Neural differentiation from human embryonal carcinoma stem cells

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Chapter Four

Activity of Sonic hedgehog signalling on the differentiation of Tera2.cl.SP12 EC cells

Section 4.1

Shh signalling in Tera2.cl.SP12 EC cell and its interactions with pathways that modify its effects

4.1.1 Introduction

Pluripotent EC cells are a valuable source of neural cells for cell biological investigation, neurodegenerative disease modelling, pharmaceutical screening and developmental modelling. If EC cells are to be harnessed effectively for these goals, it will be necessary to develop methods for directing neural commitment and reveal the mechanisms during the process. It is demonstrated that Tera2 EC cells have the ability to differentiate into neuro-ectodermal derivatives (reviewed by (Andrews, 2001)). We and others have shown that this EC cell line can differentiate into neuronal and astrocytes efficiently (Stewart et al., 2003). The most popular method is involved in RA long term induction (Przyborski et al., 2000). However, there are few reports about the underlying mechanisms and the function of other inductive factors in this process, especially one of the most important morphogens of the ventral neural tube, Shh.

The *Hedgehog* gene encodes a secreted glycoprotein that was first described in Drosophila has three counterparts in mammals: Shh, Indian hedgehog (Ihh) and Desert hedgehog (Dhh)

(Echelard et al., 1993; Pathi et al., 2001). Briefly, hedgehog molecules have different roles in development: Ihh is a mitogen for chondrocytes, Dhh plays a critical role in peripheral neural system development, and here, we focus on Shh. As we know, the secreted Shh was autocleaved from a 46KD precursor protein to release a 19KD N-terminal fragment. This 19KD N-terminal fragment mediates all the Shh signalling (Marti et al., 1995). The fragment is covalently modified by the addition of a cholesterol moiety at its C-terminal and a palmitic acid at N-terminal (Wijgerde et al., 2002). The cholesterol modification facilitates the secretion and diffusion, while the palmitoylated N-terminal may anchor the ligand to the lipid bilayer of the cell membrane and reduce the diffusivity. Although the mechanism of Shh signal transduction is not fully understood, it is known that activation of Shh signalling occurs through binding to a receptor complex which includes Patched and Smoothened. The pathway downstream of the Smoothened has remained somewhat unclear, but involves the Gli transcription factors, the homologs of the drosophila gene Cubitis interruptis (Ci), which mediate the Shh signal to the target genes (for more information see Chapter1, Section1.6.4).

Shh plays multiple roles in the formation of the CNS (Sotelo, 2004). In prospective spinal cord, Shh induces motor neurons, oligodendrocytes and a variety of interneurons; in mid-brain and hind-brain regions, Shh is involved in the induction of dopaminergic and serotonergic neurons; in forebrain, basal ganglia appear to be induced by Shh (Marti and Bovolenta, 2002). Chick embryonic spinal cord is a popular *in vivo* model as Shh organizes the developing dorso-ventral spinal cord patterning. In this model, Shh, first secreted from the notochord and then the floor plate, diffuses towards dorsal neural tube. Target cells switch at threshold values to form mature ventral neuron phenotypes, and the phenotypes from ventral to dorsall are V3-MN-V2-V1-V0 (ventral neuron subtypes). The progenitor groups form domains that are defined by specific sets of homedomain and paired box-

containing transcription factors. The activation of domain specific transcription factors is dose-dependently controlled by morphogens like Shh (Lee and Pfaff, 2001).

The Shh gradient in the ventral neural tube is antagonized by dorsally secreted BMPs from the epidermal ectoderm and roof plate. Furthermore, RA from the mesoderm has a complicated effect on Shh regulation. In chick embryos with a deficiency of RA signalling, Shh expression is highly reduced and dorso-ventral patterning is not organized properly. Therefore, Shh interactions with RA and BMPs will modify its role in developing spinal cord. Understanding these interactions holds a key to unravelling how the CNS develops by orchestrating the genomic response to excellular information and how Shh specifies the fate of responding cells.

In this study, we use Tera2.cl.SP12 EC cells as an *in vitro* model system to explore the various activities of Shh in cell proliferation, cell specification and neural differentiation, in addition, we study how its activity is modified by other dorso-ventral signalling molecules, such as RA and BMPs.

4.1.2 Methods

4.1.2.1 EC cell culture

Tera2.cl.SP12 EC cells are maintained as described in the Methods and Materials. Cells were induced as required. The inductive factor concentration: Shh 25ng/ml or as indicated, RA 10μM, BMPs 10ng/ml, cyclopamine (CP), 1μM, SANT-1, 30nM.

4.1.2.2 Immunocytochemistry (ICC) staining

The induced EC cells were collected at 1, 2, 4 and 5 week time points by fixing with 4% PFA before performing the ICC staining. The fixed cells were washed with PBS three times

and blocked with 10% HS and 1% Triton-X-100 for about 1 hour at room temperature or 4°C overnight. The properly diluted primary antibodies were applied to the cells for 1 hour at room termperature. The primary antibodies used in the study: Nestin, Tuj1, βIII tubulin, Ki67, Pax6. Apply secondary antibodies as the procedure is in Methods and Materials.

4.1.2.3 RT-PCR

RT-PCR technique was used to examine gene expression of Shh pathway and DV patterning transcription factors. Tera2.cl.SP12 EC cells were induced by Shh, RA, Shh antagonists, BMPs alone or in combination and collected at different time points as indicted in the text. The collected cells were then lysed by Trizol and total RNA was extracted and cDNA was obtained by reverse transcription and later amplified by PCR as described in Methods and Materials.

4.1.2.4 Flow cytometry

Cell surface antigens A2B5, TRA-1-60 and SSEA-3 and intracellular antigens βIII tubulin and nestin were examined by flow cytometry using cell surface and intracellular protocols described in Chapter2 Methods and Materials.

4.1.2.5 Western blotting

Cells were induced and collected as showed in the text. For Oct-4 expression, nuclear protein were extracted; for other proteins expressions, cell were lysed to get cytoplasmic proteins. The protein samples were examined by the western blotting protocol in Methods and Material.

4.1.2.6 Statistical Analysis

Statistical analysis performed involved students paired and unpaired *t*-tests, indicated with the use of Microsoft Excel to analyse the cell number. Statistical significance was set at $p<0.05$ level for the student *t*-tests analysis, with a minimum n value of 3 determinations.

4.1.3 Results

4.1.3.1 RA and Shh induce proliferation in Tera2.cl.SP12 EC cells

We first examined that the effect of RA and Shh on the growth of Tera2.cl.SP12 EC cells. RA induced cells showed no significant difference in growth rate compared to control EC cells during the first 7 days of induction, whereas Shh induced cells show a sharp increase after day5 (Figure4.1.1). Interestingly, under the condition of RA+Shh combined induction, the cells showed no proliferation, but suppression in cell number after day5 compared to control EC cells, however, at day14, the cell number in this combination is more than the other conditions (data not shown), furthermore, more cells were proliferative at 14days in RA+Shh cultures compare to RA cultures (Section3.3.10). This suggested that a predominant effect of Shh at the early stage of induction is to act to increase cell survival and/or proliferation of Tera2.cl.SP12 EC cells. The lag in cell number increase in RA+Shh induced cells is probably due to RA having an inhibitory effect on Shh induced growth effect. RA may selectively increase the proliferation of differentiated precursor cells later.

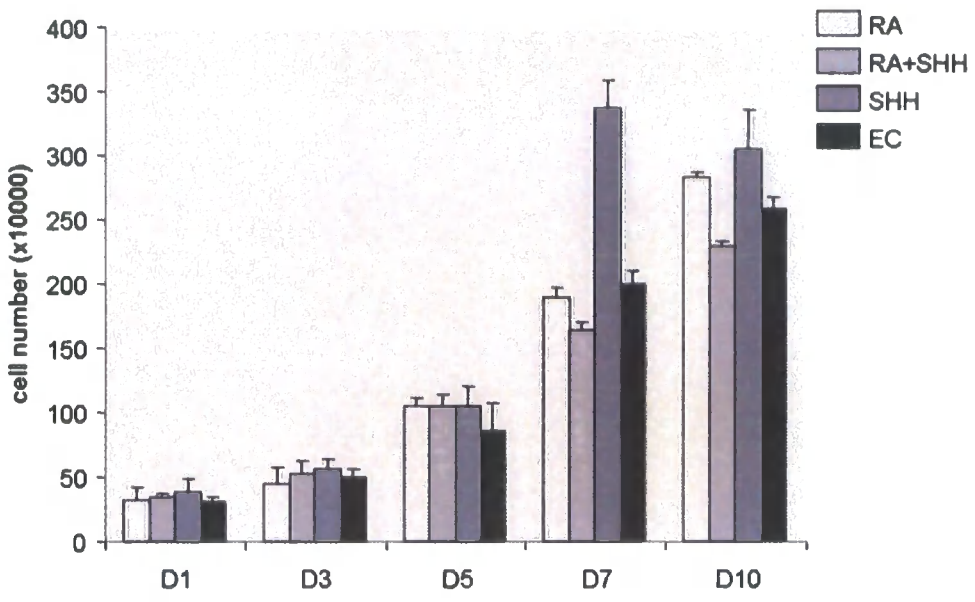

Figure 4.1.1 Shh and RA induce cell proliferation in Tera2.cl.SP12 EC cells. EC cells were seeded in the 12 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) up to 2 weeks. Cells were trypsinized and counted at the indicated time points. Cell number was determined by using hematocytometer. Note that Shh induced the most cell number at day7. Error bars are shown as SEM, n=3.

4.1.3.2 RA and Shh induce differentiation in Tera2.sp12 EC cells

To assess the potential contribution of the RA and Shh signalling pathway to EC cell fate decisions, we surveyed expression of TRA-1-60, SSEA-3 and A2B5 in non-induced and induced EC cells. By flow cytometric analysis, we found that typically around 75% of undifferentiated cells expressed the ES cell marker SSEA-3 and around 98% of the cells expressed another ES cell marker TRA-1-60, compared to about 3% of the cells adopting a differentiated cell fate by expressing A2B5 (Figure4.1.2.1). In contrast, in RA induced EC cells, there is a significant increase of A2B5 positive cells and almost elimination of SSEA-3 and TRA-1-60 expression, suggesting that most of the RA induced cells had undergone differentiation. However, in the presence of Shh, cells retained a low expression of A2B5 and a relatively high expression of SSEA-3 and TRA-1-60. In the condition of RA+Shh,

cells had an increased number of A2B5 positive cells and more SSEA-3 and TRA-1-60 positive cells compared to RA induced cells, indicating that RA signalling has the dominant effect in inducing EC cell differentiation. To further test whether Shh directly promotes EC cell differentiation, we examined the expression of nestin and βIII tubulin in these conditions. We found that Shh induced cultures had the most nestin positive cells and the least βIII tubulin positive cells, and RA induced cultures had the least nestin positive cells and moderate βIII tubulin positive cells, while RA+Shh induced cultures had a moderate amount of nestin positive cells and the most βIII tubulin cells (Figure4.1.2.2). Furthermore, Shh induced nestin positive cells were arranged in a random manner, while RA and RA+Shh induced nestin positive cells arrange as rosettes. If we block the Shh signal by the Shh antagonist cyclopamine and SANT-1, RA induced cells are highly positive for βIII tubulin but negative for nestin staining, furthermore, cells are more differentiated. Collectively, these data showed that Shh acts at the initial stages of EC cell differentiation to promote the transition into premature precursors, and RA helps the cells advance to a more mature stage.

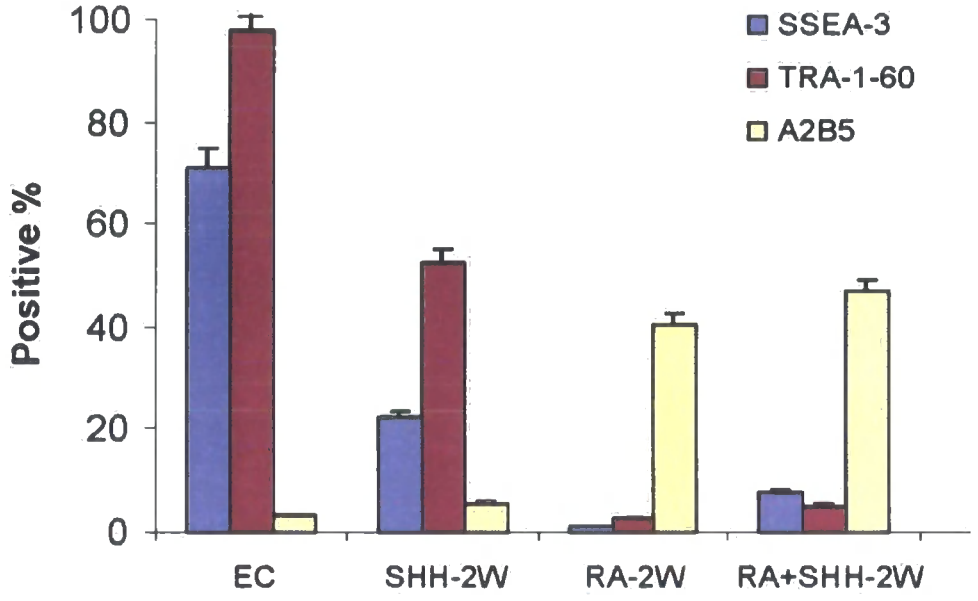

Figure 4.1.2.1 Analysis of stem cell surface markers by flow cytometry. EC cells were seeded in the tissue culture flasks at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) for 2 weeks. Cells were trypsinized to become individual cells before staining with stem cell surface markers. SSEA-3 and TRA-1-60 are typical ES stem cell surface markers, and A2B5 here serves as a marker of cell differentiation. Note that EC stem cells are high in SSEA-3 and TRA-1-60 and low in A2B5, while RA induced cells is high in A2B5 and low in stem cell markers. Error bars are shown as SEM, n=3.

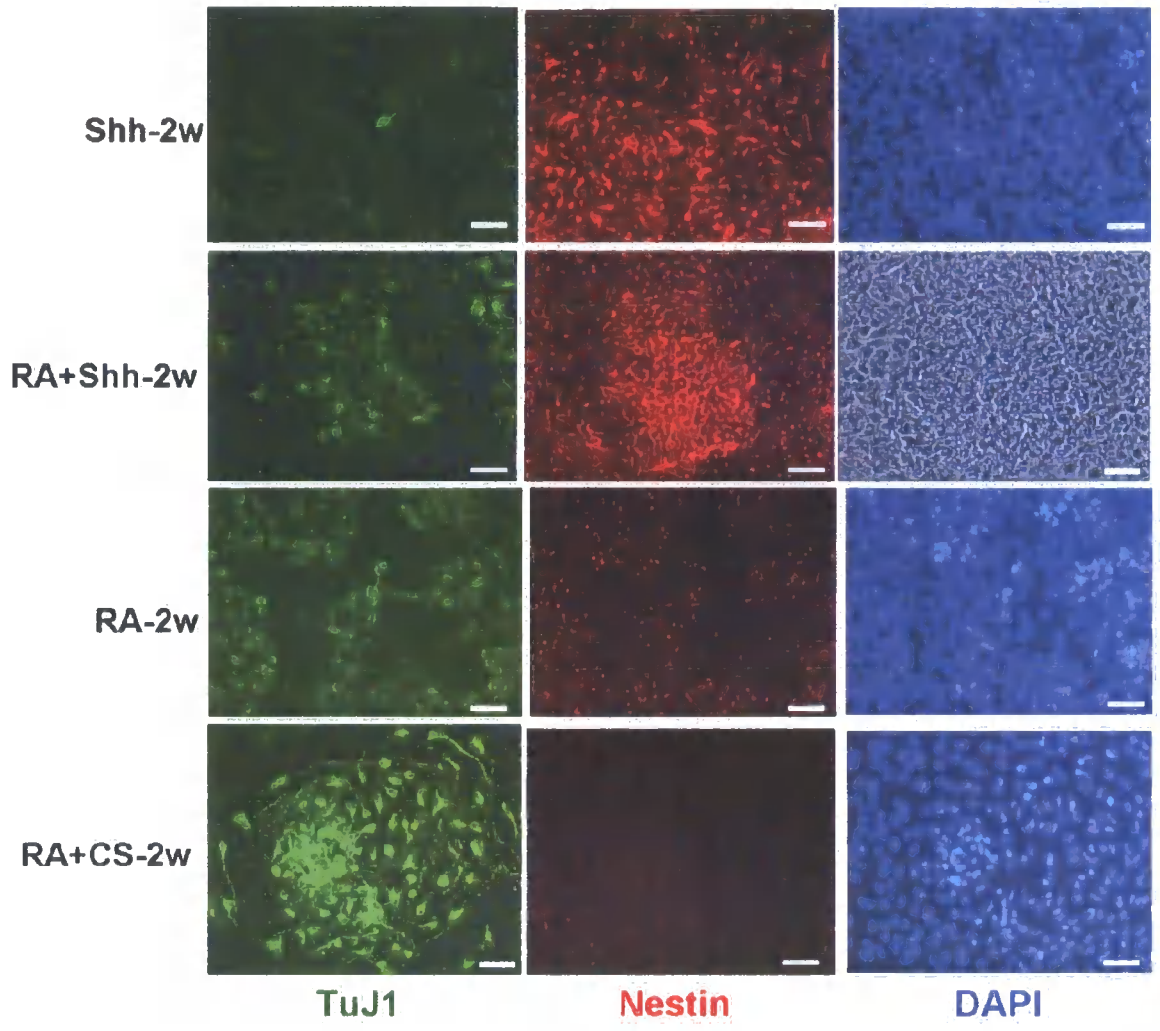

Figure 4.1.2.2 Double staining of nestin and TuJ1 in RA and Shh induced EC cells. EC cells were seeded in the 12 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) CS (cyclopamin, 1μM; SANT-1, 30nM) for 2 week before fixing with 4% PFA and double immuo-labelled with neuronal Tuj1 (green) and progenitor cell marker Nestin (red). The nuclei were counterstained with Hoechst or a phase contrast image is shown instead. Note that the staining of Tuj1 is stronger in RA induced cultures, while more Nestin positive cells in the Shh induced cultures. Scale bars = 50μm

4.1.3.3 RA and Shh induce neuro-ectodermal differentiation in Tera2.cl.SP12 EC stem cells

Recent studies have demonstrated the differentiation of human ES cells into neuro-ectoderm in cultures (Sonntag et al., 2006). In our present study, we found that the application of RA and Shh induced similar neuro-epithelial rosette formation from EC cells (Figure4.1.3.1). In RA induced cells, particularly in RA+Shh condition, the culture produced more homogeneous colonies of neuro-epithelial rosette-like structures, whereas in Shh induced cells, no rosettes appeared in the culture. Furthermore, these EC cell derived rosettes expressed the progenitor proliferative marker Ki67 in their central part and the neuronal marker Tuj1 at their edges, indicating their immature and neural differentiation properties. These rosettes are also nestin positive, comprising slender cells with scarce cytoplasm and two long processes (Figure 4.1.3.2). We also observed another nestin positive cell population in Shh induced cells with distinct morphology. The Shh induced nestin positive cells had relatively flat cell bodies with a larger cytoplasm, thick and short processes (Figure 4.1.3.2), and there were also Ki67 positive cells scattered in the Shh induced cells, suggesting that Shh induced cells are proliferating immature cells. Reports showed that such rosettes represent neural plate-like and/or neural tube-like precursor cells (Li et al., 2005), the human ES cell derived rosettes expressed neural precursor markers Nestin and the Tuj1 positive cells appeared at the edges of the rosettes (Sonntag et al., 2006). The similarity between ES and EC derived rosettes suggested that human EC cell-derived rosettes are at the neuro-ectodermal stage. The Shh treated cells may acquire a neuro-ectodermal identity but has no ability to further progress to form neuro-epithelial rosettes, suggesting that Shh has litmited neural differentiation ability.

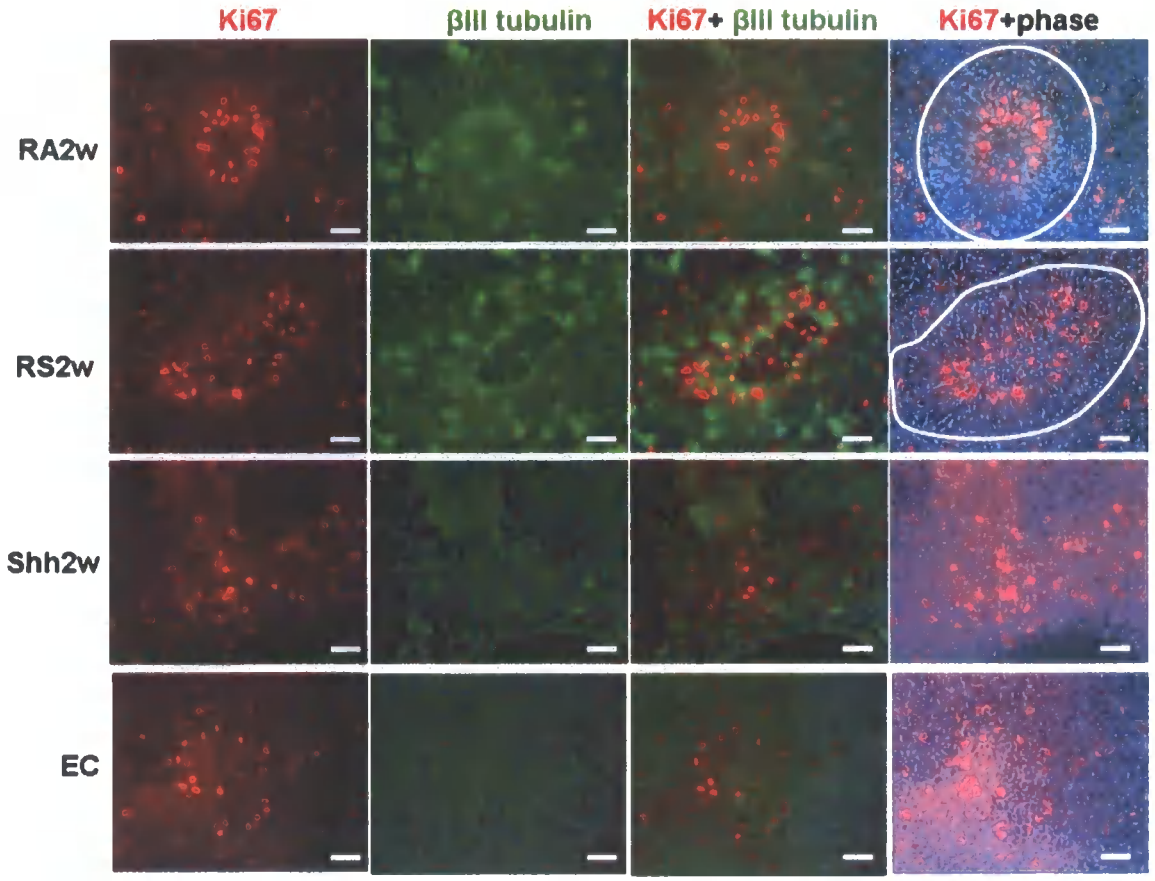

Figure 4.1.3.1 Formation of rosettes in the EC culture. EC cells were seeded in the 12 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) for 2 weeks prior to fixation with 4% PFA and double immuo-labelled with neuronal βIII tubulin (green) and the proliferation marker Ki67 (red). The phase contrast images overlaid with Ki67 staining are shown. Note that RA induced rosettes labelled with white circles, while Shh induced no rosettes formation in the culture. The proliferation marker Ki67 is positive in all four conditions, but its positive cells present at the centre of the rosettes in RA and RA+Shh induced cells, while scattered around in Shh induced and non-induced EC cells. βIII tubulin positive cells present at the edge of the rosettes. The induced rosettes may present neural tube like precursor cells. Scale bars = 50μm

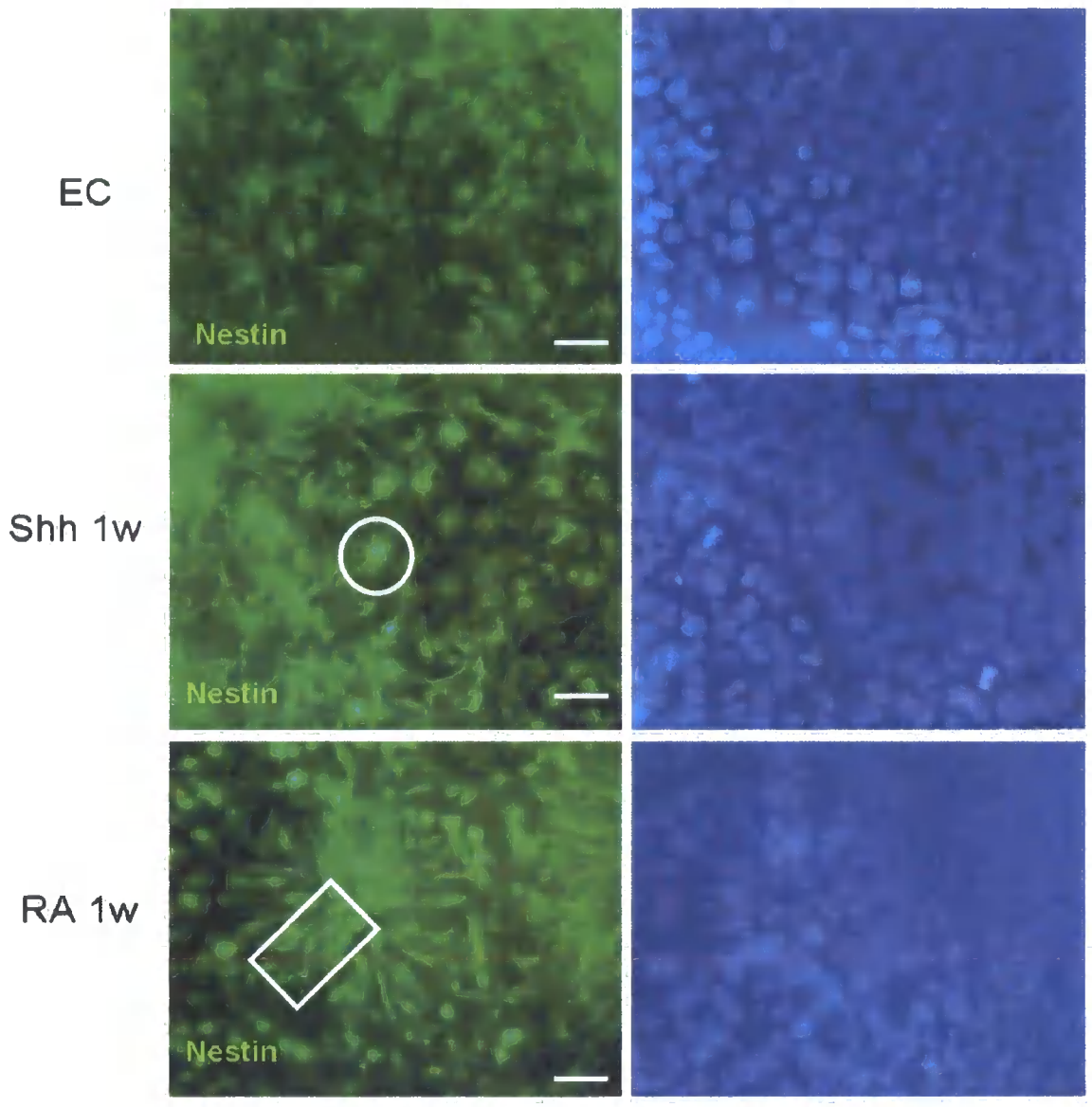

Figure 4.1.3.2 The morphology of Nestin positive cells in RA and Shh induced EC cultures. EC cells were seeded in the 12 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) for 1 week before fixing with 4% PFA and immuo-labelled with progenitor cell marker Nestin (green). The nuclei were counterstained with Hoechst. A typical Shh induced nestin positive cell is labelled with with white circle and the typical RA induced nestin positive cells are labelled with the white rectangle. Scale bars = 20μm

4.1.3.4 Regulation of Oct-4 expression in Tera2.cl.SP12 EC cells during exposure to RA and Shh induced

The POU domain transcription factor Oct-4 is one of the main determinants of the pluripotent state in the mammalian embryo, which is expressed in ES cells and down-regulated early during the loss of pluripotency and self-renewal ability (Babaie et al., 2006). Oct-4 is highly expressed in undifferentiated EC cells (Figure 4.1.4.2). Oct-4 expression here is used as tag to indicate the differentiation status of EC cells. We used western blotting analysis to determine the level of Oct-4 expression in 1 and 2 week induced EC cells (Figure 4.1.4). In 1 week induced EC cells, we detected a strong band of Oct-4 in Shh induced cells, whereas in the Shh antagonists, cyclopamine+SANT-1 condition, the Oct-4 expression was significantly reduced. Further evidence of Shh signalling increase proliferation plays a role in maintaining the pluripotency of EC cells at the early induction. In RA and RA+Shh conditions, there is only a trace amount of Oct-4 expression detected by western blotting, which means RA signalling instead of Shh signalling contributes more in the loss of pluripotency of EC cells. It is also possible that RA inhibits other pathway as well as being involved in EC cell self-renewal. We noted that there is no obvious band in 2 week induced EC cells, suggesting that EC cells undergo irreversible differentiation when grow in these induced conditions. These results are also consistent with flow cytometry results, supporting the idea that RA is the predominant differentiation factor and Shh is a much slower differentiation factor for EC cells.

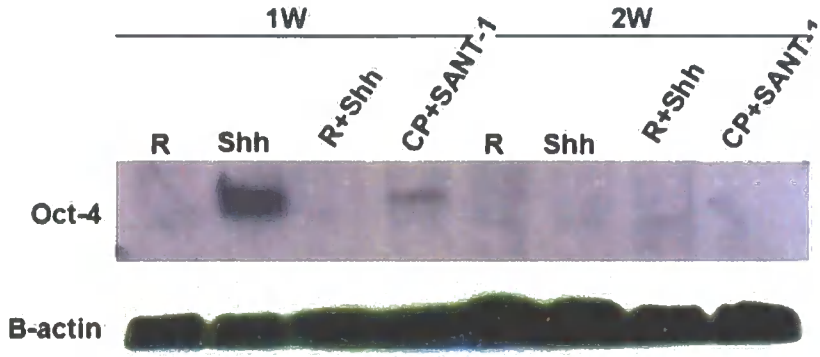

Figure 4.1.4.1 Western blotting analysis of Oct-4 expression in induced EC cells. EC cells were seeded in the 12 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) CP+SANT-1 (cyclopamin, 1μM; SANT-1, 30nM) for 1 week or 2 weeks before nuclear protein extraction, and then nuclear protein was aliquoted for western blot analysis. Oct-4, ES stem cell marker; β-actin, internal control; cyclopamine and SANT-1 are Shh antagonists. The band size of Oct4 is about 50KD, β-actin is 45KD.

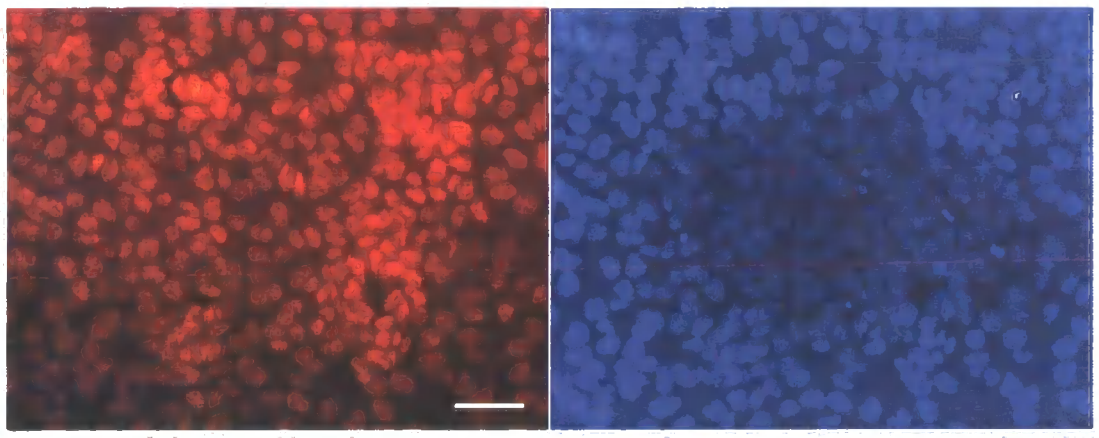

Figure 4.1.4.2 Oct-4 is highly expressed in the undifferentiated EC cells. EC cells were seeded at the density of 20,000cells/cm^2 without any inductive factors. EC cells were maintained for 7 days prior to fixation with 4% PFA and labelling with antibodies specific to Oct-4. The nuclei were counterstained with hoechst. Note that Oct-4 staining is positive for every undifferentiated EC nuclei. Scale bars = 50μm.

4.1.3.5 Regulation of the Shh pathway of 1 week and 2 weeks differentiated Tera2.cl.SP12 EC cells

To investigate the role of the Shh pathway in EC cell differentiation, we used RT-PCR analysis to examine the key genes of the Shh pathway, notably, Shh binding receptor PTC-1, and Shh signalling pathway effectors GLI-1, -2, and -3. It is known that Shh induced PTC-1 expression is a conserved response in vertebrates (Briscoe et al., 2001). The observation of PTC-1 expression in Shh induced cells by RT-PCR confirmed this (Figure 4.1.5). Shh + CP showed no expression of PTC-1 at 2 weeks indicates that Shh has a neutralizing effect on cyclopamine. To our surprise, RA showed a lower stimulation on PTC-1 expression in 1 week induced cells and a higher stimulation in 2 week induced cells compared to Shh induced cells. This suggests that RA can regulate PTC-1 expression and does so in a conserve manner to Shh. In RA+Shh condition, there is higher PTC-1 expression compared to the Shh condition. When we blocked the Shh with cyclopamine, it did not decrease the PTC-1 expression to the level of RA conditions, suggesting that RA induced PTC-1 expression without Shh signal. It also indicates that there maybe endogenous Shh in EC cells that antagonizes the RA signals during the RA alone condition. For the Shh pathway effector transcription factors GLI-1,-2, -3, there were different expression patterns in the induced EC cells (Figure 4.1.5). For GLI-3, it seems that there was not much difference between all the conditions; a bright band was detected in all conditions by RT-PCR analysis, suggesting that GLI-3 is constitutively expressed in EC cells, and it is not regulated by the inductive factors. In contrast to GLI-3, GLI-2 showed a much lower expression level in all the conditions. The reasons for that could be GLI-2 is a powerful effector, its weak expression is enough for mediating the signal, or GLI-2 is constitutively weak expressed in EC cells. GLI-1, however, showed a differented regulated expression. In Shh, CP and Shh+CP conditions, the GLI-1 expression is consistent with

PTC-1 expression. In 1 week RA induced cells, a weakly expressed band was detected, while in 2 week induced cells, a strongly expressed band was detected, indicating that RA induced GLI-1 expression. However, in RA+Shh condition, GLI-1 expression first increased greatly in 1 week induced cells but did not have a corresponding increase in 2 week condition like RA, suggesting that RA and Shh co-function at first, and later Shh signalling may antagonize the RA signal to some extent. When the Shh signal was blocked by cyclopamine for 2 weeks, there was a weak band similar to 1 week RA condition, suggesting that RA and Shh signals have a complex effect on GLI-1 expression. These results suggest that RA and Shh have antagonizing effects on each other and RA can induce GLI gene expression. The data also indicate that EC cells at different differentiation stages may have different responses to RA and Shh signalling: Shh has the predominant effect during the first week, and RA has an alternative effect after 2 weeks.

