plate [30]. When Shh was neutralized by antibody, there was inhibition of notochord mediated induction of ventral types. Also, Shh knockout mice lack floor plate and motor neurons but four other classes of ventral neurons still develop. In contrast, when explants are exposed to Shh, ectopic floor plate and motor neurons develop. Shh has two forms, membrane bound and non-membrane bound. It is presumed that the bound protein form is involved in floor plate induction while the soluble secreted form is in charge of motor neuron specification [31]. Soluble secreted Shh is also shown to be able to act as a morphogen, eliciting different cell fates at different thresholds of concentration [32]. Graded Shh activity directs neural identity through a set of homeodomain proteins that exhibit mutual cross repressive interaction. Within the ventral spinal cord, five progenitor domains are labeled by unique combinations of transcription factors. The patterns of gene regulation in the progenitor domains are established by the high-ventral to low-dorsal gradient of Shh (Figure 1.2). Shh can either induce or repress the expression of the transcription factors in progenitor cells. These graded responses (either positive or negative) to Shh lead to the patterned expression of unique combinations of factors in each progenitor cell domain. A second level of transcriptional regulation of opposing class I and class II factors are found to

have a cross-repressive interaction. Thus, mutations in one of the factors usually lead to the expansion of the opposing factor into an inappropriate domain, which is associated with cell fate conversions.

Shh is involved in motor neuron generation with distinct roles according to developmental stages. Ericson el al, used chick neural explant assays to show that there are two distinct stages of Shh signaling for motor neuron generation. During the early period, it drives naive neural plate cells into ventralized progenitors then directs these cells into motor neurons during the late periods [33]. During the early period, Shh exposure results in the extinction of pax7 in neural plate cells close to the notochord resulting in ventralized progenitors. The expression of other homeobox genes is also repressed by notochord and Shh. According to the Shh concentration during the late period, ventralized progenitors differentiate either into motor neurons or interneurons. Interneurons are characterized by subsequent expression of Lim 1/2, while motor neurons express Lim domain protein Isl1/2. Poh et al, observed similar requirements of early and late exposure of Shh in motor neuron development [34]. In addition they found that there is a critical time window for Shh requirement otherwise, cells become Shh independent. Shh applied on dorsal spinal cord explants gave rise to motor neurons and oligodendrocytes [35]. However, there is preferential expansion according to the age of the embryo explant acquired [34, 36]. Early stage embryo preferentially expands motor neuron while in explants from older embryos the oligodendrocyte lineage is preferentially expanded. Also, cell fates can be manipulated by addition of anti Shh antibodies and inactivation of its receptor Patched [37].

Sonic hedgehog acts through the Patched (PTCH) receptor. Patched binds to signal transducer of Smoothened (Smo) and inhibits its action in the absence of Shh. When Shh is

present, Smo is released from PTCH and transcription factors of Gli proteins locate into the nucleus to activate target gene transcription [38]. PTCH gene is the target gene of this pathway and Shh-induced SMO activation resulted in transcription of PTCH itself. Thus, receptor PTCH expression can serve as a biological marker of the target tissue of Shh and that exerted Shh effects will be reflected by overexpression of PTCH.

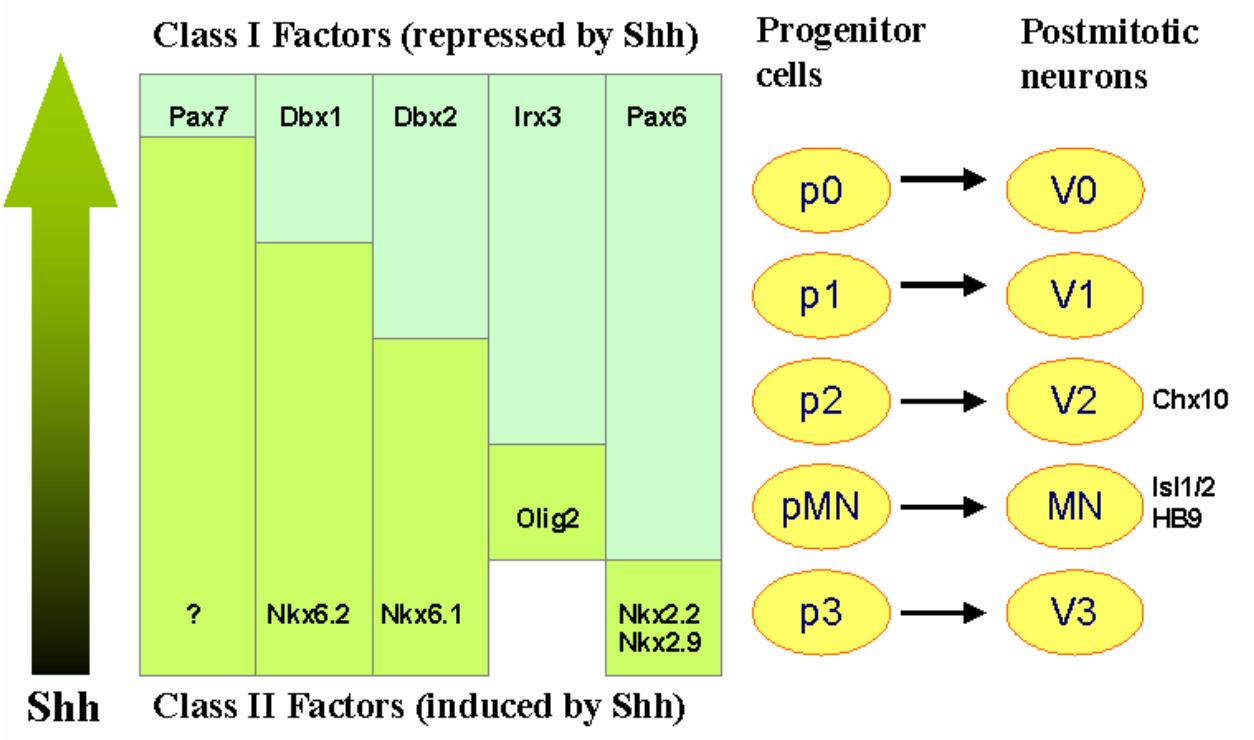

Figure 1.2 Expression of unique combinations of factors in each progenitor cell domain
Modified and adopted from Shirasaki et al. Annu rev. neurosci. 25:241-281 (2002) [39]

THE ROLES OF RETINOIC ACID

Retinoic acid (RA) is the biologically active derivative of vitamin A and it induces a variety of embryonic carcinoma and neuroblastoma cell lines to differentiate into neurons [40]. Retinoic acid acts through at least two receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) which can interact with multiple putative coactivators and corepressors to yield

a complex molecular pathway with a variety of pleiotropic effects. Retinoids are thought to function as morphogens during anterior posterior patterning in vivo. In fact, Horton et al, analyzed endogenous retinoid level in individual components of mouse embryo by high performance liquid chromatography. They detected a gradient of endogenous retinoid from the forebrain to the spinal cord. It was expressed at very low levels in forebrain and midbrain. However, there was a RA gradient expressed in hindbrain while spinal cord expressed a high level of it [41, 42]. Ectopic expression of RA to whole Xenopus embryos leads to increase in volume in the hindbrain and spinal cord with a corresponding decrease in volume of the forebrain [43]. There is evidence indicating that RA acts as a morphogen. RA affects the development of a head structure in a concentration dependent manner and can alter tail structures as well. In hindbrain, gradually increased RA makes stepwise activation of genes in gradually more posterior segments. In addition, RA is implicated in establishing regional identity within the spinal cord itself. It appears to act at sequential developmental stages to impose different rostrocaudal positional values.

Retinoic Acid also controls neurogenesis in caudal neural plate. Diez et al, showed that signals from paraxial mesoderm regulates the formation of the neurogenic zone from proliferating stem zone. Signals from somatic mesoderm induce neuronal development, whereas FGFs from presomitic mesoderm and caudal cells inhibits neurogenesis in adjacent neural tissue. They found that neuronal differentiation required both attenuation of FGF signals and neural cell fate resolving signals and proposed RA as a candidate [44]. In fact, it has been shown that somites express an enzyme that converts retinaldehyde to RA [45] and retinoids present into somites [42]. To test the RA effect on neurogenesis, Diez et al, manipulated RA signals [46]. Increased RA signals resulted in expression of NeuroM, bHLH transcription factors by newly

differentiating neurons and vanishing FGF8 transcripts. In contrast, neural development was inhibited with disrupted RA signaling both in vivo and in vitro. Mesoderm also did not induce NeuroM in cocultured caudal neural plate when RA signal was blocked.

Recently, it has been shown that RA is intensively involved in specifying neuronal fates in ventral spinal cord. Novitch et al blocked RA signaling in chicken embryos and in explant using dominant negative RA receptor and showed that cells lost Olig2 expression which is exclusively expressed in motor neuron progenitor domain as described in Figure 1.2 [47]. Consistent with this, quail embryos deficient for Vitamin A reduced their expression of Olig2 [46]. These observations suggest that RA signaling is required for Nkx6 cells to progress to Olig2 motor neuron progenitors (Figure 1.2). It was also demonstrated that RA also contributes to motor neuron specification without presence of Shh. When chicken neural explants were exposed to RA and bFGF, Class I gene expression was repressed and Olig2 and motor neuron markers were expressed even when Shh was blocked by antibody 5E1 raised against Shh [47]. This suggests that RA activates transcription for motor neuron development in concert with repressed Class I activity by FGF.

In addition, RA has been shown to increase motor neuron number through an increase in progenitor cell numbers [48]. When chicken neural explant was exposed to RA either by exogenous sources or endogenous synthesis through retinoic acid synthetic enzyme, retinaldehyde dehydrogenase-2 (RALDH2), the number of motor neurons are increased by 60%. Conversely, retinoic acid receptor antagonists block both the retinoid induced increase in motor neuron number and the generation of subtype motor neurons. Therefore, RA is one of the important candidates for motor neuron induction from NEP.

THE ROLES OF FGF

Although, FGF has been shown to play a major role as a repressor of differentiation, it has recently been shown to contribute to neural patterning. Fibroblast growth factor (including 2,4,8) inhibits differentiation of adjacent neural tissue which is opposition of the effect of RA. FGF-2,4 and 8 are generated by presomitic mesoderm and caudal cells and inhibit adjacent neural tissue from differentiation and maintain the caudal region as a stem zone [44]. They also affect patterning of ventral spinal cord by differential inhibitory action on bHLH (Olig2) and homeobox transcription factors (Figure 1.2). Experiments made with explant assays showed that FGF signaling inhibits Class I genes and partially inhibits Class II genes (Figure 1.3) [46, 47].

Novitch et al, demonstrated that forced expression of FGF in vivo neural cells resulted in marked repression in Class I, but limited repression in Class II proteins[47]. They also observed joint action of FGF and RA can induce Olig2 expression in the absence of Shh. This suggests that there is a Shh independent pathway in ventral neural pattern formation. The independent role of FGF was also observed in oligodendrocyte progenitor's induction from neocortical precursors in culture [49]. This FGF activity was not affected by cyclopamine which blocks the Shh pathway. In contrast, Shh activity was blocked by PD173074 which is the inhibitor of the FGF receptors (FGFR). It showed that constitutive activity of FGFR maintains a basal level of phosphorylated mitogen activated protein kinase (MAPK) and that Shh depends on MAPK for Olig2 induction.

Induction of neural Hoxc expression requires FGF signaling. Hoxc proteins are expressed in motor neurons and their expressions specify motor neuron column identity such as brachial, thoracic and lumber one. Fibroblast growth factor receptor inhibitor, SU5402 blocked expression of Hoxc expression in neural explants (Hoxc6, 8, 9, 10) [50, 51]. Also, graded FGF

signaling establishes the distinct expression pattern of motor neuron Hox gene in neural progenitor. Exposure of neural progenitor cells in vitro to increasing FGF levels induces the differentiation of motor neurons having a progressively more caudal Hoxc profiles. In vivo experiments showed that increasing the level of FGF signaling in the neural tube elicits a rostral to caudal switch in the profile of Hoxc protein expression [50, 52]. Brachial FGF8 expression results in a brachial to thoracic switch in the profile of Hoxc expression (extinction of Hoxc6 and onset of Hoxc9 also with decreased RALDH2 and increased BMP5). However, thoracic FGF8 expression didn't markedly influence the Hoxc profile.

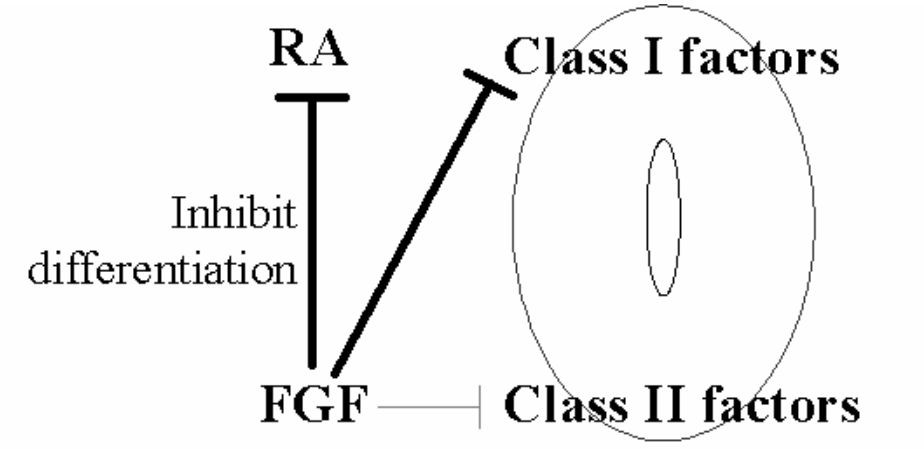

Figure 1.3 Role of FGF signals in motor neuron differentiation
Modified and adopted from Novitch et al. Neuron. 40:65-79 (2003) [47]

In summery, for neural pattern or specification, several molecules have been demonstrated to be involved and that these inductive signals are suggested to be required to be present at the right time for neural induction and specification from hESC to occur.

THE GENES RELATED TO MOTOR NEURON DERIVATION

As described in previous section, nascent NEP are differentiated into motor neurons by inductive signals. Induced motor neuron progenitors and motor neurons require and express specific genes which can be used as motor neuron markers as well. Olig2 is a bHLH factor induced by Shh in the ventral neural tube and its onset defines motor neuron progenitor domain as described in figure 1.2. At time of neural specification, Pax6 (Class 1 factor) and Nkx6.1 (Class 2 factor) induced Olig2 expression in defined motor neuron progenitor domain. Then Olig2 indirectly induces motor neuron fate through activation of the motor neuron determinants of Isl1, MNR2 and HB9 [53]. The mutant mice with homozygous inactivation of Olig2 have neuroepithelial cells in the ventral spinal cord that fail to differentiate into motor neurons or oligodendrocytes [54]. This result also indicates Olig2 is an essential regulator in motor neuron and oligodendrocyte development.

Homeodomain (HD) factors, Lhx3, Islet1 and Mnx (MNR2, HB9) have been studied as downstream effectors to set motor neuron (MN) identity in the spinal cord (Figure 1.4). The combined class I and class II factors induce motor neuron progenitor domain to express MNR2 and Lhx3 in chick. MNR2 play a role as a major determinant for motor neuron generation. It induces the expression of downstream transcription factors such as Lhx3, Islet1 and HB9. Gain of function studies demonstrated that ectopic expression of MNR2 in dorsaral progenitor cells suppressed interneuron fates and presented motor neuron identity [55]. The expression was shown Lhx3 is also expressed in motor neuron progenitors, however it is involved both in motor neurons and in interneurons generation. Another Lim protein of Islet expressed in postmitotic motor neurons contributed to this motor neuron and interneuron cell fate determination. Lhx3 expression without Islet1 triggered Chx10 transcription factor which forced cell to an interneuron

fate. However, presence of Islet1 inhibited Lhx3 from direct binding to cofactor of NLI and resulted in motor neuron with HB9 expression [56]. HB9 is another Mnx family and expressed in postmitotic motor neurons in chick and mouse. However, in mouse, HB9 was expressed both in MN progenitors and postmitotic MNs [57, 58] and both HB9 and Islet1 were expressed in MNs. As in chick, HB9 were restricted to MNs and coexpression with other interneuronal marker was not observed. Therefore, HB9 and Islet1 have been used as phenotype marker for motor neurons (Figure 1.4).

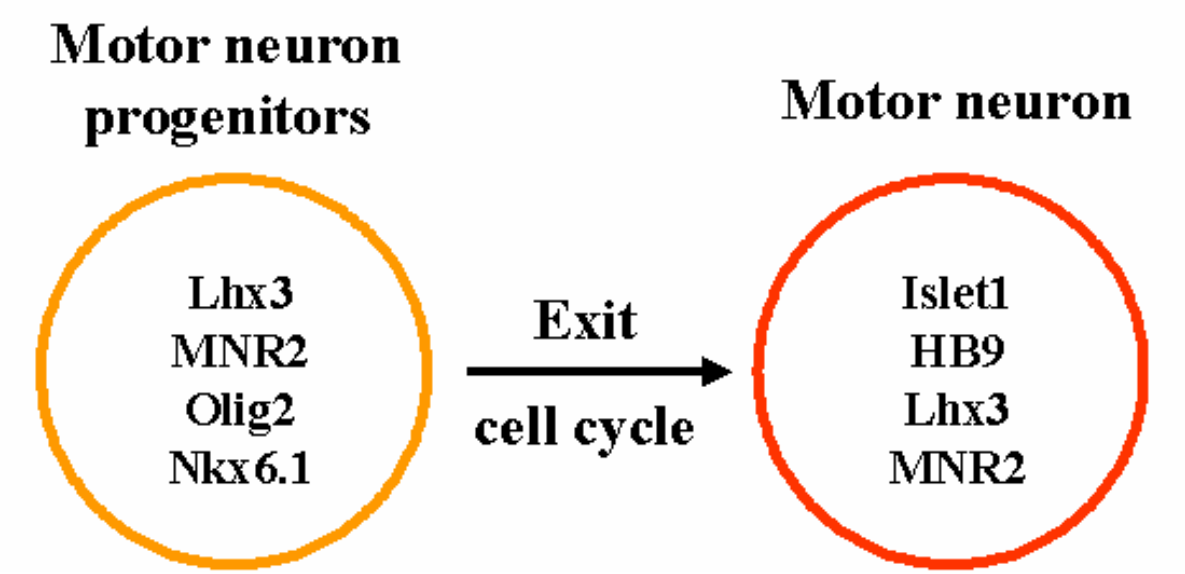

Figure 1.4 Homeodomains and Olig2 expression profiles in motor neuron progeneitors and in motor neurons
Modified and adopted from William et al. Development. 130:1523-1536 (2003) [59]

INDUCED NEURAL DIFFERENTIATION OF EMBRYONIC STEM CELLS

Currently, several different strategies have been used to induce neural differentiation in hESC. The most routine way for generating differentiated cell types has been through the three dimensional structure of the embryoid body (EB). Embryonic stem cells in this agglomerate

start spontaneous differentiation to form a sphere in suspension culture. Differentiated EBs contained neural stem cells and their proportion increases by RA exposure [60, 61]. Reubinoff et al, cultured hESC until spontaneous differentiation occurs then isolated of subpopulation to make neurospheres [62]. Pera et al, introduced BMP inhibitor of noggin in this prolonged culture condition [63]. However, EB culture has disadvantages compared to adherent culture in that phenotype observation within the sphere is not possible with standard microscopy. In addition, stochastic differentiation yielded multiple cell lineages and limited the overall yield of the desired cells [64].

Ying et al, developed a monolayer differentiation to obtain efficient neural induction on mESC. When differentiation was triggered by the withdrawal of LIF, mESC monolayer chose a neural fate in serum deprived medium [65]. This efficient neural determination did not occur in medium containing serum. Though it is uncertain whether hESC will behave as their mouse counterpart, adherent differentiation in defined culture is an attractive strategy.

Serum contains numerous undefined proteins including growth factors and molecules would stimulate differentiation to particular lineage. Wiles et al, demonstrated that growth factors in serum contributed to mesodermal cell growth in EB [66]. In chemically defined serum free medium mouse EB differentiated into neuroectoderm without commensurate expression of Brachyury. Tropepe et al, proposed neural induction as a default choice of cell fate. When mESC were dissociated to culture in serum and feeder deprived conditions, colony forming primitive neural stem cell population could be obtained [67]. Thus, defined culture will be beneficial not only to directed differentiation but also to elimination of mesodermal differentiation.

Neural induction was also induced via a feeder cell coculture system or use of conditioned medium from specific cell lines. A mouse stromal cell line, PA6, has a stromal cell derived inducing activity (SDIA) and induced efficient neuronal differentiation when mouse or non human primate ESC were culture on top of these cells. This neural inducing activity was shown to be which can be inhibited with BMP4 [68]. Coculture with this cell line was shown to be effective especially for midbrain neuron in mouse and non human primate ESC. The SDIA activity was shown to be on cell surface and molecular nature of SDIA has not been elucidated.

Conditioned medium from human hepG2 cell line (MEDII) was shown to have neural induction activity in mESC. When mESC were exposed to this conditioned medium following EB formation, the resulting cell population was preferentially neurectoderm and was positionally unspecified [69]. The neural inducing activity was also observed in non human primate and hESC. Embryoid bodies formed in MEDII medium showed more rosette structures which is acknowledged as a specified morphology for neuroepithelial stem cells [70, 71]. More studies are required to understand the components of MEDII. However, known components of LIF and fibronectin would be beneficial for neural inductions of hESC, given that LIF appeared to be effective in default neural induction model studied in mouse [67].

More advanced studies were made focused on inducing specified types of neurons derived from ESC using signaling mechanisms known to be involved neural development. Kim et al, used Shh and FGF8 to enrich dopaminergic neuron from mESC [72]. It has been shown in development that Shh from ventral neural tube and FGF8 from midbrain and hindbrain boundary interplayed to create induction sites for dopaminergic neuron [73]. Spinal motor neurons were generated from mESC by attempting to recapitulate cell signaling events during neural development. For example, Wichterle et al, derived neural progenitors in EBs and then treated

these EBs with RA, a posteriorizing factor, and Shh a ventralizing factor [74]. Mouse and non human primate neural progenitors derived by coculture with PA6 was also induced to motor neuron with the same factors [68]. These studies show that Shh, and RA would be reasonable candidate molecules to induce motor neuron differentiation from hESC.