The expression of PTC-1 and GLI-1 in EC cells is sensitive to the Shh signalling, EC cells therefore provide a faithful and physiologically meaningful model for the analysis of the Shh signaling pathway and it may also reveal how RA regulates the Shh pathway in EC cells. To further evaluate the action of Shh signalling, GLI-1 was chosen for further analysis of Shh pathway by siRNA knock-down (Section 4.3).

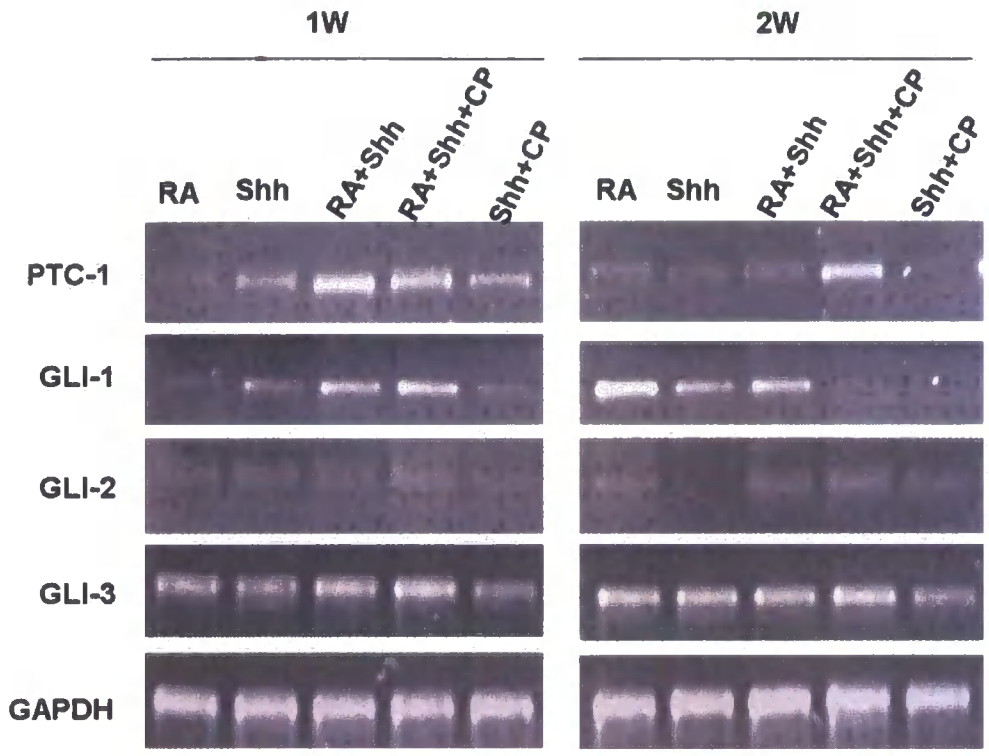

Figure 4.1.5 Regulation of the Shh pathway gene expression. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml), cyclopamin (CP, 1μM) for 1 week or 2 weeks, and then total RNA was isolated for RT-PCR. PTC-1, the Shh binding site of the receptor complex; GLI-1, -2 and -3, the Shh signalling pathway effectors; GAPDH, serves as internal control.

4.1.3.6 RA and Shh regulate neuronal differentiation in Tera2.cl.SP12 EC cells

Toinvestigate the function of RA and Shh signalling during neuronal differentiation, we studied their development over a 5 weeks period. Two key neural markers were used in this assay: nestin and βIII tubulin. Nestin was positive in all conditions at 1 week, although its distribution in the cells altered. While in RA and RA+Shh conditions, nestin positive cells adopted a slender morphology and scarce cytoplasm at 1 week, then at 2 weeks, these positive cells re-arrange to form neuro-epithelial rosette-like structures (Figure 4.1.6.1). Later at 5 weeks, nestin positive cells were significantly reduced in RA+Shh condition and no staining detected in RA condition. In Shh induced cells, nestin positive cells showed a much rounder morphology with a larger cytoplasm and thick and short processes like previous described (Figure 4.1.3.2). In the 2 weeks Shh condition, there were still numerous nestin positive cells but they did not form any rosette-like structures. Furthermore, at 5 weeks, there were more nestin positive cells compared to RA and RA+Shh conditions. These results showed that Shh induced nestin positive cells can not form neuro-epithelial rosettes, suggesting Shh induced cells do not further progress toward the neural lineage and remain immature. RA+Shh induced cells formed more rosettes and had nestin positive cells last for more than 5 weeks, suggesting that Shh helps in maintaining neural progenitor cell pool.

For βIII tubulin, we first noted that positive staining cells in RA and RA+Shh condition not only increased in cell number but also extended long processes like neuronal fibers when time progressed, suggesting that neurons differentiated in these two conditions. Consistent with nestin staining, we observed the most βIII tubulin positive cells in the RA+Shh condition, suggesting that RA+Shh condition is most efficient in neuron induction. However, in the Shh condition, there was no βIII tubulin staining at 1 week and only one or two βIII tubulin positive cells observed at 2 weeks and 5 weeks, suggesting that most of

Shh induced EC cultures stop at the nestin positive stage and have acquired cell fates other than a neuronal fate at the end.

Collectively, these data indicate that RA is a requirement to induce EC cells to differentiate into neurons. The addition of Shh promotes the neuron production, via expansion of the neuro-progenitor pool. Shh itself may help in neural specification but the cells remain at the early differentiation stage and do not advance to a mature neurons.

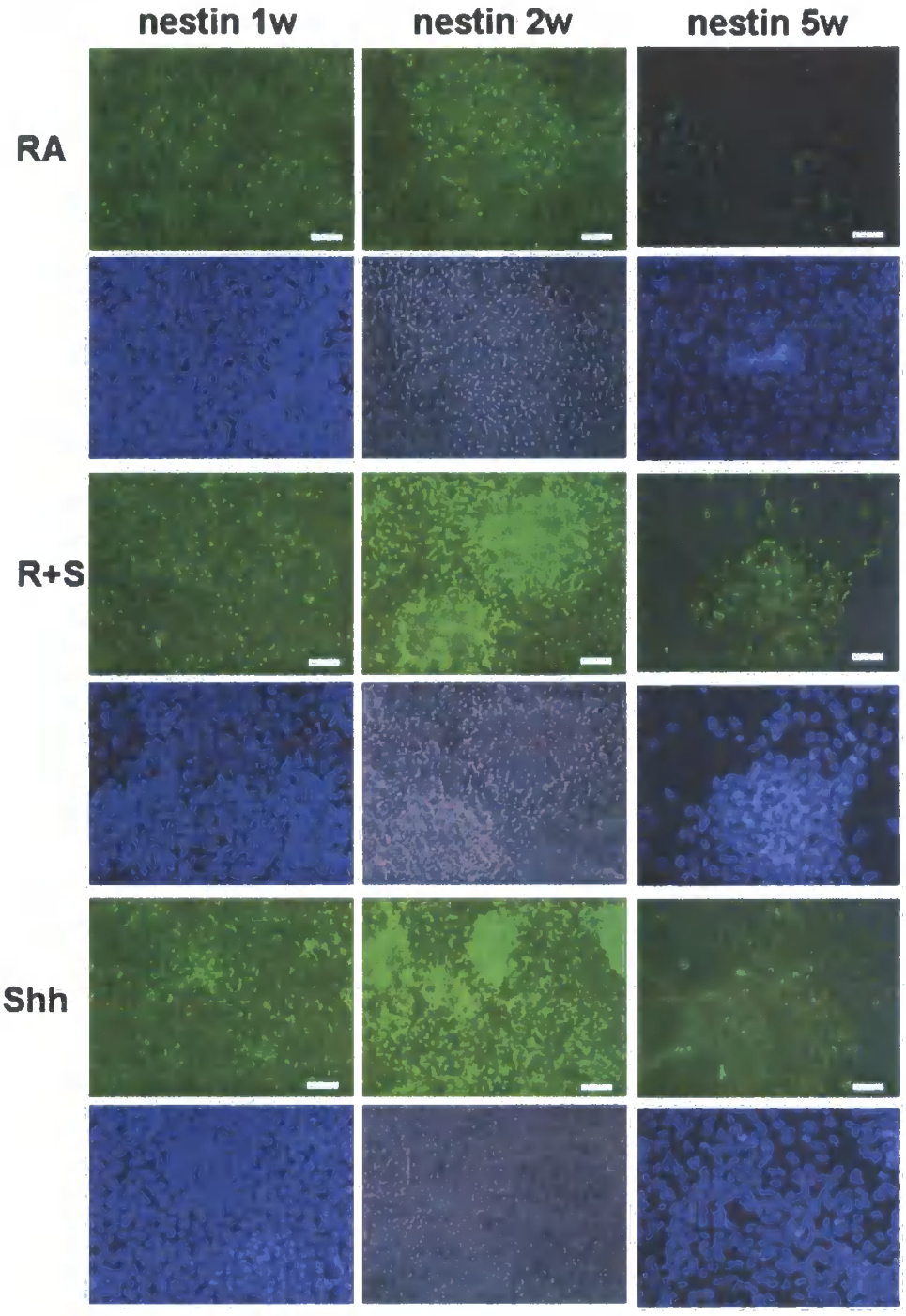

Figure 4.1.6.1 Nestin staining in RA and Shh induced EC cultures. EC cells were seeded in the 12 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) for 1, 2, 5 weeks before fixing with 4% PFA and immuo-labelled with progenitor cell marker Nestin (green). The nuclei were counterstained with Hoechst or a phase contrast image is shown instead. Note that Nestin staining lasts 5 weeks in Shh and RA+Shh cultures, while no rosettes formed in Shh induced cultures. Scale bars = 50μm

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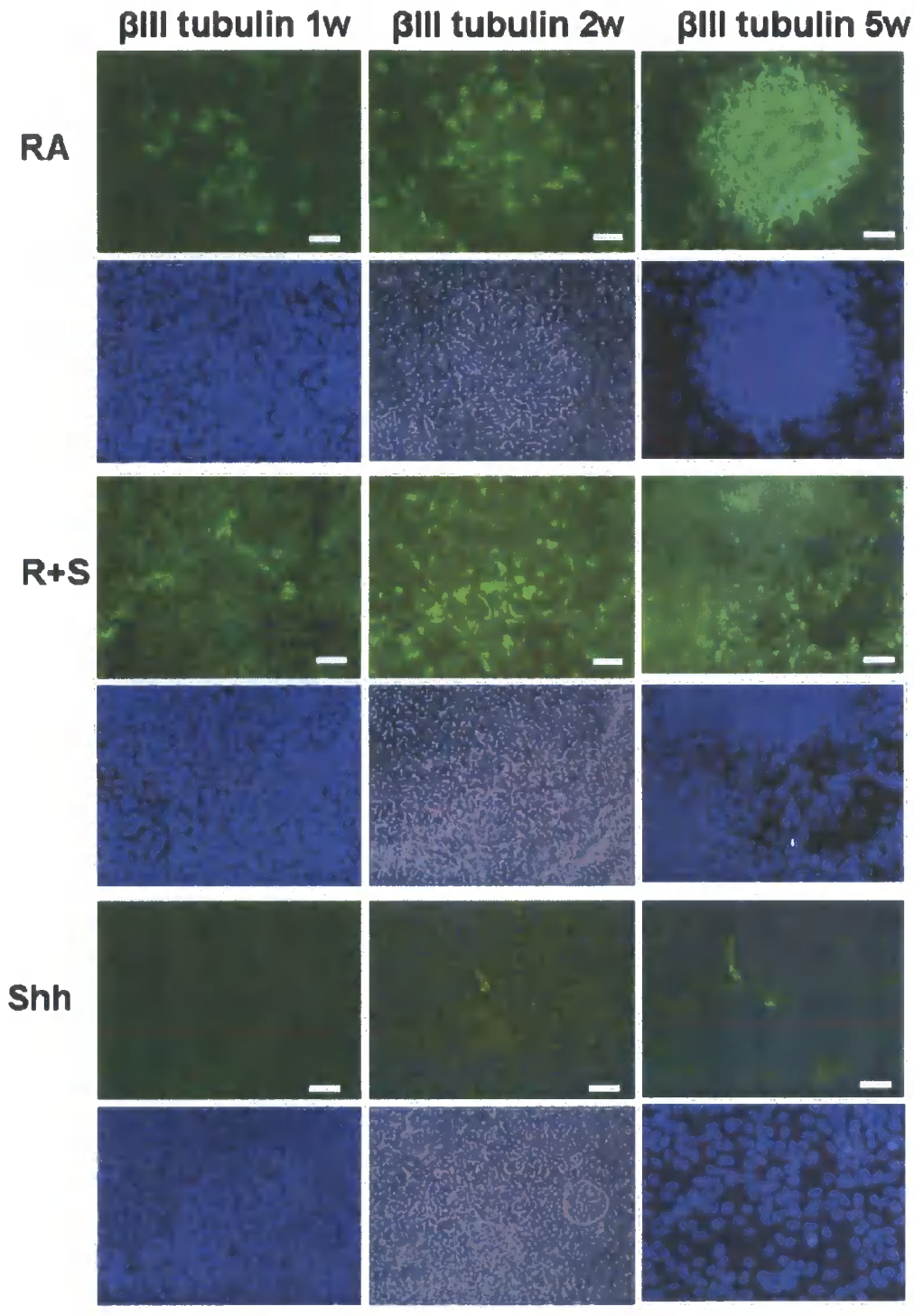

Figure 4.1.6.2 Neuronal βIII tubulin staining in RA and Shh induced EC cultures. EC cells were seeded in the 12 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) for 1, 2, 5 weeks before fixing with 4% PFA and immuo-labelled with βIII tubulin (green). The nuclei were counterstained with Hoechst or a phase contrast image is shown instead. Note that βIII tubulin staining increases over 5 weeks in RA and RA+Shh cultures, while not much staining in Shh induced cultures. Scale bars = 50μm

4.1.3.7 Ngn-2 expression in RA and Shh induced Tera2.cl.SP12 EC cells

Neurogenin-2 is a bHLH transcription factor that plays an important role in regulating neurogensis during the embryonic development (Mizuguchi et al., 2001). We investigated the Nng-2 expression in RA and Shh induced EC cells by western blotting to confirm the neurogenesis (Figure 4.1.7). At 1 week, in all Shh, RA and RA+Shh conditions, only a slight increase in Ngn-2 expression was detected compared to non-induced EC cells, however, there was a much stronger band by 2 weeks. Although there was no rosette formation in 2 week Shh induced cells and not many differentiated neurons in 5 week Shh induced cells, the expression of Ngn-2 in 2 week Shh induced cells suggested that Shh signal imposed a neural fate on EC cells at the onset of differentiation. In RA and RA+Shh conditions, the increasing expression of Ngn-2 from 1 week to 3 weeks indicated that EC cells undergo active neurogenesis under these two conditions, which is consistent with the previous results.

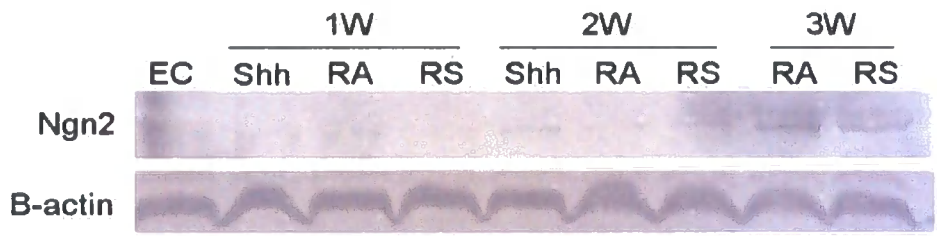

Figure 4.1.7 Western blotting analysis of Neurogenin-2 expression in induced EC cells. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) for 1, 2, 3 weeks before cytoplasmic proteins were collected for analysis. β-actin serves as internal control, is 45KD; the band size of Ngn2 is 50KD.

4.1.3.8 The cell fate determination in Shh induced Tera2.cl.SP12 EC cells

Our data suggest that Shh induced no mature neurons in EC cells and the Shh signal imposed a neural cell fate on EC cells during early stages of EC cell differentiation. Even after 5 weeks induction, there were still nestin-positive cells in Shh induced EC cells

(Figure 4.1.6.1). We set up an assay to examine the multipotency of Shh induced cells. The EC cells were induced by Shh for 3 weeks, after that Shh was withdrawn from the cultures, RA was added to the culture and cultures without additional RA were used as controls. Cells showed at least three morphologies from different lineages (Figure 4.1.8): a fraction of cells acquired neuronal morphology, displaying rounded cell bodies with long extended neurite-like processes; the second group of cells emerged as well connected membrane-like structures; the third group of cells displayed fibroblast like morphology; epithelial cells may also exist. In the control, there were no typical neuron-like cells present and there were islands of cells actively proliferating. Most of the cells still appeared to be epithelial, while the differentiated cells displayed flat cell bodies with large cytoplasms. These observations suggested that there were neural progenitor cells in the Shh induced cultures, however they needed additional RA signal to differentiate into neurons. Other lineage progenitors were also contained in Shh induced cultures, suggesting that Shh induced cells are multipotent cells, it is an inductive factor that acts on early differentiation stage.

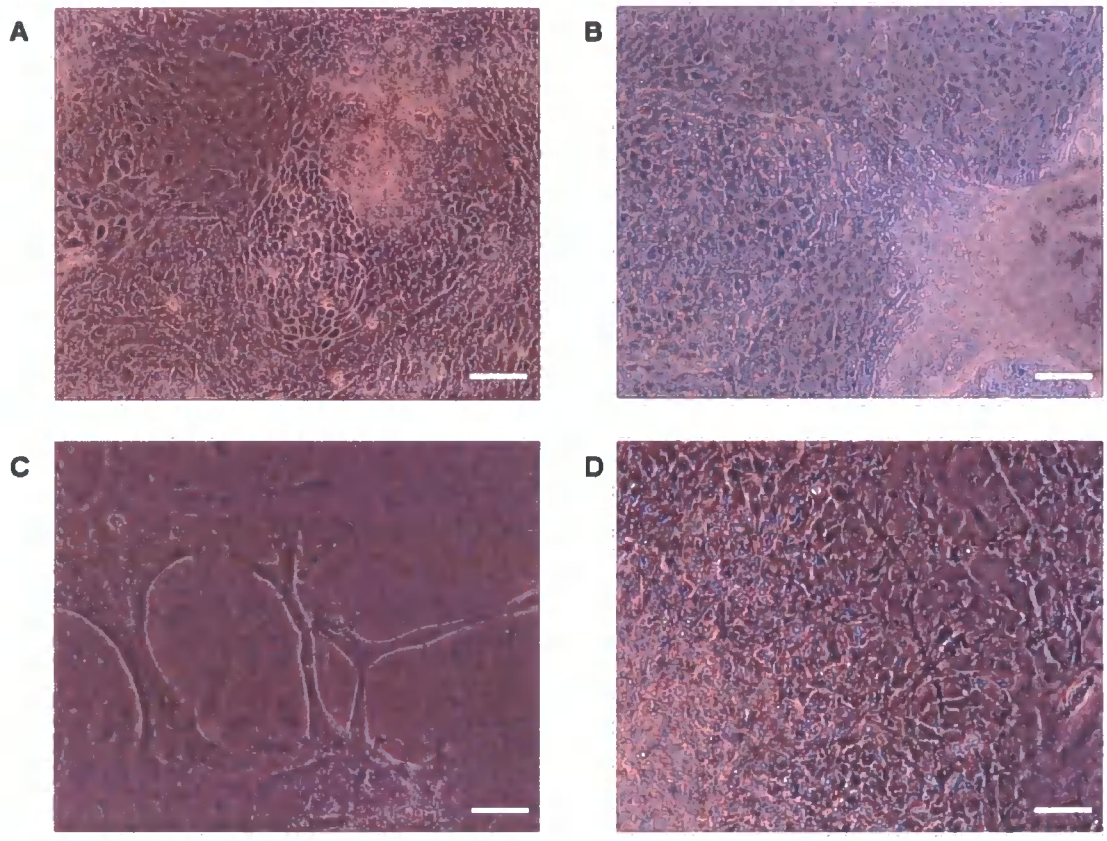

Figure 4.1.8 Phase contrast images of EC cultures induced to differentiate as stated. EC cells were seeded in the 12 well plate at 20,000 cells/cm^2 supplemented with Shh (25ng/ml) for 3 weeks, after the withdrawal of Shh, C and D were induced by RA (10μM) for a further 3 weeks, while A and B were grown without the presence of RA. Note that neuron-like cells only present in D. Scale bars =50 μm

4.1.3.9 BMPs induce proliferation and differentiation in Tera2.cl.SP12 EC cells

We examined whether BMPs have the ability to induce proliferation and/or differentiation in EC cells. Using a cell proliferation assay, we checked the cell number and viability after applying different concentration of BMP-7 on EC cells. Since the cells are healthy and proliferative, we noted an increase of optical density in the MTT assay as a response of proliferation of EC cells. All the concentrations of BMP-7 induced cultures show a proliferation after 3 days, but only cells exposed to 5ng/ml and 50ng/ml concentrations continue to have more cell number than non-induced cells on day5 (Figure 4.1.9 A). After addition of RA to the 50ng/ml BMP-7 culture, we observed no increase of reduction of proliferation until day5, suggesting that EC cells selectively respond to BMP-7 at the early induction stage. We next asked if the other BMPs behave the same way in EC cells induction. At 10ng/ml, we found that BMP-2, -4 and -7 exhibited similar growth patterns on EC cell induction (Figure 4.1.9 B). The induced cells rapidly increased in cell number from day3 and reach a peak at day7. In contrast to this result, the MTT assay showed less optical density on day5 compared to day3 in BMP-7 induction, indicating that, although the cell number is still increasing, some cells may undergo apoptosis, and also suggesting selective differentiation might be happening. BMP-6 had no obvious effect on cell proliferation compared to the other BMPs.

In the presence of BMPs, we observed no rosette-like structure formation during induction (Figure 4.1.9C). The differentiated cells showed a flat round morphology with epithelial, but no neuron characteristics. With BMP7 induction, typical epidermal cells and lipid cells are observed; however specific staining are needed to done to confirm whether or not there exists bone formation cells.

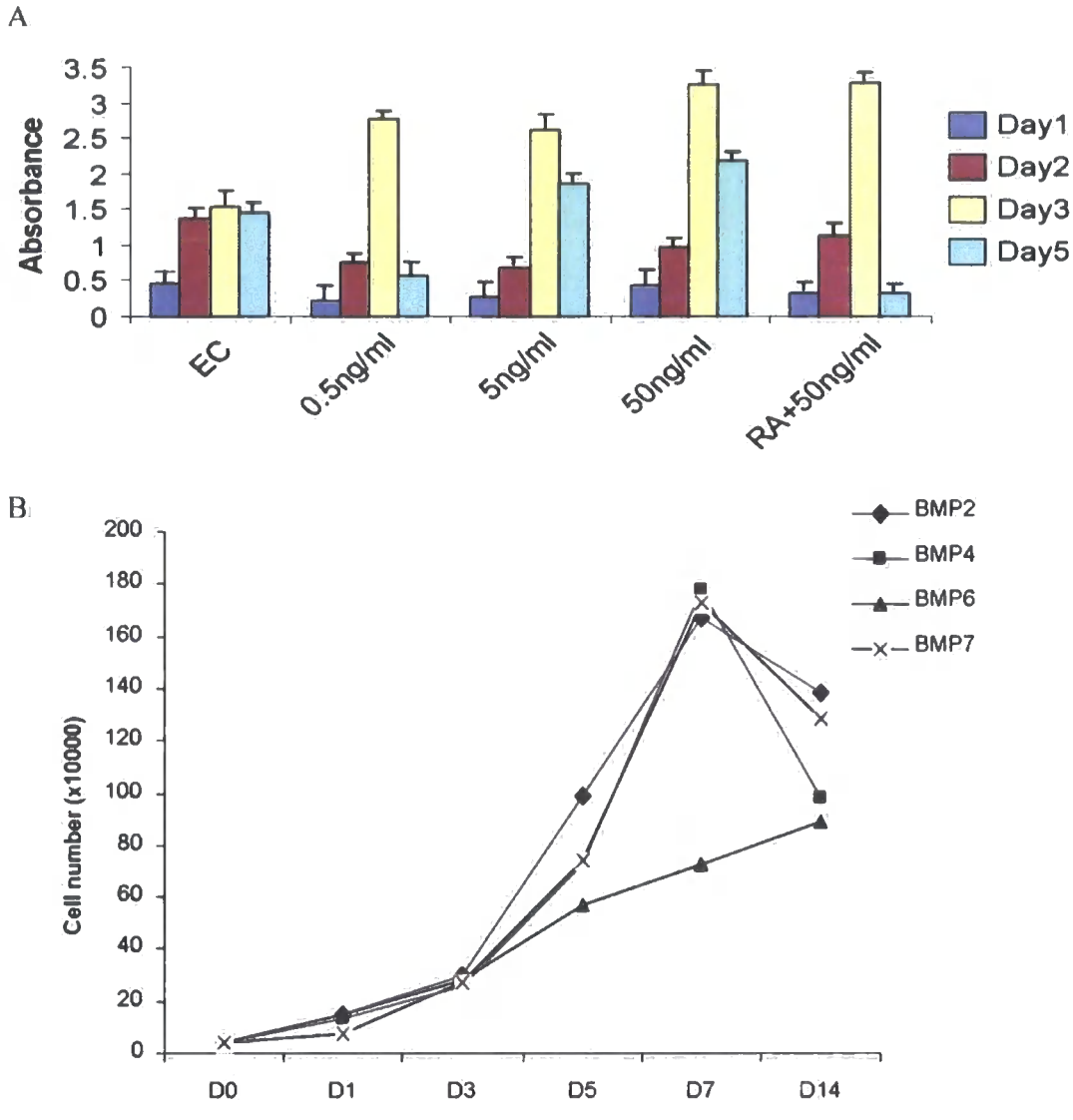

Figure 4.1.9 BMPs induce proliferation and differentiation of EC cells. A, BMP7 induced EC cell proliferation. EC cells were seeded in the 12 well plate at 20,000 cells/cm^2 supplemented with indicated concentration of BMP7 for 1 week prior to performing MTT analysis. Error bars are shown as SEM, n=3. B, Different BMP proteins induced proliferation of EC cells. Cells were induced by 10ng/ml BMP-2, -4, -6 and -7 at indicated time points before being analysed by hemtocytometer, n=3.

C

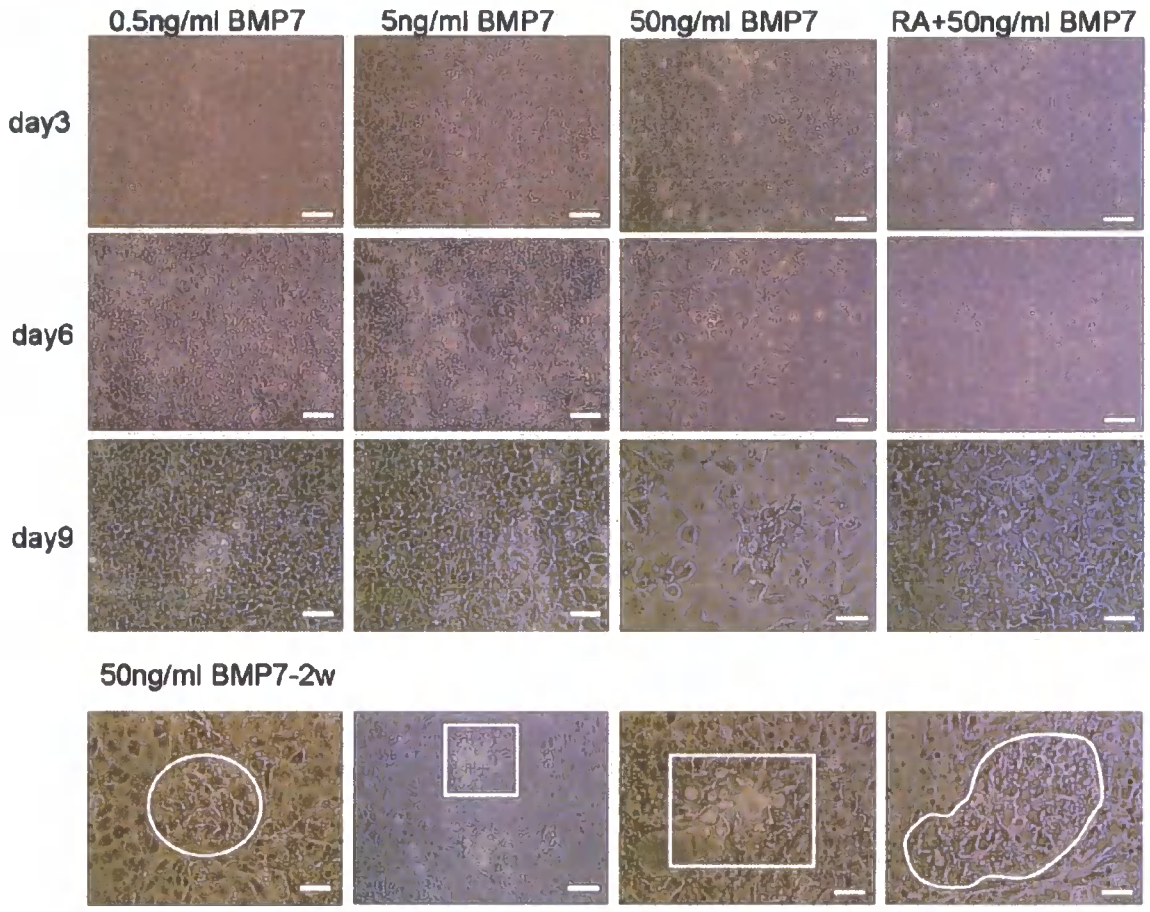

Figure 4.1.9 C, BMP7 induced EC cell differentiation toward a non-neuron fate. EC cells were seeded in the 12 well plate at 20,000 cells/cm^2 supplemented with indicated concentration of BMP7 and RA (10μM) for up to 14 days before phase constrast images were taken. Note that lipid drops (white rectangles) and epidermal like cells (while circle and irregular curve) in 50ng/ml BMP7 induced cultures for 2 weeks. Scale bars = 50μm.

4.1.4 Discussion

Shh induces a large number of diverse neuronal and non-neuronal cells during the embryogenesis (Ahlgren and Bronner-Fraser, 1999; Fan and Khavari, 1999; Zhang and Yang, 2001; Palma et al., 2005; Schnapp et al., 2005; Cayuso et al., 2006).

In Chapter3, we demonstrated that human Tera2.cl.SP12 EC cells provide an *in vitro* model of Shh signalling. In this chapter, we further explore the role of Shh in EC cell proliferation, cell specification, neuron differentiation and interaction with RA.

While one of the most important roles of Shh is the involvment in cell fate choice and patterning the CNS, Shh can also function as a mitogen, stimulating proliferation of neural progenitor cells (Rowitch et al., 1999; Cayuso et al., 2006). In the EC cell proliferation assay, we showed that Shh is the main regulator in inducing cell number increase, RA itself can also induce some extent of cell growth, however, in the combination of RA+Shh, RA seems to antagonize the proliferation effect of Shh at the early induction. In Shh, RA and RA+Shh induced cultures, the time point which elicit the peak cell number was different for each probably indicating that, while they may have common target cells, each of them acts best on their own target cell group and induce selective cell proliferation.

Using flow cytometry to examine the stem cell surface markers of the Shh and/or RA induced EC cells further confirm our speculation on selective cell proliferation. After 2 weeks, Shh induced cells still have around 40% expression of the embryonic stem cell markers SSEA-3 and TRA-1-60 and only 5% A2B5 expression, suggesting that Shh induces the least differentiation and its target cells are EC cells or very early differentiated cells. In contrast, RA induced cells have less than 5% SSEA-3 and TRA-1-60 positive cells and more than 50% A2B5 positive cells, suggesting that its target cells are the A2B5 cells and later differentiation stage cells. The western blotting of Oct-4 expression provided

evidence that Shh did induce EC cell differentiation. The loss of Oct-4 in Shh induced cells may accompany cell fate commitment, although the cell morphology did not change dramatically when examined by phase contrast images. Under the conditions of RA and RA+Shh, EC cells acquired a bipolar morphology and formed rosettes like structures, with Ki67 positive cells in the centre of the rosettes and scattered Tuj1 positive cells at the edge. Nestin positive cells formed the rosette body. The data suggest that RA instead of Shh is the key regulator in EC cell neural differentiation.

We performed RT-PCR to investigate the Shh pathway genes. Although Gli2 and Gli3 were expressed in the induced cells, their expression did not change greatly with changing induction conditions. However, the expression levels of PTC-1 and Gli-1 seem to be regulated by the different induction conditions. This is consistent with a report that Gli1 can be used as a Shh reporter, since its expression is strictly depended on Shh signal (Bai et al., 2004). Furthermore, this provides us with a model to investigate the function of the Gli1 transcription factor during neural cell specification. Later, we employed the siRNA technique to knock-down the Gli1 expression to determine its function in neural differentiation of EC cells (Section 4.3).

To explore the neural differentiation potential of Shh, we then compared 1 week, 2 week and 5 week EC cultures induced by Shh and/or RA. In contrast to RA, Shh induced cells did not differentiate into neurons after 5 weeks of culture, and cultures still contained nestin positive cells but no βIII tubulin positive cells. This demonstrated that Shh induced cells need other factors to advance to mature neurons, RA is one of these factors that promote maturation of neuronal progenitors. The RA+Shh condition provided us with the information that combination of these two factors will increase the neuron generation efficacy. If we induced EC cells by Shh for 3 weeks and then switched to RA, some of the Shh induced cells still differentiated into neurons, suggesting that Shh induced cells

contained neuron progenitors. And the BMPs induction assay confirmed that BMPs have no direct neural differentiation ability in EC induction, instead, BMPs induces epidermal differentiation of EC cells.

Taken together, we concluded that Shh is a progenitor proliferative factor. It plays a role in EC cell specification but not neuron maturation. Instead, RA is the critical factor in neuron differentiation of EC cells.

Section 4.2

Examine the role of Shh pathway using Tera2.cl.SP12 EC cell as an *in vitro* DV patterning model

4.2.1 Introduction

During embryo development, it is understood that RA, Shh and BMPs play important roles in controlling cell proliferation, cell patterning and cell type specification. Avian embryos are an excellent example of *in vivo* model to investigate these functions during the DV patterning (Poh et al., 2002).

In the developing chick embryo, a sheet of epithelial cells acquire specialised neural properties and become neural plate. At this time point the neural plate and neural tube is composed of multipotent proliferating progenitor cells. These cells display no regional identity along the DV axis and express the patterning related transcription factors in a uniform manner. A rod of axial mesodermal cells, e.g. notochord, which is immediately adjacent to the ventral-most region, secret Shh, and as its concentration decrease with the

tissue from approximately 15nM at the floor plate to 0.5nM at dorsal edge, the patterning transcription factors become regionally restricted (Briscoe and Ericson, 1999). At the DV axis, low concentration of Shh induces and confines the Pax3 and Pax7 expression at dorsal neural tube; other genes like Pax6 are only fully repressed by high concentration of Shh, so extend their expression to a ventral limit just outside the floor plate. The repression of Pax6 at its ventral limit allow the expression of Nkx2.2 domain adjacent to the floor plate(Patten and Placzek, 2000). The reciprocal repression between these two genes may act to refine and maintain p3 and pMN domains. Recently studies showed that Pax6 and Nkx genes induce Olig2 expression, and Olig2 is believed to promote cell cycle exit and acquire general neuronal identity by de-repression of Neurogenin2 and also direct the expression of Mnr2, HB-9 and Lim domain transcription factor (Islet1/2 and Lim3) that confer MN identity(Novitch et al., 2003). RA is actively involved in the neural tube patterning and the neuronal subtype specification (Wilson and Maden, 2005). Similarly, dorsal neural tube is patterned by BMP gradient concentrations through establishing specific sets of transcription factor domains, then form groups of dorsal interneurons.