SPECIFIC AIMS

Cell therapy is a prospective treatment in the replacement of diseased or degenerating cell populations, tissues and organs. Traumatic spinal cord injury is one of the most disabling conditions occurring with 11000 people per year experiencing spinal cord damage just in USA. Amyotrophic lateral sclerosis (ALS) is a progressive and degenerative motor neuron disease in the spinal cord and cerebral cortex. The goal of this dissertation was to derive motor neurons from hESC.

Hypothesis: Using hESC adherent cultures a homogenous early neuroectodermal cell type (neuroepithelial stem cells, NEP) can be derived and from these cells motor neuron differentiation is recapitulated in vitro using neural developmental inductive signals.

Specific aims:

1. Develop hESC to NEP cell differentiation and proliferation process: Adherent hESC differentiation in defined culture conditions will be examined. The differentiation process will be followed and examined immunohistochemically. Derived NEP cell population will be characterized both by differentiation capacity and by molecular characterization techniques. Subsequently, NEP will be proliferated using different medium, supplements, growth factors and oxygen tension to define optimal subculture conditions.

2. Examine NEP cell differentiation to motor neuron phenotype. The effect of inductive signals of bFGF, retinoic acid and sonic hedgehog will be examined on motor neuron differentiation. Gene expression change in motor neuron progenitors (Olig2) and motor neuron gene (HB9) will be monitored quantitatively using real time PCR.

If successful, the established system can serve as an in vitro model for the study of human neural development, motor neuron diseases and grafts in spinal cord injury.

REFERENCES

1. Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos.* Nature, 1981. **292**(5819): p. 154-6.
2. Smith, A.G. and M.L. Hooper, *Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells.* Dev Biol, 1987. **121**(1): p. 1-9.
3. Smith, A.G., et al., *Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides.* Nature, 1988. **336**(6200): p. 688-90.
4. Niwa, H., et al., *Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3.* Genes Dev, 1998. **12**(13): p. 2048-60.
5. Matsuda, T., et al., *STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells.* Embo J, 1999. **18**(15): p. 4261-9.
6. Levinson-Dushnik, M. and N. Benvenisty, *Involvement of hepatocyte nuclear factor 3 in endoderm differentiation of embryonic stem cells.* Mol Cell Biol, 1997. **17**(7): p. 3817-22.
7. Lumelsky, N., et al., *Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets.* Science, 2001. **292**(5520): p. 1389-94.
8. Maltsev, V.A., et al., *Embryonic stem cells differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types.* Mech Dev, 1993. **44**(1): p. 41-50.
9. Yamashita, J., et al., *Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors.* Nature, 2000. **408**(6808): p. 92-6.
10. Lee, S.H., et al., *Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells.* Nat Biotechnol, 2000. **18**(6): p. 675-9.
11. Thomson, J.A., et al., *Isolation of a primate embryonic stem cell line.* Proc Natl Acad Sci U S A, 1995. **92**(17): p. 7844-8.
12. Thomson, J.A., et al., *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**(5391): p. 1145-7.
13. Reubinoff, B.E., et al., *Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro.* Nat Biotechnol, 2000. **18**(4): p. 399-404.
14. Ginis, I., et al., *Differences between human and mouse embryonic stem cells.* Dev Biol, 2004. **269**(2): p. 360-80.

15. Mitalipova, M., et al., *Human Embryonic Stem Cell Lines Derived from Discarded Embryos.* Stem Cells, 2003. **21**(5): p. 521-526.
16. Amit, M., et al., *Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture.* Dev Biol, 2000. **227**(2): p. 271-8.
17. Xu, C., et al., *Feeder-free growth of undifferentiated human embryonic stem cells.* Nat Biotechnol, 2001. **19**(10): p. 971-4.
18. Amit, M., et al., *Feeder layer- and serum-free culture of human embryonic stem cells.* Biol Reprod, 2004. **70**(3): p. 837-45.
19. Richards, M., et al., *Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells.* Nat Biotechnol, 2002. **20**(9): p. 933-6.
20. Draper, J.S., et al., *Surface antigens of human embryonic stem cells: changes upon differentiation in culture.* J Anat, 2002. **200**(Pt 3): p. 249-58.
21. Carpenter, M.K., E. Rosler, and M.S. Rao, *Characterization and differentiation of human embryonic stem cells.* Cloning Stem Cells, 2003. **5**(1): p. 79-88.
22. Cooper, S., et al., *Biochemical properties of a keratan sulphate/chondroitin sulphate proteoglycan expressed in primate pluripotent stem cells.* J Anat, 2002. **200**(Pt 3): p. 259-65.
23. Oka, M., et al., *CD9 is associated with leukemia inhibitory factor-mediated maintenance of embryonic stem cells.* Mol Biol Cell, 2002. **13**(4): p. 1274-81.
24. Carpenter, M.K., et al., *Properties of four human embryonic stem cell lines maintained in a feeder-free culture system.* Dev Dyn, 2004. **229**(2): p. 243-58.
25. Liu, Y., et al., *Oligodendrocyte and astrocyte development in rodents: an in situ and immunohistological analysis during embryonic development.* Glia, 2002. **40**(1): p. 25-43.
26. Mayer-proschel, M., *Human neural precursor cells-an in vitro characterization.* Clinical Neuroscience Research, 2002. **2**: p. 58-69.
27. Kennea, N.L. and H. Mehmet, *Neural stem cells.* J Pathol, 2002. **197**(4): p. 536-50.
28. Wilson, S.I. and T. Edlund, *Neural induction: toward a unifying mechanism.* Nat Neurosci, 2001. **4 Suppl**: p. 1161-8.
29. Yamada, T., et al., *Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate.* Cell, 1993. **73**(4): p. 673-86.
30. Marti, E., et al., *Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants.* Nature, 1995. **375**(6529): p. 322-5.
31. Johnson, R.L. and C. Tabin, *The long and short of hedgehog signaling.* Cell, 1995. **81**(3): p. 313-6.
32. Ericson, J., et al., *Graded sonic hedgehog signaling and the specification of cell fate in the ventral neural tube.* Cold Spring Harb Symp Quant Biol, 1997. **62**: p. 451-66.
33. Ericson, J., et al., *Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity.* Cell, 1996. **87**(4): p. 661-73.
34. Poh, A., et al., *Patterning of the vertebrate ventral spinal cord.* Int J Dev Biol, 2002. **46**(4): p. 597-608.
35. Orentas, D.M., et al., *Sonic hedgehog signaling is required during the appearance of spinal cord oligodendrocyte precursors.* Development, 1999. **126**(11): p. 2419-29.
36. Micaglio, G., et al., *Age-dependent requirements of cultured spinal cord neurons.* Dev Neurosci, 1988. **10**(1): p. 12-6.

37. Xie, J., et al., *Activating Smoothened mutations in sporadic basal-cell carcinoma.* Nature, 1998. **391**(6662): p. 90-2.
38. Murone, M., A. Rosenthal, and F.J. de Sauvage, *Sonic hedgehog signaling by the patched-smoothened receptor complex.* Curr Biol, 1999. **9**(2): p. 76-84.
39. Shirasaki, R. and S.L. Pfaff, *Transcriptional codes and the control of neuronal identity.* Annu Rev Neurosci, 2002. **25**: p. 251-81.
40. Maden, M., *Role and distribution of retinoic acid during CNS development.* Int Rev Cytol, 2001. **209**: p. 1-77.
41. Horton, C. and M. Maden, *Endogenous distribution of retinoids during normal development and teratogenesis in the mouse embryo.* Dev Dyn, 1995. **202**(3): p. 312-23.
42. Maden, M., et al., *The distribution of endogenous retinoic acid in the chick embryo: implications for developmental mechanisms.* Development, 1998. **125**(21): p. 4133-44.
43. Hirsch, N. and W.A. Harris, *Xenopus Pax-6 and retinal development.* J Neurobiol, 1997. **32**(1): p. 45-61.
44. Diez del Corral, R., D.N. Breitkreuz, and K.G. Storey, *Onset of neuronal differentiation is regulated by paraxial mesoderm and requires attenuation of FGF signalling.* Development, 2002. **129**(7): p. 1681-91.
45. Swindell, E.C., et al., *Complementary domains of retinoic acid production and degradation in the early chick embryo.* Dev Biol, 1999. **216**(1): p. 282-96.
46. Diez del Corral, R., et al., *Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension.* Neuron, 2003. **40**(1): p. 65-79.
47. Novitch, B.G., et al., *A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification.* Neuron, 2003. **40**(1): p. 81-95.
48. Sockanathan, S. and T.M. Jessell, *Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons.* Cell, 1998. **94**(4): p. 503-14.
49. Kessaris, N., et al., *Cooperation between sonic hedgehog and fibroblast growth factor/MAPK signalling pathways in neocortical precursors.* Development, 2004. **131**(6): p. 1289-98.
50. Liu, J.P., E. Laufer, and T.M. Jessell, *Assigning the positional identity of spinal motor neurons: rostrocaudal patterning of Hox-c expression by FGFs, Gdf11, and retinoids.* Neuron, 2001. **32**(6): p. 997-1012.
51. Bel-Vialar, S., N. Itasaki, and R. Krumlauf, *Initiating Hox gene expression: in the early chick neural tube differential sensitivity to FGF and RA signaling subdivides the HoxB genes in two distinct groups.* Development, 2002. **129**(22): p. 5103-15.
52. Dasen, J.S., J.P. Liu, and T.M. Jessell, *Motor neuron columnar fate imposed by sequential phases of Hox-c activity.* Nature, 2003. **425**(6961): p. 926-33.
53. Novitch, B.G., A.I. Chen, and T.M. Jessell, *Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2.* Neuron, 2001. **31**(5): p. 773-89.
54. Takebayashi, H., et al., *The basic helix-loop-helix factor olig2 is essential for the development of motoneuron and oligodendrocyte lineages.* Curr Biol, 2002. **12**(13): p. 1157-63.
55. Tanabe, Y., C. William, and T.M. Jessell, *Specification of motor neuron identity by the MNR2 homeodomain protein.* Cell, 1998. **95**(1): p. 67-80.

56. Thaler, J.P., et al., *LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein-protein interactions.* Cell, 2002. **110**(2): p. 237-49.
57. Thaler, J., et al., *Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9.* Neuron, 1999. **23**(4): p. 675-87.
58. Arber, S., et al., *Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity.* Neuron, 1999. **23**(4): p. 659-74.
59. William, C.M., Y. Tanabe, and T.M. Jessell, *Regulation of motor neuron subtype identity by repressor activity of Mnx class homeodomain proteins.* Development, 2003. **130**(8): p. 1523-36.
60. Schuldiner, M., et al., *From the cover: effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells.* Proc Natl Acad Sci U S A, 2000. **97**(21): p. 11307-12.
61. Zhang, S.C., et al., *In vitro differentiation of transplantable neural precursors from human embryonic stem cells.* Nat Biotechnol, 2001. **19**(12): p. 1129-33.
62. Reubinoff, B.E., et al., *Neural progenitors from human embryonic stem cells.* Nat Biotechnol, 2001. **19**(12): p. 1134-40.
63. Pera, M.F., et al., *Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin.* J Cell Sci, 2004. **117**(Pt 7): p. 1269-80.
64. Dang, S.M., et al., *Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems.* Biotechnol Bioeng, 2002. **78**(4): p. 442-53.
65. Ying, Q.L., et al., *Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture.* Nat Biotechnol, 2003. **21**(2): p. 183-6.
66. Wiles, M.V. and B.M. Johansson, *Embryonic stem cell development in a chemically defined medium.* Exp Cell Res, 1999. **247**(1): p. 241-8.
67. Tropepe, V., et al., *Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism.* Neuron, 2001. **30**(1): p. 65-78.
68. Mizuseki, K., et al., *Generation of neural crest-derived peripheral neurons and floor plate cells from mouse and primate embryonic stem cells.* Proc Natl Acad Sci U S A, 2003. **100**(10): p. 5828-33.
69. Rathjen, J., et al., *Directed differentiation of pluripotent cells to neural lineages: homogeneous formation and differentiation of a neurectoderm population.* Development, 2002. **129**(11): p. 2649-61.
70. Calhoun, J.D., et al., *Differentiation of rhesus embryonic stem cells to neural progenitors and neurons.* Biochem Biophys Res Commun, 2003. **306**(1): p. 191-7.
71. Schulz, T.C., et al., *Directed neuronal differentiation of human embryonic stem cells.* BMC Neurosci, 2003. **4**(1): p. 27.
72. Kim, J.H., et al., *Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease.* Nature, 2002. **418**(6893): p. 50-6.
73. Ye, W., et al., *FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate.* Cell, 1998. **93**(5): p. 755-66.
74. Wichterle, H., et al., *Directed differentiation of embryonic stem cells into motor neurons.* Cell, 2002. **110**(3): p. 385-97.

Chapter 2

Long term proliferation of human embryonic stem cell-derived Neuroepithelial cells using defined adherent culture conditions[1]

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ABSTRACT

Research on the cell fate determination of embryonic stem (ES) cells is of enormous interest given the therapeutic potential in regenerative cell therapy. Human ES cells have the ability to renew themselves and to differentiate into all three germ layers. The main focus of this study was to examine the factors affecting derivation and further proliferation of neuroepithelial (NEP) stem cells from human ES cells. ES cells cultured in serum-deprived defined medium developed distinct tube structures that could be isolated either by dissociation or adherently. Dissociated cells survived to form colonies of cells characterized as NEP when conditioned medium from the human hepatocellular carcinoma hepG2 cell line (MEDII) was added. However, cells isolated adherently developed an enriched population of NEP-like cells independent of MEDII medium. Further characterization suggested that they were NEP cells, since they expressed markers associated with the earliest multipotent neural stem cells. They were positive for Nestin, a neural intermediate filament protein, and Musashi-1, a neural RNA binding protein, but few cells expressed further differentiation markers such as PSNCAM, A2B5, MAPII, GFAP, or O4. Further differentiation of these putative NEP cells gave rise to a mixed population of progenitors that included A2B5-positive and PSNCAM-positive cells and post-mitotic neurons and astrocytes. To proliferate and culture these derived NEP, ideal conditions were obtained using neurobasal medium supplemented with B27 and bFGF in 5% oxygen. NEP cells were continuously propagated for over six months without losing their multipotent neural stem cell characteristics and maintained a stable karyotype.

Key words: Embryonic stem cell, differentiation, neuroepithelial stem cell, defined culture

INTRODUCTION

After human embryonic stem (ES) cells were established (1, 2), there was an immediate interest in differentiating these pluripotent cell lines toward a neuronal cell fate as a promising source for replacement cell therapy. The central nervous system contains endogenous stem cells that are capable of proliferating; however, in many cases these cells are too few in number or incapable of restoring function after neuronal damage has occurred (3). Neural tissues from fetuses, immortalized cell lines and ES cells are three main candidate sources for replacement cells. Fetus-derived neural tissue has been transplanted in humans, and encouraging results were obtained (4). The outcome varied, however, depending on the age of the graft cells or the presence of subculture (5). In addition, the supply of fetal neural tissue is limited because of ethical concerns.

Neuronal stem cells derived from cancer cell lines have been considered as a potential alternative cell source with unlimited capability for cell proliferation, but there is significant concern that cancer cells may be unstable and prone to tumorigenesis (6). Furthermore, it has been shown that the range of cell types derived from immortalized cells may be quite small (7). In contrast, ES cells have a unique advantage because they can proliferate and maintain their pluripotency for years (1) and can differentiate into virtually any cell type in the body. Additionally, there is no decrease in plasticity, which is shown in neural stem cells isolated from fetal tissue (7, 8). Mouse ES cells that have been expanded and differentiated into oligodendrocyte precursors and then transplanted into an animal model of human myelin disease have resulted in effective remyelination of host axons and functional recovery (9).

Neuroepithelial stem cells (NEP) are self-renewing cells that can differentiate into neurons, oligodendrocytes and astrocytes (10). These undifferentiated non-lineage committed

cells express Nestin but not the differentiated cell markers, A2B5 and PSNCAM (11). In humans NEP form the neural tube during the third and fourth weeks of gestation (12). These cells divide symmetrically or asymmetrically to give rise to all the cells that comprise the mammalian central nervous system, including various types of neurons and glial cells (13).

Neural developmental pathways can be delineated through ES cell studies. Neuronal development in rodents is a well-documented stepwise process, much like hematopoietic stem cell differentiation. Mouse neurectoderm forms the earliest pluripotent neural stem cells, called neuroepithelial stem (NEP),cells which then differentiate further into neuronal restricted precursor cells (NRPs) or glial restricted precursor cells (GRPs) (14). PSNCAM and A2B5 are used as critical lineage markers of rodent neuronal and glial lineages, respectively. Human NEP can be isolated from the fetus (11) and also from embryonic stem cells (15). These cells form neural rosettes and are Nestin and Musashi 1 positive.

A variety of methods have been used to derive NEP from ES cells (15-17). However, most of these methods have used cell aggregation or embryoid bodies (EBs), which allows stochastic differentiation into all three germ layers, including NEP. When either mouse ES cells (18) or non-human primate ES cells (17) were cultured with conditioned medium from the human hepatocellular carcinoma hepG2 cell line (MEDII), they developed preferentially into neurectoderm. In this study, factors required for the neural differentiation of human ES cells were examined and conditions allowing further proliferation were optimized. We show that adherent cultures of human ES cells in serum-deprived medium without feeder layers gave rise to a rosette enriched population. Characterization of this population showed that the cells were multipotent NEP with proper phenotype marker profiles and that they were able to differentiate further to both A2B5-positive and PSNCAM-positive precursor cells. Thus, this study

demonstrates that derived NEP can be cultured more than six months in optimized conditions without the cells losing their capacity for neural and glial differentiation while maintaining a stable karyotype.

MATERIAL AND METHODS

Human ES Cell culture. Human ES cell lines of BG01 and BG02 used in this experiment were cultured on mouse embryonic fibroblasts (MEF) layer, inactivated by mitomycin C (19). Since there were no differences in experimental results due to ES cell lines in this study, data from both cell lines were pooled. The cells were cultured in ES medium of DMEM/F12 medium (Gibco) supplemented with 15% serum and 5% knock-out serum replacement (KSR, Gibco), 2mM L-glutamine, 0.1mM MEM non-essential amino acids, 50 U/ml penicillin, 50 ug/ml streptomycin, 4 ng/mL basic fibroblast growth factor (bFGF, Sigma) and 10ng/mL Leukemia Inhibitory Factor (LIF, Chemicon). For passage, ideal colonies were mechanically dissected into small pieces and replated on mitotically inactivated MEF and the medium changed every other day as described (19). These cell lines have maintained their distinct stem cell morphology and karyotype and remain Oct-4- and SSEA4-positive (19).

Conditioned medium preparation. HepG2 cells (ATCC HB-8065) were seeded at a density of 9.4×10^4cells/cm^2 and proliferated for 3 days in DMEM/F12 medium supplemented with 10% FCS, 2mM L-glutamine, 50 U/ml penicillin, 50 ug/ml streptomycin. To produce conditioned medium, cells were washed twice with PBS, and DMEM/F12 medium without serum supplement was added at a ratio of 0.285ml/cm^2. In 3 days, conditioned medium was collected and stored at 4°C for less than 5 weeks as MEDII.

Antibodies and immunocytochemistry. Cells plated on polyornithine/laminin coated permanox slides were washed in PBS and fixed with 4% paraformaldehyde/4% sucrose in phosphate buffered saline (PBS) for 15 min. Fixed cells were washed two times with PBS before staining. Permeabilization and blocking was carried out in blocking buffer consisting of 0.1% Triton, 3% goat serum in Tris buffer for 40 min. For cell surface antigen, permeabilization was excluded. Primary antibodies were applied in blocking buffer for 2 h at room temperature (RT) and washed three times in blocking buffer before secondary antibody application. Secondary antibodies of goat anti-mouse Alexa-conjugated, goat anti-rabbit Alexa-conjugated (Molecular Probe) were diluted at 1:1000 in blocking buffer and applied to cells for 40 min at RT. After two washes in PBS, DAPI was applied for nuclear staining for 10 min, and cells were observed under the fluorescence microscope. For flow cytometry application, cells were harvested by trypsinization and suspended in PBS to be fixed and stained using the same procedure coupled with serial centrifugation at 3000 rpm and resuspension in PBS. For negative controls, first antibodies were omitted and the same staining procedure was followed. Primary antibodies and dilutions used included the following: mouse anti-Nestin (1:100; R&D system), rabbit anti-Nestin (1:200; Chemicon), rabbit anti-Musashi 1 (1:500; Chemicon), mouse anti-beta III tubulin (1:400; Sigma), rabbit anti-Tuj (1:500; Covance), mouse anti-Hu (1:50; Molecular Probes), mouse anti-muscle actin (1:50; DAKO), mouse anti-α feto protein (1:50; DAKO), rabbit anti-GFAP(1:50; Sigma), mouse anti-O4 (1:10; Chemicon), mouse anti-PSNCAM (1:400; Abcys), mouse anti-A2B5(1:100; a gift from Mayor Proschel).

Experimental design

All experiments were replicated three times unless otherwise noted.

Experiment 1: The effect of ES, DN2 and MEDII media on differentiation of stage 1 ES cells cultured with feeder cells. The differentiation procedure is outlined in Figure 2.1. After manual passage onto fresh feeder cells, hES cells were allowed to proliferate in ES medium for seven days (stage 1). Cell differentiation was then induced with either DN2, MEDII or ES medium for another seven days (stage 2). DN2 medium is DMEM/F12-based medium supplemented with N2 (Gibco), L-glutamine, penicillin/streptomycin(P/S) and 4ng/ml bFGF**.** MEDII medium for this study is DN2 medium supplemented at 50% (unless otherwise noted) with conditioned medium (described above). To understand and follow the differentiation steps applied here, phenotype marker expression was examined at the time intervals described in Figure 2.1. At stages 1, 2 and 3, populations were harvested and the markers Musashi-1, Oct-4 and Nestin, an early NEP stem cell marker, were observed. Immunocytochemical analysis was also performed on the adherent cell population. The cells at both stages were double-stained with Nestin and Oct-4 and observed under the fluorescence microscope for immunocytochemical examination associated with morphology. Groups that displayed phenotypic difference were then subjected to quantitative analysis for these same markers using flow cytometry.