However, the relationship between RA and Shh has not been fully investigated, as well as the relationship between Shh and different BMP molecules. And we know that, during the DV patterning, Shh derived from notochord setup a gradient concentration from the ventral to dorsal spinal cord with the maximum concentration at the floor plate; similarly, BMPs derived from ectoderm also setup a gradient concentration from dorsal to ventral with the maximum concentration at the roof plate of the spinal cord (Gilbert, 2003). Tera2.cl.SP12 EC cells respond to RA, Shh and BMPs. These natural inducing factors are present in the DV patterning of embryonic spinal cord development. Based on the above information, we theoretically constructed a decreased Shh concentration to induce EC cells: Shh, cyclopamine, BMP, BMP+CP, to represent the Shh signal from ventral to dorsal part of the

spinal cord. In this section, we also investigate the role of Shh, BMPs and RA in DV patterning and some key DV patterning genes in this *in vitro* model.

4.2.2 Results

4.2.2.1 Regulated expression of patterning genes on *in vitro* model of DV patterning

By adding Shh antagonists and BMP to EC cells, we constructed a virtual Shh gradient concentration. Considering EC cells can be a source of endogenous Shh, the virtual Shh gradient concentration from high to low can be achieved in theses combination: Shh+EC, EC cells alone, EC+Shh antagonist, EC+BMP, EC+BMP+Shh antagonist. Patterning genes were examined on this model to test whether the virtual Shh gradient concentration recaptulates the *in vivo* gene expression pattern.

PAX genes are distributed over the DV axis of spinal cord, with PAX-6 mainly at ventral side and PAX-3 and -7 at the dorsal side. In this *in vitro* DV model, PAX-3, -6 and -7 were weakly expressed all of the combinations, suggesting that the distribution of Pax genes over the virtual Shh gradient concentration did not recapitulate their *in vivo* expression pattern (Figure 4.2.1B).

In BMP6 and BMP6+CP induced cells, PAX-6 gene was absent or weakly expressed. This suggested that BMP6 instead of BMP4 and BMP7 repressed PAX-6 expression. In the RA condition, we observed a significant decline in PAX-6 expression, which is inconsistent with previous reports that RA has an inductive effect in PAX-6 regulation, while PAX-3 and PAX-7 are consistent with RA regulation *in vivo* (Briscoe et al., 2000). We next investigated another DV patterning genes the mid-line marker EN-1. We observed that EN-1 had reduced expression in BMP6+CP and BMP6+CS conditions, but not in BMP6 or CP alone conditions, it also had a reduced expression in RA condition, suggesting that EN-1

did not have strong expression in dorsal most condition. However, BMP4 and BMP7 had no suppression in EN-1 expression similar to PAX-6, suggesting that regulation patterns of EN-1 are not the same between BMPs. We then examined the ventral markers from ventral most towards the mid-line, NKX-2.2, OLIG-2 and ISL-1. The most ventral interneuron progenitor marker NKX2.2, which is neighbouring to the floor plate, had strong expression in Shh induced cells, which is consistent with the recent studies (Briscoe et al., 1999). In striking contrast to the increase in Shh induced cells, cells induced with BMP6 and BMP7 showed no expression of NKX2.2, and neither in RA and cyclopamine conditions. BMP4 did not repress the expression of NKX2.2. For OLIG-2, the common marker for motor neuron and oligodendrocyte progenitors, its expression was induced by Shh and repressed by RA and BMP6, where as BMP4 and BMP7 have no inhibitory effect on its expression. Interestingly, RA induced OLIG-2 expression at first and then diminished its expression until there was no expression after 10 days induction, suggesting that OLIG-2 is involved in neural cell fate specification during the early induction and its absence was also important for RA induced neuron development. ISL-1 is a marker for motor neuron and interneuron progenitors; it can be induced by Shh like other ventral markers, however, in this study, it was also induced by BMPs and by RA, although RA showed diminishing response on the gene expression. We compared the expression of these DV patterning genes with or without RA treatment before the 1 week induction. The RA treatment causes expression of some patterning genes switch off and/or turn on, indicating that RA is a necessary factor in DV patterning regulation, the response to RA signal helps to establish the correct expression domain of the patterning gene along DV axis.

Taken together, our results provided the first evidence that DV patterning genes respond differently to different type of BMPs. In our study, BMP6 shows more inhibitory effects compared to BMP4 and BMP7 on EC cells. For RA signalling, many ventral markers

showed to be induced at the early induction stage and to be repressed later. Considering that most of the transcription factors are repressor in properties, the elimination of their expression is required by the progress of neural development, it also implicates the importance of RA signalling. That the sustained expression of these markers in Shh induced cells may also explain why Shh induced EC cell can not finally advance to mature neuron stage.

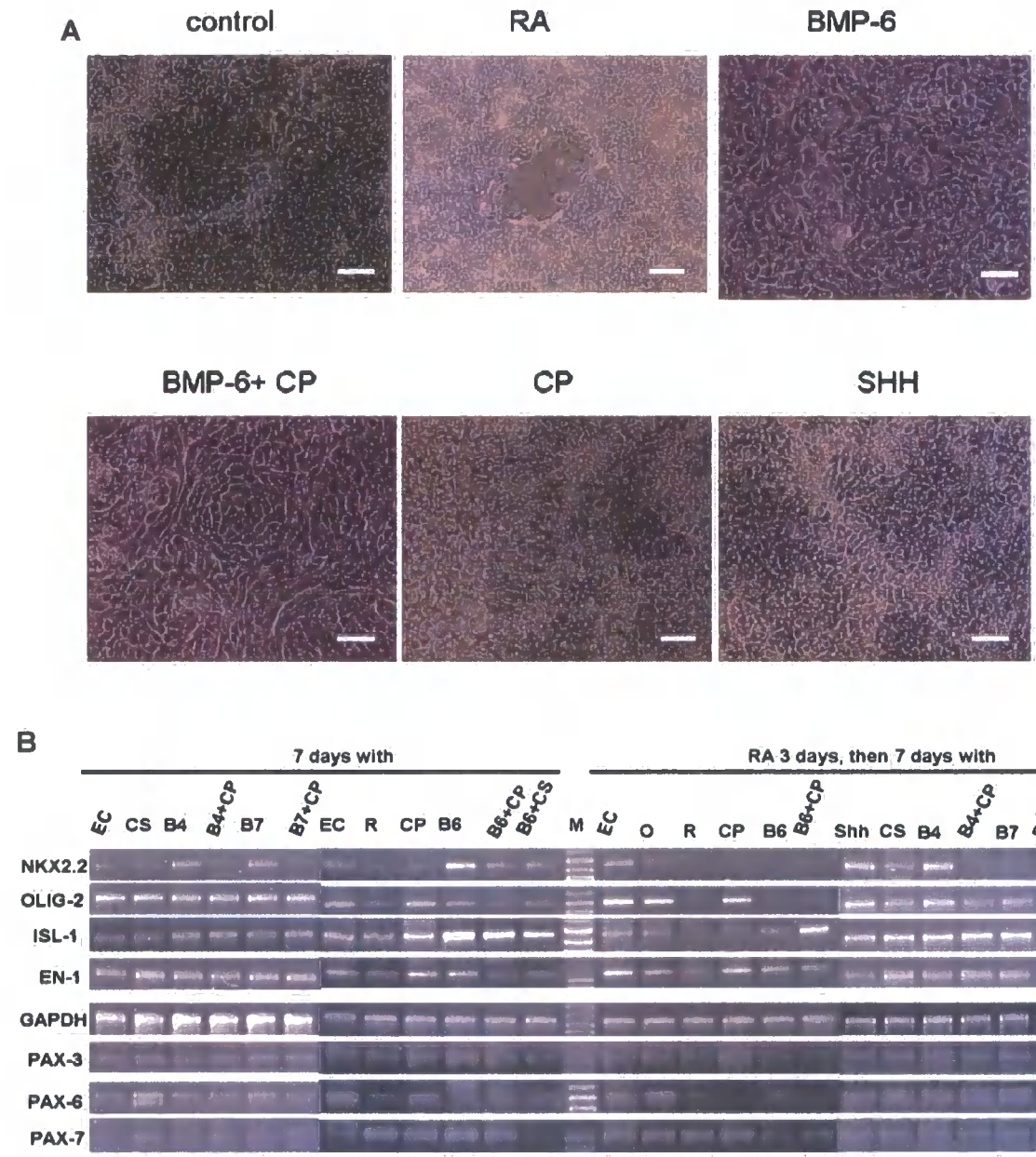

Figure 4.2.1 Analysis of Tera2.cl.SP12 EC cells as an *in vitro* model of dorso-ventral patterning. A, shows the morphology of induced EC cells before being subjected to RT-PCR analysis; EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) for 3 days before adding the inductive factors RA (10μM), BMP6 (B6, 10ng/ml), Shh (25ng/ml) and cyclopamine (CP, 1μM) for 7 days. Scale bars = 50μm. B, shows the RT-PCR analysis of the dorso-ventral patterning genes express in the induced cultures derived from A. EC cells were also induced with BMP4 (B4, 10ng/ml), BMP7 (B7, 10ng/ml), 1μM cyclopamine and 30nM SANT-1 (CS), BMP4 and cyclopamine (B4+CP), BMP6 and cyclopamine (B6+CP), BMP7 and cyclopamine (B7+CP), BMP6 and cyclopamine and SANT-1 (B6+CS) and no additional factors (O). GAPDH serves as internal controls.

4.2.2.2 Regulation of the Shh pathway in the DV patterning model

To address the influence of the Shh signal in DV patterning gene expression, we examined the components of the Shh pathway in this *in vitro* DV patterning model based on Tera2.cl.SP12 EC cells. PTC, the suppressor of the Shh pathway, is also the binding receptor for the Shh ligand (Fuse et al., 1999). *Ptc* is also a target gene of the Shh pathway, Shh up-regulates *Ptc* expression is a conserved response in vertebrates (Chuang et al., 2003). High level of PTC molecules on the target cell membrane limits the cell respond to Shh (Murone et al., 1999). In response to Shh, EC cells expressed increase level of PTC (Figure 4.2.2). However, Ptc is also a target gene of other signalling pathways such as RA and FGF (Shimizu et al., 2007) (Fogarty et al., 2007). According to our results, factors that have antagonizing effect on Shh such as RA, cyclopamine (CP) and BMP-6 had the inhibitory effect on the expression of *Ptc* (Figure 4.2.2). Although the up-regulation of *Ptc* is an indicator to the Shh gradient concentration, some recent reports suggested that *Ptc* up-regulation can be an effect independent of Shh (Banerjee et al., 2005), and this may be a reason why we detected increased expression level of *Ptc* in BMP4 and BMP7 conditions. Increased PTC without Shh ligand will block the Shh pathway by inhibiting Smo, the signalling component of the Shh receptor complex (Murone et al., 1999). Shh signals by binding to PTC receptor, which results in the recruitment of Gli transcription factors, influences the Shh transcriptional target genes. Therefore, we examined the regulation of *Gli* genes. In contrast to *Gli-2* and *Gli-3*, *Gli-1* seems to be regulated by *Ptc-1* expression; no *Gli-1* expression was detected in the conditions that no *Ptc-1* expression was induced. *Gli-2* and *Gli-3* were expressed in all conditions, indicating that their expression may be constitutive in EC cells. These results suggested that the Shh pathway is not only involved in EC cell proliferation activity but may also play a role in regulating cell differentiation and possibly the expression of DV patterning genes. .

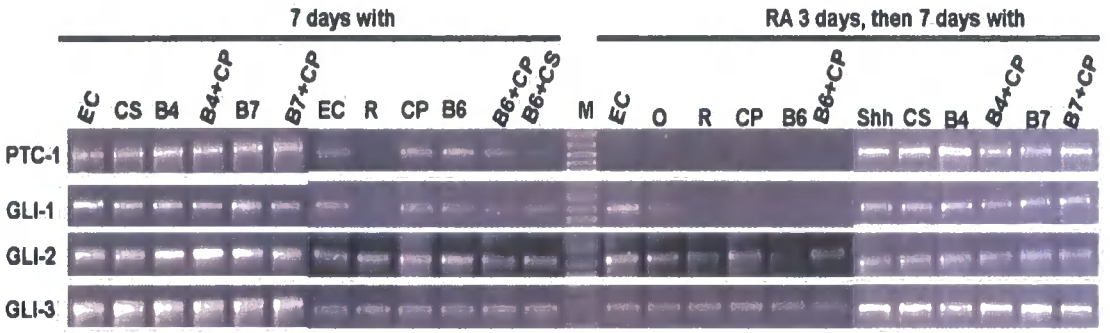

Figure 4.2.2 Analysis of the Shh pathway genes in the EC cell model of dorso-ventral patterning. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 with or without supplemented RA (10μM) for 3 days before adding the inductive factors RA (R, 10μM), BMP6 (B6, 10ng/ml), Shh (25ng/ml), cyclopamine (CP, 1μM), BMP4 (B4, 10ng/ml), BMP7 (B7, 10ng/ml), 1μM cyclopamine and 30nM SANT-1 (CS), BMP4 and cyclopamine (B4+CP), BMP6 and cyclopamine (B6+CP), BMP7 and cyclopamine (B7+CP), BMP6 and cyclopamine and SANT-1 (B6+CS) and no additional factors (O) for 7 days. Total RNA was isolated from the cultures and then subjected toRT-PCR analysis. The internal control GAPDH is shown in Figure 4.2.1.

4.2.2.3 RA acts on Shh pathway downstream of Smo during early neurogenesis by EC stem cells

To confirm that RA signalling plays an important role during the DV neurogenesis, we examined how RA regulates components of the Shh pathway, DV markers and neuronal markers during this process. Earlier in this study, our results showed that RA is a critical factor in regulating Shh pathway (Figure 4.1.5). When EC cells were induced by RA, neuronal markers like NeuroD, NCAM, Tuj1 and NSE showed increase expression (Figure 3.6.1). RA also induced expression of Pax-6, and motor neuron progenitor marker HB-9, suggesting that RA is involved in motor neurogenesis. We also provided the evidence that endogenous Shh protein and the Shh pathway downstream transcription factor Gli-1 had higher expression in RA induced cells compared with EC non-induced cells (Figure 4.2.3), suggesting that RA has the ability to regulate Shh and Gli-1 expression. The results also raised the possibility that, at least part, the RA signalling effect on EC cells is mediated via

the activation of the Shh pathway. For example, cell proliferation and the specification of early cell fate can be achieved by activation of Shh pathway in RA treated culture. To further confirm that Shh pathway mediates some of the RA's effects, we added Shh antagonists cyclopamine and SANT-1 to the RA induced cells to block the Shh signal transduction. However, we found that these exogenous antagonists can neither alter the expression of endogenous Shh nor the transcription factor Gli-1 (Figure 4.2.3), indicating that the response were controlled by some other intrinsic mechanism and appeared to resist to the inhibitory effect of cyclopamine (C) and SANT-1 (Sa), both bind to Smo to prevent the Shh signalling from reaching Gli transcription factors (Incardona et al., 1998) (Chen et al., 2002). The possible underlying mechanism is that RA first binds to its nuclear membrane receptors and then activates Shh pathway within the nucleus, which bypass the transmembrane protein Smo, the signalling initiator of the Shh receptor complex in the cell membrane. To further explore how RA regulates Shh pathway, a Gli inhibitor but not the Smo inhibitor should be applied to the system.

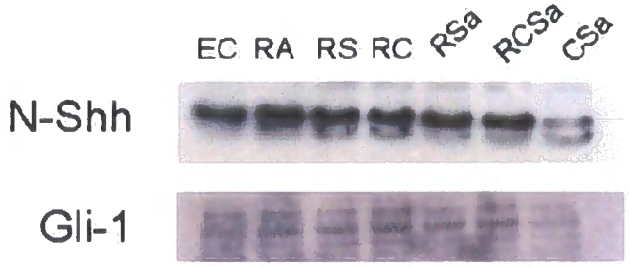

Figure 4.2.3 Western blotting analysis of endogenous Shh and Shh pathway effector Gli1. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml), cyclopamine (1 μM), SANT-1 (30nM) for 1 week before isolation of cytoplasmic protein and analysed the expression of N-terminal Shh and the Shh pathway effector Gli1 by western blot. Note that cyclopamine and SANT-1 did not affect the expression level of Gli1 and N-Shh in the presence of RA. Abbreviations: EC, EC cells without additional factors; RS, RA+Shh; RC, RA+cyclopamine, RSa, RA+SANT-1; RCSa, RA+cyclopamine+SANT-1; CSa, cyclopamine+SANT-1. The estimated band size of N-Shh is 45KD, Gli1 is 115KD.

4.2.2.4 RA and Shh signalling in motor neuron generation

RA and Shh are known to be involved in control of neuronal subtype specifications. Shh is required for dopamine neuron and serotonergic neuron specification from human ES cells (Hynes et al., 1995; Ye et al., 1998; Roussa and Krieglstein, 2004), both RA and Shh are required for motor neuron specification in ES cells (Zhang et al., 2007). EC-derived motor neurons were observed in RA+Shh culture; however, more motor neurons were obtained by adjusting these two factors in the EC cell culture.

We next investigated how RA and Shh signalling function in the generation of motor neurons (Figure 4.2.4A). By RA itself, after 4 weeks induction, we detected a moderate expression of HB-9, a key transcription factor in motor neurons, and ChAT, the enzyme involving in neurotransmitter production of motor neurons. When we added low concentration of Shh (25ng/ml) at the same time, a moderate increased level of ChAT was detected, if we increase the Shh concentration to100ng/ml, a significant increase in ChAT and HB-9 were detected. However, the addition of high Shh concentration after 2 week RA induction failed to induce an increase in ChAT and HB-9 expression, suggesting that the cells are differentially sensitive to increased concentration of Shh. Only early Shh induction promotes the efficacy of motor neuron generation. To further confirm this observation, we induced the EC cells with a high concentration of Shh for 5 days before adding RA to the culture, as we expected, an increase of ChAT and HB-9 resembles the response of 4 weeks high Shh concentration induction. Then we investigated whether the motor neuron generation is also regulated by BMP signalling. Chordin, the BMP4 antagonist, was added to RA induced cells with either high or low concentration of Shh. Enhanced ChAT and HB-9 expression were detected in both conditions compared to RA alone or RA plus low concentration of Shh, indicating that the dorsal BMP signalling is involved in ventral motor neuron generation.

To quantify the efficacy of motor neuron generation by RA and RA+Shh (25ng/ml), we generated individual cells from the differentiated neuron aggregates, which were induced by RA and RA+Shh for 4 weeks and then treated with mitotic inhibitors for about 1 week (Figure 4.2.4B). We showed that around 90% of cells were Tuj1 positive. ChAT and three transcription factors were used to label the motor neuron in this assay. RA induced neurons showed positive cells range between 44.2% of ChAT positive and 56.6% of Isl-1 positive, while RA+Shh induced neurons showed the range of positive cells between 59.9% of ChAT and 66.5% of Isle-1, indicating that RA+Shh condition induced motor neurons at least 10% more than the RA condition. It also suggested that the Shh pathway plays an important role in motor neuron specification, even though it is less potent in neuron development.

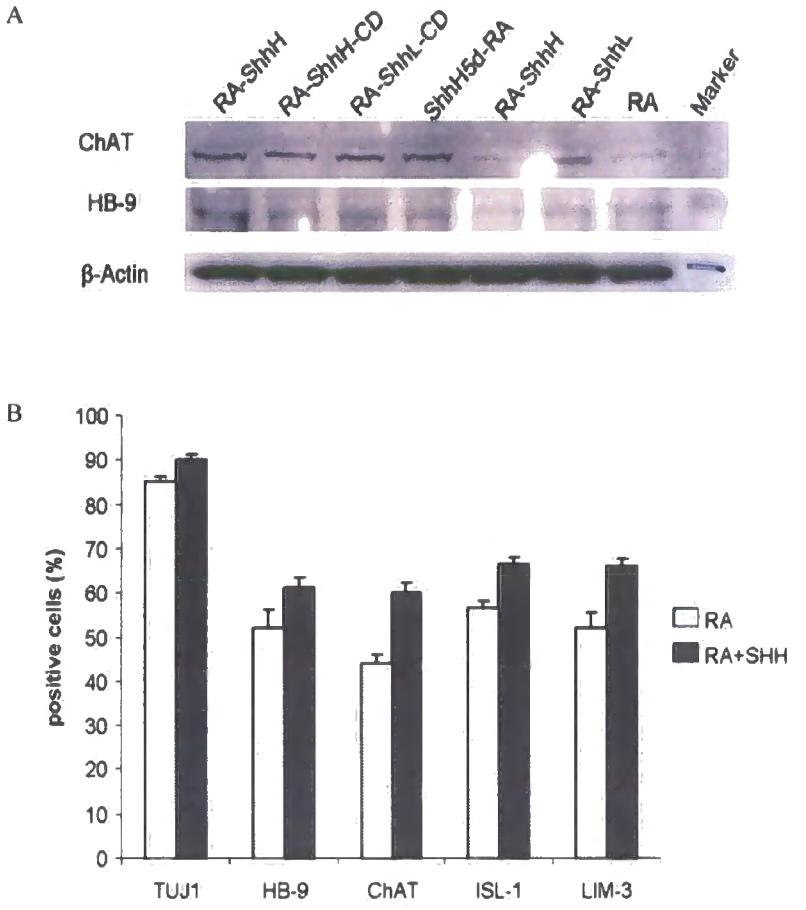

Figure 4.2.4 RA and Shh signalling in motor neuron differentiation. A, increase Shh concentration resulted in higher expression levels of motor neuron markers. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or ShhL (25ng/ml), ShhH (100ng/ml), chordin (10ng/ml) for 4 weeks before protein isolation. Motor neuron markers ChAT and HB-9 were detected by western blot. Band size is ChAT, 68-70KD; HB-9, 40KD; β-actin, 45KD. ShhH5d-RA means EC cells were treated with Shh (100 ng/ml) for 5 days before addition of RA into the culture. B, quantification of RA and RA+Shh induced neurons. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) for 4 weeks. Cells were trypsinized to become individual cells before fixation with 4% PFA and immuno-labelled with neuronal marker Tuj1 and motor neuronal markers HB-9, ChAT, ISL-1 and LIM3. Fluorescence positive cells were counted under the microscope. At least 10 fields were counted. Error bars are shown as SEM.

4.2.3 Discussion

In this section, we first constructed an artificial reduced Shh signal to mimic its reduced gradient from ventral to dorsal axis. This was achieved by applying Shh, Shh antagonist and BMPs to induce the EC cells. By RT-PCR, we examined the level of patterning transcription factors: the ventral Pax6, Nkx2.2, Isl-1; the midline En-1; and dorsal Pax3 and Pax7. Our results showed that BMP4, 6 and 7 have different abilities in the activation and suppression of different patterning transcription factors, BMP6 is a repressive factor compare to other BMPs, it can repress the ventral transcription factor expression like Pax6. Our results demonstrated that RA is another important factor that interacts with Shh and BMPs to regulate the patterning genes. RA may help setup the correct transcription factor territories and it affects most of the progenitor domains from ventral motor neuron to dorsal interneurons. Data from channel μ-slide assays also supports this view (Figure 3.2.1-3.2.9). That RA regulates the Shh pathway was supported by the observation of an increase in level of endogenous Shh expression and Gli1 expression in RA induced EC cells by western blotting. Furthermore, RA directly acts on Gli transcription factors in the nucleus since Shh antagonist cyclopamine did not have any effect on Gli1 expression of RA induced cells. Cyclopamine is said to bind to Smo, a transmembrane protein that functions as the signalling activator of the Shh receptor complex (Chen et al., 2002). Also we observed that RA induced Shh binding receptor Ptc-1's expression. This suggests that RA can act as an inhibitor for the Shh pathway by inducing PTC-1's expression. Also it can act as an activator by directly regulating Gli1 in the nucleus. Therefore, whether RA inhibits or activates the Shh pathway is depended on the cell context environment.

Studies based on the *in vivo* models suggested that Shh plays an important role in specifying motor neuron progenitors (Lewis and Eisen, 2001). Our results from western

blotting of HB-9 and ChAT supported this conclusion. However, it also suggested that RA and BMP signalling were involved in motor neuron generation. Furthermore, RA appears to be the main regulator in this process. RA itself can induce EC cells to differentiate into motor neurons, Shh promotes the efficacy, and more motor neruons were generated if chordin was added to antagonise BMP signalling. We also examined the timing for the addition of Shh signal. There is no change in motor neuronal markers expression compared to RA itself after the addition of Shh 2 weeks later than RA. On the contrary, if EC cells were induced by Shh 5 days before the addition of RA, we observed the same level of motor neuron markers expression as RA+Shh condition.

The data suggest that the culture system can serve as a good model for motor neuron generation and we can use it to study different factors affecting motor neuron differentiation, and investigate the best methods to generate motor neurons for research and cell replacement therapy.

Section 4.3

Using RNAi to study Gli1 function in Tera2.cl.SP12 EC cell model

4.3.1 Introduction

Our previous results provided the evidence that Shh regulates the early cell specification of EC cells, and furthermore, Gli1 is regulated in response to Shh and RA. Gli1 is one of the final signal transducers that mediate Shh signalling to the target genes (Lee et al., 1997). To explore the role of Gli1 during the differentiation of EC cells and in the EC DV patterning

model, we used RNAi to study the effect of suppressing Gli1 expression on EC cell proliferation and neurogenesis regulated by the related factors.

RNA interference (RNAi) is a popular technique that introduces the exogenous double stranded RNAs that are complimentary to a known mRNA into the cells and destroys that particular mRNA resulting in a diminished or abolished gene expression (Elbashir et al., 2001). It has been suggested that the RNAi machinery also play a role in antiviral defence, in which viral double stranded RNAs are target for destruction in plants and some eukaryotic organisms (Baulcombe, 1996; Scacheri et al., 2004).

When exogenous or endogenous long double stranded RNAs appear in the cell, they are recognized and cleaved by Dicer, a member of the RNase III family of double stranded RNA specific endonucleases (MacRae and Doudna, 2007). The long double stranded RNAs, which are cut by Dicer, generate small interfering RNAs (siRNA) to mediate the silencing effect, which are characterized with two nucleotide long 3' overhangs at both ends. The generated siRNAs form a ribonucleoprotein complex called RNAi silencing complex (RISC), which includes a Slicer with RNase H like domain (White-Grindley and Si, 2006). RISC first mediates the unwinding of the siRNA duplex, and then couples to a resulted single stranded siRNA. This complex will bind to a target mRNA in a sequence-specific manner. The binding mediates cleavage of the target mRNA by Slicer, the cleavage site falls in the middle of the region of siRNA complementarity (Lingel and Izaurralde, 2004). The cleaved mRNA can be recognized by the cell as foreign invader and then destroyed. Therefore, it prevents translation from happening, abolishing the expression of the gene that the mRNA was transcribed.

The single stranded siRNA that is not assocated with RISC can bind to their target mRNA in a sequence-specific way and serve as a primer for RNA dependent RNA polymerization to polymerize the anti-sense RNA strand. This amplification results in new double stranded

siRNAs and is further involved in cleavage and amplification process until the specific mRNAs are all degraded. (The mechanism of RNAi is summarised from Nature Reviews Genetics and Arkitek Studios Collaborate on RNAi Animation (http://www.nature.com/focus/rnai/animations/index.html).)

However, the long double stranded RNAs are not suitable for mammalian cells because they will trigger a non-specific degradation of mRNA. After extensive studies on this, the researchers now confirm that 21 to 23 nucleotides long double stranded RNA fragments can induce gene-specific inhibition of expression in C.elegans as well as mammalian cells (Fire et al., 1998; Elbashir et al., 2001). The application of RNAi in mammalian cells provides us a powerful tool to determine the gene function by blocking the expression of a specific mRNA (Elbashir et al., 2001). The RNAi technique, that mimics the gene knock-out in a simple, fast and specific way, also can be delivered into living organs so that opens the possibility for gene therapy.

In this study, we transfected the EC cells with 21 nucleotides long double stranded RNAs, which target human Gli-1 mRNA, ordered from QIAGEN Ltd. The target sequence of Gli1 siRNA is CCA GCC CAG ATG AAT CAC CAA. The sense sequence of Gli1 siRNA is AGC CCA GAU GAA UCA CCA A, and the antisense sequence is UUG GUG AUU CAU CUG GGC U. All the sequences are provided by QIAGEN Ltd. The transfected cells were induced by RA or Shh and assessed for their proliferation activity and differentiation ability.

4.3.2 Results

4.3.2.1 RT-PCR of Shh pathway in very early induced Tera2.cl.SP12 EC cells

To choose *Gli1* as target gene to down regulate the Shh signalling, we need information about the interaction of *Gli1* and Shh, *Gli1* and other Shh pathway components. We have shown that the Shh pathway appears to be activated during the first weekof EC cells differentiation. However, it will be interesting to know the expression of Gli1 in the very early induction. RT-PCR was performed and samples were collected after 30min, 1 hour and 4 hours induction. For the selected time points, Shh induced cells showed a clear expression of *Ptc-1*, *Gli-1* and *Gli-3*, also a weak band of *Gli-2* (Figure 4.3.1). However, we did not observe any effect on these expression profiles when we added cyclopamine to the condition at the same time, suggesting that cyclopamine did not block the Shh signalling at this early stage, suggesting that it is not appropriate to use cyclopamine to inhibit the effect of Shh at early stage of EC cell differentiation, although cyclopamine is widely used as Shh pathway inhibitor (Incardona et al., 1998; Chen et al., 2002). In RA alone induced cells, similar expression was observed, indicating that RA possibly activated the Shh pathway via its nuclear receptors. While in RA+Shh or RA+Shh+CP condition, the levels of gene expression did not significantly differ from the Shh or RA induced cells. The results suggested that Shh induced EC cells are not sensitive to RA and cyclopamine at that stage. From 1 week and 2 week induction results (Figure 4.1.5), we can see that *Ptc-1* and *Gli-1* was regulated by Shh, RA and cyclopamine, therefore, we consider that different group of progenitors have been developed from the early induced stem cells, suggesting that selective differentiation is undergoing in the induced EC cells. Gli2 and Gli3 are usually cleaved to generate repressor or activator in response to Shh induction (Ruiz i Altaba, 1998). Whether they choose to be a repressor or an activator is depended on the cell

context (Ruiz i Altaba, 1999). We did not understand the sustained expression of *Gli-2* and *Gli-3* from the early stage to the later induction, and we do not know whether the detected bands are their repressor form or activator form during the differentiation. To clarify the function of Gli2/3 and further understand the Shh pathway in EC cell differentiation, further experiments will be required.

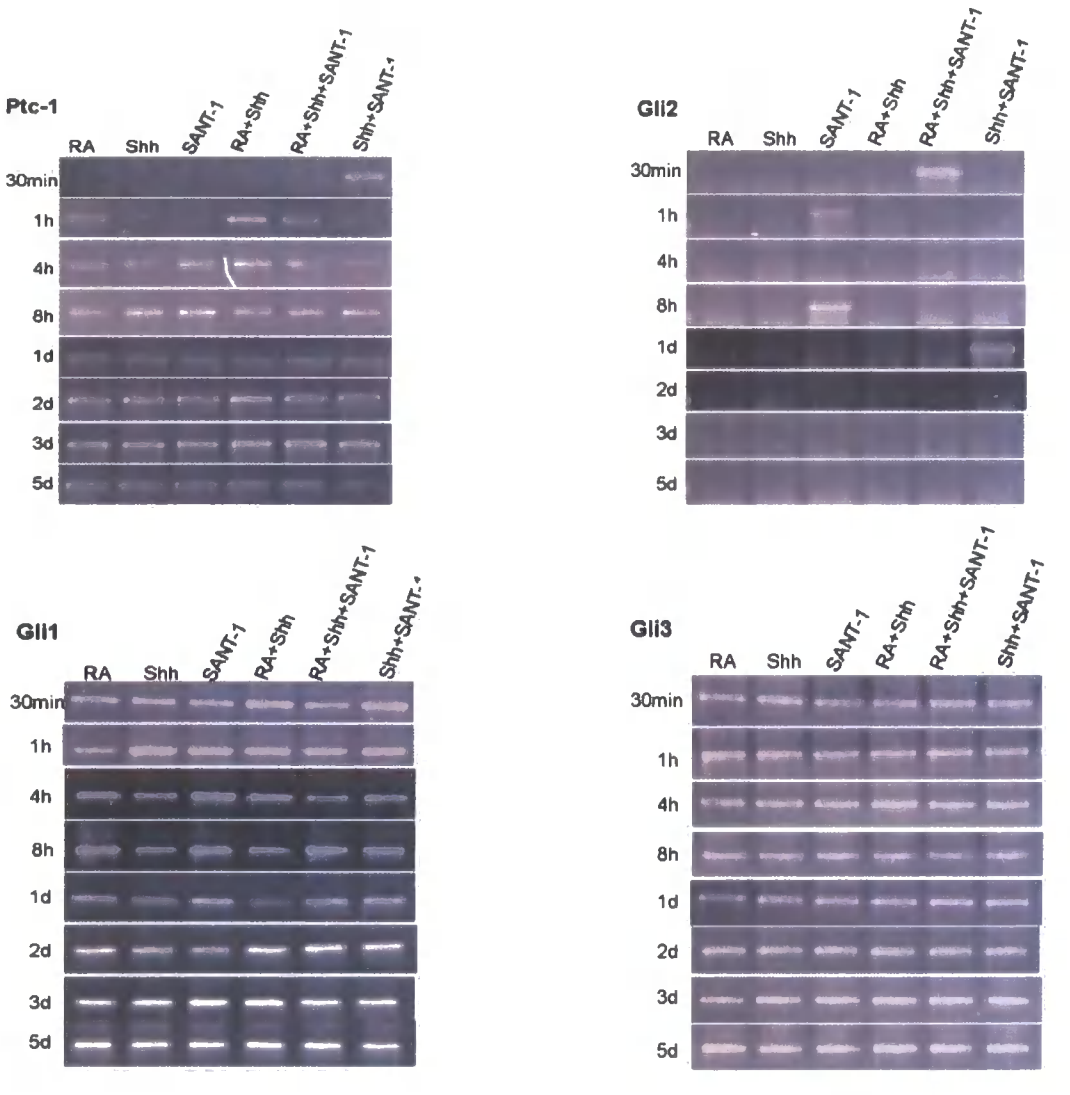

Figure 4.3.1 RT-PCR analysis of Shh pathway genes in induced EC cells during early stages of differentiation. EC cells were seeded in the 6 well plate at 100,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml), SANT-1 (30nM) for the indicated time points before isolation of total RNA and analysed the expression of the Shh pathway gene Ptc1, Gli-1, -2 and -3. EC cells express no Ptc-1 and Gli2, but are positive for Gli1 and Gli3 (data not shown). SANT-1 is Shh antagonist.

4.3.2.2 Gli-1 and endogenous Shh expression in induced Tera2.cl.SP12 EC cells

Gli1 is a Shh-inducible transcription factor in EC cells. It is important to know how it is regulated in EC cells before knocking down the expression. The results from western blotting were consistent with the 30min to 4 hour RT-PCR results described above. However, RA started showing a diminishing Gli-1 expression, from 24 hours to 5 days induction, until there is no expression at 2 weeks (Figure 4.3.2). Treatment with Shh antagonist cyclopamine and SANT-1 reduced the Gli-1 expression from day5. Furthermore, Gli-1 expression was abolished in all samples after 2 weeks of induction. We also examined endogenous Shh expression by Western blotting. We detected a strong band of N-Shh in EC cells without treatment, and a weak expression of Gli-1. Interestingly, the levels of endogenous N-Shh expression were not altered by RA but were by Shh antagonists (shown in Fig 4.2.3), suggesting that RA activates endogenous Shh expression and this activation is not affected by cyclopamine and SANT-1. Because EC cells express endogenous Shh, it is not possible to assess the function of exogenous inductive factors, such as RA and Shh and BMPs, without the interrupted effect from endogenous Shh.

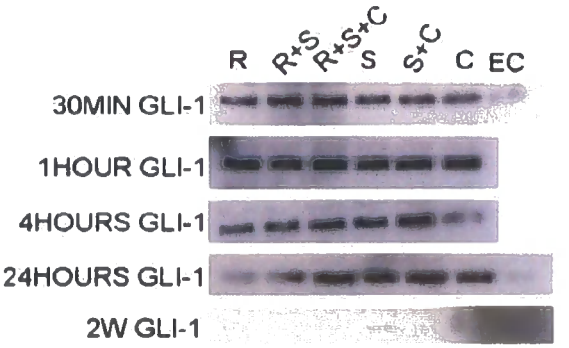

Figure 4.3.2 Western blotting analysis of Gli1 expression in induced EC cells during early stages of differentiation. EC cells were seeded in the 6 well plate at 100,000 cells/cm^2 supplemented with RA (R, 10μM) and/or Shh (S, 25ng/ml), cyclopamine (C, 1μM) for the indicated time points before isolation of cytoplasmic protein and analysed the expression of the Shh pathway effector Gli-1.

4.3.2.3 The efficacy of siRNA transfection in Tera2.cl.SP12 EC cells

To examine the transfection rate of the siRNA assay, the negative control, Non-silencing Control siRNA Alexa Fluor 488 was used to transfect the EC cells and images were taken after 48 hours. The cells with green fluorescence (Alexa Fluor 488) spots were counted under the microscope. Around 90% of cells had spots, which is consistent with the transfection rate revealed by the handbook (Figure 4.3.3).

4.3.2.4 Gli1 siRNA transfection down-regulates the expression of Gli1 and Pax6 in Shh induced Tera2.cl.SP12 EC cells

We know that Shh induces the expression of Gli1 is conserved in vertebates (Bai and Joyner, 2001; Bai et al., 2002). If we add Shh to the Gli1 siRNA transfected EC cells, it should be clear whether or not the Gli1 expression is inhibited in these cells. Non-transfected EC cells were used as positive control. After 24hours, we observed a moderate decrease in the intensity of the fluorescence staining of Gli1 in siRNA transfected EC cells compare to non-transfected cell (Figure 4.3.4.1). However, there is no significant decrease in Pax6 and nestin staining between transfected and non-transfected cells, the possible reason for that is due to the sensity of immuno-fluorescence staining (Figure 4.3.4.2). After 48hours, Pax6 staining staining was greatly down-regulated and nestin staining was also slightly down-regulated (Figure 4.3.4.5), accompanied by decreased Gli1 staining (Figure 4.3.4.4). By western blot analysis, we detected down-regulated level of GLI1 and PAX6 expression after 24 hours induction, indicating that Gli1 siRNA knocks down the expression of Gli1 expression and it also affects the expression of Pax6 (Figure 4.3.4.3). Therefore, we demonstrated that Gli1 siRNA is effective in knocking down the expression of Gli1 in EC cells. The results also suggest that Gli1 is involved in regulating transcription factor expression.

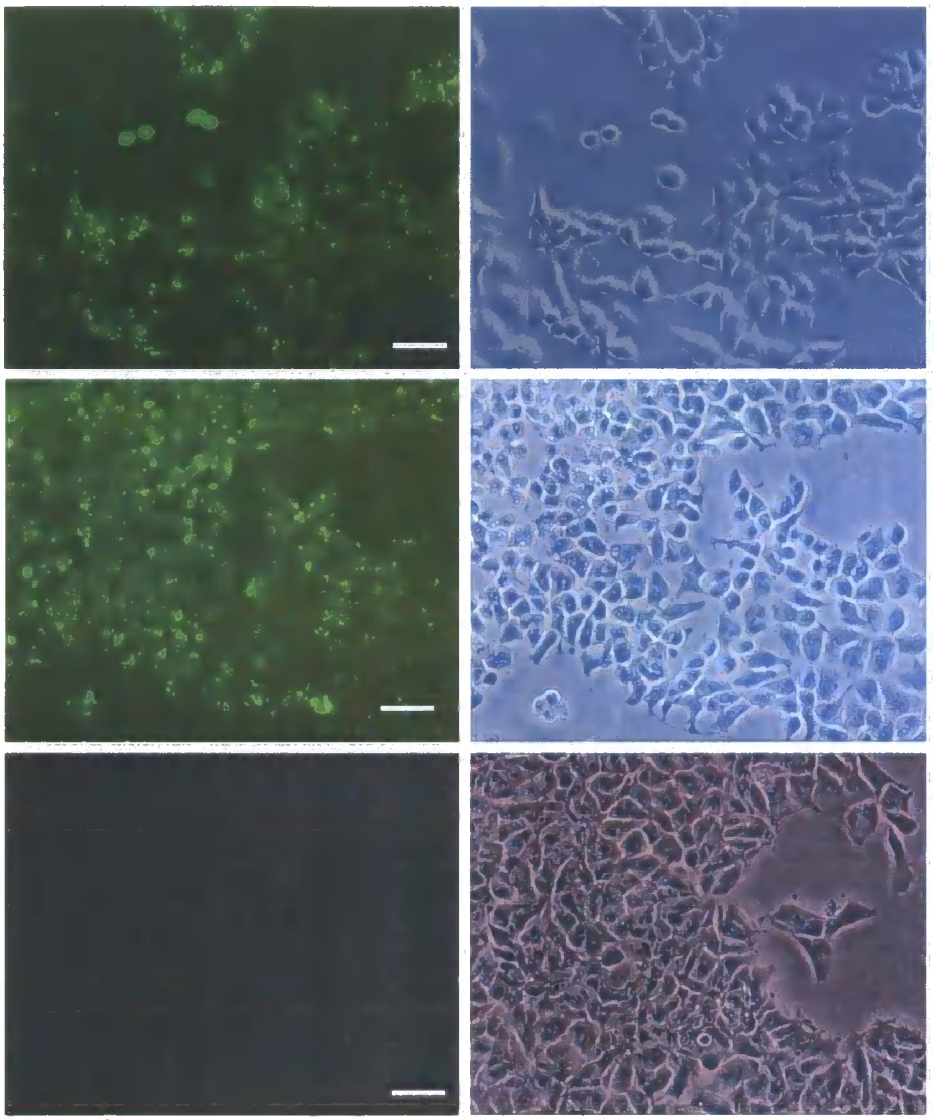

Figure 4.3.3 Non-silencing Control siRNA Alexa Fluor 488 transfected EC cells. EC cells were seeded at 100,000cells/well of a 24-well plate in 0.5ml EC cell growth medium. 37.5ng siRNA and 3µl HiPerFect transfection reagent were mixed and added to the cells, and then incubated for 48h before microscopic analysis. Note that the transfected Cells have green fluorescence spots under the microscope and transfection rate can be calculated. Non-transfected cells have no fluorescence at all. Scale bars = 50µm.

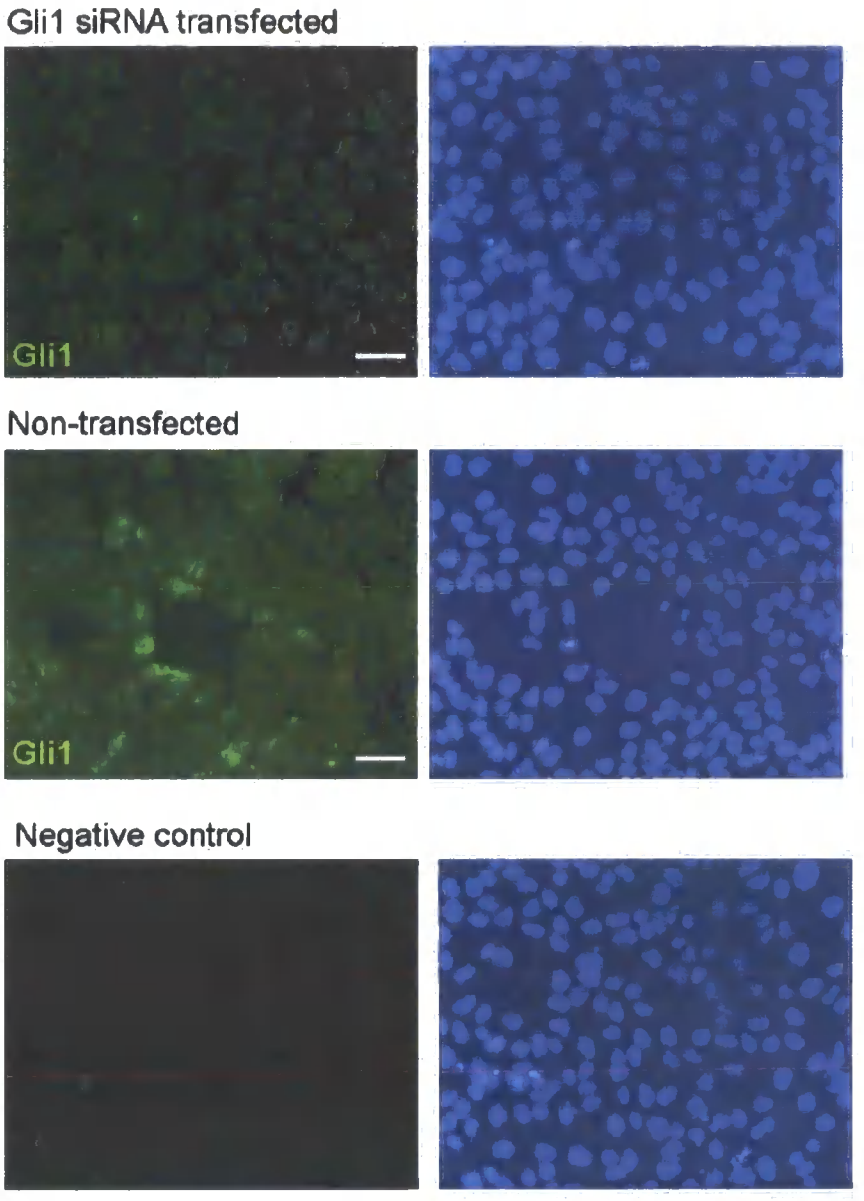

Figure 4.3.4.1 The expression of Gli1 is down-regulated in Gli1 siRNA transfected EC cells. EC cells were seeded at 100,000cells/well of a 24-well plate in 0.5ml EC cell growth medium. 37.5ng siRNA and 3µl HiPerFect transfection reagent were mixed and added to the cells, and then incubated for 24h. Cultures were exposed to Shh (25ng/ml) for 24h before fixation with 4% PFA and immuno-labelled with Gli1 (green). Note that the transfected Cells expressed less Gli1 than non-transfected cells have no fluorescence at all. Scale bars = 50µm. Cells that are stained with secondary antibodies serves as negative control.

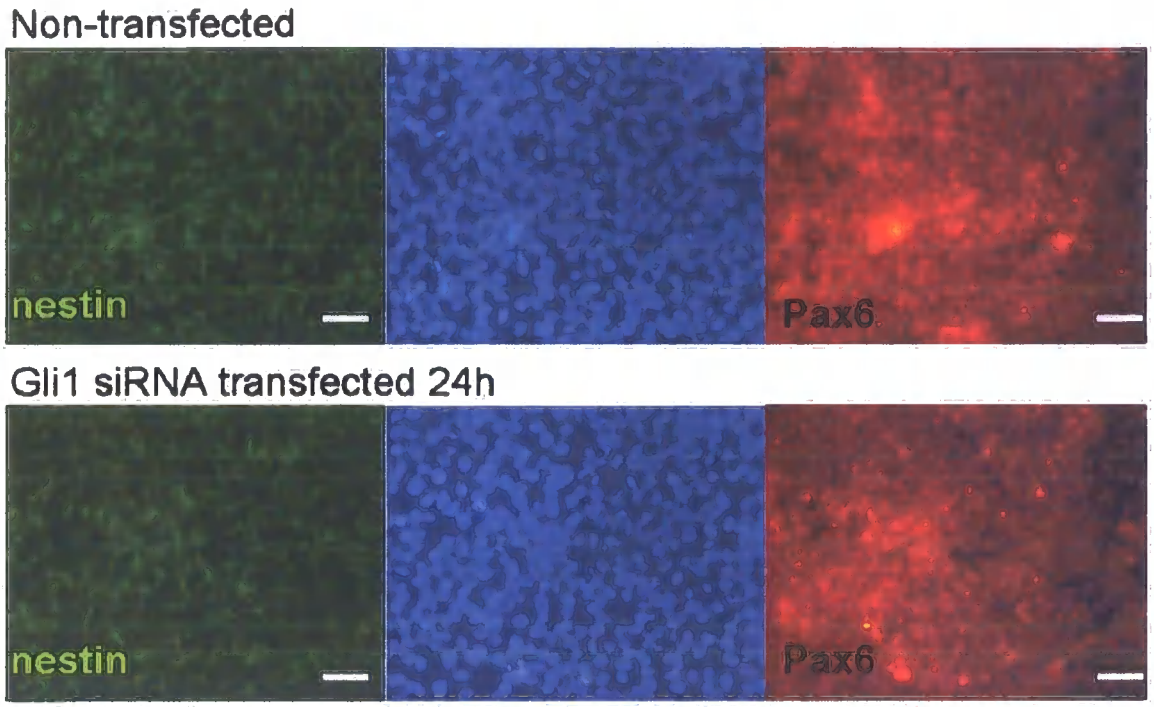

Figure 4.3.4.2 The expression of nestin and Pax6 is not significantly down-regulated in 24 hours Gli1 siRNA transfected EC cells. EC cells were seeded at 100,000cells/well of a 24-well plate in 0.5ml EC cell growth medium. 37.5ng siRNA and 3µl HiPerFect transfection reagent were mixed and added to the cells, and then incubated for 24h. Cultures were exposed to Shh (25ng/ml) for 24h before fixation with 4% PFA and immuno-labelled with Nestin (green) and Pax6 (red). Scale bars = 50µm.

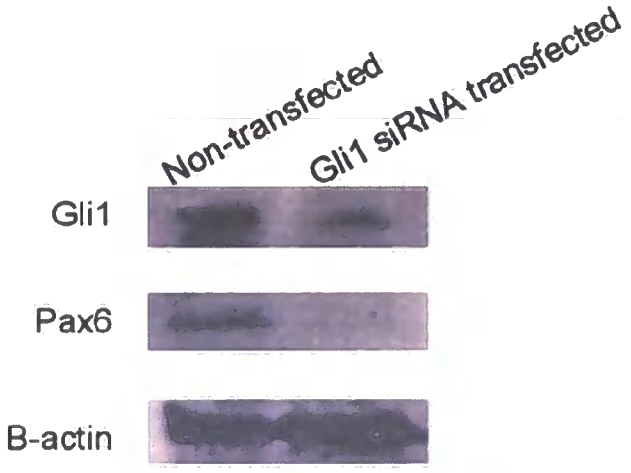

Figure 4.3.4.3 Western blot analysis of Gli1 and Pax6 expression in Gli1 siRNA transfected EC cells. EC cells were seeded at 600,000cells/well of a 6-well plate in 2.3ml EC cell growth medium. 150ng siRNA and 12µl HiPerFect transfection reagent were mixed and added to the cells, and then incubated for 24h. Cultures were exposed to Shh (25ng/ml) for 24h before western blot analysis for Gli1 and Pax6. The detected bands are Gli1 115KD, Pax6 50KD, and β-actin 45KD.

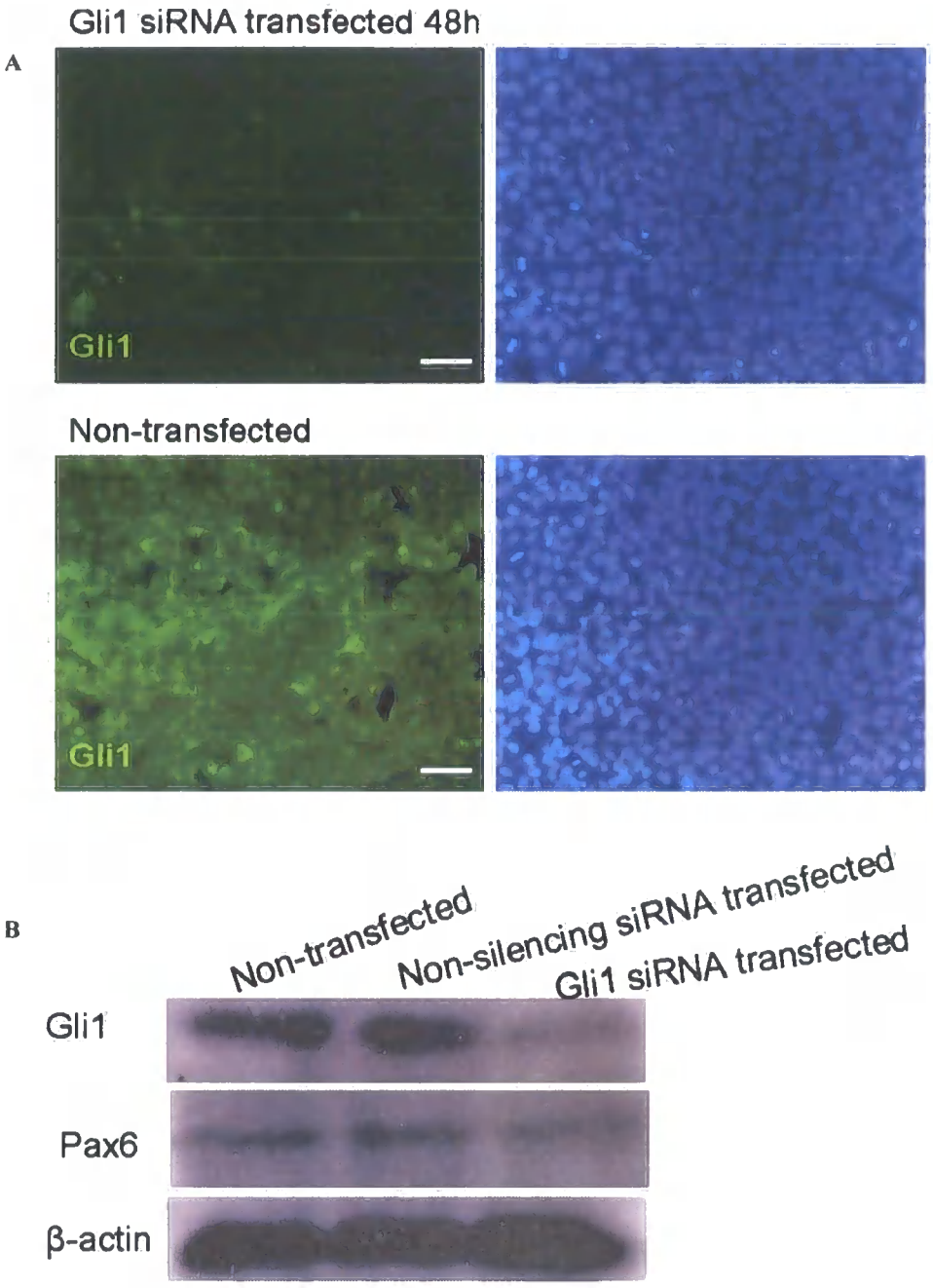

Figure 4.3.4.4 A, The expression of Gli1 is down-regulated in 48 hours Gli1 siRNA transfected EC cells. EC cells were seeded at 100,000cells/well of a 24-well plate in 0.5ml EC cell growth medium. 37.5ng siRNA and 3μl HiPerFect transfection reagent were mixed and added to the cells, and then incubated for 48h. Cultures were exposed to Shh (25ng/ml) for 24h before fixation with 4% PFA and immuno-labelled with Gli1 (green). Scale bars = 50μm. B, Cytoplasmic proteins isolated from the 48-hour Gli1 siRNA transfected cells were analysed by western blot, and non-transfected cells and cells transfected with non-silencing siRNA were served as controls. The detected bands are Gli1 115KD, Pax6 50KD, and β-actin 45KD.

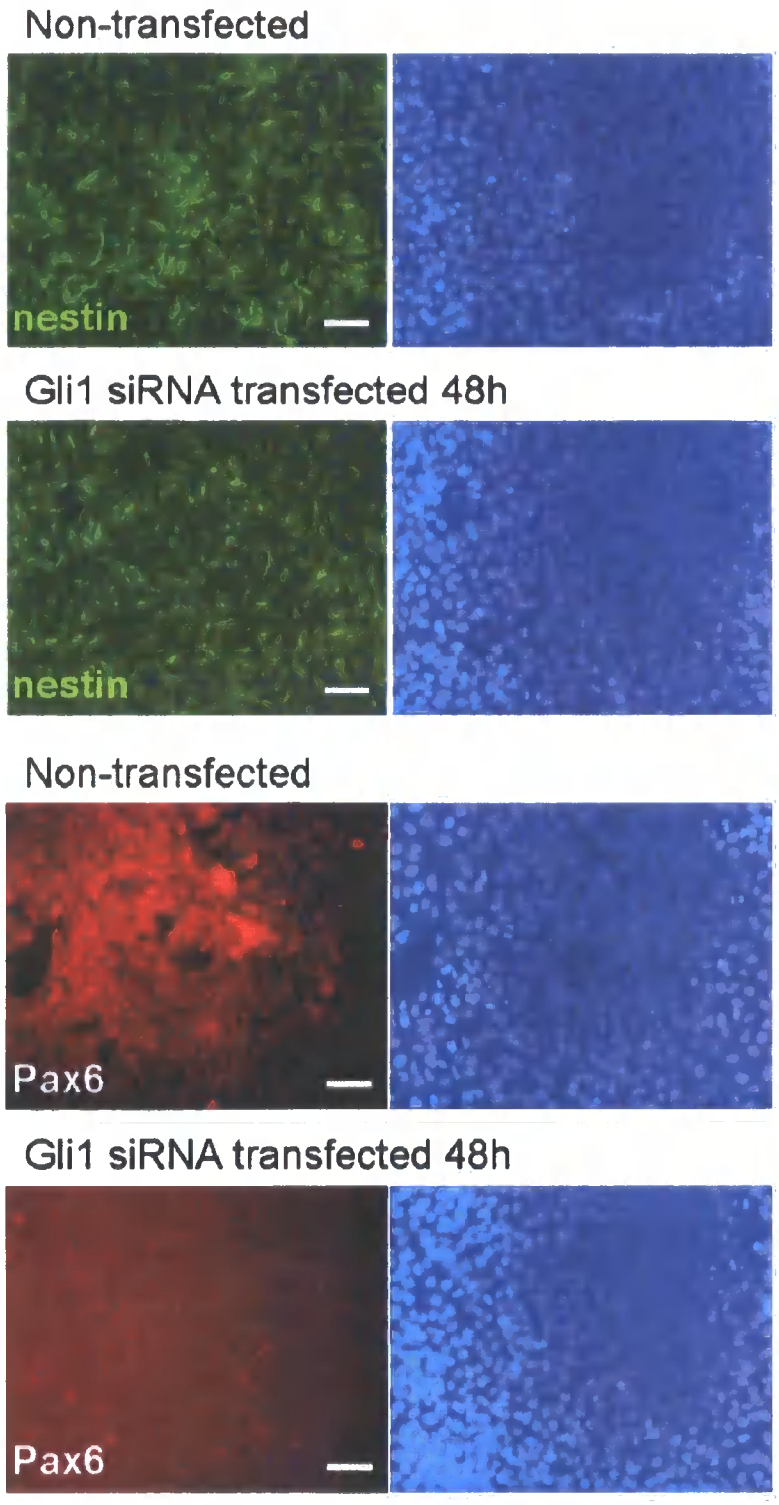

Figure 4.3.4.5 Pax6 not nesting is greatly down-regualted in Gli1 siRNA transfected EC cells. EC cells were seeded at 100,000cells/well of a 24-well plate in 0.5ml EC cell growth medium. 37.5ng siRNA and 3µl HiPerFect transfection reagent were mixed and added to the cells, and then incubated for 48h. Cultures were exposed to Shh (25ng/ml) for 24h before fixation with 4% PFA and immuno-labelled with Nestin (green) and Pax6 (red). Scale bars = 50µm.

4.3.2.5 Gli-1 transcription factor appears to mediate cell proliferation in Tera2.cl.SP12 EC cells

After the cells were transfected for 48 hours, the inductive factors like RA and Shh were added to the transfected cells. To control for this assay, we also did a set of induction with non-transfected EC cells. We did not observe a significant decline in the number of Gli-1 siRNA transfected EC cells compared to the non-transfected EC cells. The treatment with RA and/or Shh did not have any effect on the increase of cell number, however, these factors induced an increase in the non-transfected EC cells (Figure 4.3.5). It suggests that Gli-1 does mediate the proliferative activity of EC cells. Furthermore, the proliferative function of RA and Shh signalling appear to involve the Gli-1 transcription factor.

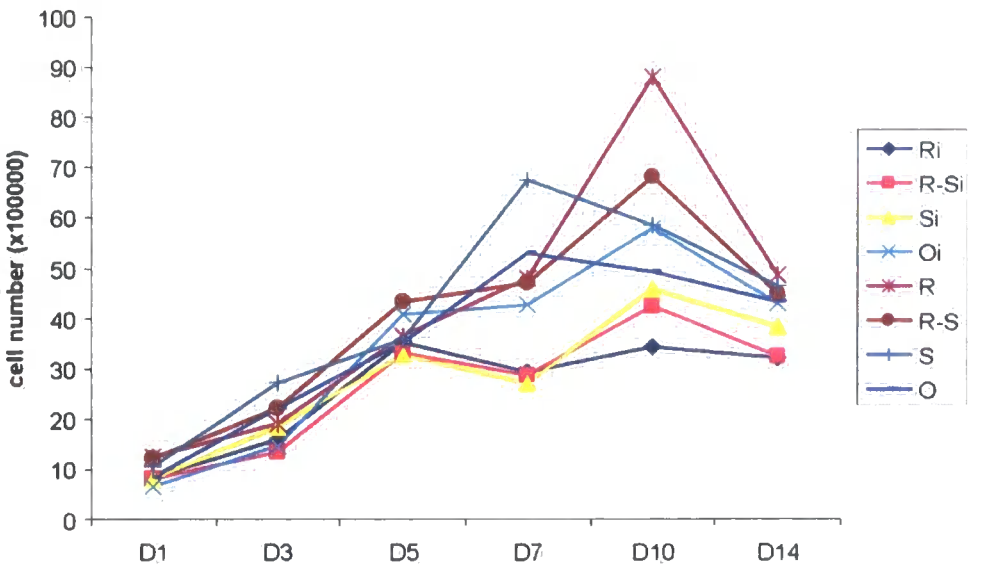

Figure 4.3.5 Gli1 siRNA transfected EC cells have decreased proliferative ability. Transfected and non-transfected EC cells were seeded at 100,000cells/well of a 24-well plate before addition of RA (R, 10μM) and/or Shh (S, 25ng/ml) or no additional factor (O). Cells were typsinized and counted using a hematocytometer at the indicated time points. n=3. Transfected conditions wer labelled as Ri, R-Si, Si and Oi.

4.3.2.6 Gli-1 transcription factor is involved in neurogenesis by Tera2.cl.SP12 EC cells

Patterning transcription factor Pax6 was used to examine the Gli1 siRNA transfected EC cells. The expression of Pax6 was all but abolished in the RA and/or Shh induced siRNA transfected cells, suggesting that Gli1 is involved in regulation of DV patterning genes (Figure 4.3.6). Ki67 is a nuclear proliferation marker, it is expressed in the center of rosette like structure in the RA and RA+Shh 2 weeek induced EC cells, however, there were no positive Ki67 cells appear in the center of the small rosettes in the Gli1 siRNA transfected EC cells under the same condition. This suggested that Gli1 was involved in mediating the proliferative activity in RA or Shh induced cells. Furthermore, even though rosettes were formed in RA and RA+Shh induced condition in Gli1 siRNA transfected EC cells, the size of the rosettes was much smaller than in the induced control populations. This indicated that inhibition of Gli1 can not prevent the cells from committing to a neuro-epithelial fate but can decrease the proliferation of committed cells. For the neuronal maker Tuj1, we observed little to no expression in the induced Gli1 siRNA transfected cells, indicating that the abrogation of Gli1 disturbed the normal neuron differentiation of EC cells. Another neural progenitor marker nestin was also affected. Fewer nestin positive cells were induced in the transfected EC cells compared to the non-transfected cells. These data further suggest that Gli1 not only increases cell number but also plays a role in neurogenesis.

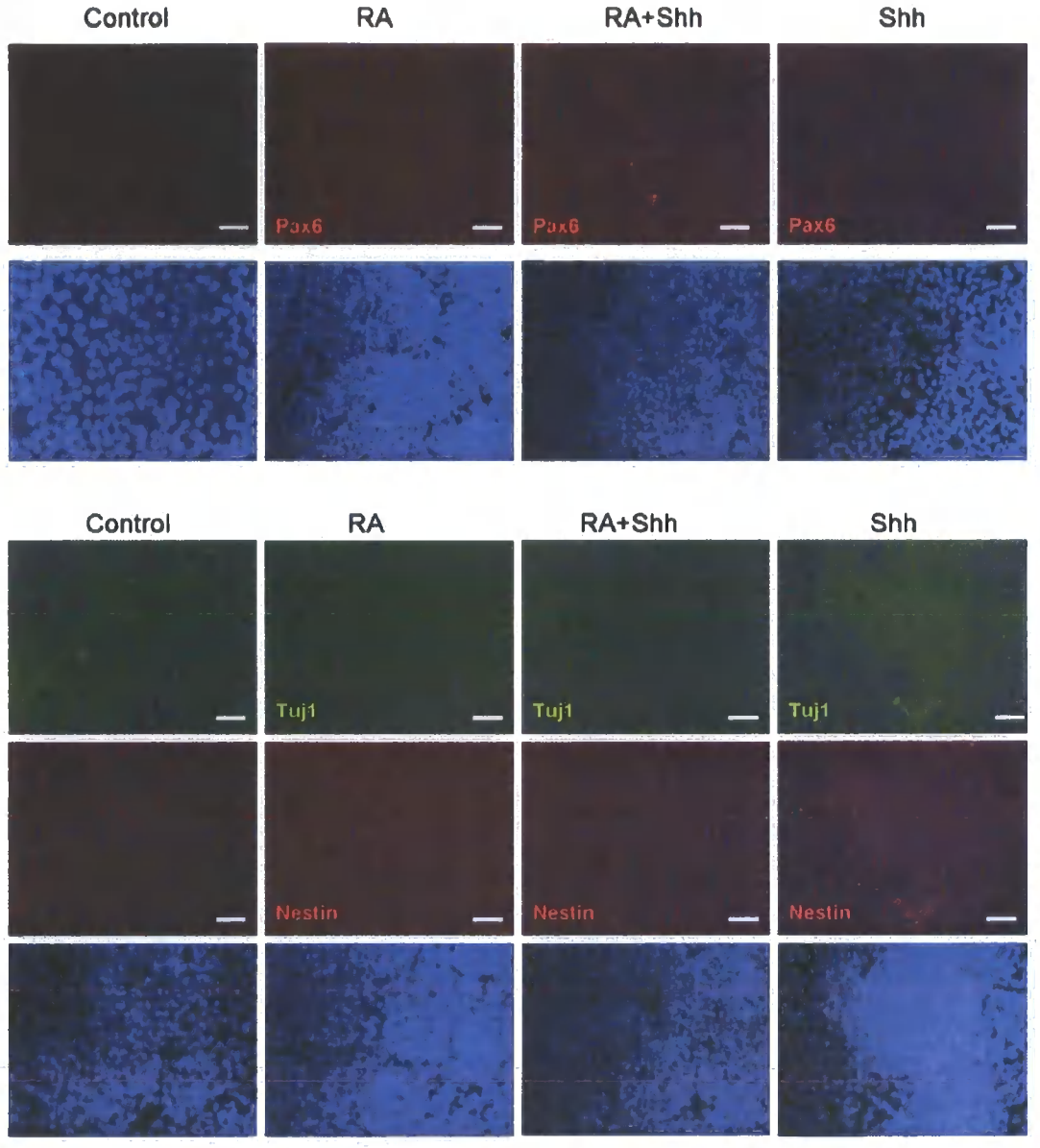

Figure 4.3.6 Pax6 and Tuj1 and Nestin are negative in Gli1 siRNA transfected EC cells after RA and/or Shh induction. EC cells were seeded at 100,000cells/well of a 24-well plate in 0.5ml EC cell growth medium. 37.5ng siRNA and 3µl HiPerFect transfection reagent were mixed and added to the cells, and then incubated for 48h. Cultures were exposed to Shh (25ng/ml) and/or RA (10µM) for 1 week before fixation with 4% PFA and immuno-labelled with Tuj1 (green) and Nestin (red). Cell proliferation marker Ki67 is also negative (data not shown). Controls were cells stained with secondary antibodies. Scale bars = 50µm.

4.3.3 Discussion

All three Gli genes are expressed in vertebrate neural tube, indicating that they play an important role in neurogenesis (Nguyen et al., 2005). The Gli proteins are the mediators of Shh signalling in the neural tube, however, it is unclear how Gli proteins mediate Shh signalling in multipotent proliferating progenitor cells and regulate cell fate and cell behaviour. From the *in vivo* models, it is known that three Gli genes function partially redundantly and cooperatively in mediating Shh signalling (Ruiz i Altaba, 1998). Gli1 behaves as a positive activator and also is strictly dependent on the Shh signal for its expression, and thus is an excellent indicator of positive Shh signalling (Bai et al., 2002); Gli2 can act as a positive activator or a negative repressor in Shh signalling, its function can be replaced by Gli1 (Bai and Joyner, 2001); Gli3 can act as both activator or repressor *in vivo*, and can cooperate with or inhibit Gli1 function *in vitro* (Tyurina et al., 2005). In this study, we found that all three Gli genes were expressed in our *in vitro* EC cell model, Gli2 and Gli3 were expressed even in the absence of exogenous Shh signal and showed no response to inhibitory regulators. In contrast, Gli1 was strictly dependent on Shh and the inhibitors for its expression, which is consistent with recent studies (Bai et al., 2002; Bai et al., 2004). As an indicator for Shh, the expression level of Gli1 increases in response to Shh induction, this provides us a tool to determine the effect of Gli siRNA.

We have clues that the combinatory effect of all three Gli genes in EC cells will lead to EC cell proliferation and cell specification, however, their individual requirement in the neurogenic program has not been determined. In this study, we used RNAi to mimic the knockdown of Gli1 to determine the requirement of the Gli1 protein in mediating Shh signalling in neurogenesis. From our results, we first noticed that Gli1 function is not completely replaced by Gli2 or Gli3, the two Shh mediators that were not knocked down in

this study. The Gli1 siRNA transfected cells showed significant difference compared to non-transfected cell: tranfected cells had reduced cell proliferation activity; in Shh-induced cultures, when Gli1 expression was reduced, their expression of Pax6 and Tuj1 was suppressed.