Experiment 2: The effect of ES, DN2 and MEDII media on differentiation of stage 2 ES cells in adherent cell culture without feeder cells. To improve NEP cell derivation, a method using adherent differentiation was exploited. It was possible to isolate subpopulations of stage 2 cells that had infiltrated under the feeder layer to attach firmly on culture plates. To test the effect of ES, DN2 and MEDII media on this derivation method, the mouse feeder layer

was physically removed from each group of stage 2 cells in calcium/magnesium-free PBS. The remained cells were cultured another three days in respective media as described in Figure 2.1 (stage 3). At stage 3, populations were harvested from each group, and morphology and phenotype marker expression of Oct-4, Nestin and Musashi 1 was observed as described in experiment 1 using flow cytometry and immunocytochemistry for Oct-4, Nestin and Musashi-1.

Experiment 3: Effect of MEDII medium and low cell density on cell survival of stage 2 differentiating cells. The effect of MEDII medium was examined using single cell passage of stage 2 cells in the medium supplemented with 4 different concentrations of MEDII. As shown in Figure 2.1, stage 2 MEDII cultured cells were obtained. The resulting adherent cells were dissociated in 0.02M EDTA containing PBS, and 10^4 cells/cm^2 were then plated on polyornithine and laminin coated dishes in different concentrations of MEDII medium (0, 25, 50, 100%). After ten days of culture in respective media, cells were harvested and derivation efficiency (resulting cell number / starting cell number X 100) was determined over four replicates

Experiment 4: Characterization and examination of differentiation capacity of derived NEP-like cells. Rosette-forming populations of stage 3 NEP cells derived in DN2 and MEDII media from experiment 2 and rosette-forming NEP populations from experiment 3 were characterized by immunocytochemistry to examine the phenotype of NEP using the phenotype markers Nestin, Musashi 1, Oct-4, muscle actin and α-fetoprotein. For terminal differentiation, NEP-like cells were cultured in neurobasal medium (Gibco) and supplemented with B27 (Gibco), L-glutamine and penicillin/streptomycin without b-FGF for 14 days. For oligodendrocyte differentiation, NEP-like cells were exposed to 5ug/ml PDGF (Upstate) and 50uM 3T3 (Sigma) for 6 days before terminal differentiation. Differentiated cells were characterized using the

restricted progenitor markers PSNCAM, A2B5 and the post-mitotic neural marker Hu, the neuron-specific tubulin, β-III tubulin, oligodendrocyte marker O4 and astrocyte-specific GFAP.

Experiment 5: The effect of medium, supplement, growth factor and oxygen conditions on proliferation and viability of subcultures of derived NEP-like cells.

Effect of culture medium. To obtain a more uniform subculture system, two different kinds of base media--DMEM/F12 (D) and neurobasal medium (N)--were tested with supplements of either N2, B27 or MEDII-conditioned media. Stage 3 NEP-like cells were allocated into four different media: DN2, NN2 (neurobasal medium supplemented with N2), NB27 (neurobasal medium supplemented with B27) and 50% MEDII in DN2 medium, as described above with the same supplement of L-glutamine, P/S and 4 ng/ml bFGF. After 12 days of culture, cells were harvested and examined for morphology and viability using the Guava ViaCount (Guava Technologies) flow cytometry assay. Briefly, the Guava ViaCount reagent combines two different DNA dyes. One dye binds to the nucleus of every cell to give a total cell number and the other dye binds differentially to only non-viable cells. The data collected include total cell number and viability of the sample.

Subculture of NEP cells. NEP derived from either DN2 or MEDII were further propagated in NB27 with L-glutamine, P/S, 10ng/ml LIF and 20ng/ml bFGF on poly-ornithine and laminin coated dishes. Cells were continuously passaged by either by mechanical trituration or by trypsin ($1\times10^5/cm^2$) to be replated. After more than 6 months in culture, NEP-like cells were characterized as described before (Exp.4) and karyotyped using standard karyotype protocols, and chromosomes were counted. Briefly, cells were treated with 0.02 μg/μl colcemide for 1.5 hours and harvested to be hydrated and fixed. Chromosomes were stained with Giemsa and then counted (15 cells).

Effect of LIF and bFGF on subcultured NEP cells. Two groups of cultured NEP cells, one less than 1month (<1 mo) and the other approximately 6 months (6 mo) in NB27 (described in previous section), were dissociated by 0.05% trypsin to obtain a single-cell suspension, and 50,000 cells/cm^2 were plated in one of the subculture media on polyornithine and laminin-coated dishes. Two concentrations of two growth factors (LIF; 0 or 10 ng/ml and bFGF; 0 or 20 ng/ml) in NB27, were applied to cells. Cells were harvested from each group and nuclei were counted by flow cytometry on days 1 and 14. Plating efficiency rate was calculated as the ratio of cells harvested to cells plated on day 1. Proliferation was measured on day 14. For each replicate, counted nuclei from the four treatment groups were added to obtain an overall total. The total cell number within each group was then divided by the overall total cell number and expressed as a percent. This data conversion was carried out to reduce biological variation due to replicate preparation.

Effect of oxygen concentration on subcultured NEP cells. To examine the effect of oxygen concentration on cell proliferation and viability, the subcultured NEP cells (described above) were dissociated by 0.05% trypsin, and 2×10^5 cells/cm^2 were plated and propagated using the NEP subculture process, except one group was cultured at oxygen concentration of 20% and the other group was cultured at 5% O2. After 7 days of culture, cells were harvested to calculate total cell number and viable cell number, as described previously (Exp. 5).

Statistical analysis. For each parameter, significance of main effects was determined using the GLM procedure of SAS 8.01. Significance of differences among individual treatment means was determined by the least square means method. Differences were considered significant at $P< 0.05$.

RESULTS

Experiment 1. The effect of ES, DN2 and MEDII media on differentiation of ES cells cultured with feeder cells. After 7 days of culture, hES cells in ES medium (stage 1) proliferated to form multi-cell layers. These cells expressed both the pluripotent marker Oct-4 and the NEP cell markers, Nestin (Figure 2.2) and Musashi-1. When expression was quantitated for each phenotype marker using flow cytometry, 74.9%, 77.5% and 88% of total cells were positive to Oct-4, Nestin and Musashi-1, respectively (Table 2.1). These results showed that in ES medium, ES cell transition to NEP occurred gradually, with intermediate stages expressing both Oct-4 and the NEP markers Musashi-1 and Nestin. This overlap in expression was observed using both flow cytometry and immunocytochemistry including double-staining for both Nestin and Oct-4 (Figure 2.2). When the stage 1 cells were cultured for an additional week in either DN2, MEDII or ES media (stage 2), resulting colony morphologies were compared and differences were observed between ES medium and DN2 or MEDII media. DN2 and MEDII-cultured stage 2 cells developed neural tube-like structures (Figure 2.3A), whereas ES medium-cultured stage 2 cells failed to form these structures (Figure 2.3B). When cells were examined under the microscope, nuclear staining indicated the distinct cell arrangement (neural tube-like structures) developed in MEDII and DN2-derived populations that was not seen in ES-derived populations. There was no morphological difference between DN2 and MEDII-derived stage 2 cells; therefore, quantitative data was obtained only for ES and MEDII-derived stage 2 cells (Table 2.1). The pluripotent cell expression marker Oct-4 decreased in both groups from 74.9% (stage1) to 32.6% and 18.8% for ES and MEDII stage 2 groups, respectively ($p<0.05$). These results indicate that DN2 and MEDII media were more effective in promoting differentiation of ES cells to NEP cells than ES medium

Experiment 2. The effect of ES, DN2 and MEDII media on differentiation of stage 2 ES cells in adherent cell culture without feeder cells. Similar to results from stage 2 cells in experiment 1, we found differences for stage 3 cells cultured in ES medium compared to cells cultured in MEDII or DN2 media after feeder cell removal. Following feeder cell removal, cell culture gave rise to enriched rosette formation in MEDII or DN2 media, characteristic of NEP cell formation (Figure 2.4A), but ES medium-derived cell culture resulted in cells with large nucleus to cytoplasmic ratios, characteristic of ES cells (Figure 2.4B). Both MEDII and DN2 groups developed a similar differentiation pattern with distinct structure of neural tube-like formation (15) and further rosette-enriched populations.

In addition to microscopic examination, quantitative data indicated differences between cell populations. When cells were differentiated in MEDII medium, the percent of cells expressing Oct-4 was decreased dramatically (74.9% at stage 1 vs. 17.4% at stage 3 ; $p<0.05$). Furthermore, stage 3 MEDII-cultured cell populations with rosette structures showed expression of the early neural stem cell markers, Nestin and Musashi-1. However, the majority of stage 3 ES medium cultured cell populations retained their Oct-4 expression even after spontaneous differentiation (62.8%). In accordance with the flow cytometry results, immunocytochemistry demonstrated that for cells cultured in ES medium, stage 3 cell populations were both Nestin and Oct-4 positive (Figure 2.5B), whereas stage 3 cells cultured in MEDII medium had only increased Nestin staining without Oct-4 expression (Nestin +/Oct-4 -; Figure 2.5A). These results indicate that in adherent cell cultures without feeder cells, DN2 and MEDII medium promote differentiation to NEP-like cells, while ES medium does not.

Experiment 3. Effect of MEDII medium and low cell density on cell survival of stage 2 differentiating cells (tube-like structure forming cells). In order to obtain enriched populations of the desired cells (Nestin +/Oct-4 -) found in Experiments 1 and 2, we attempted single-cell passaging to propagate the differentiating cells in various concentrations of MEDII. A 50% MEDII medium was used in experiment 1 and 2 based on previous mouse ES cell MEDII neural differentiation studies (20). However no previous reports have tested different concentrations of MEDII on single cell or clonal propagation of NEP cells.

Stage 2 cells forming neural tube-like structures first observed in experiment 1 were passaged in one of four concentrations of MEDII medium (Table 2.2). Without MEDII, few cells survived and/or propagated (190 cells out of 10000), because when cells were dissociated and cultured in serum-deprived medium without feeder cell support, significant cell death occurred (1.9 ± 1.2% cell survival). However, when these cultures were supplemented with as little as 25% MEDII-conditioned medium, there was a 10-fold increase in surviving colony forming cells (22.3% cell survival). Cell survival and cell propagation were further improved and optimized at the 50% MEDII level, with 40,200 cells (40.2%) of the original cells surviving or propagating over the five days in culture. Immunocytochemistry showed the derived cells had the same characteristics as stage 3 NEP (Nestin+, Oct-4-).

Experiment 4. Characterization and examination of differentiation capacity of derived NEP-like cells. Rosette forming NEP-like cells were obtained from DN2 and MEDII-derived stage 3 cells and from clonally passaged cells from experiment 3. Phenotype characterization by immunocytochemistry is summarized in Table 2.3. Nearly 100% of rosette forming cells were positive for the early NEP markers Nestin and Musashi 1 (Figure 2.6A and 2.6B) and negative for later stages of differentiation markers A2B5, PSNCAM, Hu, GFAP, O4.

Removal of FGF and LIF from the culture medium resulted in further differentiation of NEP cells to form intermediate precursors staining positive for A2B5 or PSNCAM (Figure 2.7A and 2.7B). After 14 days of culture in neurobasal medium supplemented with B27 without bFGF, terminally-differentiated cell cultures contained neurons positive for Hu and Tuj (Figure 2.8A), astrocytes stained with GFAP and Dapi (Figure 2.8B) and oligodendrocyte stained with O4 (Figure 2.8C).

Experiment 5. The effect of medium, supplement, growth factor and oxygen conditions on proliferation and viability of subcultures of derived NEP-like cells.

Effect of culture medium. The effects of base media and supplements on cell survival were determined in this experiment (Table 2.4). A higher percent of cells cultured in NN2 survived compared to cells cultured in DN2 (33.8% DN2 vs 75.4% NN2 $P<0.05$), indicating that derived NEP cells survived better in neurobasal medium than DMEM medium with N2 supplement. Furthermore, all three groups of NN2, DN2 and MEDII supplemented cultures developed rosette structures. Also, the addition of MEDII to DN2 medium increased cell survival rate from 33.8% to 77.6% ($p<0.05$). In contrast, there was no difference in survival rate or the morphology of cells between N2 and B27 supplement when added to the neurobasal medium.

Subculture of NEP cells. These derived NEP cells have been cultured for more than 6 months without losing this characteristic and maintained a normal karyotype. Cells retained expression of Nestin and Musashi-1 (Figure 2.9A), and when terminally differentiated in medium lacking bFGF and LIF, the cell population included both neurons and glial cells (data not shown). When NEP cells were karyotyped with Giemsa staining, all 15 samples examined were stable with 46 XY chromosome numbers (Figure 2.9B).

Effect of LIF and bFGF. NEP cells propagated in NB27for approximately one month or 6 months were subjected to different concentrations of LIF and bFGF and cell survival was determine as well as cell proliferation over 14 days (Table 2.5). For early NEP cells (1 mo) the addition of LIF, bFGF or LIF + bFGF had no effect on plating efficiency and was only about 50%, indicating a relatively high rate of cell death. In contrast, the presence of bFGF increased cell proliferation more than four-fold (8.9% vs. 38.5% $p<0.05$), while LIF had no effect on proliferation of NEP cells either in the presence or absence of bFGF. After 6 months in LIF supplemented culture (described in Exp. 5), LIF, bFGF and the combined groups exhibited a higher plating efficiency than the control. bFGF had a greater effect on cell proliferation than LIF ($p<0.05$) for both the short-term (<1 mo) and long-term (6 mo) NEP cultures, However, only long term cultured NEP cells demonstrated increased proliferation rate for both LIF and bFGF individually and in combination.

Effect of oxygen concentration. After 7 days of culture in NB27 medium, total NEP-like cell number was approximately 25% greater in 5% oxygen compared to 20% oxygen ($p<0.05$) (Table 2.6). Considering that the plating efficiency was 50% when NEP-like cells were dissociated, we estimated that there was approximately a 2.5-fold increase in cell proliferation for 5% oxygen and a 1.96-fold increase for 20% oxygen.

DISCUSSION

The overall objective of these experiments was to obtain efficient neural differentiation of hES cells and to develop a defined medium that would be supportive of NEP stem cells and allow enzymatic passage, thereby facilitating more controlled and refined future studies. In contrast to previous reports, we employed both immunocytochemistry and flow cytometry

analysis in order to obtain both quantitative and morphological information on NEP formation at various stages of in vitro differentiation and culture conditions. Immunocytochemistry makes it possible to identify specific markers on cells; however, quantification is difficult and often subjective, and sample preparation can add bias due to selection from limited cell populations. The benefit of immunocytochemistry is that it allows co-localization of markers with the associated cell morphology, while flow cytometry analysis provides more objective quantification for marker expression.

The majority of studies investigating mouse and human ES cell differentiation to neural progenitors have used methods involving cell aggregation or embryoid body (EB) formation. EB formation in serum-containing medium included cells differentiated into NEP (15, 21), but also led to stochastic differentiation yielding multiple cell lineages, thus limiting the overall yield of the desired NEP cells (22). Dang et al compared EB differentiation cultures to adherent differentiation culture and reported that cell number limitation was not a factor in adherent differentiation cultures. In addition, they showed that adherent differentiation seemed to exclude cell differentiation toward hematopoietic development. Ying et al used adherent differentiation with mouse ES cells and obtained efficient neural commitment (23). In our study, hES cells were allowed to differentiate adherently in serum free medium, and we were able to obtain efficient neural differentiation. In our system feeder cells were present during the first 14 days, allowing hES cells to proliferate and differentiate. Subpopulations of stage 2 cells infiltrated underneath the feeder cell layer to attach firmly on culture plates. Serum deprivation apparently is crucial for ectodermal derivation (24) and removal of feeder cell layer produced homogenous rosette formation from homogenous spread of cells in adherent culture conditions.

In an attempt to follow the spatial and temporal differentiation of ES cells to neural lineages we divided the process into three stages. We found that Oct- 4 expression gradually decreased with the onset of expression of the NEP cell markers Nestin and Musashi-1. At an initial stage (stage 1), when cells were allowed to proliferate in ES medium, the majority of cells were positive for both pluripotent and NEP cell markers. Further differentiation resulted in morphological changes, including neural tube-like structures, when cells were cultured in either DN2 or MEDII-supplemented media but not in ES medium. Visual inspection indicated that in both DN2 or MEDII groups, cell populations developed rosette-like structures in over 70% of the total culture area, and there was little difference in rosette numbers or appearance between these two groups. The neural tube-like structures and rosettes have been previously identified as characteristic morphology of NEP (15).

In experiment 2 we found that removal of LIF, nonessential amino acids, KSR and undefined factors in serum forced ES cells to choose a neurectodermal fate. Rosette formation was not promoted when cells were cultured in ES medium with these factors included. Instead, cells retained their Oct-4 expression and delayed progression to a more differentiated state. This finding is similar to that seen with spontaneous differentiation. For example, Reubinoff et al showed that over four weeks of culture was required for ES cells to differentiate into NEP–like cells, and their system also resulted in endodermal and mesodermal differentiation (16).

MEDII added to DN2 medium did not improve tube-like structure formation (stage 2) or subsequent progression to stage 3 adherent colonies. The effect of MEDII was distinct, however, on low cell density NEP-like cell derivation. When tube structure-forming cells were dissociated and passaged in DN2, more than 98% of cells died and failed to form NEP cell colonies. This finding is similar to results obtained with mouse cells. Tropepe et al. (24)

reported that just 0.2% of the starting cell population was able to form neurospheres and supplement with LIF can improve this process. When we supplemented the dissociated cell cultures with MEDII medium, a higher proportion of cells attached to the substrate, and then subsequently proliferated to form NEP colonies. This finding was expected, because two of the known components of MEDII are fibronectin and LIF (20). When cells retained their cell contact and maintained attachment to the substrate in experiment 2, supplement of MEDII had no beneficial effects over DN2 medium. Using just morphological analysis, when cells were not disaggregated and their cell to cell contact remained in experiment 2, a more uniform and enriched rosette formation was obtained after another three to four days of culture in either DN2 or MEDII than cells passaged as single cells in experiment 3. Rosette enriched stage 3 from DN2 and MEDII groups and colonies developed from dissociated stage 2 cells were characterized to determine phenotype markers, and further differentiation capacity was examined to determine whether these cells correspond to NEP. NEP is designated as an unrestricted neural stem cell population based on Nestin expression, and these cells are non-immunoreactive to any restriction markers such as A2B5 and PSNCAM (11). Our results showed that the derived NEP-like cells had the same phenotype profile as rodent NEP or human NEP purified from fetal tissue. They were not immunoreactive to restriction markers or to specific differentiation cell markers of neurons or glial cells, but they were immunoreactive to Nestin and Musashi-1. In addition, the rosette enriched population was not immunoreactive to Oct-4 or mesodermal or endodermal markers. Mayer-Proschel showed that neural cells derived from fetal tissue were heterogeneous, with 50% of the population expressing A2B5 (11). Another step of immunopanning was required in order to obtain an enriched NEP population. In our study, enriched NEP cell populations were obtained through an efficient differentiation protocol.

As differentiation progressed, cells expressing precursor markers of PSNCAM or A2B5 appeared (Figure 2.7), and terminal differentiation resulted in neurons that expressed Hu and Tuj, oligodendrocytes that expressed O4 and astrocytes that expressed GFAP (Figure 2.8).

Experiment 5 was conducted to further define medium requirements that would support NEP cells and allow enzymatic passage and long term culture of these cells. We tested two base media, DMEM/F12, which has been used for various cell cultures including somatic cell lines and ES cell culture, and neurobasal medium, which was formulated for long term culture of rat hippocampal neurons (25). We also tested three supplements: MEDII, N2 and B27. N2 is a chemically defined concentrate developed to support growth of neural cell lines and includes insulin, transferrin, progesterone, putrescine and selenite. B27 is an optimized serum substitute for low density plating and growth of CNS neurons. We found that the serum free base medium DN2 did not support these NEP-like cells. In this medium cells lifted off the plate around day 7 of subculture and were trypan blue positive. Although cells cultured in DN2 supplemented with MEDII showed increased viability, a complex conditioned medium like MEDII can confound and limit the examination of candidate growth factor effects. In this study, comparison of DMEM/F12 and neurobasal medium showed that neurobasal medium supported NEP stem cell culture when supplemented either with N2 or B27. It also supported the survival of dissociated cells and allowed them to proliferate. Therefore, neurobasal medium supplemented with B27 was chosen as proliferation medium and further experiments were conducted using NEP cells cultured in this medium. This medium has been shown in previous studies to support survival and expansion of both adult neural stem cells and fetal and postnatal brainstem neurons in vitro (26, 27).

We also tested the effects of the growth factors LIF and bFGF on subculture of NEP-like cells. Mouse neural stem cells have been shown to be dependent on bFGF, and it was critical for neurosphere formation (24). The presence of LIF also supports and increases neurosphere formation; however, whether it acts by inducing differentiation of ES cells or by enhancing proliferation is not clear(24). In fetus-derived human neural stem cells, supplementing with both hLIF and bFGF enhanced proliferation rate (28). In our study done with short term cultured NEP (<1 mo), bFGF appeared to promote cell proliferation but supplement with LIF had little effect, nor was there a synergistic effect when LIF was combined with bFGF. Zhang reported that LIF had no effect on proliferation of derived NEP after 14 days culture (15). However, we found that after six months culture in LIF-containing medium increased cell responsiveness and cell proliferation was improved.

Physiological oxygen concentration does not exceed 5%; however, in conventional cell culture, oxygen concentration is maintained at 20%. In rat CNS stem cell culture, it has been reported that reduced oxygen concentration helped to improve cell proliferation and to reduce apoptosis (29). We tested whether reduced oxygen concentration produces the same advantage on the growth of NEP-like cells derived from human ES cells. In agreement with this previous study, low oxygen concentration improved cell proliferation rates approximately 25% after one week of culture. Because there was no difference in viability as measured by flow cytometry, the increased cell numbers do not appear to be due to increased initial cell survival.

CONCLUSION

In this study, we showed that NEP cells can be derived from human ES cells efficiently by adherent differentiation in defined medium. Derived NEP was broadly characterized with

phenotype markers and phenotype profile; in addition, differentiation capacity was similar to that of in vivo purified human NEP (11). Further NEP subculture conditions were optimized and cells were propagated successfully for over 6 months without loss of differentiation potential or stable karyotype. Our efficient derivation and proliferation of NEP demonstrates that this system can serve as an in vitro model for the examination of human neural development. A defined culture system would be ideal for further studies of effects of extrinsic factors on neuronal cell fate decision. In addition, long term cultured NEP may be good candidates for replacement cell therapy with little possibility of pluripotent cell contamination.

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REFERENCES

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science. Nov 6 1998;282(5391):1145-1147.

2. Reubinoff BE, Pera MF, Fong CY, et al. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol. Apr 2000;18(4):399-404.

3. McKay R. Stem cells in the central nervous system. Science. Apr 4 1997;276(5309):66-71.

4. Bjorklund A, Lindvall O. Cell replacement therapies for central nervous system disorders. Nat Neurosci. Jun 2000;3(6):537-544.

5. Jain M, Armstrong RJ, Tyers P, et al. GABAergic immunoreactivity is predominant in neurons derived from expanded human neural precursor cells in vitro. Exp Neurol. Jul 2003;182(1):113-123.

6. Borlongan CV, Tajima Y, Trojanowski JQ, et al. Transplantation of cryopreserved human embryonal carcinoma-derived neurons (NT2N cells) promotes functional recovery in ischemic rats. Exp Neurol. Feb 1998;149(2):310-321.

7. Morrison SJ. The last shall not be first: the ordered generation of progeny from stem cells. Neuron. Oct 2000;28(1):1-3.

8. Amit M, Carpenter MK, Inokuma MS, et al. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol. Nov 15 2000;227(2):271-278.

9. Liu S, Qu Y, Stewart TJ, et al. Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. Proc Natl Acad Sci U S A. May 23 2000;97(11):6126-6131.

10. Kalyani A, Hobson K, Rao MS. Neuroepithelial stem cells from the embryonic spinal cord: isolation, characterization, and clonal analysis. Dev Biol. Jun 15 1997;186(2):202-223.

11. Mayer-Proschel M. Human neural precursor cells-an in vitro characterization. Clinical Neuroscience Research. 2002 2002;2:58-69.

12. Kennea NL, Mehmet H. Neural stem cells. J Pathol. Jul 2002;197(4):536-550.

13. Alvarez-Buylla A, Garcia-Verdugo JM, Tramontin AD. A unified hypothesis on the lineage of neural stem cells. Nat Rev Neurosci. Apr 2001;2(4):287-293.

14. Mujtaba T, Piper DR, Kalyani A, et al. Lineage-restricted neural precursors can be isolated from both the mouse neural tube and cultured ES cells. Dev Biol. Oct 1 1999;214(1):113-127.

15. Zhang SC, Wernig M, Duncan ID, et al. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol. Dec 2001;19(12):1129-1133.

16. Reubinoff BE, Itsykson P, Turetsky T, et al. Neural progenitors from human embryonic stem cells. Nat Biotechnol. Dec 2001;19(12):1134-1140.

17. Calhoun JD, Lambert NA, Mitalipova MM, et al. Differentiation of rhesus embryonic stem cells to neural progenitors and neurons. Biochem Biophys Res Commun. Jun 20 2003;306(1):191-197.

18. Rathjen J, Lake JA, Bettess MD, et al. Formation of a primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors. J Cell Sci. Mar 1999;112 (Pt 5):601-612.

19. Mitalipova M, Calhoun J, Shin S, et al. Human embryonic stem cell lines derived from discarded embryos. Stem Cells. 2003;21(5):521-526.

20. Rathjen J, Haines BP, Hudson KM, et al. Directed differentiation of pluripotent cells to neural lineages: homogeneous formation and differentiation of a neurectoderm population. Development. Jun 2002;129(11):2649-2661.

21. Schuldiner M, Yanuka O, Itskovitz-Eldor J, et al. From the cover: effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A. Oct 10 2000;97(21):11307-11312.

22. Dang SM, Kyba M, Perlingeiro R, et al. Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. Biotechnol Bioeng. May 20 2002;78(4):442-453.

23. Ying QL, Stavridis M, Griffiths D, et al. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat Biotechnol. Feb 2003;21(2):183-186.

24. Tropepe V, Hitoshi S, Sirard C, et al. Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. Neuron. Apr 2001;30(1):65-78.

25. Brewer GJ, Torricelli JR, Evege EK, et al. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res. Aug 1 1993;35(5):567-576.

26. Wachs FP, Couillard-Despres S, Engelhardt M, et al. High efficacy of clonal growth and expansion of adult neural stem cells. Lab Invest. Jul 2003;83(7):949-962.

27. Kivell BM, McDonald FJ, Miller JH. Serum-free culture of rat post-natal and fetal brainstem neurons. Brain Res Dev Brain Res. Apr 14 2000;120(2):199-210.

28. Carpenter MK, Cui X, Hu ZY, et al. In vitro expansion of a multipotent population of human neural progenitor cells. Exp Neurol. Aug 1999;158(2):265-278.

29. Studer L, Csete M, Lee SH, et al. Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. J Neurosci. Oct 1 2000;20(19):7377-7383.

Table 2.1. Phenotype marker expression changes over time. Cells positive to each phenotype marker were calculated to obtain a percent (Mean ± SE) of total cell number.

	Stage 1	Stage 2		Stage3	
marker/groups	ES medium	ES medium	MEDII	ES medium	MEDII
Oct-4	74.9±3.0	32.6±3.5	18.8±8.4	62.8±3.5a	17.4±8.3b
Musashi 1	88.0±2.9	53.3±2.2a	76.6±6.1b	76.9±4.0	66.0±4.9
Nestin	77.5±7.4	30.9±11.9	50.14±3.3	79.7±5.9	70.9±5.5

[ab] Different superscripts within each parameter and stage are significantly different, $P<0.05$

Table 2.2. Effect of MEDII supplement on percent cell survival in serum and feeder cell-deprived culture conditions (Mean ± SE).

	0%MEDII	25%MEDII	50%MEDII	100%MEDII
Mean+SE	1.9±1.2%[a]	22.3±8.1%[ac]	40.2±10.9%[bc]	32.6±12.1%[bc]

[abc] Different superscripts within each parameter(row) are significantly different, $P<0.05$.

Table 2.3. Phenotype marker expression of rosette forming neuroepithelial stem cell (NEP)-like cells.

Antigens	NEP like cells
Nestin	>90%
Musashi	>90%
β III tubulin	rare
A2B5	rare
PSNCAM	rare
GFAP	-
Hu	-
O4	-
Muscle actin	-
α Fetoprotein	-
Oct-4	-

Table 2.4. Percent of viable cells (Mean ± SE) cultured in different media[1].

DN2	**NN2**	**NB27**	**MEDII**
33.8±6.23% [a]	75.4±2.01% [b]	74.6±4.21% [b]	77.6±4.09%[b]

[ab] Different superscripts are significantly different, P<0.05.

[1] DN2 (DMEM based medium supplemented with N2); NN2 (Neurobasal medium based medium supplemented with N2); NB27 (Neurobasal medium based medium supplemented with B27); MEDII (DN2 + 50% DMEM based condition medium).

Table 2.5. Effect of basic firoblast growth factor (bFGF) and Leukemia Inhibitory Factor (LIF) supplementation on plating efficiency and proliferation of neuroepithelial stem cell (NEP)-like cells (Mean ± SE).

		-/-	bFGF/-	-/LIF	bFGF/ LIF
<1 month culture	Plating Efficiency (% plated cell number)	51267±13487 (51.3±13.5)[a]	53733±11293 (53.7±11.3)[a]	50767±11305 (50.8±11.3)[a]	51400±8713 (51.4±8.7)[a]
	Proliferation (% of total cell number)	61516±10155 (8.9±1.9)[a]	308274±68538 (38.5±4.2)[b]	40365±4303 (6.9±2.0)[a]	347927±79011 (45.8±4.5)[b]
6 month culture	Plating Efficiency (% plated cell number)	70480±2500 (35.2±1.3)[a]	93013±8623 (46.5±4.3)[b]	92072±876 (46.0±0.4)[b]	100326±8573 (50.2±4.3)[b]
	Proliferation (% of total cell number)	123154±3398 (7.4±0.2)[a]	501150±37743 (30.3±2.4)[b]	278611±4585 (16.8±0.2)[c]	753847±41196 (45.5±2.4)[d]

[abcd] Different superscripts within each parameter (row) are significantly different, P<0.05.

Table 2.6. Effect of oxygen (O_2) concentration on viability and proliferation of neuroepithelial stem cell (NEP)-like cells (Mean ± SE).

	Viability	**Cell number (% of total)**
High O2	80 ± 4%[a]	196268 ± 18736 (44.19 ± 1.00% [a])
low O2	83 ± 3%[a]	250657 ± 7605 (55.81 ± 1.00%[b])

[ab] Different superscripts within each parameter (column) are significantly different, P<0.05.

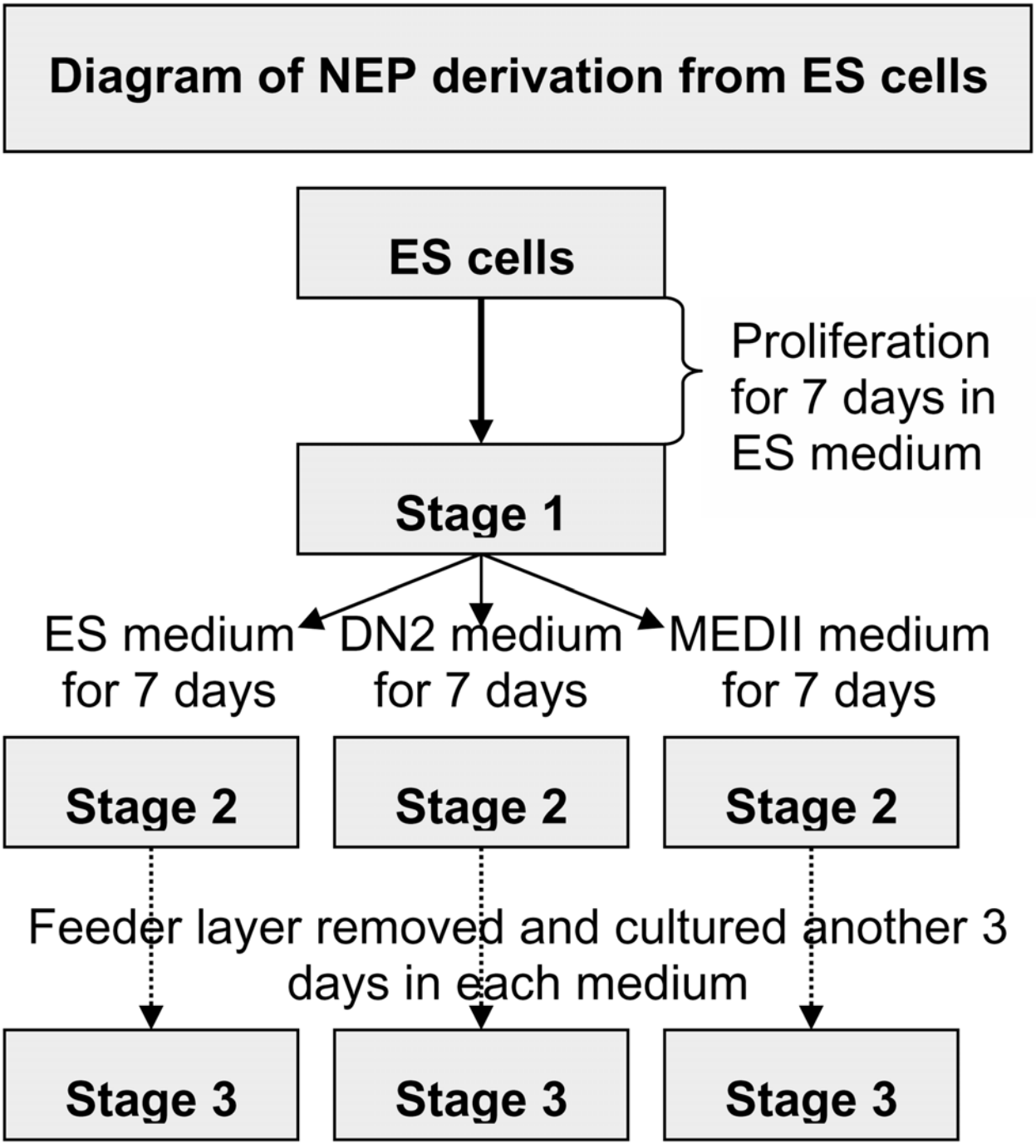

Figure 2.1. Procedure for adherent derivation of human embryonic stem cells (hES) into neuroepithelial stem cells (NEP).

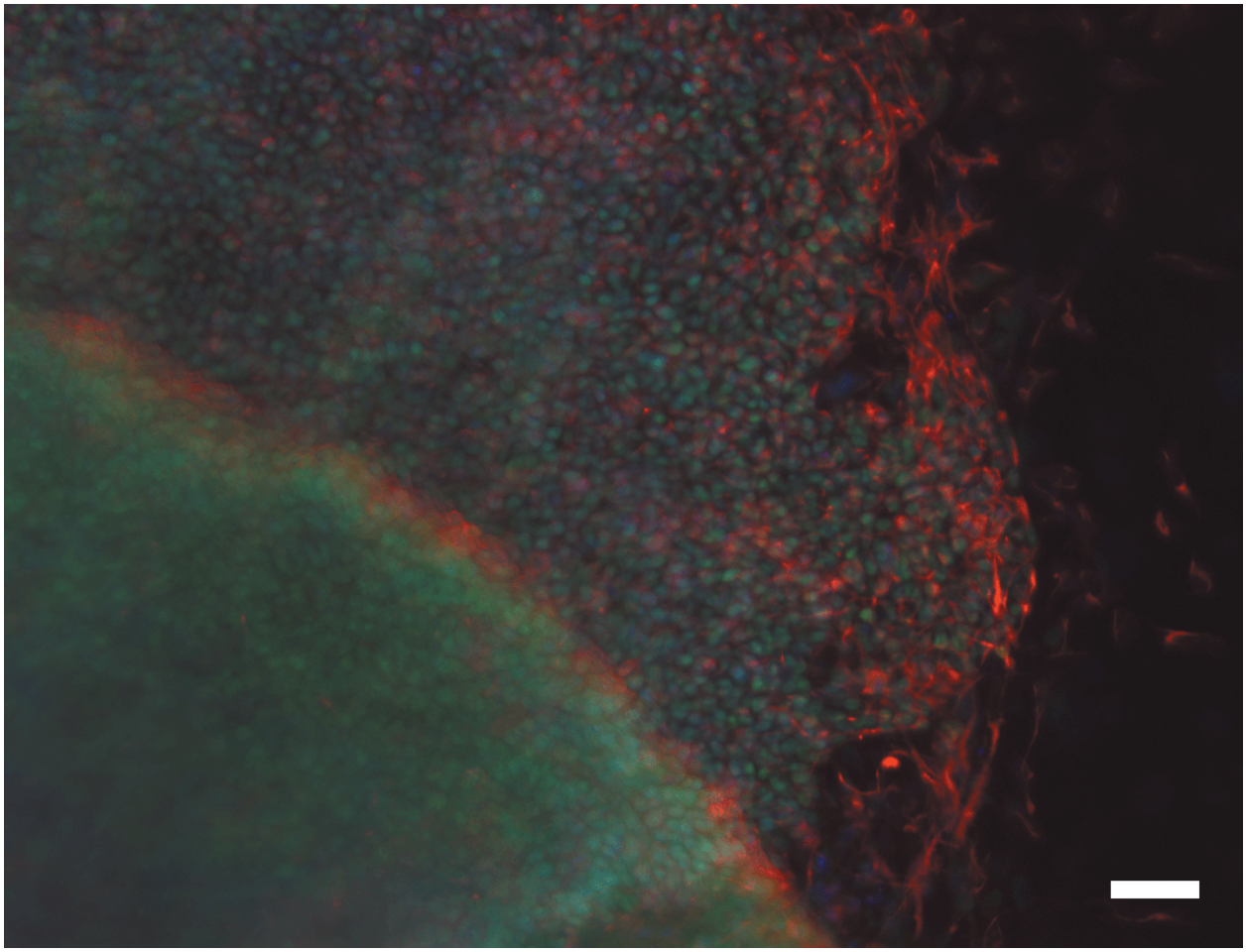

Figure 2.2. Phenotype marker expression of stage 1 cells counter-stained with Oct-4 (green), Nestin (red) and Dapi (blue). Bar=100um.

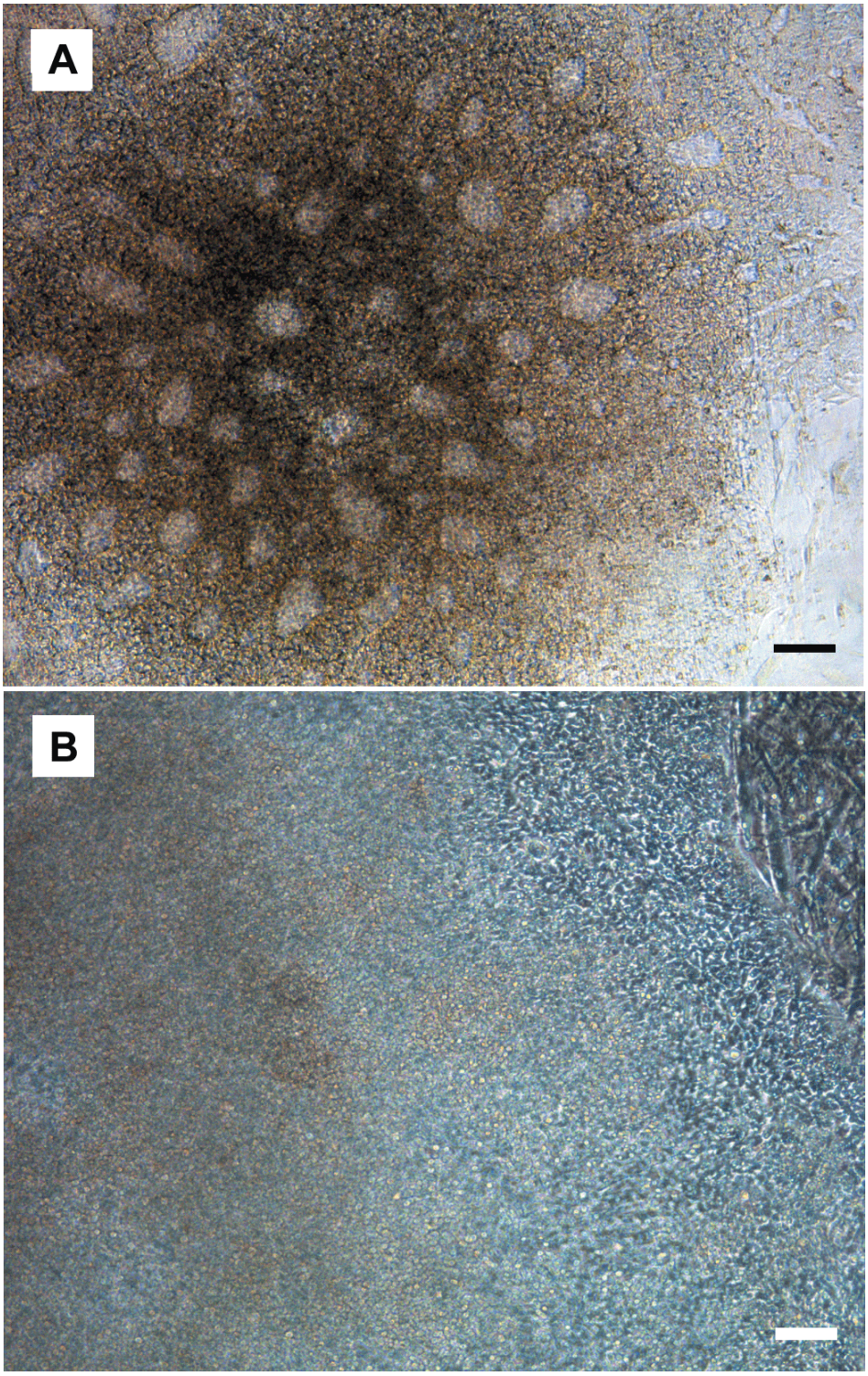

Figure 2.3. Phase contrast image of (A) Stage 2 cells cultured in MEDII medium (DN2-cultured cells were similar, so the data is not shown) (B) Stage 2 cells cultured in ES medium. Bar=100um.