The transfected cells showed that the addition of Shh failed to influence cell proliferation. This was consistent with expression of the proliferation marker Ki67, which is not expressed in Shh induced transfected cells.

It has been shown in this study that RA and RA+Shh induced EC cells from neuro-epithelial rosettes and express Pax6 confined in the nuclei. Although these two conditions can still induce rosettes to form in cultures of Gli1 siRNA transfected EC cells, the size of the rosettes were significantly smaller. Furthermore, rosettes showed decreased Ki67 staining cells in their center of rosettes, which is present in control cultures. It is demonstrated that Gli1 activate the downstream genes that will regulate cell proliferation, Gli1 siRNA transfected cells expressed no Ki67 suggesting that cells can not enter the cell cycle to divide without Gli1 activation. Furthermore, no expression of Pax6 in the Gli1 siRNA transfected cells suggested that Gli1 has the ability to induce or maintain the Pax6 expression, which is a patterning transcription factor and plays important role in motor neuron specification. In the control cultures, RA and RA+Shh induce Tuj1 positive cells after 2 weeks differentiation, however in Gli1 siRNA transfected cells, the expression of the neuronal marker Tuj1 was all but abolished, suggesting that without the Gli1 signal the progress of neuron differentiation is disturbed.

Based on these data, we conclude that Gli1 is an important regulator of cell proliferation in EC cells by promoting the cells to enter the cell cycle. Furthermore, we provide evidence that Gli1 plays a role in DV patterning and regulates Pax6 expression. The formation of neural rosettes in RA induced Gli1 siRNA transfected cells, suggests that RA plays a role

in the advancement of EC cell differentiation towards neurons. However, the abolition of Tuj1 expression indicates that Gli1 also contributes to neural differentiation. Thus, we provide evidence here that Shh signalling plays an important role in the differentiation of human EC stem cells into neuroectoderm.

Chapter Five

Functional assessment of motor neuron phenotypes differentiated from Tera2.cl.SP12 EC cells

5.1 Introduction

The function of the nervous system is to receive stimuli from both the internal and external environment to produce proper co-ordinated responses in effector organs after analysis and integration (Wheater, 2000). This function depends on the intercommunication network that connects neurons to neurons and neurons to target cells. The synapses, the site of cell-cell inter-communication, are specialised structures where depolarized neurons release neurotransmitters to initiate an action potential in other neurons. Neurotransmitters not only mediate neuron to neuron transmission but also act as chemical inter-mediates between the nervous system and effector organs. Neuromuscular junctions, which are motor neurons synapsed with skeletal muscle fibers, have long been served as models of synaptogenesis to investigate synaptic structure, function and development, because of its large size, easy accessibility and relative simplicity (Grinnell, 1995a).

Myogenesis

Before the formation of nerve-muscle contacts, myogenesis and neurogenesis are two individual processes. It is known that skeletal muscle of the vertebrate embryo derives from somites, segments of paraxial mesoderm that form on either side of the neural tube and notochord (Gilbert, 2003). The paraxial mesodermal cells that commit to a specific muscle lineage are called myogenic progenitors. Although these cells are not terminally differentiated, their normal cell fate is muscle. Depending on their locations, they become different groups of muscle precursors: cells located at cephalic mesenchyme and pre-chordal plate will differentiate into head muscles; cells at dorso-medial somite will form back muscles; cells at dorso-lateral somite will form body wall ventral muscle, appendicular and tongue muscles (Grinnell, 1995b). The muscle formation is regulated by

the signals from the surrounding tissues (Hollway and Currie, 2003). The ordered progression of myogenesis requires β-catenin-dependent Wnt signalling from the dorsal ectoderm, especially Wnt-4, -6 and -7a, which play a crucial role in stimulating myogenesis and MyoD activation (Muntoni et al., 2002). Inhibitory signals from lateral-plate mesoderm are TGF-β family members, such as Activin A limits the proliferation of progenitor cells to prevent excessive muscle growth, follistatin prevents excessive muscle cell fusion and hypertrophy, and BMP-2, -4 and -7 prevent premature activation of MyoD (Whittemore et al., 2003). The formation of slow muscle fibers depends on Hedgehog signalling acting through Gli transcription factors from notochord. FGF-8 signaling is not only required for MyoD expression but also the differentiation of a subset of fast muscle fibers. Retinoic acid signalling ensures the symmetric left-right somitogenesis also mediated by FGF-8 signaling (Parker et al., 2003).

Myogenic differentiation proceeds through irreversible cell cycle arrest of precursor cells--myoblasts(Buckingham et al., 2003). Myoblasts are proliferating cells that are fully determined to myogenic phenotype and capable of terminal differentiation. In adulthood, they exist in the form of muscle satellite cells. Myoblasts usually express muscle regulatory factor genes including MyoD, Myf-5, myogenin (Myf-4), and Myf-6 (Mrf-4, Herculin) (Blais et al., 2005). A gradual increase in expression of these genes leads to fusion of myoblasts into multinucleate myotubes which grow and elongate by cell fusion. Myoblasts condense into myotubes in two waves (Grinnell, 1995b). The first wave produces primary myotubes, served as scaffold for the second burst of myoblast fusion that produces a much larger number of secondary myotubes under the basal lamina of the primary muscle fibers. The first muscle fiber is formed from primary myotube when the primary myotube exhibits cross-striated myofibrils and was surrounded by basal lamin. Most of the primary muscles become slow muscle fibers, while the secondary muscle fibers acquire features of fast muscle fibers. The motor axons innervate all of the primary myotubes; the number of the primary myotubes approximate the number of motor axons, because the motoneuron cell death occurs at about this time, the success in innervating primary muscle fibers may play a significant role in determining the motoneuron survival. Treatment with α-bungarotoxin prevents innervation, causing reduced number of primary fibers and severely reduced secondary fibers. Furthermore, in the mouse muscular dysgenesis (mdg) mutant, that has hyperinnervated muscles, showed that the number of secondary myotubes is more than twice of the wild type (Ashby et al., 1993b). This indicates that the development and

differentiation of muscle fibers especially from secondary myotubes are dependent on innervations.

In the embryo, myogenesis represents a complex network of interactions between factors, which control the cell cycle, cell determination and differentiation, cell migration, cell contacts and re-organization of the intracellular structures (Parker et al., 2003). Myogenic regulatory factor genes and the upstream Pax genes have important roles in regulating the entry of a cell into the myogenic program (Buckingham et al., 2003). While MyoD and Myf-5 are necessary for the determination of the muscle cells, Myf-6 and especially Myf-4 are essential for later processes of the fusion of myoblasts into myotubes. Mice deficient in Myf-4 exhibit a failure of secondary myogenesis and myoblast fusion with only some residual myotubes were observed (Buckingham et al., 2003). In the mice lacking both MyoD and Myf-5, a complete absence of myoblasts was observed (Rudnicki et al., 1993). The role of MyoD in myogenic differentiaiton appears to be more complex, since it acts as transcriptional repressor as well as activator. The ChIP-on-chip analysis identified that MyoD in growing myoblasts bound to a set of genes involved in synapse specification and utilization and neuromuscular function like Semaphorins, Rapsn (receptor associated protein of synapse), Musk (muscle, skeletal, receptor tyrosine kinase), nicotinic cholinergic receptor poly-peptides, furthermore, MyoD in myotubes bound those genes related to muscle development and contraction, such as desmin, Musk, troponin, myosin (Muntoni et al., 2002). Together with the $MyoD^{-/-}$ mutant mice, it is confirmed that muscle cells have their own intrinsic transcriptional program for establishing synapses and that these are controlled at least in part by the muscle regulatory factors (Wang et al., 2003).

Post-synaptic preparation for NMJ formation

The formation of the neuromuscular junction is characterized by the progressive accumulation of nicotinic acetylcholine receptors (nAChRs) in the postsynaptic membrane facing the nerve terminal, induced predominantly through the muscle-specific kinase (MuSK) signaling cascade. There are four major components which function in this process: agrin, a heparin sulphate proteoglycan secreted from motor nerve; MuSK; AChR; and rapsyn, a peripheral membrane component required for the aggregation of the AChR in the muscle membrane (Finn et al., 2003).

Agrin is a large molecule with numerous polypeptide domains. Its N-terminal has a metalloproteinases-1 domain, which mediates high affinity binding to laminins and helps

anchor agrin to the basement membrane. The C-terminal region binds to integrin, dystroglycan, and heparin, and the final laminin-AG like domain in C-terminus is necessary for induction of AChR clustering (Ngo et al., 2007). The nerve derived agrin triggers AChR clustering or maintains clusters through the coalescence of lipid rafts, which play a pivotal role in the assembly of the post-synaptic membrane at the NMJ during agrin signalling (Stetzkowski-Marden et al., 2006a; Stetzkowski-Marden et al., 2006b). Lipid rafts are microdomains on plasma membranes. These domains are enriched in sphingo-lipids and cholesterol and fromed islands in the plasma membrane. The lipid rafts can diffuse laterally in the plasma membrane. The proposed model is, after agrin stimulation, MuSK translocates into lipid rafts to initiate signalling machineries necessary for AChR clustering, concomitantly, the AChR is recruited to the raft microdomains, where it interacts with rapsyn, which is constitutively localized in lipid rafts (Zhu et al., 2006). There is no doubt that MuSK is the post-synaptic organizer; and we know that MuSK also mediates agrin-independent AChR clustering. An unresolved question is whether the nerve derived agrin is actually required for AChRs clustering. Many studies have shown that AChR clusters formed in the central region of the myotubes, which is destined to be synapse-rich, before axons extention and even when axon extension is prevented. Further time-lapse imaging *in vivo* revealed that pre-existing clusters on early-born slow muscle fibers were incorporated into NMJs as axons advanced (Walsh and Lichtman, 2003). Axons were required for the subsequent remodelling and selective stabilization of synaptic clusters and precisely appose post- to presynaptic elements. Thus, motor axons are dispensable for the initial stages of post-synaptic differentiation but are required for later stages.

There are three alternative splice sites (X, Y and Z) presented in the C-terminal of the agrin gene. The y-agrin has an insertion of four amino acids into site Y and moderately increases the AChR clustering activity. Z-agrin, the neuronal isoform, has an extra eight amino acids inserted at site Z. This small insert enhances the AChR clustering activity by 10,000 fold (Ma et al., 1994). Agrin is also expressed in myotubes and Schwann (glia) cells as well as motoneurons (Schwander et al., 2004). However, muscle and other tissues synthesize less active forms of agrin. Post-synaptic specilization is initiated but not maintained in the absence of neuronal agrin, which selectively included the z-exon, which is for the maximal agrin bioactivity. In z-agrin deficient mutants, the number and size of AChR clusters are much lower than those controls (Li et al., 1999). Particularly, at E14.5 embryos, the number of AChR clusters is similar to the control; the average size and area of individual AChR

clusters are similar between genotypes. Over the next few days, AChR clusters in control become larger and more numerous. Mice mutants lack neuronal and non-neuronal agrin have similar AChR, AChE and rapsyn clustering at both E14.5 and E18.5, indicating that neither neural nor non-neural agrin is required to initiate post-synaptic differentiation (Pun et al., 1997). Therefore the initiation of post-synaptic development is independent of nerve derived factors. The muscles have intrinsic capacity to initiate post-synaptic specialization. In the absence of motor nerve, AChR clusters still present in mutant diaphrams and form an end-plate band in the center of the muscle suggesting that at early stage of post-synaptic differentiation, AChR clusters occur even in the absence of nerve (Sohal, 1988). At later stages, AChR clusters become smaller, dimmer, less numerous in the innervated but agrin deficient mutant. Nerve supplies muscle not only with agrin to maintain AChR clusters but also with an agrin-independent signal that disperses post-synaptic specialization that not stabilized by nerve-derived agrin.

Agrin does not bind to AChR directly, but in the presence of Ca^{++}, interacts with specific receptors in the muscle membrane. Agrin binds with high affinity to a 156KDa extracellular proteoglycan, α-dystroglycan, which in turn is bound to β-dystroglycan (Hoch, 1999). The cytoplasmic tail of β-dystroglycan is associated with dystrophin and utrophin. Dystrophin and utrophin are anchored to the muscle cytoskeleton.

MuSK is the central organizer of post-synaptic differentiation. MuSK is sufficient for clustering synaptic proteins and inducing "synaptic genes" because ectopic expression of agrin or constitutively active MuSK stimulates AChR clustering and AChR transportation. MuSK mutant has no detectable AChR clusters at early and later stages. Agrin/MuSK signalling set up multiple loops feeding back to maintain an elevated synapse specific expression of their own components as well as of AChRs in electrically active muscle fibers (Lacazette et al., 2003). Initial stages of synaptogenesis may be determined by muscle-intrinsic mechanisms. Later in development, the establishment of stable neuromuscular synapses requires the stable interaction of nerve derived agrin with MuSK, whereas AChR clusters remaining nerve-free are dispersed. In the absence of the nerve, the basal extrasynaptic expression of MuSK, NRG, and ErbBs in the adult is not sufficient to induce ectopic postsynaptic membranes in the absence of agrin. When agrin is present, the coupling of stable MuSK activation to MuSK synthesis appears to be sufficiently strong to enable ectopic agrin to induce an ectopic postsynaptic like membrane in spite of the low level of MuSK initially present in electrically active muscle. The agrin/MuSK signalling

pathway can stabilize the developing NMJ via three biological mechanisms (Ngo et al., 2007). One mechanism is the neural derived agrin activation of the MuSK which drives the transcription of genes of the AChR ε subunit, utrophin and MuSK via JNK and transcription factors GABPα/β in the immobilised nuclei beneath the post-synaptic membrane. Another mechanism involves numerous signalling pathways activation after neural agrin binding to MuSK. For example, Dishevelled binds to MuSK and couples p21-activated kinase to MuSK resulting in re-organisation of the actin cytoskeleton during the AChR cluster formation; Abl and Src kinase act downstream of MuSK providing a positive feedback loop to stabilise the AChR clusters; Dok-7 and Casein kinase 2 also can induce MuSK phosphorylation to influence AChR clustering. The third mechanism is that postsynaptic signalling downstream of MuSK activation is necessary to presynaptic nerve terminal differentiation. In mice embryos lacking agrin, MuSK and rapsyn, motor neuron axon growth cones fail to stop and form a branched nerve terminal, suggesting MuSK signalling can transform the outgrowing nerve terminal into a stable differentiated nerve terminal.

AChR at junction folds reaches 10,000/um^2 to 1000/um^2 in the depths of the folds (Grinnell, 1995b). In extrajunctional membrane a few microns distant from the terminal, AChR is about 10/um^2. At the time of initial innervation, the AChR has a density of 100/um^2 over their entire surface. Both embryonic and mature AChRs consist of 5 subunits: α2, β, δ and γ in the embryonic receptor; α2, β, γ and ε in the mature receptor. The shift in gene expression from γ to ε forms of AChR requires innervation and is driven both by muscle fiber electrical activity and by neural trophic factors. Before innervation all nuclei in the myotubes express mRNAs for α, β, γ and δ subunits. On functional innervation, myotube nuclei that are located at a significant distance of NMJ synthesize mRNA for all subunits (Grinnell, 1995b). A group of nuclei clustered immediately below the synapse greatly increase their expression of α, β and δ subunit mRNAs and shift from producing the γ subunit to producing the ε form. AchR clusters on cultured myotubes are generally plaque-shaped. 'Aneural pretzels' shared remarkable similarities to mature NMJs (Ashby et al., 1993a; Macpherson et al., 2006). They are oblong broken at one side, are elaborately branched and are approximately the size of adult NMJs.

Another motor neuron derived factor, neuregulin, was once believed to be an important postsynaptic inducer in NMJ. Neuregulins (Nrgs) are a family of proteins containing an epidermal growth factor (EGF) like motif that activate membrane-associated tyrosine

kinases related to the EGF receptor- ErbB1 (Zhu et al., 1995). Initially Nrg-1 was found to induce proliferation of Schwann cells and transcription of AChR at NMJ. More recently, Nrgs were found to regulate early cell fate determination, differentiation, migration and survival of satellite cells, Schwann cells and oligodendrocytes. In neurons, Nrgs promote neuronal migration, and selectively increase the expression of other neurotransmitter receptors. At least four genes encoding Nrgs have been identified in vertebrates (Loeb, 2003). Alternate RNA splicing further enhance the diversity of Nrg family. Nrg1-4 binds preferentially to the Erb-3 and -4 receptors, which then form homo- or heterodimers by recruiting ErbB1 or ErbB2 co-receptors to propagate signalling. However, recent studies showed that neuregulin-ErbB signalling to muscle is dispensable for postsynaptic development (Buonanno and Fischbach, 2001). Neuregulin maybe play a modulatory role in inducing AChR gene expression, but this signalling pathway is clearly not involved in transcriptional specialization of synaptic nuclei as previous thought.

Presynaptic preparation at NMJ

It has been identified that lamininβ2, WNT-7a, Neuroligin, SynCAM, FGF22 (also FGF-7 and FGF10) and Thrombospondin1/2 (TSP1/TSP2) act as presynaptic organizers (Fox and Umemori, 2006). Lamininβ2 is one of the subunits of the large heterotrimeric laminin glycoprotein in ECM. It is exclusively located in synaptic basal lamina of NMJ. Deletion of lamininβ2 results in a lack of active zones, Schwann cell processes insertion in synaptic cleft and neurotransmitter release is impaired (Knight et al., 2003). Lamininβ2 promotes presynaptic differentiation by binding and clustering the voltage-gated calcium channels (VGCCs), which in turn induce the clustering of synaptic vesicles. *In vivo* assay demonstrated that lamininβ2-VGCC interactions are critical for active zone formation and maintenance (Nishimune et al., 2004). WNT-7a is a soluble morphogen which belongs to the WNT family, which regulates key aspects of embryonic development, including cell fate determination, tissue patterning, cell migration and cytoskeletal changes. WNT-7a is expressed and released by dorsal ectoderm in embryos and epithelial and many epithelial derived tissues. Soluble WNT-7a can alter axon morphology and induce synaptic vesicle clustering, while deletion of WNT-7a results in a transient decrease in vesicle accumulation and reduced complexity of nerve terminals (Hall et al., 2000). In Drosophila, DFrizzled2 (DFz2) expresses at the presynaptic boutons and the postsynaptic muscle fibers, suggesting that DFz2 mediates Wg-induced presynaptic differentiation and synaptic maturation

(Ataman et al., 2006). However, the role of vertebrate frizzled in synaptic differentiation remains unknown. TSP1/TSP2 is adhesion ECM molecules expressed and released by glial cells in both CNS and PNS. Purifed TSP1/TSP2 can induce synaptic vesicle accumulation and more importantly, can regulate adhesion between pre and post-synaptic cells to promote synaptic maintemance (Christopherson et al., 2005). In TSP1/TSP2 deficient mice, synaptic vesicles accumulation is reduced (Christopherson et al., 2005).

In CNS, neuroligin is a potential candidate presynaptic organizer. Neuroligin is the endogenous receptor for neurexins, which are a novel family of cell-surface receptors with extracellular domains that similar to agrin and laminin. Neuroligin is expressed throughout the CNS at the peak time of synaptogenesis (Yamagata et al., 2003). The facts that neurexin and neuroligin form a stable link between pre and post-synaptic neurons, and neurexin and neuroligin signalling induces synaptic vesicle accumulation suggested a role for these molecules in not only presynaptic but also in postsynaptic differentiation. SynCAM, a transmembrane molecule which serves as cell surface receptors containing three extracellular Ig domains and one intracellular PDZ domain binding motif, induces spontaneous and evoked neurotransmitter release. SynCAM is specifically expressed in CNS: both in the pre and post-synaptic membrane (Yamagata et al., 2003).

Maturation of NMJ

In the embryo, shortly after synapse formation between motor neurons and primary myotubes, a large number of motor neurons die (Grinnell, 1995b). The good correlation between the number of primary muscle fibers and the number of survival motor neurons suggests that motor neuron survival at least in part depends on the presence and amount of innervation targets available. After the period of motor neuron cell death, secondary muscle fibers continue to form and axonal arborisations increase, myofibers are often innervated by more than one motor neuron axon at the same synaptic locus. As postsynaptic development progresses, all but one axon withdraw from the innervated myofiber. With maturation, synapse elimination and new muscle fibers being added to some muscles refine the motor neuron and muscle fiber connections (Thompson, 1985). The NMJ becomes much more homogeneous through this synapse elimination/rearrangement process. Furthermore, the NMJ neurotransmitter ACh determines the final differentiation of mature synapses. ACh released by branching motor nerves causes AChR induced postsynaptic potentials that determine the endplate localization. The regulatory role of ACh during embryonic

development was learned from the ChAT knockout mice. Pre-patterned AChR clusters, aberrant nerve growth and excessive branching, and multiple endplates formed on single myofiber were observed in the mutant mouse embryos (Brandon et al., 2003). Although the mutant mice died at birth, the model supports that the initial axon pathfinding is independent on ACh secretion and ACh mediated post-synaptic specialization. However, the maintenance and the organization of nerve terminal branches and stabilization of NMJ require ACh. In the postsynaptic membrane, ACh induced signals lead to the dispersion of non-synaptic AChR clusters and possibly loss of excess AChRs.

Summary

Over the last two decades, a rapidly increasing body of experimental evidence, obtained from *in vivo* and *in vitro* models, shed insights on the formation, maintenance, and plasticity of synaptic connections of the NMJ. Despite all the exciting progress being made, we are far from a complete understanding of the mechanisms underlying this specialized intercellular signalling connection. For example, little is know about the spatial and temporal dynamics of axonal pathfinding, pre- and postsynaptic specialization, nerve-muscle contact, elimination and maturation of NMJ, moreover, the molecules that are involved in these events. The co-culture model established in this chapter can be useful to investigate the development of NMJ *in vitro*.

5.2 Methods

5.2.1 Cell culture

The EC cell and muscle cell lines are maintained and induced as described in Chapter2. The neuron-myoblast co-culture is established as shown in Figure 5.3.2. After 4 or 5 days, twitching myotubes appear in the co-culture and the co-culture can be used for pharmologically manipulation. Or the co-cultures can be fixed and analysed by ICC after 2 weeks differentiation.

5.2.2 Immunocytochemistry (ICC) staining

The fixed co-cultures were washed with PBS before immunocytochemical staining. Primary antibodies used in this study included β III tubulin (Covance, 1:1000), synapsin I (Calbiochem, 1:500), ChAT (Chemicon, 1:100), HB-9 (Chemicon, 1:400), Islet (40.2D6)

and Lim3 (67.4E12) (Developmental Studies Hybridoma Bank, 1:50). For labelling acetylcholine receptors, coverslip cultures were incubated with Alexa Fluor 594 conjugated α-bungarotoxin (Molecular Probes, 1:500) at 20°C for 30min. Images were collected using a Nikon fluorescence microscope or a confocal microscope (Zeiss LSM 510).

5.2.3 Quantification of contractile events

Neuron aggregates and C2C12 myoblasts were seeded in 12-well plates. After myoblasts differentiated into myotubes, contractile events were counted under the Nikon phase contrast microscope at X 20 objective lense. Only the twitching myotubes were counted in each well, each condition was repeated at least three times.

5.2.4 Pharmacologically manipulation

After counting the twitching myotubes in each well of 12-well plates, AChR agonists were added to the medium, and the twitching myotubes were counted again like before. When AChR agonists were added to the medium, video clips were recorded at the same time. The AChR antagonists used in this assay were: curare (Sigma, 50μM), atropine (Sigma, 50 μM), and the AChR agonists used in this assay were: ACh (Sigma, 2 μM), Carbachol (Sigma, 2μM), Nicotine (Sigma, 2 μM), Muscarine (Sigma, 2 μM).

5.2.5 Statistical Analysis

Statistical analysis performed involved Students paired and unpaired *t*-test, with the use of Microsoft Excel to analyse the contratile events. Statistical significance was set at $p<0.05$ level for the *t*-tests analysis, with a minimum n value of 6 determinations.

5.3 Results

5.3.1 Myotube differentiation assay

Mouse C2C12 myoblast and human RD myoblast cell lines were obtained from Dr. Bashir of Durham University. To investigate the ability of these two cell lines to differentiate into myotubes, 70% confluent C2C12 and RD cells were grown in 2% horse serum DMEM instead of 10% fetal calf serum DMEM for 4 or 5 days on laminin coated flasks. C2C12 myoblasts differentiated into long large myotubes (Figure 5.3.1), while RD cells showed no fusion of myotubes (data not shown). Therefore, the C2C12 cell line was chosen to study the function of differentiated motor neurons from Tera2 cells.

5.3.2 Development of the co-culture system to test motor neuron activity

To setup a co-culture system (as shown in Figure 5.3.2) Tera2 EC cells were induced by RA or RA+Shh for about 4 weeks. Many rosette-like structures were formed during the induction, suggesting Tera2 EC cells go through a neuro-epithelial stage to differentiate into neuroblasts (Figure5.3.2A). After 4 weeks, the induced Tera2 EC cells were trypsinized and replated on poly-D-lysine and laminin coated flasks for 24 hours to allow cells to attach the flasks prior to the addition of mitotic inhibitors. Cells were grown in the presence of mitotic inhibitors for about 1 week to form neuronal aggregates. These neuronal aggregates were well connected with each other by long neurites; other differentiated non-neuron cells undergo apoptosis (Figure5.3.2B). Neuronal aggregates were collected and mixed with C2C12 myoblasts before seeding on laminin and poly-D-lysine coated 12-well plates or coverslips (for ICC). After 4 or 5 days, myotubes were differentiated and start twitching (Figure5.3.2C). The co-cultures were used to analyse the contractile events or neuronal properties by immunocytochemistry staining and SEM.

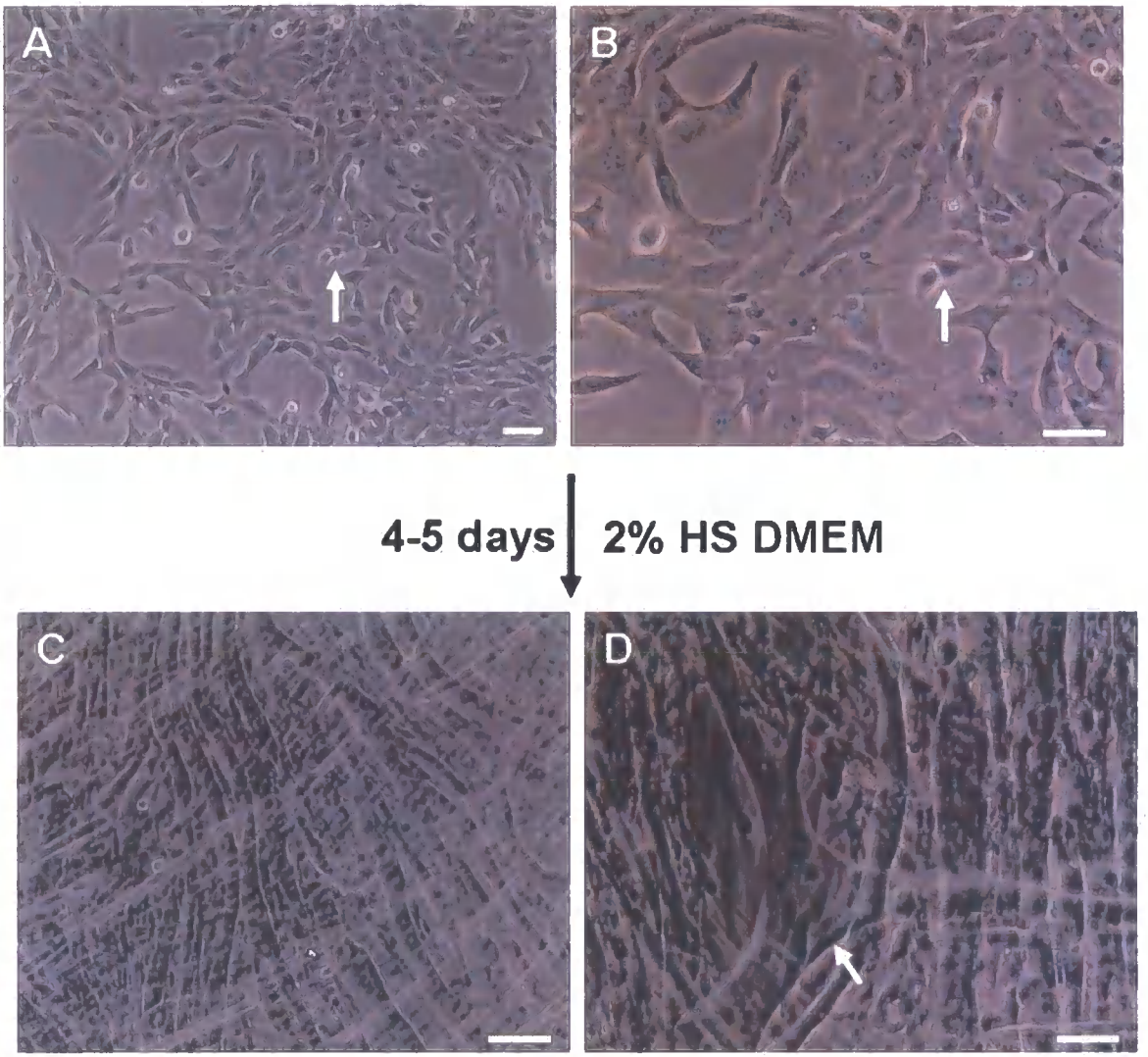

Figure 5.3.1 Myotube differentiation from C2C12 myoblasts. C2C12 were maintained in 10% FCS DMEM in A and B, note that the white arrows in A and B are pointing to the dividing myoblasts. Differentiated myotubes are shown in C and D, when cells reach 90% confluency, 10% FCS DMEM growth medium was replaced to 2% Horse Serum DMEM myotube differentiation medium. Myoblasts were differentiated into well-differentiated myotubes after 4 -5 days exposure to myotube differentiation medium. Note the white arrow in D pointing to a differentiated giant myotube. Scale bars = 50μm.

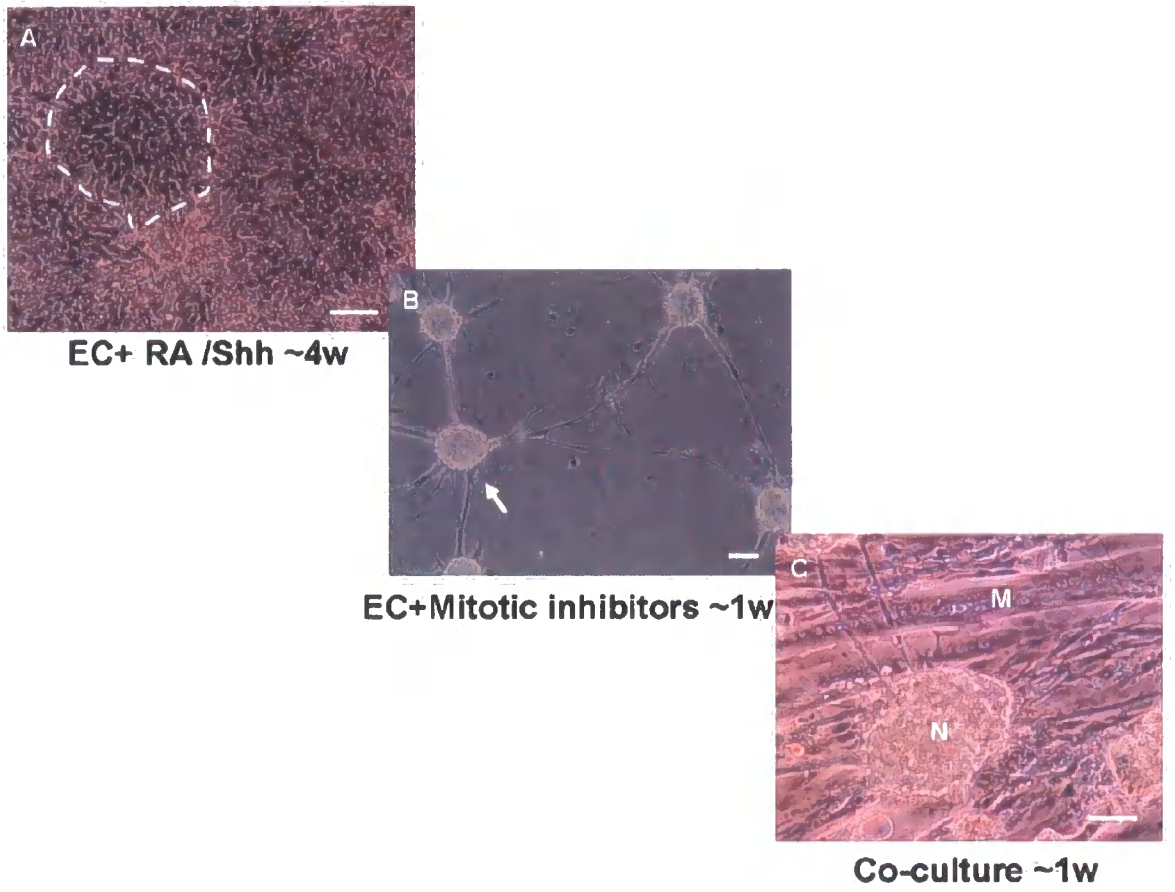

Figure 5.3.2 Schematic showing the development of co-culture system. A, motor neuron specification from EC stem cells. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) for 4 weeks. Note that rosettes are formed during the specification (labelled with white dash curve). B, neuronal aggregates differentiation. The 4 weeks induced EC cultures were trypsinized and replated to a laminin-poly-D-lysine coasted 6 well plate in the presence of mitotic inhibitors for 1 week to allow neuronal aggregate formation. Note that neuronal aggregates are connected to each other in B (labelled with white arrow). C, neuron-myoblast co-cultures. Neuronal aggregates were collected and mixed with C2C12 myoblasts on laminin and poly-D-lysine coated 12 well plate, the mixture were grown in myotube differentiation medium for 1 week before further analysis. A letter 'M' was used to label the differentiated myotube, and a letter 'N' labelled the neuronal aggregate in the co-culture in C. Scale bars = 50μm.

5.3.3 The identity of neurons derived from Tera2.cl.SP12 EC cells prepared for co-culture

To confirm the neuron identity of the differentiated neuron aggregates, we used general neuronal markers such as βIII tubulin, neurofilament 68, TUJ1 and MAP2 to analyse the aggregates before and after setup of the co-culture. All these general neuronal markers are strongly positive in the Tera2 differentiated aggregates in either RA induced (Figure 5.3.3) or RA+Shh induced aggregates (data not shown).

5.3.4 The purity of the neuronal like aggregates in co-culture

From previous work in the lab, we know the differentiated aggregates collected in this way consist mainly of neurons (Stewart et al., 2003). Some studies pointed out that oligodendrocyte and astrocyte may express some general neuronal markers (Wharton et al., 1998). To rule out the contaminant of glia cells in our co-culture system, we performed GFAP and Myelin staining in the co-culture. We found that there was no expression of GFAP and Myelin in the co-culture, while there was expression of βIII tubulin and NF-68 in RA and RA+Shh induced cells (Figure 5.3.4). This indicates that there are no oligodendrocytes or astrocytes in our co-culture system.

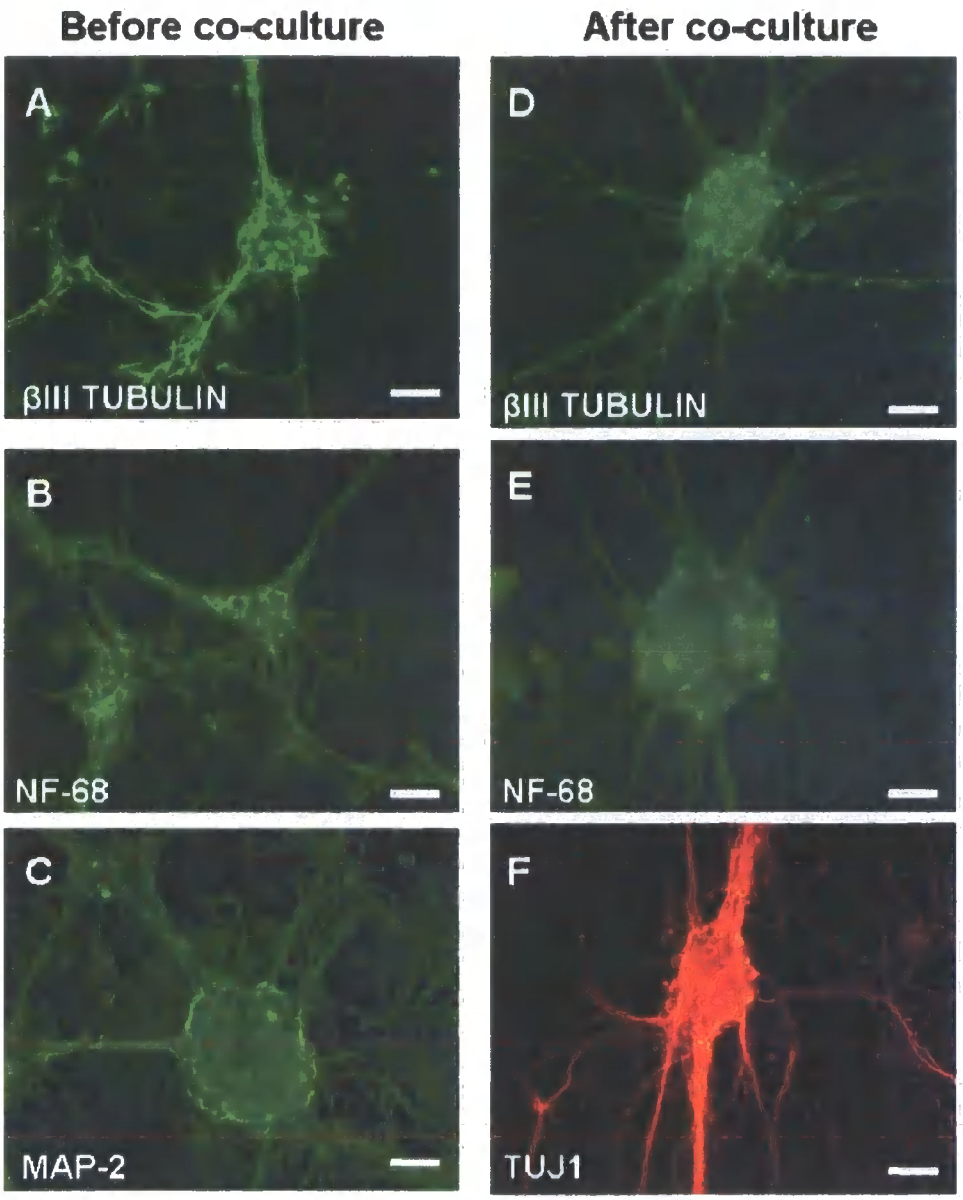

Figure 5.3.3 RA induced neuron aggregates are positive for general neuronal markers before and after co-culture with myoblasts. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) for 4 weeks. The 4 weeks induced EC cultures were trypsinized and replated to a laminin-poly-D-lysine coasted 6 well plate in the presence of mitotic inhibitors for 1 week to allow neuronal aggregate formation. The neuronal aggregates were immuno-labelled with general neuron markers βIII tubulin (A), NF-68 (B) and MAP-2 (C) before co-culture with myoblasts. After co-culture with myoblasts, the neuronal aggregates are still positive for βIII tubulin (D), NF-68 (E) and Tuj1 (F). RA+Shh induced neuron aggregates are also positive in staining with these neuronal markers (data not shown). Scale bars = 50μm.

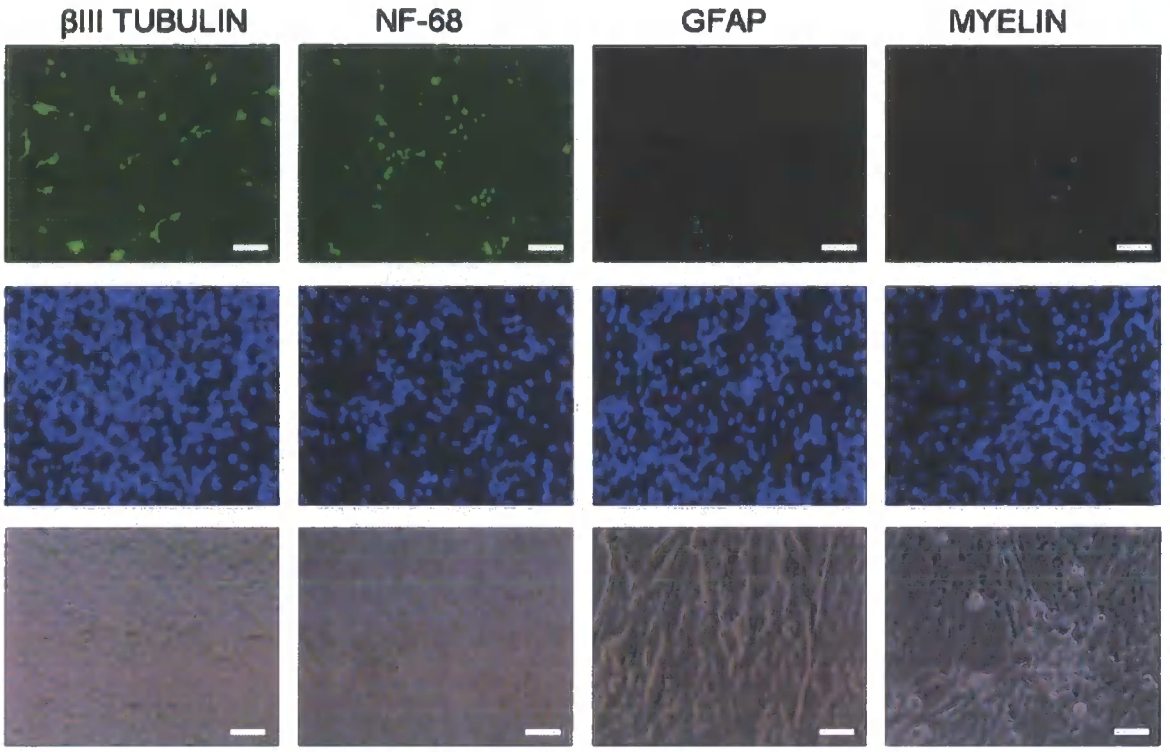

Figure 5.3.4 RA induced neurons in the co-culture system are negative for astrocyte marker GFAP and oligodendrocyte marker myelin but positive for neuronal markers βIII tubulin and NF-68. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) for 4 weeks. The 4 weeks induced EC cultures were trypsinized and replated to a laminin-poly-D-lysine coasted 6 well plate in the presence of mitotic inhibitors for 1 week to allow neuronal aggregate formation. The neuronal aggregates were trypsinized to get small aggregates before mixing with myoblasts and immuno-labelled with general neuron markers βIII tubulin, GFAP, NF-68 and myelin after co-culture with myoblasts for 3 days. RA+Shh induced neuron aggregates are also positive in staining with these neural markers (data not shown). Scale bars = 50μm.

5.3.5 The motor neuron identity of the differentiated aggregates

We observed that differentiated neurons induce the contraction of myotubes in the co-culture. To further confirm the identity of motor neurons, motor neuron markers such as HB-9, Islet-1 and Lim-3 were used to stain the differentiated neuron aggregates. We found that most of the nuclei of the cells in the aggregates are positive for these three markers (Figure 5.3.5). However, we also noted a difference in the variability of fluorescent intensity between cells in the aggregates. When staining for ChAT, the enzyme used by motor neuron to synthesize the motor neurotransmitter—ACh, the cell bodies and the processes of the aggregates are positive (Figure3.6). These results are consistent with the quantification of motor neuronal markers in 4-week Tera2 differentiated cells in Chapter4 (Figure4.2.4).

5.3.6 The interaction between aggregates and myotubes

During the C2C12 myoblasts differentiated into myotubes, the neurites of the neuron aggregates extend along the newly formed myotubes. To reveal the interaction between the neuron aggregates and the differentiated myotubes, we used myosin or phalloidin to stain the myotubes and β III tubulin to stain the neurites of the neuron aggregates (Figure 5.3.6). The double staining of the co-cultures showed that the neuron aggregates extended very long neurites and some neurites form close contact with myotubes. Confocal microscopy was also employed to study the close interaction of neurites and myotubes. The growth cones are observed at the ending of the neurites, suggesting that the differentiated myotubes may have the ability for axon guidance.

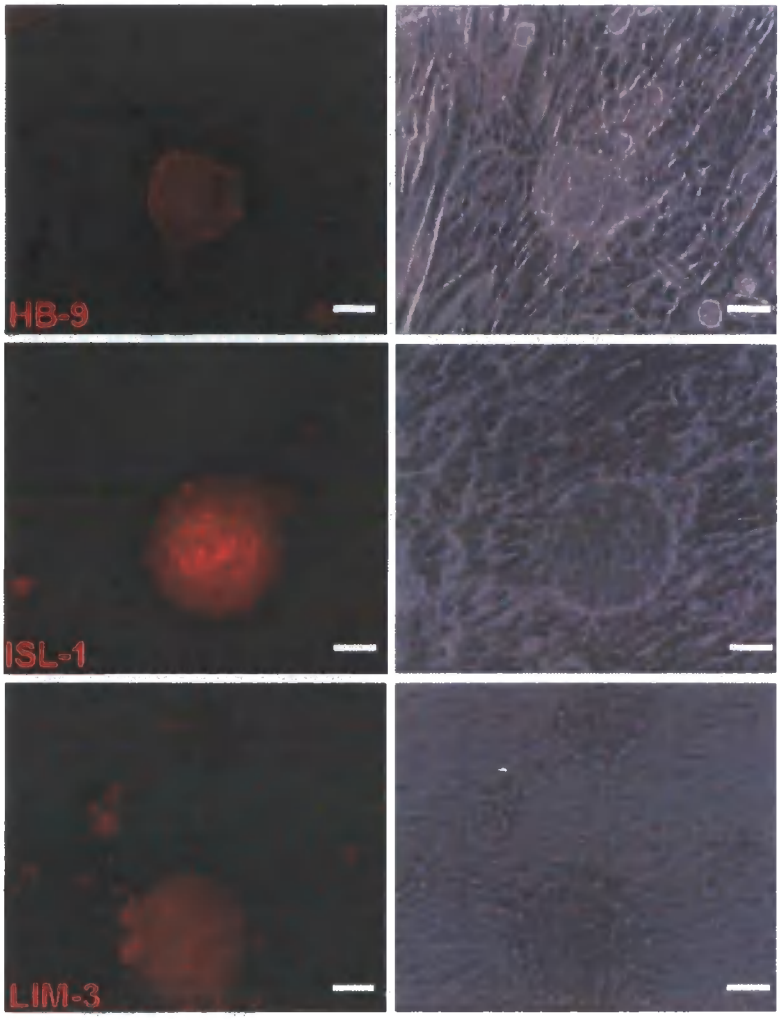

Figure 5.3.5 The neuron aggregates in the co-culture are positive for motor neuron markers. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) for 4 weeks. The 4 weeks induced EC cultures were trypsinized and replated to a laminin-poly-D-lysine coasted 6 well plate in the presence of mitotic inhibitors for 1 week to allow neuronal aggregate formation. The neuronal aggregates were co-culture with myoblasts for 1 week before fixation with 4% PFA, and immuno-labelled with motor neuron markers HB-9, ISL-1 and LIM3. RA+Shh induced neuron aggregates are also positive in staining with these motor neuron markers (data not shown). Scale bar = 50μm. Negative controls were cultures stained with secondary antibody (as shown in Figure2.1).

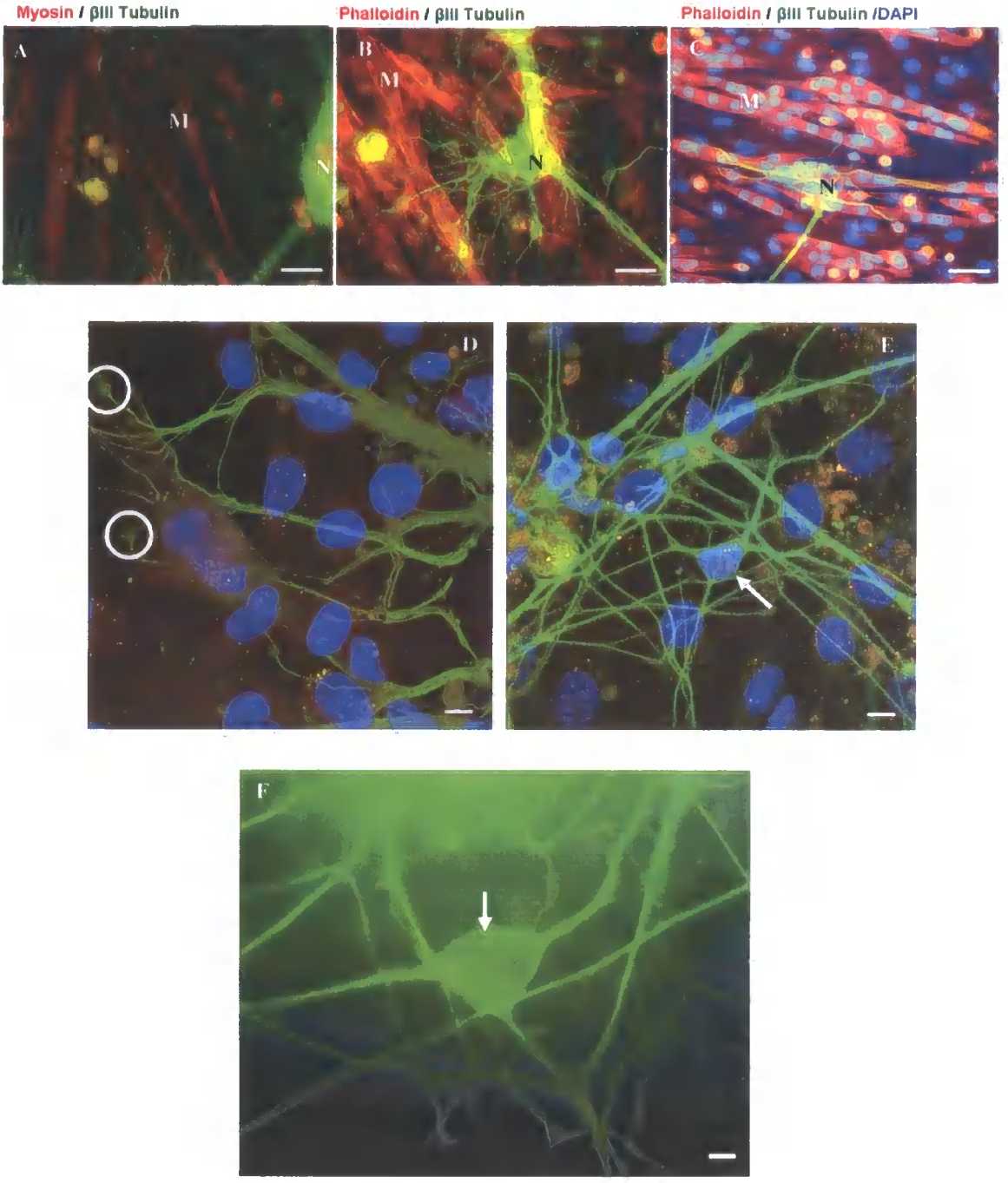

Figure 5.3.6 The interaction between neuron aggregates and differentiated myotubes. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with 10μM RA (A,C) alone or RA plus Shh (25ng/ml) (B, D, E and F) for 4 weeks, and then trypsinized and grown in the presence of mitotic inhibitors for 1 week to allow neuronal aggregate formation. The neuronal aggregates were co-cultured with myoblasts for 2 weeks before fixation with 4% PFA, and immuno-labelled with myosin (red), phalloidin (red) and βIII tubulin (green). The nuclei were counterstained with Hoechst. 'N' is used to label neuronal aggregates and 'M' label the myotubes (A-C), while circles in D marked the growth cone, white arrows in E and F pointed to the motor neurons. Scale bars = 50μm.

5.3.7 Synapses form between aggregates and myotubes

To examine whether there are contacts between the neurites and myotubes, we performed a double staining for synapsin I and β III tubulin. Synapsin I is a member of the synapsin family. Synapsins are neuronal phosphoproteins which associate with the cytoplasmic surface of synaptic vesicles (Goold and Baines, 1994). And they are implicated in synaptogenesis and the modulation of neurotransmitter release via exocytosis. The role of synapsin I in synaptogenesis is to act as a substrate for protein kinases and phosphorylation in the nerve termina. The result showed many particulate positive spots on aggregates and along the extended neurites (Figure 5.3.7). Furthermore, the pre-synaptic membrane protein synaptophysin was also positive in the co-culture (data not shown).

5.3.8 SEM imaging of neurites and neurite endings in the co-culture

To study the interaction between the differentiated neuron aggregates and the differentiated myotubes, SEM was used to investigate the neurons in co-culture and focused on the contact sites between these cell types. The results from SEM show that neurites from neuron aggregates form close contacts with myotubes (Figure 5.3.8). Also neurite growth-cones could be seen on the myotubes, suggesting that some of the neurite-myotube contacts are still in the early developmental stage of NMJ.

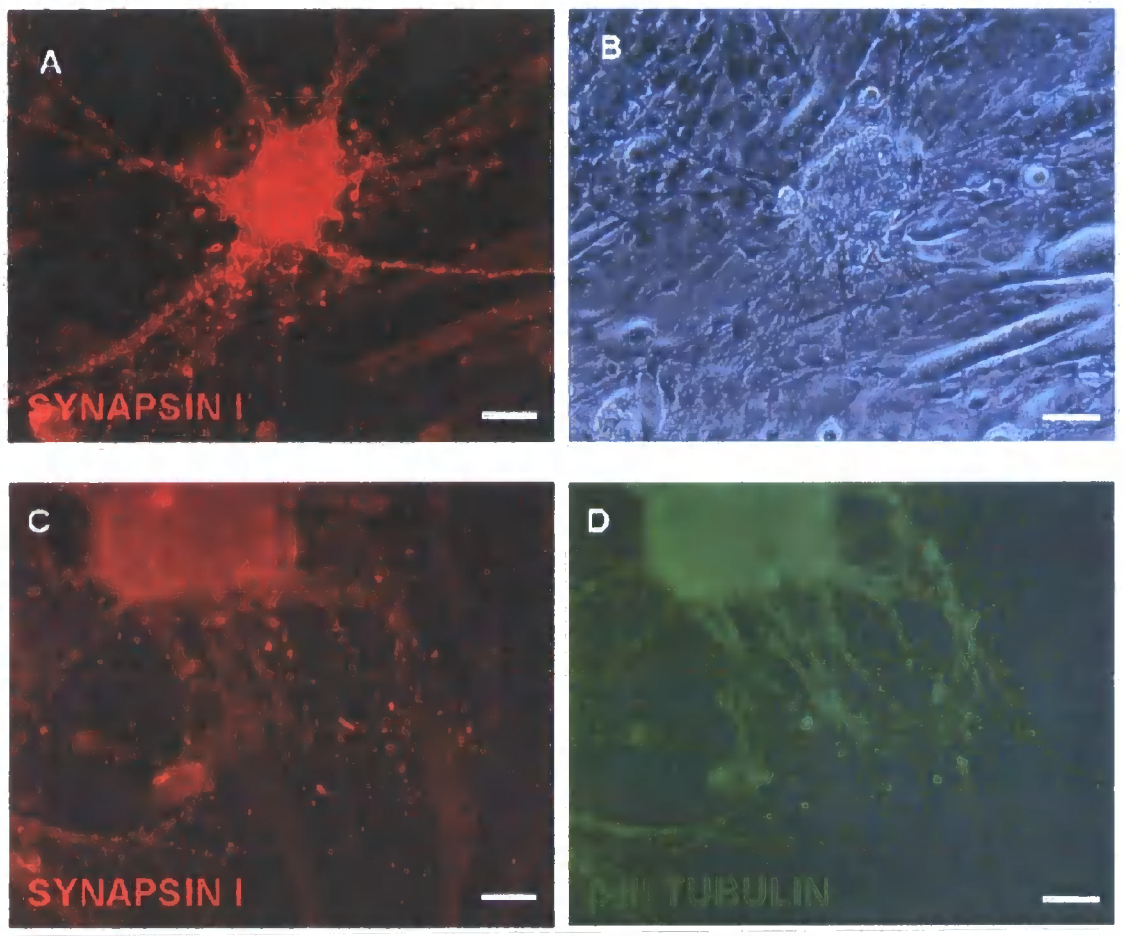

Figure 5.3.7 The neuron aggregates in the co-culture express high level of synapse protein synapsin I. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with 10μM RA (A) or RA plus 25ng/ml Shh (C) for 4 weeks, and then trypsinized and grown in the presence of mitotic inhibitors for 1 week to allow neuronal aggregate formation. The neuron aggregates were co-cultured with myoblasts for 2 weeks before fixation with 4% PFA, and immuno-labelled with synapsin I (red) and βIII tubulin (green). The phase constrast image is shown. Scale bars = 50μm.

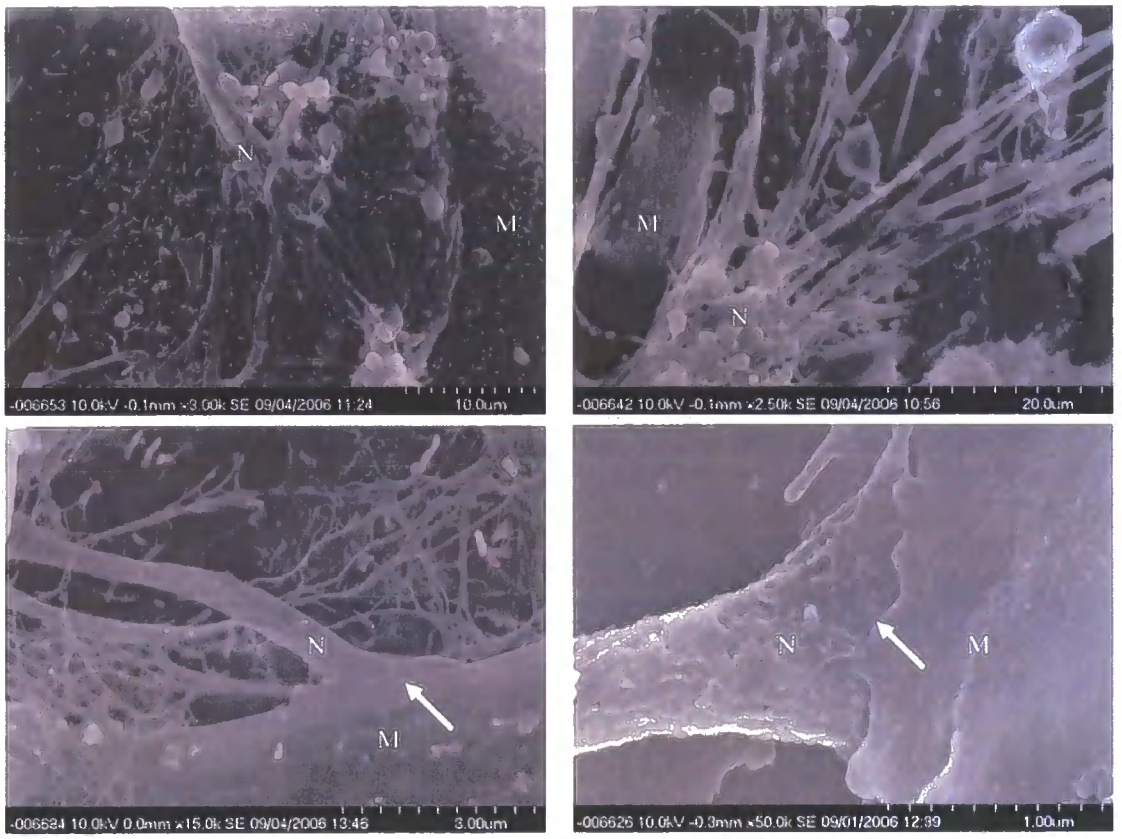

Figure 5.3.8 Examples of SEM images of neurites in co-culture. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) for 4 weeks, and then trypsinized and grown in the presence of mitotic inhibitors for 1 week to allow neuronal aggregate formation. The neuron aggregates were co-cultured with myoblasts for 1 week before fixation SEM analysis. Note that the physical contact of neurite endings and the myotubes pointed by white arrows. Labels: N-neurite ending; M-muscle; arrows - contact sites of neurite and muscle.

5.3.9 Possible formation of NMJ in the co-cultures between motor neurons and muscle cells

We nest examined the ultra-structure of contact sites between the neurites and the myotubes in the co-culture using TEM. To find evidence of NMJ formation in the co-cultures, we circled the contact sites of the twitching myotubes under the phase contrast microscope before fixing the co-culture and proceeding to section. Because it is difficult to pinpoint the exact area after embedding and sectioning, the presence of NMJ could not be demonstrated by TEM. It is known that the α-bungarotoxin is snake venom, specifically binds to the receptors for Ach with high affinity (Marques and Santo Neto, 1998). AChRs accumulate in the junction fold of NMJ and could reach 1,000 to 10,000/μm^2 compared to only about 10/μm^2 outside the NMJ (Grinnell, 1995b). Therefore, α-bungarotoxin was used to stain NMJ. Immunocyotchemistry for α-bungarotoxin and β III tubulin showed that there were many positive patches of α-bungarotoxin on the myotubes at the ending of the neurites which made contacts with the myotubes. The higher magnification of the fluorescence microscopy images showed that some neurites end with bouton like structures (Figure 5.3.9D-F). Furthermore, the boutons co-localised with clusters of AChR, which are stained by α-bungarotoxin, suggesting that boutons have induced AChR clusters on the corresponding membrane of myotubes. Furthermore, it is known that motor neuron axons *in vivo* form specialised boutons when making functional connections with muscle fibers. Accordingly, there is good evidence to suggest that the bouton like structures may be the possible NMJ in the co-culture that mediate the functional contraction of the muscle cells. Refer to Figure5.3.9.

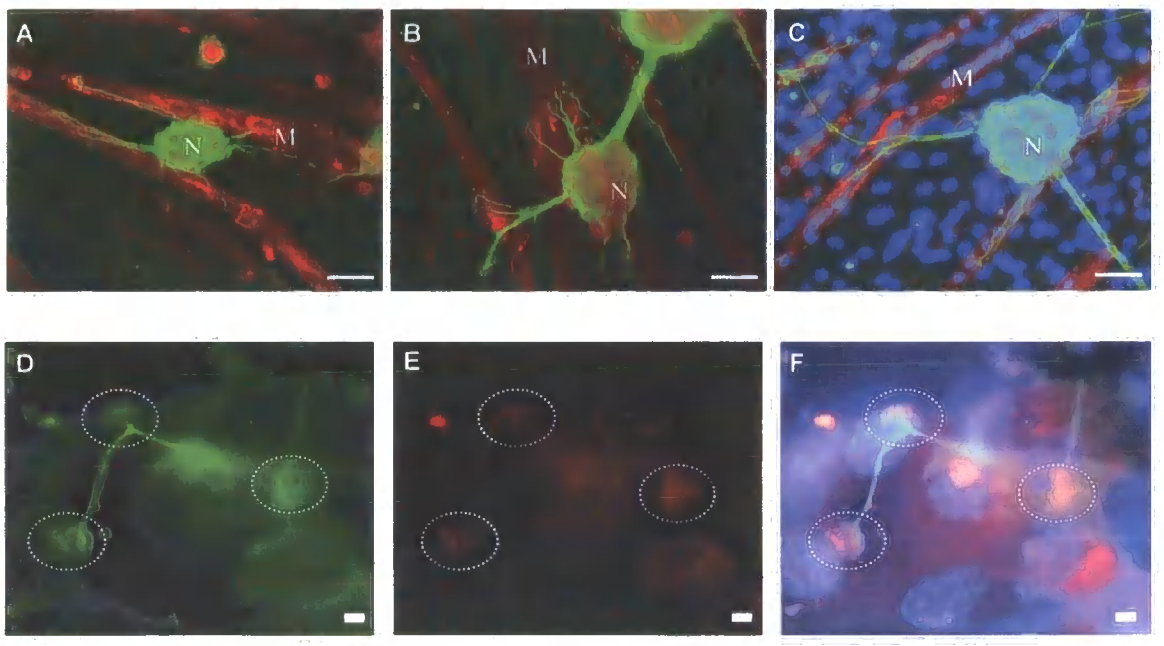

Figure 5.3.9 Neuron aggregates induced AChR clusters on the myotubes in the co-cultures. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with 10μM RA (A, C) or RA plus 25ng/ml Shh (B, D) for 4 weeks, and then trypsinized and grown in the presence of mitotic inhibitors for 1 week to allow neuronal aggregate formation. The neuron aggregates were co-cultured with myoblasts for 2 weeks before fixation with 4% PFA, and immuno-labelled with α-bungarotoxin (red) and βIII tubulin (green). The nuclei are counterstained with hoechst. Note that the red patches of α-bungarotoxin appear on the myotube membrane where neurites contact the myotubes (A-C), also the bouton like neurite endings circled in D are co-localised with AChR clusters circled in E, images were overlaid in F. Scale bars = 50μm.

5.3.10 RA and RA+Shh induced EC stem cells result in neurons capable of inducing contractile events in co-culture

From Day4 of co-culture, we observed that there were contractile events in the co-cultures. However, we observed no twitching events if myoblasts differentiate into myotubes in the absence of neuron aggregates. This is consistent with previous observations (Dr.Bashir, unpublished work). Neuronal aggregates used in the co-culture system originated from RA and RA+Shh induced Tera2 cells. We differentiated myoblasts with the same number of neurons induced by RA and RA+Shh conditions, and then counted the number of contractile events. Our results show that RA+Shh induced neurons clearly produced more contractile events compared to RA alone induced neurons (Figure 5.3.10). This indicates that RA+Shh might induce more motor neurons compared to RA induced cultures.

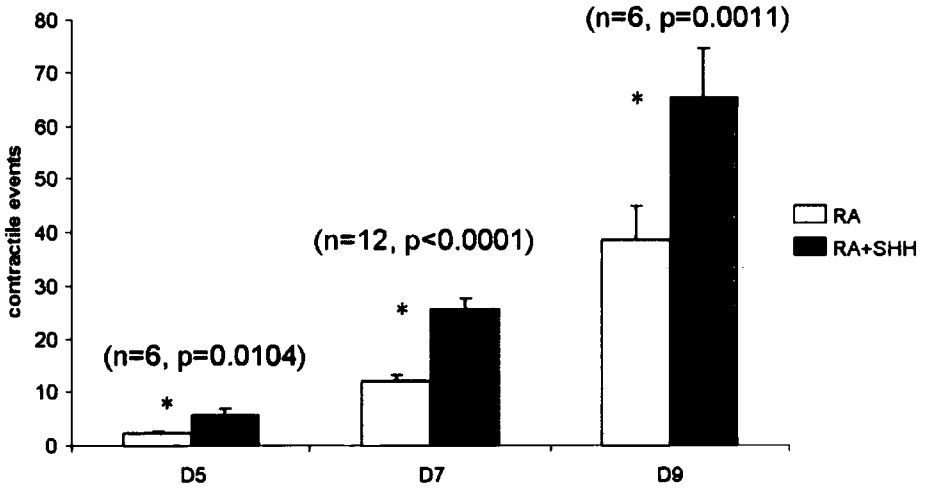

Figure 5.3.10 Quantification of contractile events stimulated by RA and RA+Shh induced neurons. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) for 4 weeks, and then trypsinized and grown in the presence of mitotic inhibitors for 1 week to allow neuronal aggregate formation. The induced RA and RA+Shh neuronal aggregates were co-cultured with myoblasts respectively in 12 well plate for 5 days before counting the contractile events. Note that there is significance (*) in D5 ($p<0.05$), D7 ($p<0.001$) and D7 ($p<0.005$). Error bars are shown as SEM. D, days after co-culture; n, number of repeats.

5.3.11 Non-selective AChR agonists induced more contractile events in co-culture

ACh and carbachol are non-selective AChR agonists; they stimulate both nicotinic and muscarinic AChRs to induce contraction of skeletal muscles (Choo et al., 1986). When ACh or Carbachol was added to the co-culture, both could increase the contractile events in myotubes grown with RA or RA+Shh neuron aggregates. Although the co-culture can be stimulated by non-selective AChR agonists; it is not certain which pathway is used in stimulating myotubes in the co-culture (Figure 5.3.11).

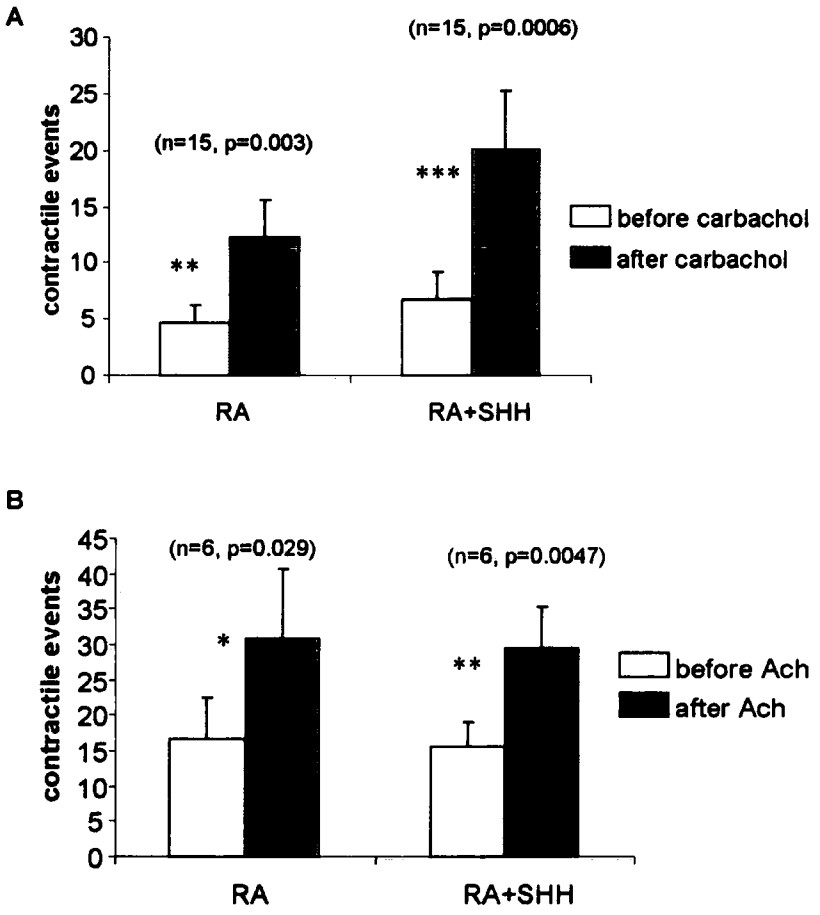

Figure 5.3.11 Quantification of contractile events stimulated by AChR antagonists ACh and carbachol. The induced RA and RA+Shh neuronal aggregates were co-cultured with myoblasts respectively in 12 well plate for 5 days before counting the contractile events. Note that there is significance (**, $p<0.005$; ***, $p<0.001$) between RA and RA+Shh induced contractile events before and after carbachol (A) and also significance (*, $p<0.05$; **, $p<0.005$) before and after ACh (B). Error bars are shown as SEM. n, number of repeats.