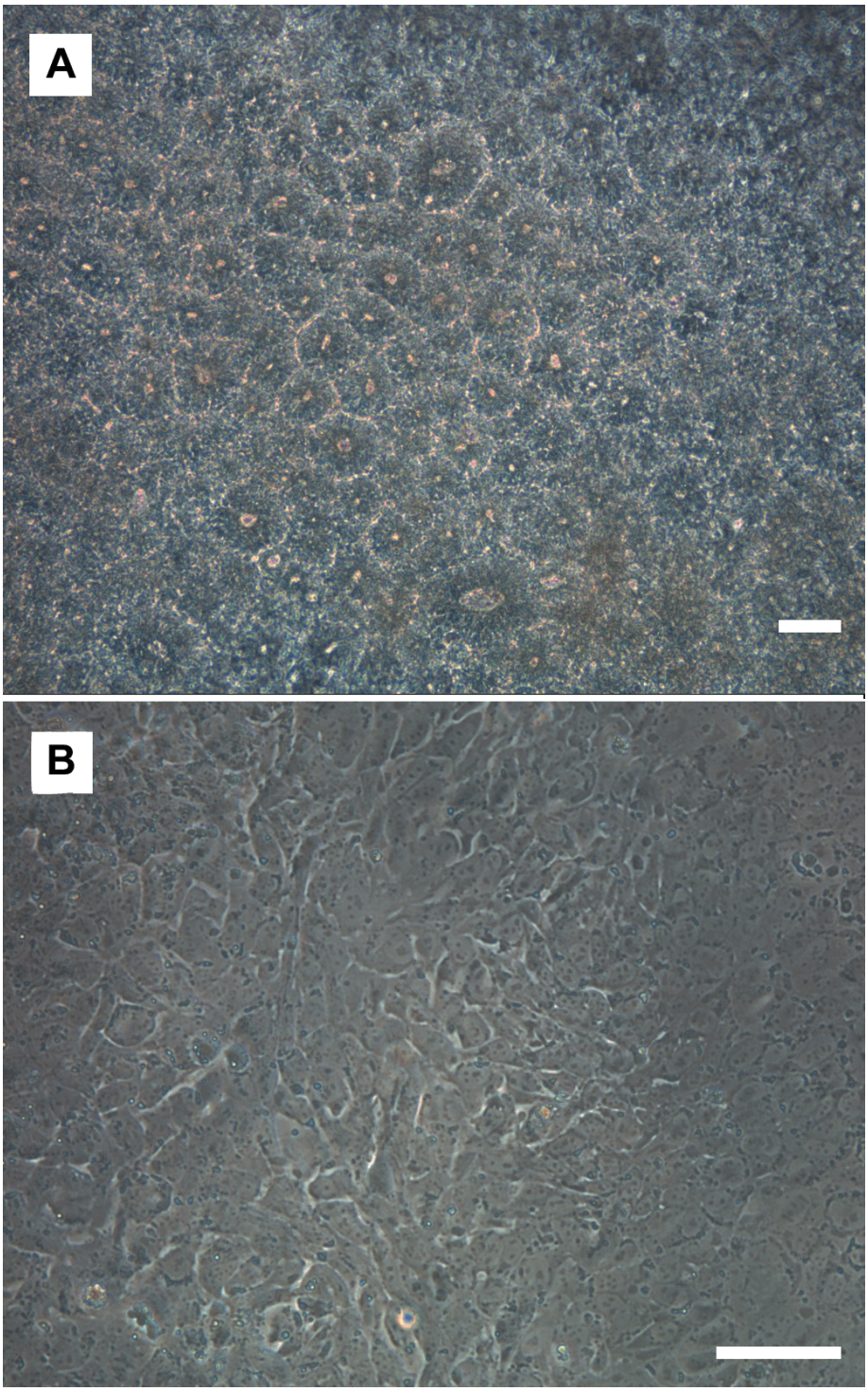

Figure 2.4. Phase contrast image of Stage 3 neuroepithelial stem cell (NEP)-like cells in adherent cell culture without feeder cells. (A) Cells cultured in MEDII medium (DN2-cultured cells were similar, so the data is not shown); (B) Cells cultured in ES medium. Bar=100um.

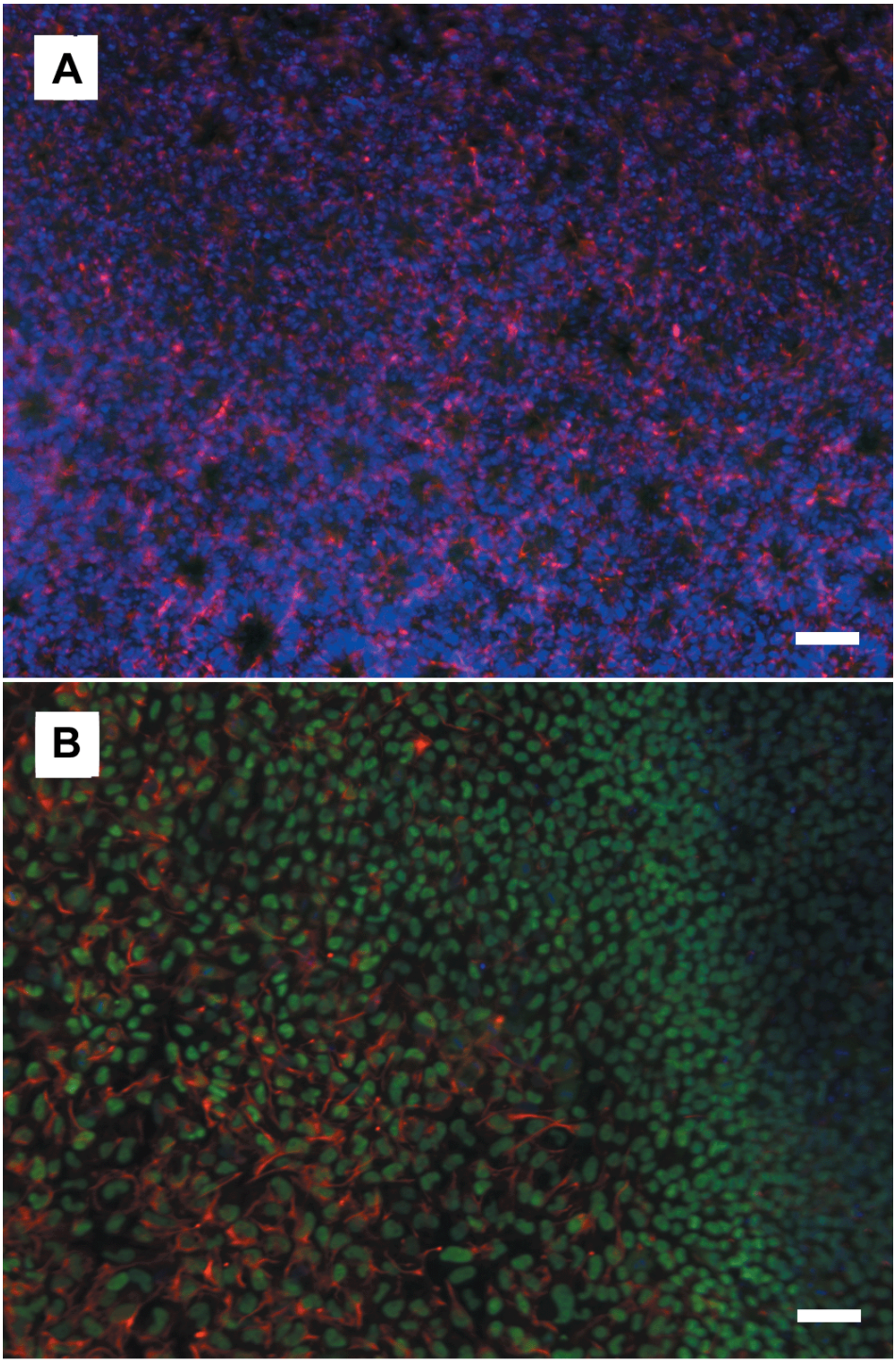

Figure 2.5. (A) Phenotype marker expression of stage 3 cells counter-stained with Oct-4 (green), Nestin (red) and Dapi (blue) developed in DN2 medium (MEDII-cultured cells were similar, so the data is not shown). (B) Phenotype marker expression of stage 3 cells counter-stained with Oct-4 (green), Nestin (red) and Dapi (blue) developed in ES medium. Bar=100um.

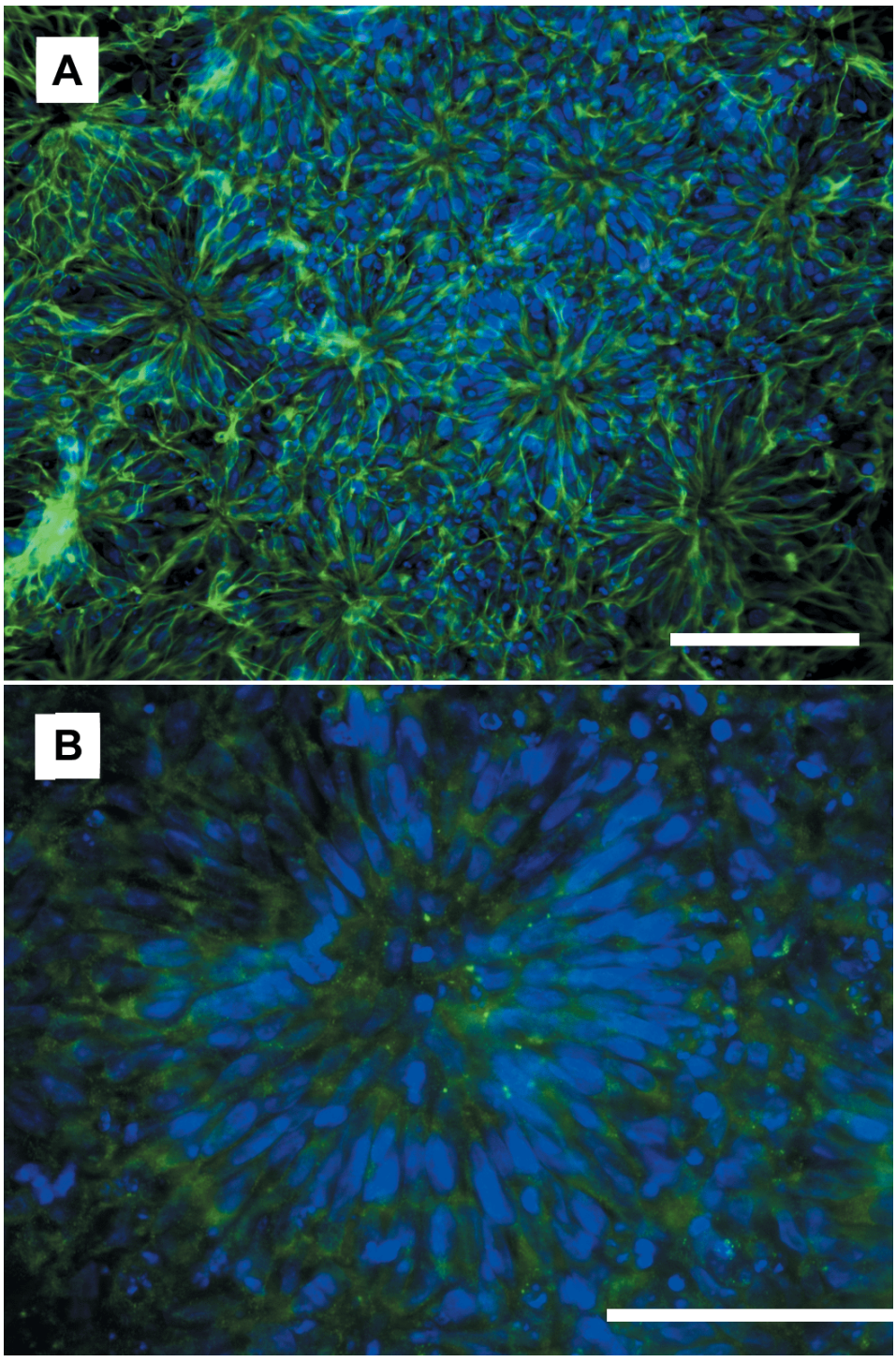

Figure 2.6. Rosette-forming neuroepithelial (NEP) cells stained with (A) Nestin (B) Musashi. Bar=100um.

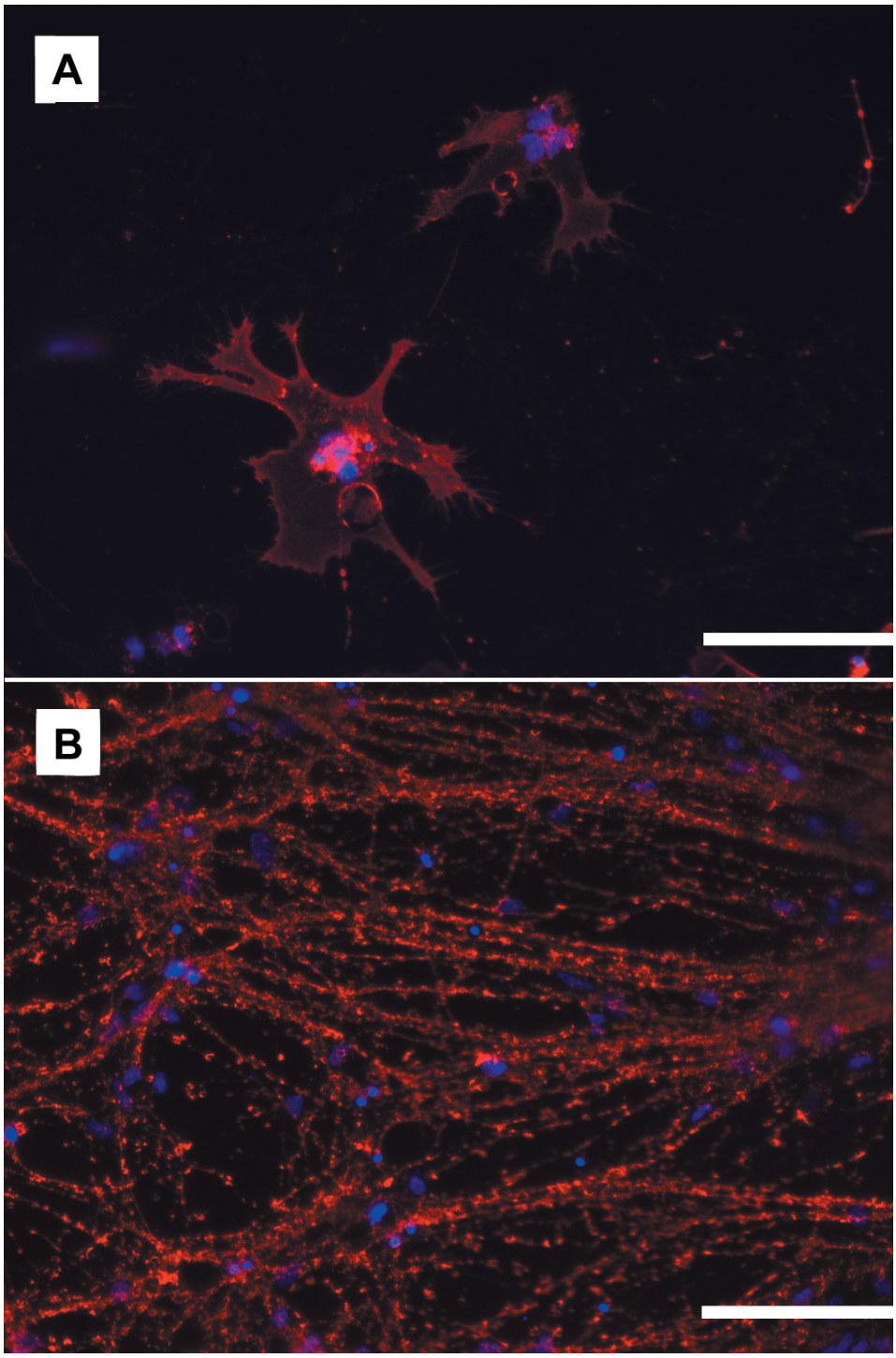

Figure 2.7. Intermediate precursor cells following removal of basic fibroblast growth factor (bFGF) and leukocyte inhibitory factor (LIF) from the culture medium. (A) Cells stained for A2B5 (red) and Dapi (blue). (B) Cells stained for PSNCAM (red) and Dapi (blue). Bar=100um.

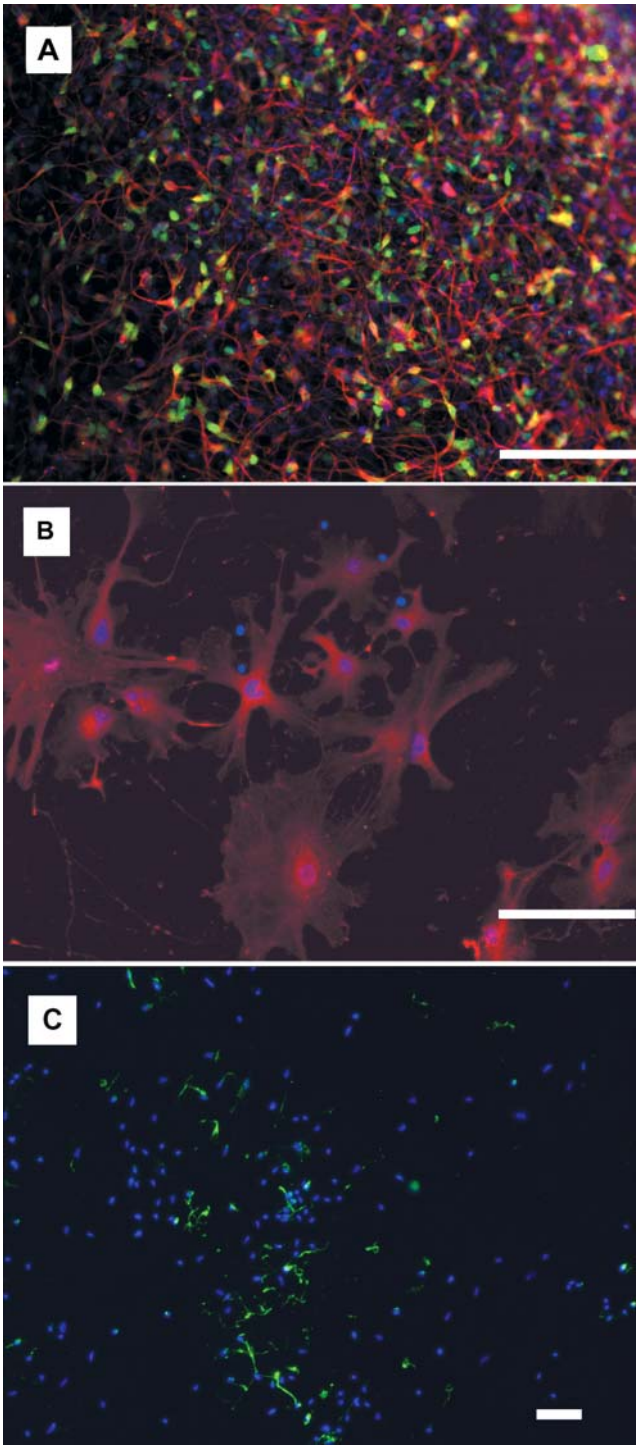

Figure 2.8. Terminally differentiated neurons and astrocytes after 14 days culture in neurobasal medium supplemented with B27 and L-glutamine, without basic fibroblast growth factor (bFGF). (A) Neurons double stained for Hu C/D (green) and Tuj (red). (B) Astrocyte stained with GFAP (red) and Dapi (blue). (C) Oligodendrocyte stained with O4 (green) and Dapi (blue) Bar=100um.

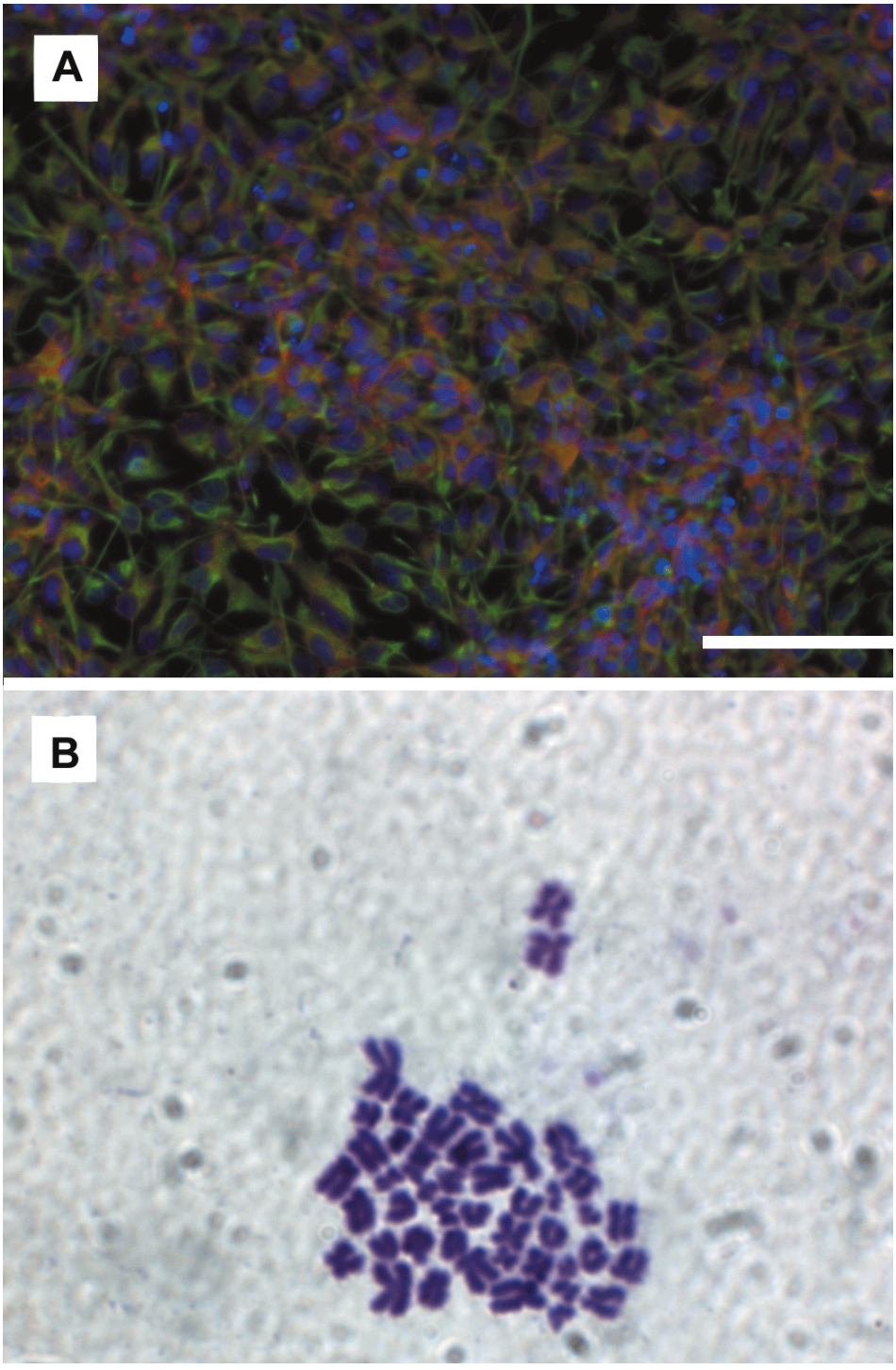

Figure 2.9. (A) Long-term (10 mos) cultured neuroepithelial (NEP) cells stained with Nestin (green), Musashi (red) and Dapi (blue). (B) 46XY chromosome stained by Giemsa. Bar=100um.