5.3.12 AChR agonists induce myotube contractions in the absence of neuronal aggregates

From our observation, we noted that myotubes did not contract if no neuron present in the culture. However, some myotube contractions were observed when AChR agonists such as carbachol, nicotine and muscarine are added to the myotube cultures. Nicotine is a selective ligand that binds to nicotinic AChRs, while muscarine specifically binds to muscarinic AChRs. With these selective and non-selective agonists, we observed a few myotube contractions in the absence of neuronal aggregates (Figure5.3.12). This suggests that it is the neurotransmitter secreted from the neuons that induce the contractile events in the co-cultures.

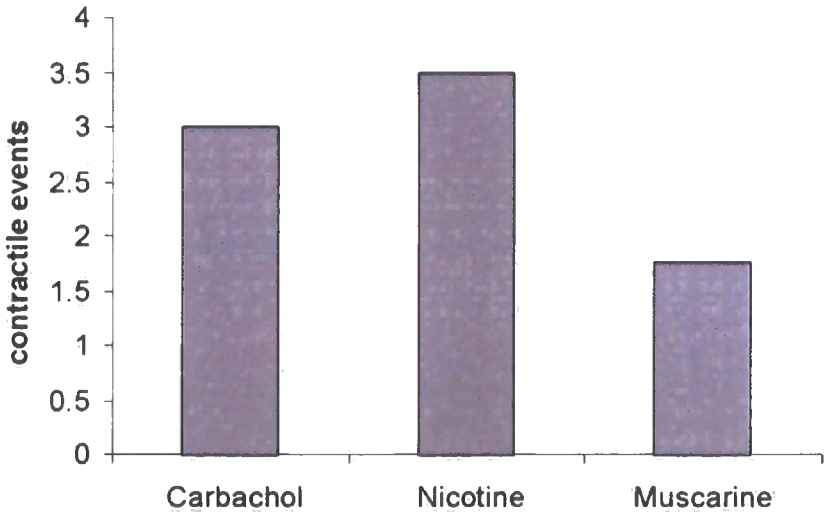

Figure 5.3.12 AChR agonists induce myotube contractions without neuronal aggregates. When C2C12 myoblasts reach 90% confluency, 10% FCS DMEM growth medium was replaced to 2% HS DMEM myotube differentiation medium. Myoblasts were differentiated into well-differentiated myotubes after 4 -5 days exposure to myotube differentiation medium. AChR agonists carbachol (2μM), nicotine (2μM) and muscarine (2μM) were added to the 5 days differentiated myotubes respectively and the contractile events were counted. The results are shown as mean of four repeats.

5.3.13 Contractile events in the co-culture can be blocked by AChR antagonists

Curare is a dart poison which exclusively blocks nicotinic AChRs on the post synaptic membrane of NMJ and induces muscle relaxation (Rang and Rylett, 1984). When introducing Curare to the co-culture, all the contractile events were blocked in a very short time. Furthermore, atropine, which is a chemical that blocks muscarinic AChRs, also stops myotube contractions when added to the co-cultures. Another chemical α-bungarotoxin also has the same effect (Data shown in video clips). At least three repeats are done for each AChR antagonist.

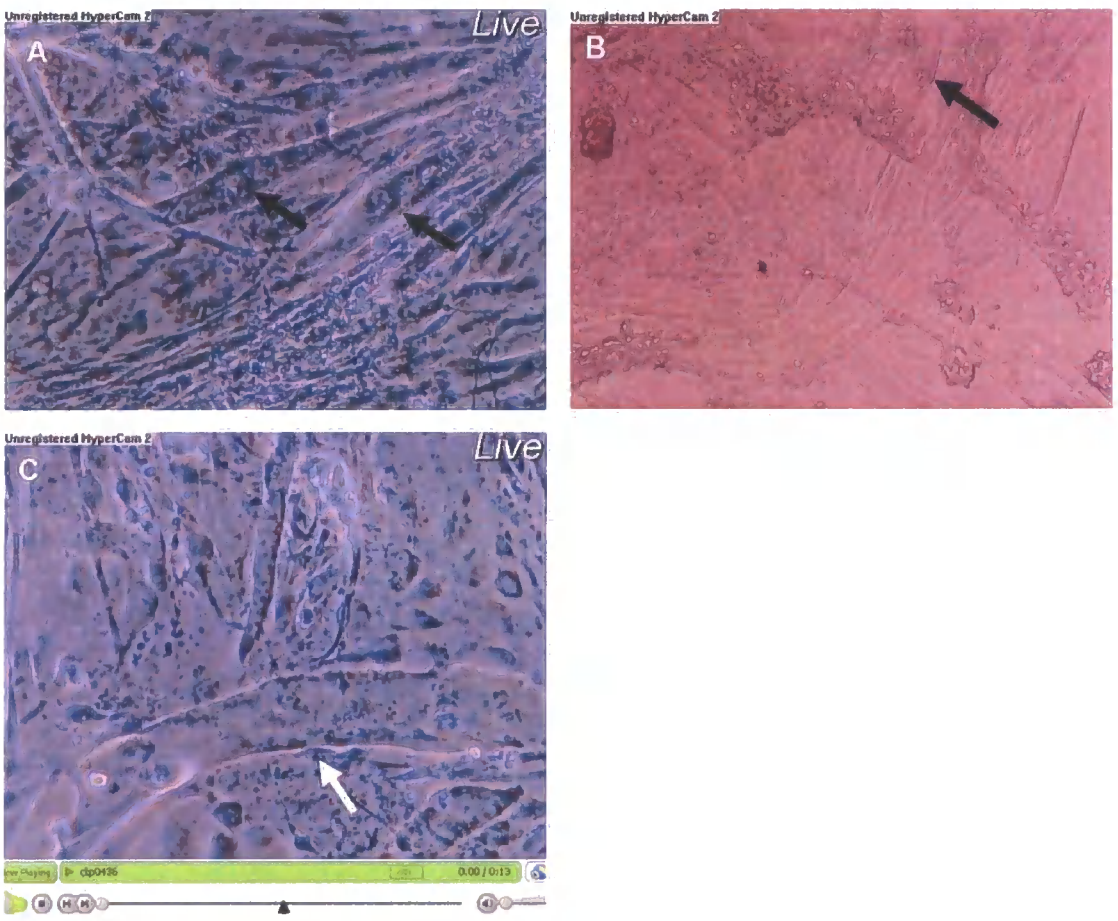

Figure 5.3.13 Pictures of video clips. A, the neurons induce myotube contraction, the contracting myotubes are labelled with black arrows. B, ACh induces myotube contraction in the co-culture, the arrow marks the twitching myotube. C, Curare blocks the contracting myotube, the contracting myotube is labelled with white arrow, and the the small black triangle marks the stop of contraction.

5.4 Discussion

To define a specific class of neurons, one must use a combination of molecular, cellular and functional properties. In chapter 3 and 4, we first noted that spinal motor neuron can be generated from Tera2.cl.SP12 embryonal carcinoma cells, which are human pluripotent stem cells, upon RA+Shh and RA induction. In this chapter, we investigated whether or not the EC cell derived motor neurons have real function using *in vitro* techniques. In particular, we were interested in whether or not they could form functional synapses with cultured muscle cells and induce muscle contraction.

We chose mouse C2C12 myoblast cell line to study the function of EC derived motor neurons because it can be easily differentiated into elongated, multinucleated myotubes after switching to a simple and efficient differentiation medium for 4 or 5 days. Also, the differentiated myotubes are free of spontaneously contraction. The recent studies have also used mouse C2C12 myoblasts to setup co-culture system with human ES cell derived motor neurons. Most of these experiments first differentiated the C2C12 myoblasts into myotubes before co-culture with differentiated neurons (Li et al., 2005). In our present study, myotubes are not allowed to form before the co-culture, we instead mixed myoblasts with differentiated neurons before plating onto poly-D-lysine and laminin coated plates. By this means, we propose that neurons and myoblasts have more opportunities to interact with each before the myoblasts differentiate into myotubes.

From the immunocytochemistry results, we confirm that the differentiated aggregates have general neuronal characteristics such as NF-68, MAP-2 and TUJ1 positive staining. Furthermore, they also expressed the typical transcription factors of motor neurons. Especially, highly positive ChAT staining suggested that the differentiated neurons with motor neuron markers have the ability to synthesize ACh, the motor neuron

neurotransmitter. Because the glial markers myelin and GFAP were negative, it was ruled out that the ACh comes from glial cells.

In embryos, the formation of NMJ is a hallmark of motor neuron innervation. It was a prevailing belief that AChR clustering depends on the activation of MuSK via agrin released from motor axon growth cone. In contrast to this, recent studies showed that initial AChR aggregation occurs along skeletal muscle fibers independent of neural signals in a MuSK dependent manner. Consistent with these studies, our results showed that pre-patterned AChRs formed clusters on the differentiated myotubes instead of being evenly spread, even without the addition of differentiated neurons. Although the initial AChR clusters form well in advance of innervation, the maintenance of these clusters requires neural agrin and other neurally secreted signals. Observations in our study also support this idea, after 2 weeks co-culture, some bright positive α-bungarotoxin patches did not disappear but directly contacted with neurite endings of the neuron aggregates. The *in vivo* imaging of the spatial and temporal dynamics of motor axon growth cones and nascent terminals showed that motor axon growth cones preferentially target pre-patterned AChR clusters and form synapses at those sites. Furthermore, the aneural innervated AChR clusters dispersed and incorporated into the newly formed synapses. Our results showed that, with the help of high magnification confocal microscopy, many α-bungarotoxin patches were passed along the bundle of neurites extension, but the axon growth cones generally did not contact with those α-bungarotoxin patches. This may indicated that many axons still stay at NMJ early developmental stage even after 2 weeks co-culture. However, at later stages, the neurite endings without obvious growth cone morphology did closely contact with α-bungarotoxin staining patches.

Since there is no contraction in C2C12 differentiated myotubes alone, and there are no visible glial cells in the co-culture, we concluded that EC derived neurons are the only

reason for the induction of myotube contraction in the co-culture and thus act as a convenient experimental system for monitoring cholinergic neuron generation. Although we did not gather solid evidence of NMJ ultra-structure from TEM, the results of immunocytochemistry, SEM and the myotube contraction strongly suggest that these cells may serve as functional cholinergic neurons. Furthermore, these myotube contractions can be manipulated pharmacologically. While the non-selective agonists ACh and Carbachol are both efficient in stimulating myotube contraction, the nicotinic AChR antagonist curare and muscarinic AChR antagonist atropine both stop myotube contraction. Besides chemicals which work on post-synaptic membrane AChRs, the pre-synaptic neurotoxin α-bungarotoxin can also block myotube contraction.

We then use the co-culture system to assess the functional neurons derived from Tera2 EC cells. Comparing the ability to induce the number of contractile events, we demonstrated that RA+Shh induced neurons significantly induced more contractile events than those induced by RA alone. This indicated that there was more ACh secreted by neurons in the RA+Shh condition, and that RA+Shh is more efficient in inducing cholinergic neurons. We have been able to demonstrate that these EC derived neurons acquire the typical morphology of motor neurons, that they express molecular markers characteristic of motor neurons, especially the typical combined transcription factors expressed by motor neurons, and that they secrete the functional neurotransmitter ACh which can stimulate myotube contraction. Our confocal microscopy analysis results also indicated that it is possible that some of these neurons did innervate the differentiated myotubes in co-culture.

Although we can count the contractile events to determine the number of cholinergic neurons and use high magnification microscope to look at the evidence of innervation, we need to conduct physiological assessment of synapse formation to confirm the effectiveness of the connection between neurons and myotubes. It is also necesarry to reveal how they

develop ionic currents necessary for appropriate action potentials, after they receive synaptic inputs. Furthermore, to examine if the EC cell derived motor neurons acquire electrophysiological properties characteristic of embryo derived motor neurons will be valuable to complete our functional study.

An *in vivo* assay chould be carried out, involving transplantation of the EC-derived motor neurons into paralysed animals to assess their ability to make valid connections to muscles and other neurons in appropriate neural circuits. Before we pursue cell replacement therapy for EC derived motor neurons, more should be done to obtain pure motor neurons of a particular subtype, because subpopulations of neurons that express a given transmitter can differ dramatically with respect to size, ion channels, receptors, projection patterns and physiological function.

Chapter Six

Oligodendrocyte and interneuron differentiation from Tera2.cl.SP12 EC cells

6.1 Introduction

Oligodendrocytes form the myelin sheath around the axon in the CNS to support the conduction of nerve impulses. Oligodendrocytes damage will cause severe disorder, such as multiple sclerosis, stroke, spinal cord injury and other traumas. Recent studies have shown that oligodendrocyte precursor transplantation can restore function by enhancing remyelination of the damaged axons (Bambakidis and Miller, 2004). Spontaneous remyelination in the CNS is limited; therefore, an exogenous oligodendroctye resource is required for the cell replacement therapy. To produce large and enriched numbers of human oligodendrocytes for cell therapy, it is necessary to understand the mechanisms underlying gliogenesis both *in vivo* and *in vitro*. The developing spinal cord has served as a valuable model for the study of oligodendrocyte origins.

During the mammalian development spinal cord, neurons and glia are generated from common neuro-epithelial progenitor cells and the processes are tightly regulated by a complex interaction of a variety signalling factors. In the mouse embryo, neurogenesis starts at E12, peaks at E15, and finishes at birth; astrocytes are first detected around E16 and oligodendrocytes around birth (Fu et al., 2002). Along the DV axis, the Shh gradient at the ventral neural tube specifies five distinct classes of neuronal progenitor domains, which are characterized by a combinatorial expression of transcription factors. The fist wave of

oligodendrocytes emerges from the ventral region that generates motor neurons. Oligodendrocytes and motor neurons are not produced at the same time: motor neurons occur first and oligodendrocytes emerge when the neurongenesis is completed in the neural tube. Platelet derived growth factor α (PDGFRα) is one of the best-studied and earliest markers for oligodendrocyte progenitors in developing neural tube. PDGFRα oligodendrocyte progenitors first appear in a highly restricted region of the ventral neuro-epithelium around E14 in the rat (Pringle and Richardson, 1993). If cells from ventral and dorsal halves of E14 neural tube are cultured separately, oligodendrocytes develop only in the ventral culture, suggesting that oligodendrocyte progenitors originate from ventral neuro-epithelialium (Hall et al., 1996). After the initial appearance of PDGFRα cells in the ventral neural tube, these cells soon increase in number and begin to disperse, at E15.5, cells disperse through the ventral neural tube and at E17-18, cells migrate to all regions of the neuro-epithelium (Woodruff et al., 2001).

Olig1 and Olig2 are basic helix-loop-helix gene regulatory proteins, involved in the earliest stages of oligodendrocyte lineage specification. In the mouse embryo, the earliest Olig2-expressing cells appear in the ventral neural tube at E9 and develop into motor neurons not oligodendrocyte progenitors (Wu et al., 2006); however, there are neither motor neurons nor oligodendrocytes if Olig2 is inactivated (Lu et al., 2002) (Figure 6.1.1). By E11.5-12, Olig2 expression is restricted to the PDGFRα expression region that appear at E13, suggesting that this Olig expression marks the earliest stage of oligodendrocyte specification (Lu et al., 2000). By E13, Olig1, Olig2, Sox10, PDGFRα and NG2 proteoglycan are all expressed in the same region of the ventral spinal cord that generates oligodendrocyte progenitors (Zhou and Anderson, 2002). During migration over to the CNS, cells first express galactocerebroside (GalC) at E17 (Calver et al., 1998). Myelin

proteolipid protein (PLP) and its alternative spliced isoform DM20 are also expressed on the cells at a similar stage (Woodruff et al., 2001).

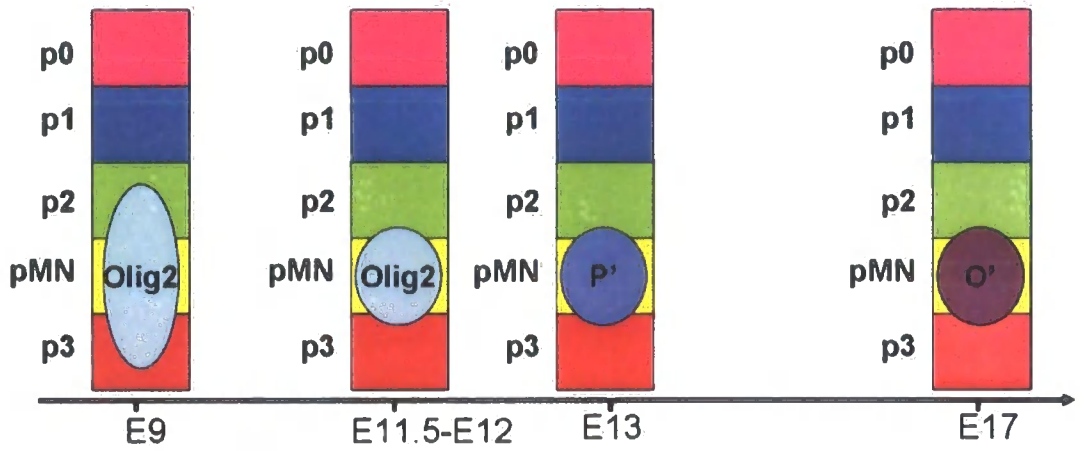

Figure 6.1.1 Schematic showing the change of oligodendrocyte progenitor domain during the mouse spinal cord development. Olig2 expression is restricted at E11.5-12, P' domain is positive for Olig1, Olig2, Sox10, PDGFα and NG2 from E13. O' domain mark the oligogenesis and is positive for oligodendrocyte markers GalC, PLP and DM20 from E17.

Based on Billon's work with ES cell culture experiments, we can follow the normal timing of oligodendrocyte development from neuro-epithelial cell stage (Billon et al., 2002a). Oct-4 expression is extinct in the ES cells after 4 days RA treatment as embryoid bodies and at the same time Sox1 is present, decreased SSEA-1 expression and increased Nestin are detected, suggesting that ES cells are differentiated into neuro-epithelial cells. When neuro-epithelial cells are treated with FGF-2 and Shh for 5 days, oligodendrocyte progenitor markers like Olig1, Olig2, PDGFRα and NG2 were expressed in the induced cells and the expression of these markers continued to increase up to day7. When treated with PDGF-AA and thyroid hormones (TH), oligodendrocyte characteristic morphology then appeared in the induced cells. On Examination of the neuron marker neurofilament and oligodendrocyte progenitor markers, they found that the neural differentiation sequence of ES cells is neurons appears first and is then followed by oligodendrocytes resembling that in normal

neural development in spinal cord. Furthermore, the timing of oligodendrocyte specification in ES cells is similar with *in vivo* time table.

Many signal factors are important in regulating the differentiation of oligodendrocyte progenitors. *In vivo*, oligodendrocyte progenitor specification is dependent on signals that regulate ventral cell type specification. Shh is the major signal factor of the ventral neural tube, it is secreted from ventral floor plate cells and diffuses towards the dorsal neural tube. In both spinal cord and forebrain, oligodendrocyte progenitors arise in close proximity to localised sources of Shh signalling suggesting that Shh signalling is required for oligodendrocyte production *in vivo* (Woodruff et al., 2001). Shh influences the timing of neuron-glia cell fate switching, this is demonstrated in Shh-/-;Gli3-/- mutants, the extended expression of neurogenin2 in Olig2 progenitor domain results in the initial delay an reduction of oligodendrocyte progenitor generation (Saeock Oh, 2005). Oligodendrocyte differentiation is also completely suppressed in Shh-/-;Gli3-/- mutants, implying that Shh is required for oligodendrocyte progenitors to emerge at the appropriate developmental stages and for subsequent terminal differentiation into mature oligodendrocytes (Tan et al., 2006). Once generated, oligodendrocyte progenitors have to follow a developmental program that involves changes in cell morphology, migratory capacity and sensitivity to extra-cellular trophic factors before becoming mature myelinating cells. PDGF-AA is a potent regulator of oligodendrocyte progenitor migration and proliferation (Dubois-Dalcq and Murray, 2000). In the presence of PDGF, oligodendrocyte progenitors tend to keep dividing and fail to differentiate, however in the absence of PDGF, cells rapidly stop dividing and differentiate (Barres et al., 1994). Cells acquire PDGFRα only 1 to 1.5 days after Olig2 expression is restricted to the oligodendrocyte progenitor domain in the neural tube. An investigation into these PDGF responsive precursors from the ventral neural tube have shown that PDGF-AA induced the cell proliferation in a dose-dependent manner and

PDGF-AA combined with FGF-2 maintained the self-renewal in these cells. These cells are bi-potent and can give rise to oligodendrocytes and interneurons but not astrocytes; these cells also have the ability to repond to trophic factors like thyroid hormone, BMPs and CNTF (Chojnacki and Weiss, 2004).

RA is another regulator present in the ventral spinal cord: it also influences the differentiation of oligodendrocytes. It is suggested that RA acts as an early regulator of oligodendrocyte differentiation by inhibiting the maturation of oligodendrocytes and permitting their dispersal throughout the entire spinal cord (Noll and Miller, 1994). Our work and other studies showed that RA induced A2B5 expression in neuro-epithelial cells, suggesting that RA contributes to the specification of A2B5 immuno-reactive oligodendrocyte precursors. However, RA did not induce A2B5 cells to become O4 immuno-reactive oligodendrocyte precursors suggesting that RA prevents oligodendrocyte differentiation progress is at a more mature stage (Noll and Miller, 1994). Additionally, RA partially inhibits the proliferation of oligodendrocyte precursors induced by FGF signalling; RA also keeps them at an immature stage by effectively inhibiting the myelin basic protein mRNA expression in these cells (Laeng et al., 1994). The fact that oligodendrocyte precursors are constitutively expressed RARα and are inducible expressed RARβ suggests that RA plays a critical role in the control of the development of oligodendroctyte (Laeng et al., 1994).

Both TH and RA can induce oligodendrocyte precursors to stop cell division and differentiation *in vitro*, TH also regulates the normal timing of oligodendrocyte development *in vivo* (Ahlgren et al., 1997). Myelination is delayed in hypothyroid animals and accelerated in hyperthyroid animals; furthermore, perinatal hypothyroidism decease the number of oligodendrocytes in the optic nerve, while normal TH is required for the onset of myelination in CNS and the commitment of CNS stem cell to the oligodendrocyte lineage

(Billon et al., 2002b). The effect of TH is mediated by thyroid hormone receptors TRα and TRβ in the nucleus. TRα is ubiquitously expressed from early in development, whereas TRβ is expressed in a more restricted range of tissues and later development. It is reported that TRα is expressed in both oligodendrocyte precursors and mature oligodendrocytes while TRβ is expressed only in oligodendrocytes (Billon et al., 2001), suggesting that TRα mediates the timing of oligodendrocyte development. In TRα-/- mutants, oligodendrocyte precursor proliferation is decreased and results in less oligodendrocytes, whereas over-expression of TRα promotes the oligodendrocyte differentiation in culture.

The general effects of FGF-2 in oligodendrocyte differentiation include: a, induce progenitor proliferation and self-renewal; b, inhibit progenitor terminal differentiation; c, suppress myelin protein transcription; d, stimulate progenitor migration; e, induce cell cycle re-entry and f, increase processes elongation in mature oligodendrocytes (Fortin et al., 2005). Multiple studies have showed that the development of oligodendrocyte lineage cell is influenced by FGF-2 signalling through its receptors FGFR-1, -2 and -3 (Bansal et al., 2003). The expression of FGFR at different stage of oligodendrocytes development indicates that FGFR1 is expressed from early progenitors to differentiated oligodendrocytes but not in myelin, FGFR2 is only expressed at later stages such as differentiated oligodendrocytes and myelin; whereas FGFR3 is only expressed in early stages like early progenitors and late progenitors (Fortin et al., 2005). The developmental switch in the expression of FGF receptors determines the cell response to FGF signalling, and the interaction of specific FGF/FGFR pairs results in distinct physiological responses during oligodendrocyte development (Fortin et al., 2005).

BMPs were shown to inhibit the oligodendrocyte differentiation *in vitro* and oligodendrocyte precursors treated with BMPs differentiate into astrocytes. However, investigation of stage specific effects of BMPs on oligodendrocyte lineage showed that

BMPs affect cell lineage decisions at earlier stages and inhibit cell specialization at later stages (Judith B. Grinspan, 2000; See et al., 2004). Oligodendrocyte progenitors and precursors treated with BMPs will result in astrocytes, but in different subtypes respectively, mature oligodendrocytes treated with BMPs show increased process extension and complexity, normally a feature of oligodendrocyte maturation (See et al., 2004).

Taken together, we conclude that the maturation of oligodendrocytes is regulated by multiple secreted factors present in the CNS during critical stages of development and a deep understanding of when and how these factors regulate oligodendrocyte behaviour during the maturation will help us generate oligodendrocytes efficiently *in vitro*.

Interneuron makes up the major neuron subtype in the neural tube. There are V3, V2, V1 and V0 subtypes in the ventral neural tube, and D1, D2 and D3 in the dorsal neural tube. The inter-neurons within the dorsal spinal cord connect sensory input from the periphery to spinal cord motoneurons and brain. In addition to the important roles that Shh and BMPs signalling play in interneuron generation, Wnt signalling is another factor involved in interneuron specification. Mouse embryos lacking Wnt-1 and -3a are defective at determining dorsal interneurons and BMPs cannot restore the interneurons (Muroyama et al., 2002). Cell explant experiments from dorsal telencephalon showed that Shh induced a large number of GABAergic interneurons while BMP-4 reduced the production of dorsal interneurons, they also suggested that Shh may work by antagonizing BMPs signalling to promote interneuron generation but without a direct requirement for Shh (Gulacsi and Lillien, 2003). Shh is not only required for ventral interneuron specification but also required for maintaining the identity of interneuron progenitors; however, it is not crucial in the interneuron migration and maturation (Xu et al., 2005). A deep understanding of how inductive factors regulate the generation of interneurons will help us reduce the by-product neurons when a specific neuron subtype is generated.

6.2 Methods

6.2.1 Oligodendrocyte differentiation protocol

Tera2.cl.SP12 EC cells were grown as either aggregates or monolayer with RA and/or Shh as previous described in Methods and Materials. After 2 weeks, aggregates and monolayer are trypsinized to produce individual cells before replated to a new tissue culture plate. Cells are grown in oligodendrocyte differentiation medium in the absence of RA and Shh but in the presence of FGF-2 and PDGF-AA for 1 week and then PDGF-AA alone for another week. Cells are induced by PDGF-AA continuously but supplemented with T3 for 1 week, then changed to CNTF and T3 for another week. Oligodendrocyte differentiation medium is derived from SATO modified medium: 5μg/ml NT-3, 1x ITS, 100 μg/ml BSA, 60 μg/ml progesterone, 60 μg/ml N-acetyl-cysteine, 16 μg/ml putrescine, 5 μM forskolin and the supplemented growth factors: FGF-2 20ng/ml, PDGF-AA 10ng/ml, CNTF 10ng/ml, T3 30nM.

6.2.2 Interneuron differentiation protocol

Tera2.cl.SP12 EC cells are grown as monolayer in the presence of RA or RA+Shh for 4 weeks before being trypsinzed to produce individual cells, cells are split at a ratio of 1:5 when seeded in a 6-well plate. Instead of growing in the presence of mitotic inhibitors, cells are grown in 2% HS DMEM for at least 2 weeks before analysis for interneurons. Cells are fixed and stained with specific interneuron antibodies. Images were collected using a Nikon fluorescence microscope or a confocal microscope (Zeiss LSM 510).

6.2.3 Quantification of differentiated oligodendrocytes and interneurons

The differentiated oligodendrocytes and interneurons were collected and produced individual cells before being fixed with 4% PFF and 0.1% glutaraldehyde. Cells were permeablized and stained with primary and secondary antibodies like previous described in intracellular marker staining for flow cytometer. Cells were also stained with DAPI before being counted under the fluorescence microscope. At least 6 fields were counted for each examined specific markers.

6.3 Results

6.3.1 Differentiation of oligodendrocytes from Tera2.cl.SP12 EC cells

There is evidence suggesting that Tera2 EC cells can differentiate into neurons and astrocytes (Stewart et al., 2003). However, we found that no studies have examined the potential of oligodendrocyte generation from the Tera2 EC cells. EC cells cultured under RA and RA+Shh induction for 2 weeks have been shown to develop into rosettes, which represent neural tube-like cells, and we know neural tube progenitors are able to differentiate into neurons and glia *in vivo*. During the neuronal differentiation, we detected the neuro-epithelial like cells expressed Dlx2, A2B5 and Olig2 in Chapter 3 and 4, these oligodendrocyte lineage markers are present during the oligodendrocyte progenitor specification. We therefore tried to generate oligodendrocytes by providing the signalling factors that are required for oligodendrocyte differentiation.

We grew the 2 week RA or RA+Shh induced cells in oligodendrocyte differentiation medium modified from SATO medium supplemented with 10% fetal calf serum without growth factors. The cell clusters settled down and adherent cells emerged around the cell clusters. At this stage the cells actively undergo mitosis, the cell number increased rapidly

in both cell clusters and the surrounding adherent cells. The morphology of some adherent cells changed into columnar epithelial cells by extention of a long cell body. Cells with typical neuron morphology appeared from some of the columnar epithelial cells. At this point the cells were replated in oliogendrocyte differentiation medium supplemented with 5% fetal calf serum and horse serum and cytokine PDGF-AA for 1 week. Cells treated with PDGF-AA did not differentiate into neurons. After 1 week, cells were plated onto laminin coated 6-well plates at a very low cell density and grown in the same medium but supplemented with CNTF and PDGF-AA. Cells were maintained in this medium until they showed multiple processes (about 2-3 weeks). Only a few of the multiple processes cells developed the mature morphology of oligodenrocytes after 6 week of induction. This suggested that the signals in the medium that direct the oligodendrocyte maturation may not sufficient enough. The mature oligodendrocytes identified in our study were O4 positive and possessed structure typical of an oligodendrocyte thus confirming their identity.

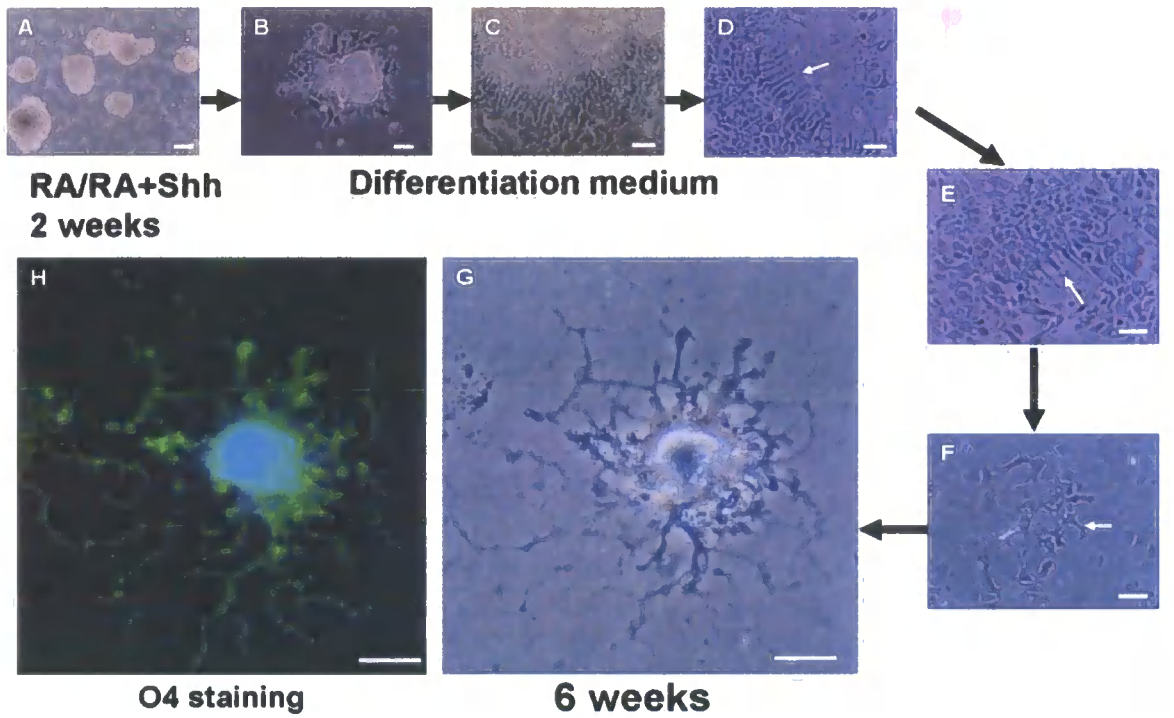

Figure 6.3.1 schematic showing the process of oligodendrocyte differentiation from EC cells. EC cells were seeded at 20,000 cells/cm^2 into bacteriological Petri dishes and treated with RA (10μM) and/or Shh (25ng/ml) for 2 weeks to form suspension aggregates. The suspension aggregates were replated to tissue culture flask in oligodendrocyte differentiation medium for another 4 weeks to produce mature oligodendrocytes. Cells were grown in oligodendrocyte differentiation medium supplemented with FGF and PDGF-AA for the first week, PDGF-AA alone for the second week, PDGF-AA and T3 for the third week, and then CNTF and T3 for the fourth week. White arrows in D, E, and F mark the cell morphology, cells go through columnar epithelial, bipolar, and multiple processes before they are differentiated into mature oligodendrocytes. Note that the mature oligodendrocyte is highly dendritic and branched, the typical oligodendrocyte processes are O4 (green) positive. Scale bars = 50μm.

6.3.2 Staining of differentiated oligodendrocyte derived from Tera2.cl.SP12 EC cells

To confirm the identity of the multiple processes cells grown in the oligodendrocyte differentiation medium, we stained the cells with a range of oligodendrocyte lineage markers: CNPase, O4 and A2B5 (Figure6.3.2). CNPase is one of the earliest known myelin-associated proteins synthesized in developing oligodendrocytes and is induced to a higher expression level at later stage of oligodendrocyte differentiation (Glaser et al., 2004). The differentiated cells with long processes are positive for CNPase (Figure6.3.1G-H), and many of the cells that are CNPase positive extend numerous processes that are usually shorter than those neurite-like processes, but they are not typical mature oligodendrocyte processes either, as shown in Figure6.3.2.

O4 is an antigen expressed from late progenitor oligodendrocytes to mature myelinating oligodendrocytes in rodents. Approximately 10% of differentiated cells in our study were O4 positive (Figure6.3.6). Some positive cells were highly dendritic and branchd, but others were not. This indicates that the differentiated cells are not a homogeneous population or at alternative stages of development.