Chapter 3

Motor neuron differentiation in neuroepithelial cells cultures derived from human embryonic stem cells[1]

[1]Soojung Shin, Steven Dalton, Steven L. Stice 2004. Motor neuron differentiation in neuroepithelial cells cultures derived from human embryonic stem cells. Submitted to *Lancet* as research letter, 10/6/04

ABSTRACT

Neuroepithelial stem cells (NEP) have been generated from embryonic stem cells, fetal and adult tissue. The ability of these NEP to further differentiate into neurons, astrocytes and oligodendrocytes is likely regulated by extracellular signals. The objective of this study was to quantitatively examine the effect of inductive signals of Sonic Hedgehog (Shh) and Retinoic acid (RA) on motor neuron differentiation of human embryonic stem cells (hESC) derived NEP. NEP were derived from hESC and proliferated in defined medium. Freshly isolated (early) and propagated (more than three months; late) cultures of NEP were characterized for further exposure to inductive signals. SOX1, SOX2 and SOX3 and the Shh receptor Patched 1 (PTCH), genes were expressed in both groups. When allowed to spontaneously differentiate, a portion of the NEP from both groups exhibited motor neuron phenotype (Islet1, ChAT positive). We used bFGF, RA and Shh to more directly induce motor neuron progenitor (OLIG2) and motor neuron (HLXB9) specific gene expression. Basic FGF alone increased OLIG2 gene expression in NEP (2.64 fold increase vs. nontreated cells $p<0.01$) and combined RA and Shh resulted in 4.15 fold increase ($p<0.01$). Further, exposure of both early and late NEP to RA gave rise to approximately two fold increase in HLXB9 expression ($p<0.01$). However, combined Shh differentially affected HLXB9 expression in early and late NEP groups (8.49 vs. 3.07 fold change compared to respective control, $p<0.01$). One potential contributing factor to this result may be up-regulation of PTCH gene expression by Shh in early NEP (1.79 fold change, $p<0.01$), whereas there is no increases in late NEP. This study suggests that NEP derived from hESC can form a motor neuron phenotype and that Shh, bFGF and RA can differentially affect expression of motor neuron associated genes in the NEP.

Key words: Human embryonic stem cell, Sonic hedgehog, Retinoic Acid, neuroepithelial stem cell, motor neurons

INTRODUCTION

Neuroepithelial stem cells (NEP) are self-renewing cells that form the neural tube and divide symmetrically or asymmetrically to give rise to all the cells that comprise the mammalian central nervous system, including various types of neurons and glial cells (27). NEP can be obtained from mouse embryonic stem cells (mESC) (23), and propagated for extended periods without losing the potential to differentiate into neurons and glial cells(36). These undifferentiated non-lineage committed mouse NEP express the phenotype marker Nestin but not neural or glial differentiation markers (16, 23). Human NEP have been derived from hESC and Nestin is also the primary antigen used as a marker of human NEP (28, 41). We have derived and propagated NEP for 10 months without overt changes in Nestin expression or differentiation capacity to neuron and glial cells (Shin manuscript submitted). If cultured human NEP can be propagated in this manner without losing their potential to form more differentiated phenotypes then they may become an ideal source of cells for regenerative cell therapies. The goal of this study was to examine motor neuron differentiation capacity of NEP and further to determine whether human NEP were responsive to factors known to induce mESC towards motor neurons.

Nestin has been the primary antigen used as a marker of NEP (28, 41). However, Nestin expression is not exclusive to NEP but is widely expressed in developing embryos. For example, Nestin is expressed in endocrine progenitor cells, vascular endothelial cells (14), testis (8) and skeletal muscle (30). In vivo expression studies in the mouse and chicken indicated that SOX1, SOX2 and SOX3 are predominantly expressed in the undifferentiated cells of NEP in CNS (6, 37). Human and mouse SOX genes are highly conserved to have over 95% homology and human embryonic brain moderately abundant human SOX1 (19). Also, it has been shown that SOX2 and SOX3 expression was modulated during neural differentiation of human

embryonic carcinoma cell line NTERA2 (32). SOX2 expression has also been observed in hESC (4, 11) and we also observed SOX2 and SOX3 in proliferating hESC (data not published). Therefore, NEP that are negative for the ESC marker oct-4 and positive for nestin in combination with SOX1, SOX2 and SOX3 expression may be a useful identification criteria for hESC derived NEP.

In the development of the mammalian CNS, neural induction begins before gastrulation, and regulatory molecules influence cells to differentiate into specific neuronal cell types. Among the signaling molecules, Shh is a well characterized morphogen. During neural development, Shh secreted from the notochord serves as a morphogen to ventralize cells in the neural tube, including motor neurons (15). The spinal cord is subdivided according to the concentration gradient of Shh, giving rise to specified neuronal subtypes. The Shh signaling pathway involves two transmembrane proteins, Patched 1(PTCH) and smoothened (SMO) (24) and both transcription and translation of PTCH are up-regulated as an early response to Shh signaling (12). Therefore in order for NEP cells to respond to Shh, PTCH must be present and is likely upregulated in response to Shh. Sonic hedgehog can either induce expression of class II genes, one being, OLIG2 or repress class I genes in progenitor cells. OLIG2 is exclusively expressed in the spinal motor neuron column of the spinal cord where motor neurons and oligodendrocytes are generated (35). In addition, the homeobox genes HLXB9 (HB9) and Islet 1 transcription factor are expressed by motor neurons in the developing vertebrate CNS. In embryonic chick spinal cord, ectopic expression of HB9 is sufficient to trigger motor neuron differentiation and its essential role in motor neuron differentiation has been demonstrated (1). Furthermore chick neural explants experiments have shown that Islet 1 expression is affected not only by the presence of Shh but also temporal events (26). Islet 1 positive motor neurons were

induced if there was Shh signal within less than 12 hours after initiation of explant culture. However, if Shh was absent during this time, the motor neuron were not present and the explant differentiated into interneurons. Therefore, OLIG2, HB9, Islet1 and Choline acetyltransferase (ChAT) expression in a neural population is indicative of motor neuron differentiation and is dependant to some extent on proper temporal extrinsic cues. Retinoic acid is highly expressed in developing mouse spinal cord and has been shown to have caudalizing effects, giving ectodermal cells their spinal positional identity (38). Retinoic acid inhibits WNT3A, which is involved in mesoderm formation (5). In the mouse, RA was shown to stimulate rostral neural progenitors to acquire spinal positional identity (22), and coexposure with Shh, ventralized spinal progenitor cells, resulting in differentiation into motor neurons (2). Similar effect of Shh and RA was demonstrated in mESC. When mESC were induced to neural fate and exposed to Shh and RA, the differentiating population was preferentially directed to motor neuron fate (Islet 1, HB9 and Tuj1 positive) (21, 38). However, bFGF alone, can drive long term culture fetal human neural stem cell to cholinergic neuron (39), which suggests bFGF may also induce hESC derived NEP to a neural fate independently of Shh or RA. Therefore, hESC to spinal motor neuron formation may require several differentiation cues and factors. Previously we and others (28, 41) (Shin manuscript submitted) have derived Nestin positive NEP from hESC. However, SOX gene expression and temporal gene expression changes associated with factors known to induce a motor neuron phenotype has not yet been described in hESC.

The aims of this study, was to 1) further define the starting population of NEP using SOX1, SOX2 and SOX3 expression and characterize Shh and RA effects on recently isolated (early) and propagated (late) human NEP (> 3 months). 2) derive a motor neuron phenotype from the NEP. Here we demonstrate that Islet 1 and ChAT positive motor neurons can be

produced from NEP and that inductive signaling in NEP may not only affect on the presence or absence of extrinsic factors, but also how long the NEP are cultured in vitro prior to being exposed to these factors.

MATERIAL AND METHODS

Derivation and culture of NEP. NEP were derived from BG01 and BG02 hESC lines (20) and outline in Figure 1. Briefly, after one week of culture on mouse feeder cell layer, hESC were fed with the derivation medium, which is DMEM/F12 medium (Gibco) supplemented with 2mM L glutamine, 50U/ml penicillin, 50ug/ml streptomycin, N2 (Gibco) and 4ng/ml of basic fibroblast growth factor (Sigma) for 7 days. The mouse feeder layer was then removed, allowing NEP to attach to the culture dish and develop rosettes after 3 days in derivation medium (early NEP). Derived NEP were propagated further on polyornithine and laminin coated dishes in neurobasal medium (Gibco) supplemented with L glutamine, penicillin, streptomycin, B27 (Gibco), 20ng/ml of bFGF (Sigma) and 10ng/ml of Leukemia inhibitory factor (LIF, Chemicon) and continuously passaged either by mechanical triturating or by trypsin. Under these conditions NEP could be cultured greater than 10 months retaining NEP marker expression and differentiation capacity to neurons and glial cells if growth factors were removed from culture medium. Further characterization of these NEP are described in Shin 2004 (submitted).

Antibodies and immunocytochemistry. Cells plated on polyornithine and laminin coated permonox slides were washed in PBS and fixed with 4% paraformaldehyde and 4% sucrose in PBS for 15 min. Fixed cells were washed two times with PBS before staining. Permeabilization and blocking was done in blocking buffer of 0.1% Triton, 5% FBS in Tris

buffer for 40mins. Primary antibodies were applied in blocking buffer for 2 h at RT and washed three times in blocking buffer before secondary antibody application. The secondary antibodies, goat anti-mouse Alexa conjugated, donkey anti-goat Alexa conjugated, and goat anti-rabbit Alexa conjugated (Molecular Probes), were diluted at 1:1000 in blocking buffer and applied to cells for 40 min at RT. After two washes in PBS, DAPI was applied for nuclear staining for 10 min, and cells were observed under the fluorescence microscope. For negative controls, first antibodies were omitted and the same staining procedures were followed. Primary antibodies and dilutions used were mouse anti- Islet 1 (1:100; DSHB), goat anti-ChAT (1:100; Chemicon), rabbit anti-Tuj1 (1:500; Covance) and mouse anti-Oct4 (1:200; Santa Cruz biotech).

Experimental design

Experiment 1. Characterization of the early and late NEP and their spontaneous motor neuron differentiation potential. Early and late NEP, two candidate populations for motor neuron induction were characterized first both by their SOXs gene expression and by their differentiation capacity to motor neurons. To examine SOXs gene expression, RNA was isolated from early and late NEP. Samples were washed once with PBS and total RNA was extracted using Trizol. For reverse transcription PCR, 2ug of total RNA from each sample was treated with DNase (Promega). One µg RNA was converted to cDNA by using the Superscript III kit (Invitrogen) using oligo dT as a primer, and 1µg was prepared without reverse transcriptase (noRT) to serve as control for exclusion of genomic amplification. ReadyMix REDTAQ (Sigma) was used and 50ng of cDNA was added for the PCR reaction. For SOX1, commercial primer and probe for real time PCR were used and 25ng of cDNA was subjected to

real time PCR according to the manufacturer's instructions. After amplification, products were separated on 2% agarose gel and visualized using ethidium bromide (EtBr) staining under UV light. Primer sequences (forward and reverse), size of the product and PCR condition are described in table 1.

In order to investigate factors involved in the motor neuron induction we first needed to determine whether the NEP were capable of generating motor neuron. We removed bFGF and LIF from culture medium of early and late NEP. After two weeks of culture, cells were fixed and motor neuron phenotype was examined as described in antibody and immnunocytochemistry section. In addition, Oct4 expression was examined in both early and late NEP to exclude the possible SOXs gene expressions by hESC.

Experiment 2. Expression of the Shh receptor Patched 1 (PTCH) in NEP. In order to respond to Shh, the target tissue needs to have receptor for the ligand. In this experiment, Shh receptor of PTCH was examined in early and late NEP. Cells were harvested from early and late NEP and RNA was extracted as described in experiment 1. For reverse transcription PCR, 1ug of total RNA from each sample was treated with DNase (Promega); 500ng was converted to cDNA by using the Superscript III kit (Invitrogen) with oligo dT as a primer and the other 500ng was prepared without reverse transcriptase (noRT) to serve as control for genomic amplification. Twenty five (25) ng of resulting cDNAs were subjected to real time PCR using specific primer and probe (ABI, Hs_00181117_m1), and gene expression was visualized as an amplification curve using ABI 7700 sequence detection system, and cycle number when amplification exceeded threshold (Ct values) was obtained.

Experiment 3. bFGF effect on OLIG2 gene expression in NEP. Basic FGF has been used to proliferate NEP and shown to drive long term cultured fetal human neural stem cell

to cholinergic neuron (39). To investigate the effect of bFGF on motor neuron induction, OLIG2 expression change was monitored using late NEP because of its preferential expression in motor neuron progenitor domain. Late NEP were divided into four groups and cultured in four different media (with or without bFGF × with or without RA and Shh). After 1 day of culture, total RNA was extracted, treated with DNase, and converted to cDNA as described in experiment 2. Twenty five (25) ng of each cDNA was subjected to real time PCR, target gene of OLIG2 expression was normalized using Human β Actin (ABI, 4326315E) as a reference gene, and then quantitative gene expression in treated and untreated groups was compared. If not specified, each experiment included three identical replicates and two independent experimental replicates. Relative gene expression was shown as fold change (ratio) in gene expression using Comparative Ct method (17).

Experiment 4. Shh and RA affect HB9 gene expression in NEP. HB9 expression, a transcription factor for spinal motor neuron was used to investigate the effects of RA and Shh on motor neuron differentiation of NEP. Based on result from experiment 3, bFGF was maintained at time of Shh and RA exposure for 7days. Early and Late NEP were cultured for 7 days with or without Shh (1ug/ml) and RA (2uM) then fully differentiated by culture in neurobasal medium (Gibco) supplemented with L Glutamine, penicillin, streptomycin and B27 (Gibco) for two weeks. For quantitative gene expression, real time PCR was carried out (ABI 7700 system). Primers were designed to flank the exon and intron boundary for specific amplification (Table 1). PCR products generated after real time PCR were separated on 2% agarose gel to verify product size and expression of HB9 was normalized to reference gene of GAPDH. Relative gene expression was shown as fold change like as experiment 3.

Experiment 5. Combined Shh affects PTCH expression in NEP. In this experiment, response to combined Shh was examined by monitoring up regulation of PTCH which is a target gene of Shh. Both Early and late passaged NEP were divided into two groups and one from each were exposed to Shh (500ng/ml; R&D system) and RA (1uM; Sigma) and the other groups were served as control respectively. After 24hr exposure, RNA were extracted both from control and treatment groups and converted to cDNA for subsequent real time PCR as described in experiment 2. Target gene of PTCH expression was normalized using Human β Actin as a reference gene, then quantitative gene expression in treated and untreated groups was compared.

Statistical analysis. All target gene Ct values in each parameter were normalized by reference gene Ct value to have delta Ct value (target gene Ct – reference gene Ct). For statistical analysis, delta Ct values of control and treatment group were subjected to one-tailed T-test. Significant differences between the treatments were defined as $P < 0.01$.

RESULTS

Experiment 1. Characterization of the early and late NEP and their spontaneous motor neuron differentiation potential. Before the exposure to inductive signal, starting material of early and late NEP was characterized by their SOXs gene expression and motor neuron formation potential. First, we examined SOX1, SOX2 and SOX3 gene expressions in early and late NEP. Both early and late NEP expressed SOX2 and SOX3 (Figure 2A). By using real time PCR, the SOX1 gene was amplified and the amplicon was visualized by EtBr staining. The SOX1 gene was also expressed in both cell groups (Figure 2B). To exclude the possibility of SOXs gene expression from hESC remnants, Oct4 expression was examined in

both early and late NEP. The result showed that there were no Oct4 positive cells in neither of both populations (Figure 3A-D).

We then showed that both NEP cell groups could spontaneously differentiate into mature neuron whose subpopulations were immunopositive to Islet1, Tuj1 and ChAT (Figure 3E, 3F). However we did not observe any obvious differences in the potential of early or late NEP populations to spontaneously differentiate into NEP cells.

Experiment 2. Expression of the Shh receptor Patched 1 (PTCH) in NEP. PTCH gene expression, receptor for the Shh, was examined in NEP to determine whether this portion of the Shh signaling pathway is active in our NEP groups. Both groups were shown to have RNA expression and the amplification exceeded the threshold at amplification cycle around 30 (Table 2). This amplification was not observed from negative control where reverse transcriptase was omitted at the time of cDNA preparation (noRT). The negative control Ct value was 40, this is the total cycle number that real time machine was set for signal detection suggesting no detectable signal. Similar to the negative control, amplification was not present in RNA sample prepared from the mouse embryonic fibroblast feeder cells.

Experiment 3. bFGF effect on OLIG2 gene expression in NEP. Basic FGF has been used to propagate NEP. We needed to examine the effect of bFGF on Shh and RA role in motor neuron induction. To investigate the effect of bFGF on motor neuron induction, motor neuron progenitor transcription factor Olig2 expression change was monitored using late NEP. Basic FGF increased OLIG2 expression in treated late NEP to 2.64 fold ($p<0.01$) compared to the nontreated late NEP of control group, and expression was further increased to 4.15 fold ($p<0.01$, Figure 4) by combining bFGF with RA and Shh. However, RA and Shh treatment did not have a significant effect on OLIG2 expression in the absence of bFGF (1 vs. 1.16, $p>0.01$).

Experiment 4. Shh and RA affect HB9 gene expression in NEP. Based on result from experiment 3, bFGF was included at time of RA or RA combined Shh exposure and HB9 gene expression change was monitored to investigate their effect on motor neuron induction. Retinoic acid exposure increased the HB9 expression level up to 2.20 fold in early NEP ($p<0.01$) and the expression level was increased to 8.49 fold ($p<0.01$) by combined Shh treatment (Figure 5A). In late NEP, RA treatment increased HB9 expression to 2.42 fold ($p<0.01$) similar to the result obtained in early NEP. However, combined Shh slightly increased the expression of HB9 (3.07 fold vs. 2.42 fold, $p>0.01$, Figure 5B) which was not statistically different from RA exposed late NEP.

Experiment 5. Shh differentially affects PTCH expression in NEP. In order to explain differential effect of Shh on motor neuron induction in early and late NEP, response to combined Shh was examined by monitoring up regulation of PTCH, Shh target gene. Increased expression of the PTCH gene according to combined Shh was observed in early NEP. After 24 hr exposure to Shh and RA, expression increased up to 1.79 fold ($p<0.01$, Figure 6A) compared to untreated early NEP. However, there was no increased but decreased PTCH expression in Shh and RA treated late NEP compared to untreated late NEP ($p<0.01$, Figure 6B).

DISCUSSION

Neuroepithelial stem cells are the cell population from which all the cells comprising the CNS arise. Among characterization markers, Nestin and Musashi 1 have been primary phenotype markers for these cells (28, 41). Previously we showed that the NEP used in this experiment express these markers and differentiate into neural and glial phenotypes (Shin, manuscript submitted). The present study further examined early and late NEP with their SOXs

gene expression. In mouse, SOXs were mainly expressed in developing nervous system and SOX1 has been used as target gene for neural stem cell isolation in mESC differentiation (40). Additionally, human SOX1, SOX2 and SOX3 were isolated whose sequences were highly conserved to mouse counterparts (19, 33). Among these SOXs, SOX2 has been shown to be expressed also in hESC (4, 11) and we also observed that proliferating hESC expressed SOX2 and SOX3. Therefore, SOX2 and SOX3 gene expression alone does not exclude the rare possibility of the expression from remnant of hESC. However, we showed in this study that NEP cultures without containing Oct4 positive cells expressed SOXs gene. Both early and late NEP expressed SOX1, SOX2 and SOX3 and that expression difference were not observed between two populations. These results indicate that SOXs gene expression in the absence of Oct4 can be used as further verification for NEP.

In order to direct early and late NEP into motor neuron differentiation, developmental inductive signal of Shh and RA were selected. Sonic Hedgehog is involved in motor neuron generation by establishing a set of homeodomain proteins in motor neuron progenitor domain. To use this well-defined morphogen, we examined first whether NEP have receptor for Shh. Gailani and Bale showed that the vertebrate homolog of Drosophila PTCH is expressed in all known target tissues of Shh and that expression of this receptor can be a useful biological marker in screening tissue for Shh treatment (10). It has been also shown that PTCH was expressed in the mouse neural tube (3, 9). In the present study, early and late NEP were shown to have PTCH gene expression indicating plausible responsiveness to Shh. However, it is important to note that the expression of PTCH does not necessarily represent functional responsiveness to Shh for motor neuron cell specification. Therefore, we next examined the changes in motor neuron progenitor and motor neuron gene to test Shh role in motor neuron induction.

Retinoic acid is widely used to induce differentiation. During development, RA is produced by presomitic mesoderm and somites and is involved in neurogenesis and specific neuronal fate determination (18, 34). Its role in neurogenesis has been shown in the frog neural plate (7, 31) and in mESC (29, 38). In addition, RA has been shown to contribute to ventral spinal cord patterning and motor neuron specification (25). RA directly enhanced the expression of Class I genes such as PAX6, and subsequently, OLIG2 and Mnx homeodomain gene class (Mnr2 and HB9) expression. In this study, OLIG2 was selected first as early target gene for motor neuron induction and combined effect of Shh and RA were examined. The results showed that combined Shh and RA exposure of late NEP increased OLIG2 expression in the presence of bFGF. However, the effect of combined Shh and RA was not shown without bFGF, which is similar to results by Novitch et al (25). They showed OLIG2 expression in chicken neural explant culture by combined bFGF, RA and Shh. As a result, bFGF was combined with RA and Shh exposure for motor neuron induction in further HB9 experiment.

In this study, early and late NEP were exposed to RA and increased HB9 expression approximately two fold for both young and old NEP compared to untreated control respectively. However, the increased gene expression by combined Shh was only observed in early NEP. For the late NEP, the increased HB9 expression was only observed by RA, and exposure to Shh did not have a significant effect compared to treatment with RA alone. In addition, the PTCH over expression was shown only in early NEP by combined RA and Shh exposure. If Shh is present, PTCH doesn't antagonize signal transducer SMO, thus resulting in increased target gene transcription. Because PTCH is a target of Shh, the abundance of the PTCH transcript is regulated by Shh (12). Therefore, we examined the responsiveness of early and late NEP by monitoring PTCH overexpression. Interestingly, although both populations had receptors for

Shh, the increased expression of PTCH did not appear in late NEP. Patched expression fold change was greater when Shh was introduced during NEP derivation (data not shown), and the degree of expression change was decreased in derived NEP according to the duration of the culture period. The results showed that combined Shh resulted in upregulation of both PTCH and HB9 compared to either untreated control or RA alone treated group in freshly derived NEP whereas the effect of combined Shh was not shown in long term cultured NEP. Two other groups also suggested limited Shh effects on Islet 1 positive neuron formation. In a previous experiment with explants from chicken ventral neural plate, there also appeared to be a critical time period in response to Shh (26). Induction of Islet1 marker, an indicator of motor neuron derivation, was only observed when there was Shh-N signal within less than 12 hours after initiation of explant culture. Another study reported similar results in their long term expanded primary fetal human neural stem cells (39). Along with our results from late NEP, they found Shh did not have effect on Islet1 positive cell formation from their neurospheres differentiation culture. Therefore, our results indicate that NEP from hESC has limited time period for Shh action and during in vitro culture, cell changed their responsiveness to Shh while retaining NEP markers and SOXs gene expression.

In our spontaneous differentiation, both early and late NEP resulted in motor neuron with the expression of Islet 1, Tuj1 and ChAT. Wu et al, showed that long term cultured fetus derived neurospheres could not generate Islet 1, ChAT positive neurons without priming factors. They had to prime neurospheres with laminin to spread out cells, with bFGF and heparin to increase biological activity of bFGF to generate Islet 1, ChAT positive neuron before differentiation step. However, we have used adherent culture to proliferate our NEP using bFGF and LIF as mitogen in culture medium, which is similar to their priming strategy.

Although underlying mechanisms remain to be defined, our NEP may be developed tendency to motor neuron differentiation already, therefore spontaneous differentiation was enough to generate motor neurons. In our untreated control groups of early and late NEP, NEP indeed had expression of OLIG2 and HB9 expression, which supports this possibility. In addition, screwed fate to motor neuron may explain low level of expression change by inductive molecules.

In our study, bFGF alone increased expression of OLIG2. One study by Kessaris et al (13) demonstrated similar results using mouse neocortical precursors. They showed that Shh and bFGF can both induce expression of OLIG2 and that bFGF appears to act via a Shh independent pathway. In our study, late NEP was not influenced by Shh and bFGF alone increased OLIG2 expression. This result supports the possibility of Shh independent pathway for motor neuron differentiation. Another study of Gabay et al, demonstrated that for a subtype population of spinal cord stem cells, bFGF caused endogenous Shh expression (9). The NEP cells used in our study remained exposed to bFGF in culture medium. If endogenous Shh production is also enhanced by bFGF supplement in subculture medium, late NEP would be continuously exposed to Shh. It is likely, thus, NEP progresses to the next step after Shh exposure and does not react any more to extrinsic Shh for that pathway. However, the underlying mechanism for Shh irresponsiveness of late NEP and the role of bFGF in OLIG2 expression need further study for clarification.

In this study, we have shown motor neuron differentiation from early and late NEP derived from hESC and further examined the effects of extrinsic factors on motor neuron induction of the NEP. We demonstrated that motor neuron induction was affected not only to the kinds of factors applied, but also the application time at which NEP cells were exposed to the factors. It has been shown that bFGF was required for motor neuron differentiation and for the

action of combined RA and Shh. While RA could force both early and late population to increase expression of HB9, Shh was only effective in early NEP. This study has shown established culture systems can serve as an in vitro model for the study of human neural development. In addition, study of derived motor neurons will expedite the elucidation of molecular mechanisms that regulate survival of spinal cord neurons and also can serve as a model system for drug screening for motor neuron disease and spinal cord injury.

REFERENCES

1. Arber, S., Han, B., Mendelsohn, M., Smith, M., Jessell, T. M., and Sockanathan, S. 1999. Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. *Neuron* **23:** 659-674.
2. Briscoe, J., and Ericson, J. 2001. Specification of neuronal fates in the ventral neural tube. *Curr Opin Neurobiol* **11:** 43-49.
3. Carpenter, D., Stone, D. M., Brush, J., Ryan, A., Armanini, M., Frantz, G., Rosenthal, A., and de Sauvage, F. J. 1998. Characterization of two patched receptors for the vertebrate hedgehog protein family. *Proc Natl Acad Sci U S A* **95:** 13630-13634.
4. Carpenter, M. K., Rosler, E. S., Fisk, G. J., Brandenberger, R., Ares, X., Miura, T., Lucero, M., and Rao, M. S. 2004. Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. *Dev Dyn* **229:** 243-258.
5. Catala, M. 2002. Genetic control of caudal development. *Clin Genet* **61:** 89-96.
6. Collignon, J., Sockanathan, S., Hacker, A., Cohen-Tannoudji, M., Norris, D., Rastan, S., Stevanovic, M., Goodfellow, P. N., and Lovell-Badge, R. 1996. A comparison of the properties of Sox-3 with Sry and two related genes, Sox-1 and Sox-2. *Development* **122:** 509-520.
7. Franco, P. G., Paganelli, A. R., Lopez, S. L., and Carrasco, A. E. 1999. Functional association of retinoic acid and hedgehog signaling in Xenopus primary neurogenesis. *Development* **126:** 4257-4265.
8. Frojdman, K., Pelliniemi, L. J., Lendahl, U., Virtanen, I., and Eriksson, J. E. 1997. The intermediate filament protein nestin occurs transiently in differentiating testis of rat and mouse. *Differentiation* **61:** 243-249.
9. Gabay, L., Lowell, S., Rubin, L. L., and Anderson, D. J. 2003. Deregulation of dorsoventral patterning by FGF confers trilineage differentiation capacity on CNS stem cells in vitro. *Neuron* **40:** 485-499.
10. Gailani, M. R., and Bale, A. E. 1997. Developmental genes and cancer: role of patched in basal cell carcinoma of the skin. *J Natl Cancer Inst* **89:** 1103-1109.
11. Ginis, I., Luo, Y., Miura, T., Thies, S., Brandenberger, R., Gerecht-Nir, S., Amit, M., Hoke, A., Carpenter, M. K., Itskovitz-Eldor, J., and Rao, M. S. 2004. Differences between human and mouse embryonic stem cells. *Dev Biol* **269:** 360-380.

12. Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A., and Scott, M. P. 1996. Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Dev* **10:** 301-312.
13. Kessaris, N., Jamen, F., Rubin, L. L., and Richardson, W. D. 2004. Cooperation between sonic hedgehog and fibroblast growth factor/MAPK signalling pathways in neocortical precursors. *Development* **131:** 1289-1298.
14. Lardon, J., Rooman, I., and Bouwens, L. 2002. Nestin expression in pancreatic stellate cells and angiogenic endothelial cells. *Histochem Cell Biol* **117:** 535-540.
15. Lee, S. K., and Pfaff, S. L. 2001. Transcriptional networks regulating neuronal identity in the developing spinal cord. *Nat Neurosci* **4 Suppl:** 1183-1191.
16. Liu, Y., Wu, Y., Lee, J. C., Xue, H., Pevny, L. H., Kaprielian, Z., and Rao, M. S. 2002. Oligodendrocyte and astrocyte development in rodents: an in situ and immunohistological analysis during embryonic development. *Glia* **40:** 25-43.
17. Livak, K. J., and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25:** 402-408.
18. Maden, M., Sonneveld, E., van der Saag, P. T., and Gale, E. 1998. The distribution of endogenous retinoic acid in the chick embryo: implications for developmental mechanisms. *Development* **125:** 4133-4144.
19. Malas, S., Duthie, S. M., Mohri, F., Lovell-Badge, R., and Episkopou, V. 1997. Cloning and mapping of the human SOX1: a highly conserved gene expressed in the developing brain. *Mamm Genome* **8:** 866-868.
20. Mitalipova, M., Calhoun, J., Shin, S., Wininger, D., Schulz, T., Noggle, S., Venable, A., Lyons, I., Robins, A., and Stice, S. 2003. Human embryonic stem cell lines derived from discarded embryos. *Stem Cells* **21:** 521-526.
21. Mizuseki, K., Sakamoto, T., Watanabe, K., Muguruma, K., Ikeya, M., Nishiyama, A., Arakawa, A., Suemori, H., Nakatsuji, N., Kawasaki, H., Murakami, F., and Sasai, Y. 2003. Generation of neural crest-derived peripheral neurons and floor plate cells from mouse and primate embryonic stem cells. *Proc Natl Acad Sci U S A* **100:** 5828-5833.
22. Muhr, J., Graziano, E., Wilson, S., Jessell, T. M., and Edlund, T. 1999. Convergent inductive signals specify midbrain, hindbrain, and spinal cord identity in gastrula stage chick embryos. *Neuron* **23:** 689-702.
23. Mujtaba, T., Piper, D. R., Kalyani, A., Groves, A. K., Lucero, M. T., and Rao, M. S. 1999. Lineage-restricted neural precursors can be isolated from both the mouse neural tube and cultured ES cells. *Dev Biol* **214:** 113-127.
24. Murone, M., Rosenthal, A., and de Sauvage, F. J. 1999. Sonic hedgehog signaling by the patched-smoothened receptor complex. *Curr Biol* **9:** 76-84.
25. Novitch, B. G., Wichterle, H., Jessell, T. M., and Sockanathan, S. 2003. A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. *Neuron* **40:** 81-95.
26. Poh, A., Karunaratne, A., Kolle, G., Huang, N., Smith, E., Starkey, J., Wen, D., Wilson, I., Yamada, T., and Hargrave, M. 2002. Patterning of the vertebrate ventral spinal cord. *Int J Dev Biol* **46:** 597-608.
27. Rao, M. 2004. Stem and precursor cells in the nervous system. *J Neurotrauma* **21:** 415-427.
28. Reubinoff, B. E., Itsykson, P., Turetsky, T., Pera, M. F., Reinhartz, E., Itzik, A., and Ben-Hur, T. 2001. Neural progenitors from human embryonic stem cells. *Nat Biotechnol* **19:**

1134-1140.

29. Schuldiner, M., Eiges, R., Eden, A., Yanuka, O., Itskovitz-Eldor, J., Goldstein, R. S., and Benvenisty, N. 2001. Induced neuronal differentiation of human embryonic stem cells. *Brain Res* **913:** 201-205.
30. Sejersen, T., and Lendahl, U. 1993. Transient expression of the intermediate filament nestin during skeletal muscle development. *J Cell Sci* **106 (Pt 4):** 1291-1300.
31. Sharpe, C. R., and Goldstone, K. 1997. Retinoid receptors promote primary neurogenesis in Xenopus. *Development* **124:** 515-523.
32. Stevanovic, M. 2003. Modulation of SOX2 and SOX3 gene expression during differentiation of human neuronal precursor cell line NTERA2. *Mol Biol Rep* **30:** 127-132.
33. Stevanovic, M., Lovell-Badge, R., Collignon, J., and Goodfellow, P. N. 1993. SOX3 is an X-linked gene related to SRY. *Hum Mol Genet* **2:** 2013-2018.
34. Swindell, E. C., Thaller, C., Sockanathan, S., Petkovich, M., Jessell, T. M., and Eichele, G. 1999. Complementary domains of retinoic acid production and degradation in the early chick embryo. *Dev Biol* **216:** 282-296.
35. Takebayashi, H., Nabeshima, Y., Yoshida, S., Chisaka, O., and Ikenaka, K. 2002. The basic helix-loop-helix factor olig2 is essential for the development of motoneuron and oligodendrocyte lineages. *Curr Biol* **12:** 1157-1163.
36. Tropepe, V., Sibilia, M., Ciruna, B. G., Rossant, J., Wagner, E. F., and van der Kooy, D. 1999. Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Dev Biol* **208:** 166-188.
37. Uwanogho, D., Rex, M., Cartwright, E. J., Pearl, G., Healy, C., Scotting, P. J., and Sharpe, P. T. 1995. Embryonic expression of the chicken Sox2, Sox3 and Sox11 genes suggests an interactive role in neuronal development. *Mech Dev* **49:** 23-36.
38. Wichterle, H., Lieberam, I., Porter, J. A., and Jessell, T. M. 2002. Directed differentiation of embryonic stem cells into motor neurons. *Cell* **110:** 385-397.
39. Wu, P., Tarasenko, Y. I., Gu, Y., Huang, L. Y., Coggeshall, R. E., and Yu, Y. 2002. Region-specific generation of cholinergic neurons from fetal human neural stem cells grafted in adult rat. *Nat Neurosci* **5:** 1271-1278.
40. Ying, Q. L., Stavridis, M., Griffiths, D., Li, M., and Smith, A. 2003. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat Biotechnol* **21:** 183-186.
41. Zhang, S. C., Wernig, M., Duncan, I. D., Brustle, O., and Thomson, J. A. 2001. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol* **19:** 1129-1133.

Table 1. Primer information and size of the products.

Gene	Primers	Product size(bp)	Annealing T	Cycles
SOX2	5’-AGT CTC CAA GCG ACG AAA AA-3’ 5’-GCA AGA AGC CTC TCC TTG AA-3’	141	55°C	35
SOX3	5’-GAG GGC TGA AAG TTT TGC TG-3’ 5’-CCC AGC CTA CAA AGG TGA AA-3’	131	55°C	35
HLXB9	5’-GCT GGA GCA CCA GTT CAA GT-3’ 5’-CGG TTC TGG AAC CAA ATC TT-3’	111	60°C	40
GAPDH	5’-GAA GGT GAA GGT CGG AGT C-3’ 5’-GAA GAT GGT GAT GGG ATT TC-3’	226	60°C	40
SOX1	Hs00534426_s1 (Applied biosystems)	NA*	60°C	40
PTCH	Hs00181117_m1 (Applied biosystems)	NA*	60°C	40
OLIG2	Hs00377820_m1 (Applied biosystems)	NA*	60°C	40
β actin	4326315E (Applied biosystems)	NA*	60°C	40

* Company refuses to disclose primer and probe sequence nor size of product. However, confirmed to range under 200bp for each primer.

Table 2. Real time PCR analysis to examine the expression of Shh receptor of PTCH.

Tested Group	Ct value	NoRT Ct value
early NEP	30.5	40.0
late NEP	30.3	40.0

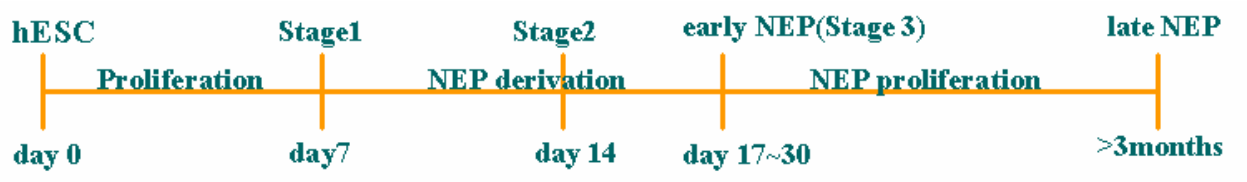

Figure 1. Derivation and proliferation of NEP from hESC.

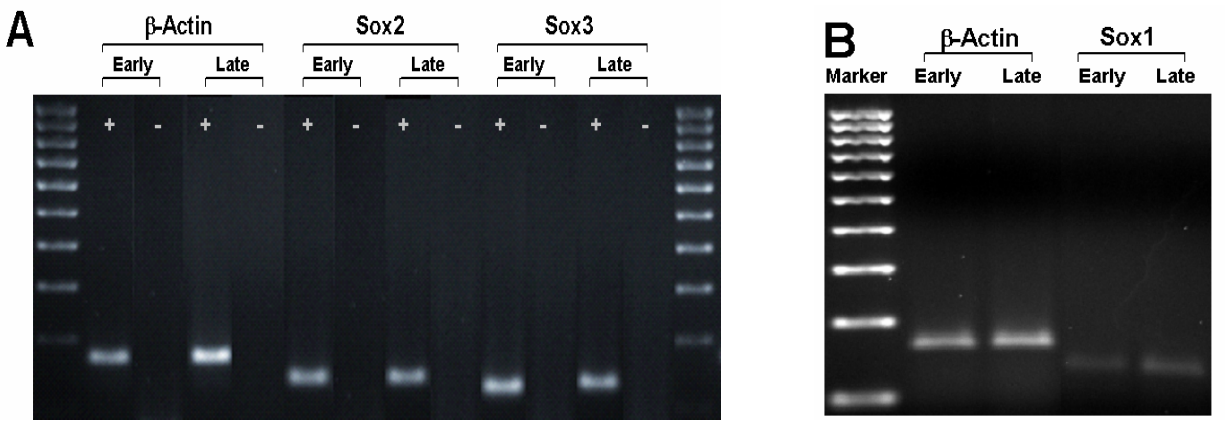

Figure 2. Both early and late NEP cells express Sox1, 2 and 3. RT PCR analysis of the expression of SOX1 (B), SOX2 and SOX3 (A). Panels show 2% agarose gels stained with ethidium bromide. Genomic contamination was monitored by sample prepared without reverse transcriptase (-). For size marker 1kb DNA ladder was used. The size of SOX2 is 141 and SOX3 is 131.

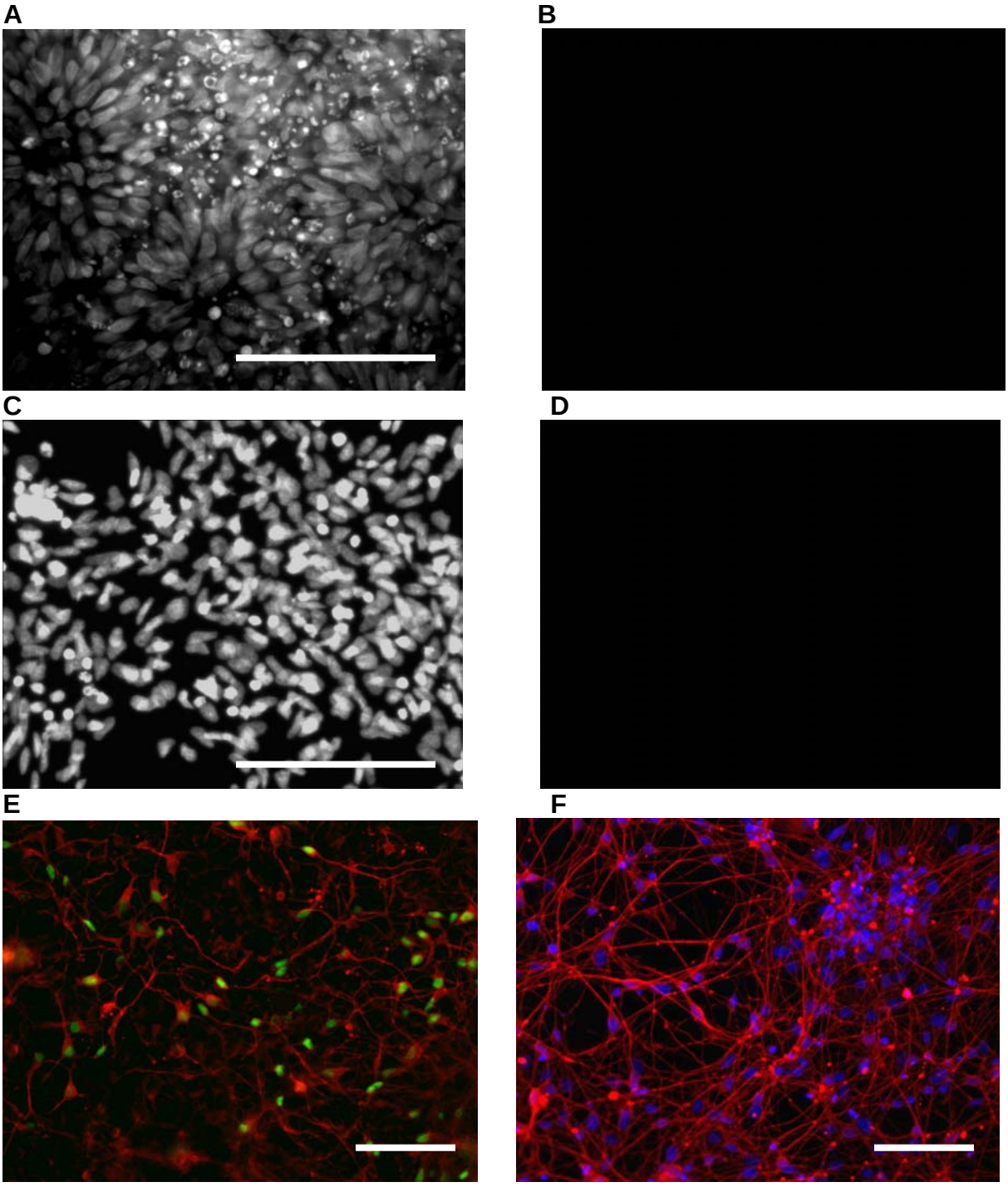

Figure 3. Nuclei stained by DAPI in early (A) and late (C) NEP. Oct4 expression in early (B) and late (D) NEP. Both NEP did not express undifferentiation phenotype marker of Oct4. Phenotype marker expression of motor neuron derived from early NEP counter-stained with Islet1 (green) and Tuj1(red) (E). Phenotype marker expression of motor neuron derived from early NEP counter-stained with ChAT (red) and DAPI (blue) (F). Bar=100um.

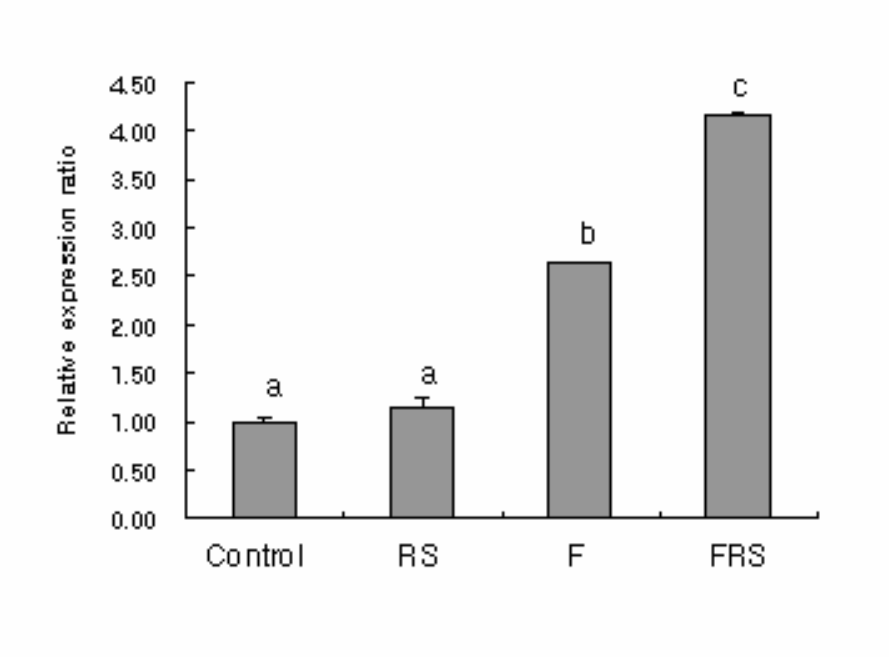

Figure 4. OLIG2 expression change according to bFGF (F), Retinoic acid (R) and Sonic hedgehog (S). Different letters denote significant difference ($p<0.01$).

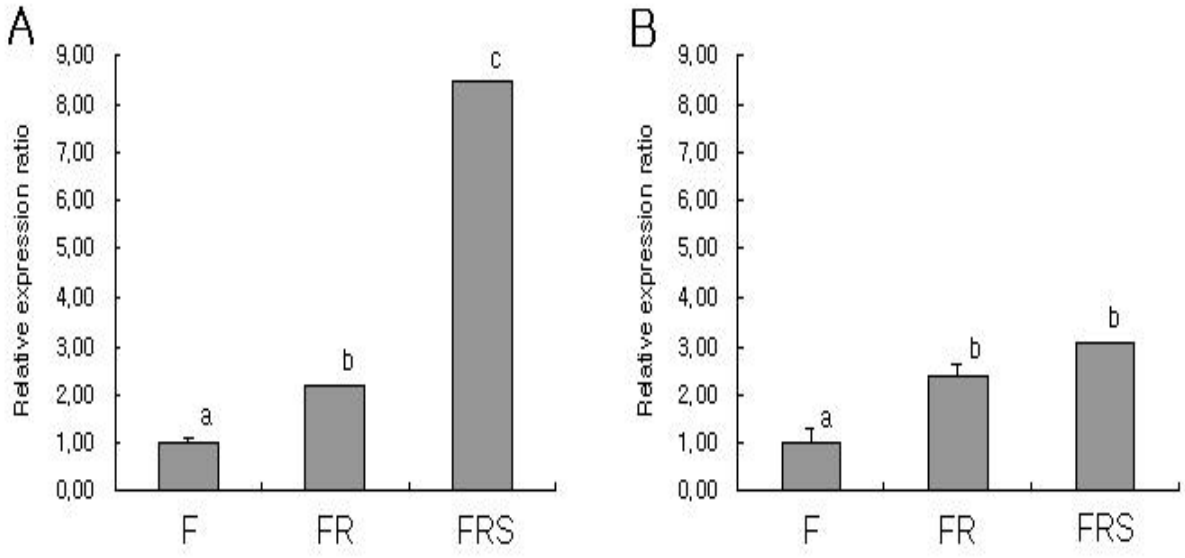

Figure 5. HLXB9 expression change according to Retinoic acid (R) and Sonic hedgehog (S) in early (A) and late (B) NEP. Control group of F prepared without R or S but with bFGF (F). Different letters denote significant difference ($p<0.01$).

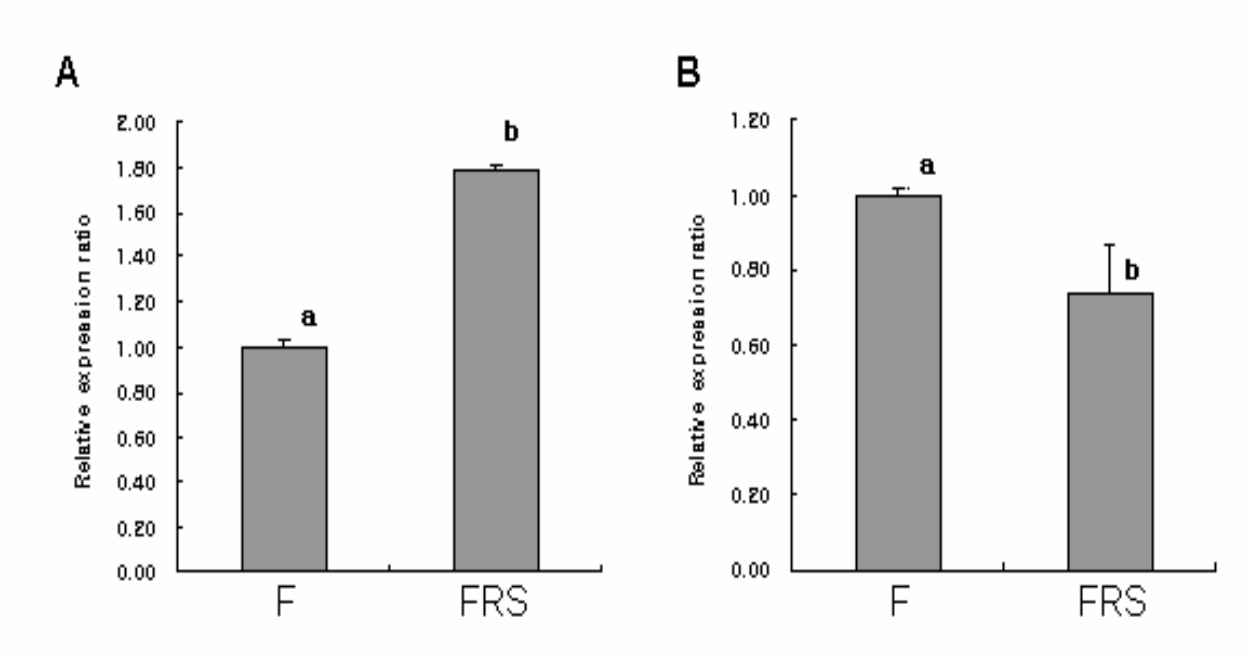

Figure 6. PTCH expression change of early NEP (A) and late NEP (B) according to combined Sonic hedgehog (S) and Retinoic acid (R). Control group was prepared with bFGF (F). Different letters denote significant difference ($p<0.01$).

Conclusion

The goal of this dissertation was to study the process of motor neuron differentiation using hESC. To achieve this goal we first focused on differentiation of hESC into early neuroectodermal cell populations of neuroepithelial stem cells (NEP). This NEP cell population was characterized both by morphological and by molecular characterization techniques to show similarities to its in vivo counterpart. Optimal subculture conditions were established for proliferation of NEP, then, NEP populations were exposed to inductive signals to enhance motor neuron differentiation.

Previous studies indicated that neuronal differentiation could be obtained from mouse [1] and human ESC [2, 3]. However, directed differentiation and isolation of desired cell types remain a pending question. It has been shown that defined medium prevented mesodermal differentiation of mESC [4] and adherent differentiation was introduced for efficient homogenous differentiation [1]. In addition, inductive signals in in vivo development have been shown to induce motor neuron differentiation in mouse and non-human primate ESC [5, 6]. In this dissertation, an optimized process of adherent differentiation in defined medium was exploited to establish homogenous populations of NEP cells. In addition, the effects of bFGF, RA and Shh on motor neuron differentiation were examined using quantitative gene expression change of motor neuron progenitor and motor neuron genes.

STUDY I: DERIVATION AND PROLIFERATION OF NEUROEPITHELIAL STEM CELLS FROM HUMAN EMBRYONIC STEM CELLS

The main focus of first study was to establish Neuroepithelial stem cells from hESC. Accordingly, we examined the factors affecting derivation and further proliferation of neuroepithelial (NEP) stem cells from hESC. Embryonic stem cells cultured in defined medium, developed a distinct canal structure which could be isolated by two methods, dissociation and adherent separation by physical feeder removal. Dissociated cells formed colonies comprised of cells characterized as NEP in a MEDII dependent manner. However, adherently isolated cells developed enriched NEP like cells independent of exposure to MEDII. Further characterization indicates that these cells expressed markers associated with the earliest multipotent neural stem cells and can thus be characterized as NEP. A majority of the cells were positive for Nestin, a neural intermediate filament protein, and Musashi-1, a neural RNA binding protein, while few cells expressed further differentiation markers such as PSNCAM, A2B5, MAPII, GFAP, and O4. Further differentiation of these putative NEP cells over eight days gave rise to a mixed population of progenitors that included A2B5 positive and PSNCAM positive cells. When fully differentiated, cell populations contained postmitotic neurons, astrocytes and oligodendrocytes. Neurobasal medium supplemented with B27, bFGF and LIF at low oxygen conditions was identified as an optimal culture conditions for proliferating and culturing the NEP. Cells have been proliferated successfully under these conditions for over six months without losing their multipotent neural stem cell characteristics and maintenance of stable karyotype.

STUDY II: MOTOR NEURON DIFFERENTIATION OF NEUROEPITHELIAL STEM CELLS BY INDUCTIVE SIGNALING MOLECULES

After successful derivation and proliferation of NEP, we next studied their differentiation into specific types of neurons. To differentiate NEP into motor neurons, specific morphogens that have been demonstrated as important in development were introduced to short and long term cultured NEPs. These included sonic hedgehog (Shh), retinoic acid (RA) and basic fibroblast growth factor (bFGF). First, freshly isolated (less than one month, early) and propagated (greater than three months, late) cultures of NEPs were characterized by examination of candidate genes expressed in the developing CNS. Both groups expressed the genes SOX1, SOX2 and SOX3 in addition to the Shh receptor Patched 1 (PTCH). Furthermore, both were able to generate cells with motor neuron phenotype when spontaneously differentiated. Therefore, both populations were exposed to inductive signals for the stimulation into motor neurons. It was shown that bFGF was required for induction of Shh and RA mediated increases in the motor neuron progenitor gene OLIG2 expression using late NEP. Subsequently, the RA and Shh role in motor neuron derivation was shown by examining the motor neuron gene HLXB9 (HB9) expression in early and late NEPs. Increased HB9 expression was shown in both early and late NEPs by RA. However, NEP differed in their responses when combined with Shh. Expression changes of the motor neuron gene HB9 in response to Shh combined with RA were greater in early NEP than in late NEP. In addition, downstream target gene over-expression was shown only in early NEP, whereas there was no over expression change in late NEP. This study suggests that NEP derived from hESC can form a motor neuron phenotype and that Shh, bFGF and RA can differentially affect expression of motor neuron associated genes in the NEP.

FUTURE DIRECTIONS

It is certain that hESC are an invaluable material in many ways; developmental studies, pharmaceutical aspects, better understanding of human disease and replacement therapies. Since 1998, when the first hESC were established, researchers have made good progress in spite of government restrictions. Among the seventy eight different National Institutes of Health (NIH) registered lines, limited biological data are available for twenty six lines and just 19 lines are available for research purposes which can be supported by the federal government [7]. Two of these lines, BG01 and BG02, were used in this dissertation. However, with greater scientific demands, more cell lines have been established which is inspiring for fundamental human stem cell biology [8]. Even in well established mESC, it has been noted that there is a difference in isolation and propagation of lines [9, 10]. In fact, even the hESC lines generated by one researcher under the same conditions don't have exactly the same gene expression profiles [11]. Some differences in human cell lines have been described [7, 12]. In addition, variation in LIF receptor expression was observed among the cell lines. The newly developed hESC line I-6, expressed significant levels of LIF receptor (LIFR), while H1, H7, H9 and bulk passaged BG1 and BG2 expressed low or no level of LIFR [11, 13]. It is generally accepted that hESC do not require LIF in culture to maintain pluripotency. However, this variation may be explained by underlying differences in biology and needs to be determined. So, basic hESC biology needs to continue to answer what are the mechanisms that maintain stem cells and how can observed differences among the cell lines be explained.

One major project in stem cell research is to direct pluripotent ESC toward a limited cell fate. Upon spontaneous differentiation, the resulting structure contains mixed cell populations of ectodermal, mesodermal, endodermal and even trophoectodermal lineages. For therapeutic

purposes, two main things need to be considered. One is to exclude pluripotent cells which can differentiate into undesired cell types after transplantation. The inclusion of ESC in graft material has been a concern because of teratoma formation or unwanted chaotic differentiation to non neural lineage. The other is that the grafts need to act functionally in the transplant recipient. In this dissertation, several factors were combined to establish enriched populations of NEP. Along with the result from mESC, adherent differentiation in defined culture medium turned out to be efficient to generate homogenous NEP from hESC. Without sphere formation, it was easy to monitor cell phenotype changes and resulting homogenous rosette formation could be separated for proliferation. The time course monitoring of Oct4 phenotype demonstrated that pluripotent cells lost their expression of pluripotent markers when exposed to defined medium. Therefore, hESC will differentiate under these conditions preventing pluripotent cells from existing. However, a more definitive method of enriching for select populations includes genetic selection or fluorescence activated cell sorting (FACS), magnetic activated cell sorting (MACS) combined target cell isolation or undesired cell removal. Among cell surface antigens of hESC, SSEA3 and SSEA4 would be candidate antigens for ESC removal from the graft material. With these techniques, the choice of gene and cell surface markers needs to be specific and expressed or present at high enough levels for separation, especially since embryonic and neural stem cells share similar gene expression profiles [14]. In this study, SOXs gene expression was examined on NEP to be positive to all SOX1, SOX2 and SOX3. It has been shown that hESC also expressed SOX2, which was also confirmed in our proliferating ESC. They were positive to SOX2 and SOX3 but not SOX1. Therefore, like in mouse, SOX1 would be a candidate for neuroepithelial cell target gene. Keyoung et al, showed Musashi1 and Nestin were expressed in fetal human ventricular zone and used these two genes as their targets

to enrich NEP from fetal human brain [15]. However, as stated previously, Nestin expression was not restricted to NEP but expressed in other cell organ such as testis, which is also the case for Musashi1. Currently, specific markers just for NEP are not available. Consequently, more in-depth studies will be needed to characterize and to find out specific gene and surface markers for NEP. Recently, the microarray has been introduced as a way to define molecular phenotype of target cells [16-19] and analysis of these data would give more clues to find the right genes and markers. However, purified NEP is imperative to get unbiased results. It should be noted that the NEP generated in this study would be great material for this purpose.

Eventual cell therapies will require research to determine which cells and at what stage they could be transplanted. Neuroepithelial stem cells have been shown to migrate and undergo differentiation following transplantation into the developing brain [19, 20]. However, this plasticity is not easily obtained in adult CNS. Several studies have shown that the local environment is the predominant determinant of the differentiated fate of engrafted cells. When cells were transplanted into non-neurogenic regions of adult CNS, most cells primarily differentiated into the glial fate [21, 22]. Therefore, more studies are needed to elucidate local instructive signals related to lineage restriction. In addition, undifferentiated NEP tended to remain as stem cells rather than differentiate into desired neuron after transplantation. In contrast, if this undifferentiated NEP were differentiated to express early neuronal phenotype markers before transplantation, more neuronal differentiation was obtained in the transplant host [23, 24]. It is damaged CNS which needs replacement, so it is difficult to expect the host environment to have molecular cues for directed differentiation of desired cell types. Therefore, it may be beneficial to restrict NEP to the neuronal lineage before transplantation. There are two main approaches to accomplish this; exploiting inductive signals and genetic approaches.

Yamamoto et al, tried to express genes related to neuronal development and obtained partial success [25]. In this dissertation, Shh, RA and bFGF were used to enrich populations with motor neurons. However, they achieved a mixed population that included astrocytes, oligodendrocyte, Islet1 negative neurons, motor neurons as well as NEP. Therefore, for therapeutic or drug screening models, it would be beneficial to purify motor neurons from other types of cells. One way to do this includes labeling motor neurons with a reporter. Currently, HB9 seems to be the most reliable transcription factor for spinal motor neuron identification. If we establish ESC or NEP whose HB9 expression is modified for identification, we can first enrich them with motor neurons using inductive signals and then motor neurons can be purified based on reporter expression. As a reporter system, HB9 driven fluorescence expression (GFP, EGFP, YGFP) for sorting or antibiotic resistance for survival would be considered. However, major expression of HB9 is observed in postmitotic neurons. In chicken, MNR2 is expressed in chicken motor neuron progenitor and motor neurons. A mammalian identical molecule which has the functions of MNR2 is not currently available. If this gene is identified, a knock-in in NEP would be sufficient to direct cell fate to motor neuron.

Among the inductive molecules examined in this dissertation, Shh showed increased target gene expression of Patched1 as well as the motor neuron gene HB9 in early NEP. In mammalian, Shh pathway was mostly deduced from drosophila Hedgehog pathway. Though basic features are similar between hedgehog and Shh, human homologs have not been identified for all of the signal components. In addition the role of each transcription factor of Glis remain to be answered. Therefore early NEP would be a good model system to answer these questions. Exploring the effect of RNAi targeting of each Gli transcription factors on Shh pathway activity such as Patched1 overexpression can be included.

In conclusion, a stable process of adherent differentiation in defined medium was developed to establish homogenous population of NEP cells. Extensive characterization demonstrated that derived the NEP cell population was similar to its in vivo counterpart and NEP could be proliferated under ideally combined culture conditions without losing their differentiation capacity to neurons and glial cells. Furthermore, it has been demonstrated that NEP could generate motor neurons which can be enhanced by bFGF and RA and early exposure of Shh. As stated in this chapter, there are many questions to be answered before using stem cells for therapeutic purposes. Still, it is certain that hESC and derived NEP and motor neurons are powerful tools and I believe it is not far away before we are blessed with this technology.

REFERENCES

1. Ying, Q.L., et al., *Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture.* Nat Biotechnol, 2003. **21**(2): p. 183-6.
2. Reubinoff, B.E., et al., *Neural progenitors from human embryonic stem cells.* Nat Biotechnol, 2001. **19**(12): p. 1134-40.
3. Zhang, S.C., et al., *In vitro differentiation of transplantable neural precursors from human embryonic stem cells.* Nat Biotechnol, 2001. **19**(12): p. 1129-33.
4. Wiles, M.V. and B.M. Johansson, *Embryonic stem cell development in a chemically defined medium.* Exp Cell Res, 1999. **247**(1): p. 241-8.
5. Wichterle, H., et al., *Directed differentiation of embryonic stem cells into motor neurons.* Cell, 2002. **110**(3): p. 385-97.
6. Mizuseki, K., et al., *Generation of neural crest-derived peripheral neurons and floor plate cells from mouse and primate embryonic stem cells.* Proc Natl Acad Sci U S A, 2003. **100**(10): p. 5828-33.
7. Carpenter, M.K., E. Rosler, and M.S. Rao, *Characterization and differentiation of human embryonic stem cells.* Cloning Stem Cells, 2003. **5**(1): p. 79-88.
8. Cowan, C.A., et al., *Derivation of embryonic stem-cell lines from human blastocysts.* N Engl J Med, 2004. **350**(13): p. 1353-6.
9. McWhir, J., et al., *Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a non-permissive genetic background.* Nat Genet, 1996. **14**(2): p. 223-6.
10. Kumar, A., et al., *Nodal signaling uses activin and transforming growth factor-beta receptor-regulated Smads.* J Biol Chem, 2001. **276**(1): p. 656-61.
11. Zeng, X., et al., *Properties of pluripotent human embryonic stem cells BG01 and BG02.* Stem Cells, 2004. **22**(3): p. 292-312.
12. Heins, N., et al., *Derivation, characterization, and differentiation of human embryonic*

stem cells. Stem Cells, 2004. **22**(3): p. 367-76.
13. Ginis, I., et al., *Differences between human and mouse embryonic stem cells.* Dev Biol, 2004. **269**(2): p. 360-80.
14. Ivanova, N.B., et al., *A stem cell molecular signature.* Science, 2002. **298**(5593): p. 601-4.
15. Keyoung, H.M., et al., *High-yield selection and extraction of two promoter-defined phenotypes of neural stem cells from the fetal human brain.* Nat Biotechnol, 2001. **19**(9): p. 843-50.
16. Sperger, J.M., et al., *Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors.* Proc Natl Acad Sci U S A, 2003. **100**(23): p. 13350-5.
17. Carpenter, M.K., et al., *Properties of four human embryonic stem cell lines maintained in a feeder-free culture system.* Dev Dyn, 2004. **229**(2): p. 243-58.
18. Rao, R.R. and S.L. Stice, *Gene Expression Profiling of Embryonic Stem Cells Leads to Greater Understanding of Pluripotency and Early Developmental Events.* Biol Reprod, 2004.
19. Rosler, E.S., et al., *Long-term culture of human embryonic stem cells in feeder-free conditions.* Dev Dyn, 2004. **229**(2): p. 259-74.
20. Brustle, O., et al., *Chimeric brains generated by intraventricular transplantation of fetal human brain cells into embryonic rats.* Nat Biotechnol, 1998. **16**(11): p. 1040-4.
21. Cao, Q.L., et al., *Pluripotent stem cells engrafted into the normal or lesioned adult rat spinal cord are restricted to a glial lineage.* Exp Neurol, 2001. **167**(1): p. 48-58.
22. Chow, S.Y., et al., *Characterization and intraspinal grafting of EGF/bFGF-dependent neurospheres derived from embryonic rat spinal cord.* Brain Res, 2000. **874**(2): p. 87-106.
23. Shin, J.J., et al., *Transplanted neuroblasts differentiate appropriately into projection neurons with correct neurotransmitter and receptor phenotype in neocortex undergoing targeted projection neuron degeneration.* J Neurosci, 2000. **20**(19): p. 7404-16.
24. Vescovi, A.L. and E.Y. Snyder, *Establishment and properties of neural stem cell clones: plasticity in vitro and in vivo.* Brain Pathol, 1999. **9**(3): p. 569-98.
25. Yamamoto, S., et al., *Proliferation of parenchymal neural progenitors in response to injury in the adult rat spinal cord.* Exp Neurol, 2001. **172**(1): p. 115-27.