A2B5 is a cell surface ganglioside epitode that is expressed on oligodendrocyte precursor cells. It is reported that O-2A bi-potential progenitor cells express A2B5 and later will give rise to A2B5 and GFAP positive type-2 astrocytes and oligodendrocytes (Armstrong et al., 1990; McKinnon et al., 1990). Our study showed that the differentiated cells are A2B5 negative, a small fraction of cells are GFAP positive and no cells are both A2B5 and GFAP positive. This indicates that the differentiated astrocytes are not type-2 astrocytes but type-1 astrocytes. The oligodendrocytes and astrocytes differentiated in our study are not derived from the common O-2A progenitors, suggesting that oligodendrocytes and astrocytes have independent progenitors derived from the neuro-epithelial cells which are induced by RA and RA+Shh. However, we can not exclude the possibility that there is a common

progenitor cell population that give rise to both oligodendrocytes and astrocytes in differentiation EC cultures. However, our data indicate that these progenitor cells are not O-2A progenitor cells.

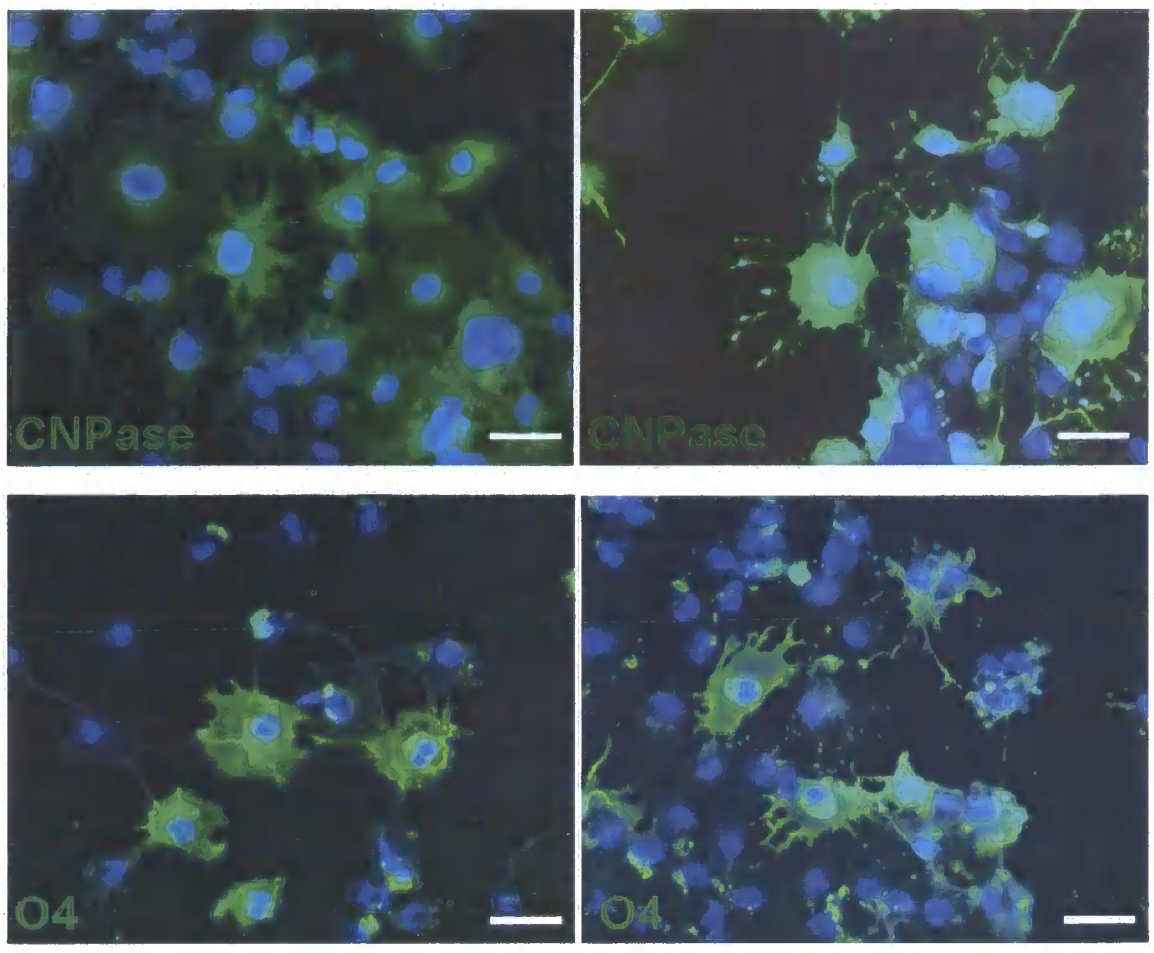

Figure 6.3.2 CNPase and O4 staining of the differentiated oligodendrocytes. EC cells were seeded at 20,000 cells/cm^2 into bacteriological Petri dishes and treated with RA (10μM) and/or Shh (25ng/ml) for 2 weeks to form suspension aggregates. The suspension aggregates were replated to tissue culture flask in oligodendrocyte differentiation medium for another 4 weeks to produce mature oligodendrocytes prior to fixation with 4% PFA and stained with oligodendrocyte markers CNPase and O4 staining. Scale bars = 50μm.

6.3.3 Oligodendrocyte precursor differentiation from Tera2.cl.SP12 EC cells

In order to define the oligodendrocyte precursors, we tried several oligodendrocyte lineage markers in 2 week RA and RA+Shh induced EC cells. Dlx2 is transcription factor that plays an important role in early oligodendrocyte specification, this is demonstrated in Dlx1/2 null mice, in which oligodendrocyte development is disrupted (Yung et al., 2002). We only detected Dlx2 expressing cells in the RA+Shh condition, these cells did not co-expressed βIII tubulin. RA induced cells had a peak expression of Dlx2 during the first week of induction. In RT-PCR experiments, we detected RA induced cells first expressing Olig2 at day14, whereas RA+Shh induced cells expressed Olig2 persistently from the beginning of induction. It is suggested that Dlx2 is an upstream transcription factor for Olig2. During development, Olig2 is expressed predominantly in the CNS, and in the adult its expression is most abundant in oligodendrocyte-rich areas (white matter predominantly) (Woodruff et al., 2001). In the developing neural tube, Olig2 expressing cells are thought to be a common progenitor of oligodendrocyte and motor neuron (Lu et al., 2002). The extinction of Olig2 is essential for the activation of downstream motor neuron specific transcription factors, while the persistent expression of Olig2 as well as Nkx2.2 could be a property of migratory oligodendrocyte progenitors (Woodruff et al., 2001). The expression of Nkx2.2 in both conditions together with Olig2 suggested that there were oligodendrocyte progenitors in the induced cells. NG2 positive cells appear to co-localize to O-2A progenitors *in vivo* (Nishiyama et al., 1996), however, we did not detect any NG2 positive cells in the induced cells, which is consistent with the previous results which showed that no type-2 astrocytes differentiated under the condition. However, we did detect GalC positive cells in the 2 weeks culture (Figure 6.3.3), although a small fraction of these positive cells co-localized with nestin positive cells raising the hypothesis that not all the GalC positive cells in the culture are oligodendrocyte progenitors. Therefore, double

labelling of Olig2 and Nkx2.2 will be required to more accurately identify oligodendrocyte progenitors in the future.

6.3.4 The possible signalling pathway in oligodendrocyte differentiation from Tera2.cl.SP12 EC cells

To identify the possible signalling pathway that helps switch the cell fate from neuron to oligodendrocyte, we collected cytoplasmic protein from cells after 1 week PDGF-AA induced the neuro-epithelial cells (differentiated from 3 week RA induced EC cells) to perform western blotting (Figure 6.3.4). We detected no Gli1 protein and no expression of Smad-1 and p-Smad-1, suggesting that neither the Shh pathway nor BMP pathway was involved in this process. However, ERK-1 and p-ERK are positive by western blotting, which suggests that the ERK pathway might be involved in oligodendrocyte generation. The ERK pathway is an important pathway in ES and EC stem cells (Smith et al., 2004), which is required for cellular proliferation, differentiation and survival. The ERK could be an effector of FGF, NGF and other cytokines activated pathways. The rapid effect phosphorylation of ERK is involved in RA stimulation, which is involved in neuronal survival (Canon et al., 2004). Yim et al (2001) observed that the ERK pathway is activated during the oligodendrocyte differentiation (Yim et al., 2001). It is possible that this pathway is activated during the oligodendrocyte differentiation of EC cells.

Western blotting analysis also showed that cells induced by PDGF-AA did not express motor neuronal marker ChAT nor interneuronal marker GAD65/67, which is consistent with the observation that there were no neurons under this condition.

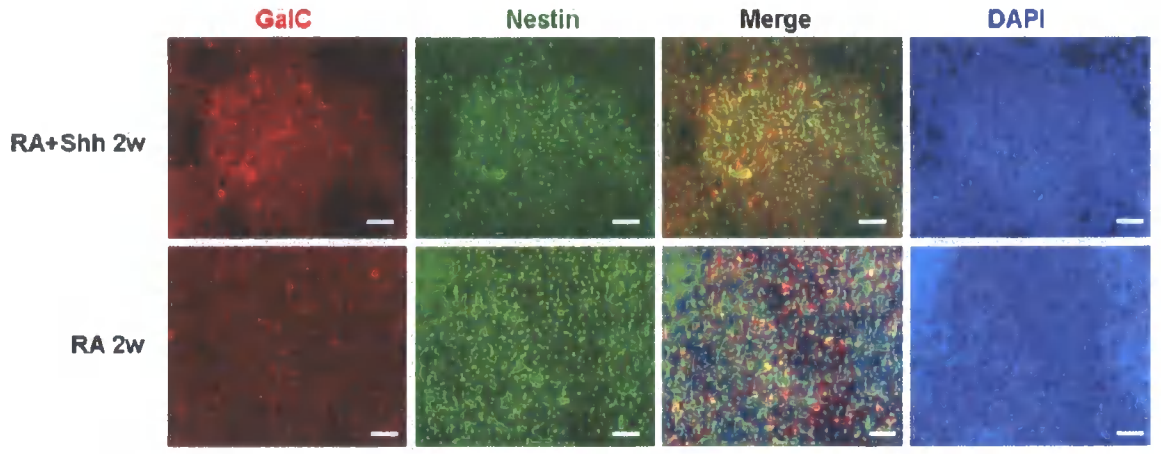

Figure 6.3.3 Analysis of oligodendrocyte progenitor markers in RA and RA+Shh induced EC cells. EC cells were grown with RA (10μM) and/or Shh (25ng/ml) for 2 weeks as monolayer cultures prior to fixation with 4% PFA and stained with oligodendrocyte precursor markers GalC (red), progenitor cell marker Nestin (green). The nuclei are counterstained with Hoechst. The induced cells at this stage are weak at Dlx2 and negative for NG2 (data not shown). Scale bars = 50μm.

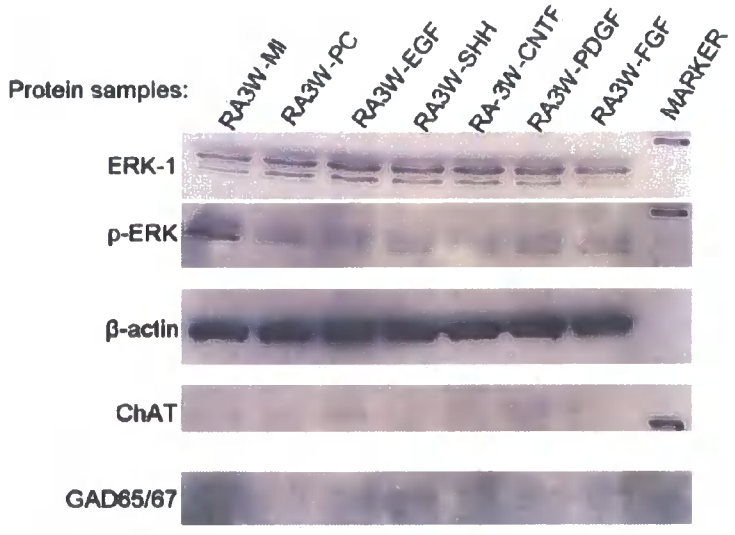

Figure 6.3.4 Western blot analysis of the signalling pathway in induced EC cells. EC cells were grown with RA (10μM) for 3 weeks and then incubated with various growth factors for 1 week before protein extraction. Samples are negative for Shh pathway mediator Gli1 and BMP pathway mediator Smad1 (data not shown). Note that p-ERK is strong in RA3W-MI condition, which directed cells differentiation into neurons. Abbreviations: MI, mitotic inhibitors; PC, PDGF+CNTF. Band size: ERK-1 (p-ERK), 42 and 44KD; ChAT, 68-70KD; GAD65/67, 65 and 67KD; β-acin, 45KD.

6.3.5 Differentiation of interneurons from Tera2.cl.SP12 EC cells

Interneuron functions in neural circuits in spinal cord to receive inputs from sensory neurons and out put excitatory or inhibitory signals to effector neurons (Murashov et al., 2005). To generate pure interneurons from Tera2 EC cells, we modified the differentiation protocol described previously after 4 weeks induction by RA or RA+Shh. Cells were typsinized after 4 weeks induction, but instead of growing them in the presence of mitotic inhibitors in 10% fetal calf serum DMEM, we replated the cells at a low density on 6-well plates in 2% horse serum DMEM. Cells were maintained for at least 2 weeks in this condition before they were harvested and analysed for interneuron markers.

The neurons were positive for GABA, the neuro-transmitter of interneurons; and they were also positive for GAD65/67, an interneuron enzyme responsible for producing GABA from L-glutamic acid (Figure 6.3.5).

We setup a co-culture assay to test these interneurons. The collected interneurons were mixed with C2C12 myoblasts, and the co-cultures were differentiated like previously described in Chapter5. As we expected, we observed no contractile events in the interneuron co-culture system, suggesting that these cells are motor neuron free cells and they produce no ACh to induce myotube contraction.

We also observed the growth of large cells with thin cytoplasm under the interneurons during the differentiation, which might play a role in interneuron generation. A possible mechanism is that these big flat cells produce BMPs to induce the neurons to differentiate into dorsal interneurons, but further experiments are required to confirm this.

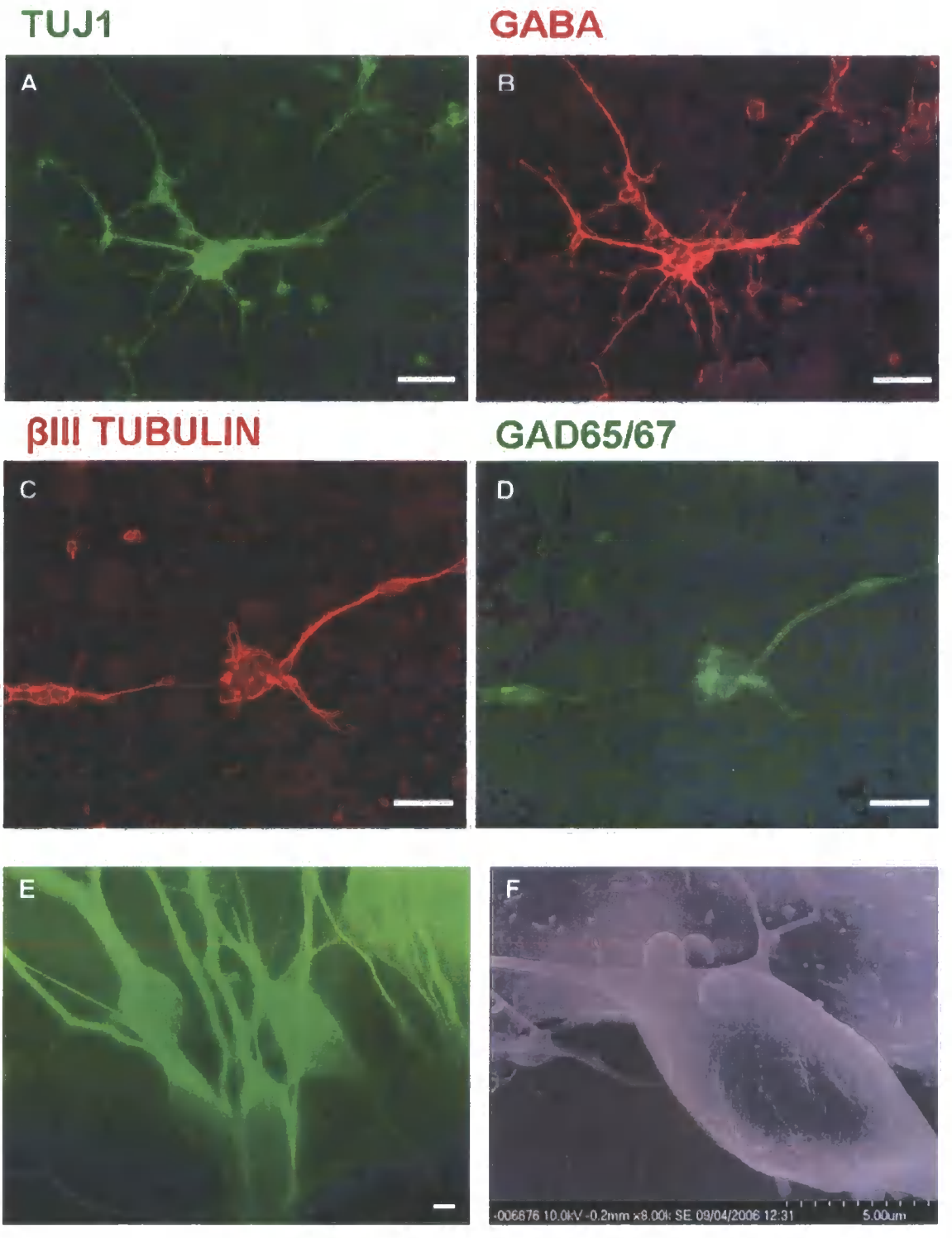

Figure 6.3.5 Images of the differentiated interneurons from EC cells. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) for 4 weeks and then replated in 2% HS DMEM for 2 weeks prior to immuno-labelling. Double staining of GABA (red) and Tuj1 (green) in A and B; double staining of βIII tubulin (red) and GAD65/67 (green) in C and D; typical interneuron morphology βIII tubulin stained (green) in E and SEM of interneuron in F. Scale bars =50μm.

6.3.6 Quanitification of the oligodendrocytes and interneurons derived from Tera2.cl.SP12 EC cells

Tera2.cl.SP12 EC cells were induced by RA and RA+Shh for 2-3 weeks, harvested the cells and replated them according to the oligodendrocyte or interneuron differentiation procedure respectively. After differentiation, the cells were collected and analysed using oligodendrocyte markers O4 and CNPase and interneuron marker GABA. For GABA staining, we washed the cells briefly before fixed with 4% PFA and 0.1% glutaraldehyde. In this way, we can remove cell surface GABA but preserve the GABA in the cell body (images shown in figure 6.3.6). Cells were permeablized with 1% triton-x-100 before staining with the primary antibodies. Positive cells were counted and graphed as shown. There are fewer O4 positive cells than CNPase positive cells, about 10% verses 28%. A possible explanation is that CNPase may be present in the more mature cells in differentiated oligodendrocytes. For the GABA staining procedure, the washing step is very critical, because GABA is a small soluble molecule, which is immobilized by cross linking to the adjacent large protein molecules. Intense washing before fixing with PFA and glutaraldehyde will result in no GABA staining in the interneurons. We observed more than 50% GABA neurons by this means, however, specific additional staining needs to be done to further identify whether they are dorsal or ventral interneurons.

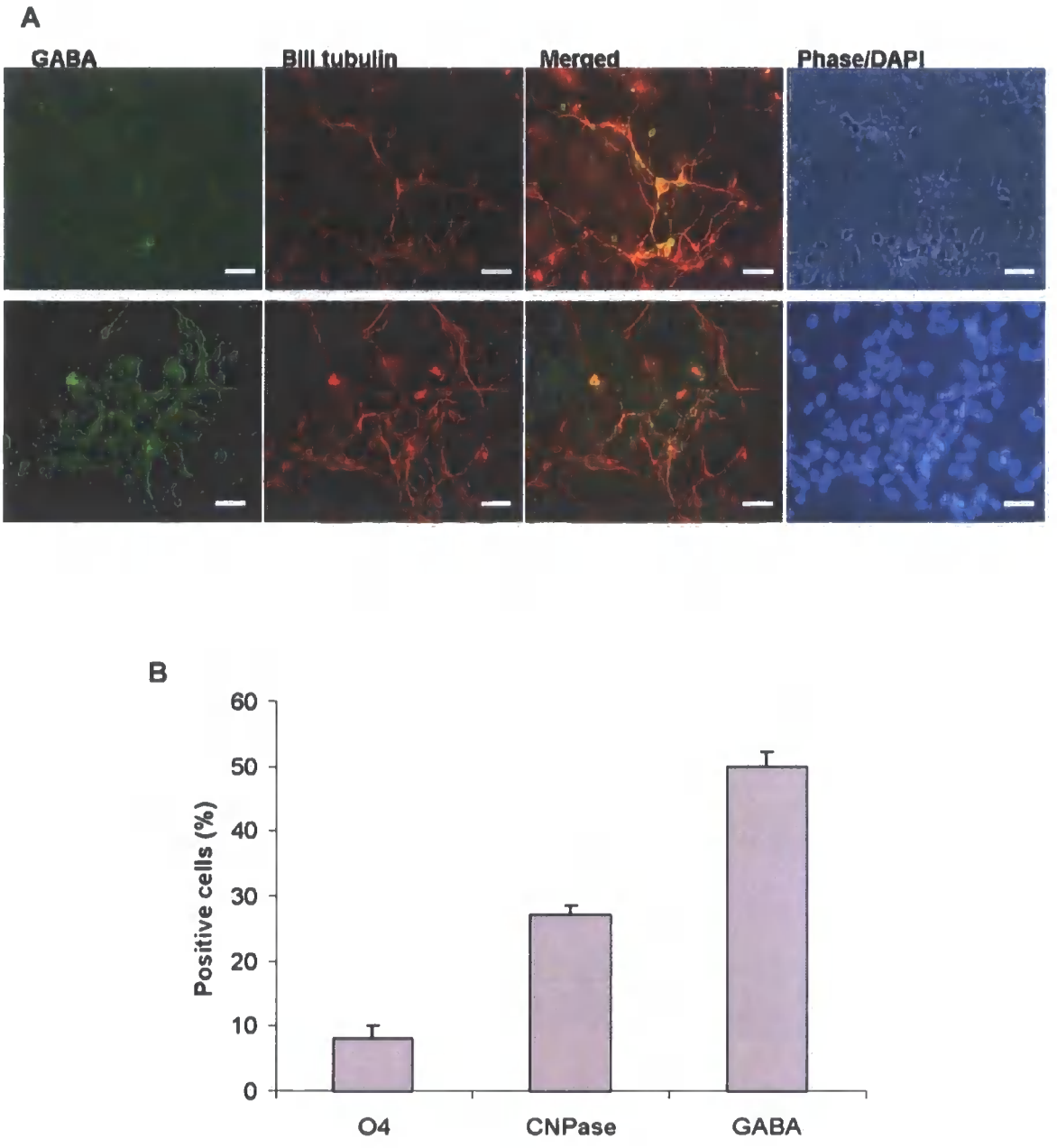

Figure 6.3.6 Typical GABA staining images (A) and the quantification of the differentiated oligodendrocytes and interneurons (B). A, showed the GABA staining of the differentiated interneurons. EC cells were seeded in the 6 well plate at 20,000 cells/cm^2 supplemented with RA (10μM) and/or Shh (25ng/ml) for 4 weeks and then replated in 2% HS DMEM for 2 weeks prior to immuno-labelling with GABA (green) and βIII tubulin (red). B, show the quantification of O4, CNPase positive cells in the differentiated oligodendrocytes, and GABA positive cells in the differentiated interneurons. Error bars represent SEM, n=6. Scale bars = 50μm.

6.4 Discussion

During development, the proliferation and differentiation of oligodendrocytes from cells of the neural tube is closely regulated by intrinsic and extrinsic factors. During their cellular maturation, oligodendrocyte precursors go through a series of discrete differentiation steps which can be identified by the expression of particular cell surface epitopes and proliferative responses. A fraction of neuro-epithelial cells that express A2B5 will differentiate into oligodendrocyte pregenitors; subsequently, oligodendrocyte precursors begin to express antigens recognized by O4; this intermediate stage is replaced oligodendrocytes that express GalC, a major myelin glycolipid, before they mature into myelinating oligodendrocytes. This expression sequence helps us to recognize oligodendrocyte differentiation stages in our *in vitro* study. RA and RA+Shh can both induce EC cells to express A2B5 after 2 weeks induction, suggesting that these A2B5 positive cells have the capacity to differentiate into neurons, astrocytes, and oligodendrocytes. We treated these cells with inductive factors that favour oligodendrocyte differentiation and attempted to generate oligodendrocytes from them.

In the late embryonic CNS, oligodendrocyte precursors intrinsically possess all the information required for their subsequent differentiation, because the differentiation into GalC positive cells does not appear to require positional information or the presence of other neural cell types. It is suggested that once the A2B5 positive cells acquire an oligodendrocyte identity at an early specification stage, the intrinsic mechanism will direct them to differentiate into terminal oligodendrocytes.

However, even if all the information necessary for oligodendrocyte differentiation is present in the precursors, the temporal progression through the stages of maturation appears to be influenced by cellular environmental factors. It provides us with an opportunity to

induce cell proliferation, to obtain enough mature cells, it also provides us methods to delay or accelerate cell maturation. PDGF can stimulate oligodendrocyte precursor proliferation, although it will delay the cells progression to mature oligodendrocytes, it selectively increases the cell number of oligodendrocyte precursors. The withdrawal of PDGF stops cell division and stimulates the cells to exit cell cycle to undergo differentiation. CNTF also has an effect on differentiated oligodendrocytes and induce processes elongation, and TH stimulates the expression of various myelin protein genes. Therefore, we applied these secreted factors that function in the critical stages of oligodendrocyte maturation *in vivo* to produce oligodendrocytes from human EC cells. After sequential treatment with these factors, we demonstrated that differentiated oligodendrocytes do appear to exist in our cell culture. Further testing these factors on EC cells to investigate their exact function and to refine the concentration and timing of application will help us to generate oligodendrocytes efficiently.

It has been reported that oligodendrocytes can be efficiently generated from ES cells. These reports can be divided into two categories: one is genetically modified the ES cells differentiate into purified oligodendrocytes (Billon et al., 2002a), the other is sequential application of those factors that function in oligodendrocyte maturation after inducing the ES cells into neural precursor cells. In the latter case, ES cells were differentiated into neural rosettes, detached to form spherical neural mass on bacterial Petri dishes and expanded in the presence of FGF-2 and N2 supplement. After 5 days, cells were plated onto matrigel-coated dishes and sequentially treated with EGF, PDGF and T3 to generate mature oligodendrocytes (Kang et al., 2007). We found that this method is somewhat similar to the protocol used here: we also grow the EC cells into neural rosettes first, and then trypsinized the neural rosettes to plate in a new culture dish and then applied EGF, PDGF and T3 sequentially to the cells. Although we do not generate oligodendrocytes at a very high

efficiency, we demonstrated that, like ES cells, EC cells follow a similar pathway in oligodendrocyte development *in vivo*. This also suggests that the EC cell system may be especially useful for studying the specification of neuro-epithelial cells towards the oligodendrocyte lineage.

Our results also indicated that regulators such as Shh, RA, FGF and PDGF only function at an early stage of oligodendrocyte specification. Once oligodendrocytes have acquired their identity, they possess all the information required for subsequent maturation. Therefore, we were unable to detect the activation of any Shh and BMPs pathway signalling mediators by western blot while the cells were undergone maturation. The only active pathway detected in this process by western blot was ERK, which can be the downstream pathway of FGF and PDGF signalling. There are many factors involved in oligodendrocyte differentiation, and their exact functions are still controversial. We have yet to identify a factor that is sufficient and necessary in oligodendrocyte maturation in our study. Furthermore, the underlying mechanism that how the precursors acquire an oligodendrocyte identity and how these precursors control progress toward mature cells is still unclear. Therefore, if we could identify the transcription factors that regulate this process and how the environmental factors regulate these transcription factors, this would help to reveal the underlying mechanisms. There is also need to investigate the effect of these factors on EC cells to improve the efficiency of oligodendrocyte generation.

Development of the neural tube is guided by concentration gradient of RA, Shh and BMPs along the dorso-ventral axis. Upon treatment with RA and Shh, EC cells express Pax6 and then HB-9 that direct the cells to differentiate into motor neurons. While differentiation of EC cells into motor neurons may be successfully achieved by treatment with RA and Shh, the potential to derive GABAergic interneuron from EC cells has not yet been tested.

After treated with 4-/4+ protocol, to induce differentiation of ES cells, they were grown with RA, Shh, BMP-2 and Wnt3A for 4 days and then replated in optimal medium for neuron differentiation (Zhou et al., 2005). Analysis of the differentiated neurons showed that more than 50% of neurons were dorsal interneurons. Compared to our protocol, EC cells were replated in 2% horse serum medium for 3 weeks after 4 weeks RA or RA+Shh treatment. One factor is common: RA is the critical signalling factor required to induce neuraliztion in ES or EC cells. The protocol we used here is similar to the motor neuron generation protocol, however, after cells had been replated in 2% horse serum for several days, we observed a non-neuronal cell layer appear underneath the neurons. The neruons grown on this cell layer for three weeks do not induce myotube contraction when co-cultured with myoblasts. It is suggested that these cells contain no motor neurons and secrete no motor neuron neurotransmitter ACh. It is also suggested that contact with the non-neuronal cell layer prevents motor neuron generation in the culture. The GABA and GAD65/67 staining results suggest their interneuron identity. However, we do not know whether or not they are dorsal interneurons without further testing the dorsal interneuron specifc markers. If we consider the role of BMPs in the specification of interneuron in the neural tube, the source of BMPs during the interneuron differentiation could be the non-neuronal cell layer underneath, or even the EC cells themselves; since EC cells have ectodermal properties (ectoderm secreted BMPs during embryogenesis). Future studies are required to generate specific classes of interneurons and to investigate the molecular mechanisms that control the development of interneuron.

We are the first to demonstrate that Tera2.sp12 EC cells have the capacity to generate oligodendrocytes and interneurons. These results thus show that Tera2.sp12 EC cells can act as a convenient experimental system for monitoring neural induction.

Chapter Seven

Summary discussion and future direction

The overall aim of this project was to use Tera2.cl.SP12 human embryonal carcinoma stem cells as an *in vitro* model to study neurogenesis along the dorso-ventral axis. Neurogenesis in the developing neural tube requires morphogens such as RA, Shh and BMPs to provide the positional information and differentiation cues.

The human stem cell model we use in this study is Tera2.cl.SP12 EC cells; this cell line has the ability to differentiate into epidermal cells and neural cells, suggesting that these cells may represent the cells of primitive ectoderm (Stewart et al., 2005). It is known that cells of primitive ectoderm give rise to the neural plate and later form the neural tube. *In vivo*, Shh is secreted from notochord, and RA from somites, together they coordinate the neural tube formation and specify the progenitor domains of the ventral neural tube. However it can be difficult to distinguish the individual effects of RA and Shh during neurogenesis in the complicated *in vivo* models. By using the Tera2.cl.SP12 cell line, we can study the roles of RA and Shh during neurogenesis at the cellular level as well as in a human *in vitro* model. We can also test whether or not the mechanisms of neural development derived from lower species are conserved in man.

Tera2.cl.SP12 cells responded differently to RA and Shh induction. Cells induced with RA form rosettes were first observed after 2 weeks culture, while cells treated with Shh formed no rosettes. Staining with the proliferation marker Ki67 showed that cells grown in both conditions remain proliferative; under RA induction, the Ki67 positive cells were concentrated at the centre of the rosettes, while the positive cells in the Shh condition were scattered around in the whole culture. We have shown that RA and Shh act as mitogen in

neural differentiation, since cells from both conditions are positive for the neural progenitor marker nestin. However, there are two types of nestin positive cells: RA-induced nestin-positive cells are long thin bipolar cells with typical neural progenitor morphology, while Shh-induced positive cells possess a rounder cell body with a larger cytoplasm. Collectively, these data suggest that although cells in both conditions are committed, the RA-induced cells progress to a more advanced stage of neural differentiation. Staining with neuronal marker βIII tubulin was also consistent with this observation. There are many βIII tubulin positive cells in the RA condition, and the cells appear at the edge of the rosettes, while there are very few positive cells in Shh condtion. This is also consistent with the observation that RA induces pro-neuronal transcription factors NeuroD/M, which promotes the pan-neuronal identity of neuronal progenitor cells *in vivo*. By contrast Shh had no effect in regulating of this group of transcription factors (Wilson and Maden, 2005). Furthermore, we also investigated the combined effects of RA and Shh on these cells. RA+Shh induce similar cell responses to that of the RA condition. Taken together, the data suggest that RA and Shh have different roles during neurogenesis, they induced different cell behaviours, and RA is responsible for the predominant effect in neural differentiation compared to Shh. After treatment with Shh for 2 weeks, Tera2.cl.SP12 cells are over-grown, but formed no rosettes, and the cell morphology remains exclusively epithelial cells. We observed a significant increase in cell number in the Shh induced cells at 7 days compared to other conditions, and these cells express high levels of Oct4. High levels of Oct4 are related to pluripotency and the self-renewal ability of embryonic stem cells (Babaie et al., 2006). It suggests that after 7 days Shh induced cells remain pluripotent and capable of self-renewal. However, after exposure to Shh for 2 weeks, these cells show a great reduction in Oct4 expression even though they remain the epithelial morphology. Further examination of cell surface markers by flow cytometry shows that stem cell markers SSEA-3 and TRA-1-60

are also reduced in these cells, but the low level of expression differentiation marker A2B5 may explain the slow change in morphology of these cells. On the contrary, RA and RA+Shh induce a peak of cell number at later a time point (14days and 18days, respectively), after 2 weeks time, treatment with either RA or RA+Shh the cells are more differentiated, which is demonstrated by very low level of Oct-4, SSEA-3 and TRA-1-60, and a high level of expression of differentiation marker A2B5. This suggests that Shh also induces cell differentiation of Tera2 cells but at a lower rate, compare to RA.

It is known that the Shh pathway plays multiple roles in neural development *in vivo* (Patten and Placzek, 2000). Previous studiess have demonstrated that Shh binds to its receptor complex (Ptc and Smo) on the target cell membrane (Murone et al., 1999). In the absence of Shh, Ptc-1, the suppressor of the pathway, always inhibits the phosphorylation of Smo. Once Shh binds to Ptc-1, Ptc-1 releases its inhibition on Smo, the activator of the pathway. Smo becomes phosphorylated and to initiate the activation signal which is passed to the downstream pathway components in the cytoplasm. Although the cytoplasmic components are as yet unidentified, the effectors of the pathway have been demonstrated to be Gli transcription factors. Gli factors mediate the Shh signalling in the nucleus by activating other transcription factors to induce cell proliferation and specification. In Tera2.cl.SP12 cells, Shh induces Ptc-1 and Gli1, which is a conserved response in vertebrates and invertebrates. If we block the Shh pathway with the Shh antagonist cyclopamine, there is no expression of Ptc-1 or Gli1 after two weeks induction. RA induces a different expression pattern of Ptc-1 and Gli1; it induces low expression of Ptc-1 and Gli1 at the first week, but much higher expression at the second week, suggesting that RA has the ability to regulate the Shh pathway, in Tera2.cl.SP12 cells. On the contrary to Shh, RA treatment inhibited the expression of Ptc-1 and Gli1at first, and activates the pathway later. The treatment with RA+Shh seems to have had the combined effect of RA and Shh respectively, indicating that

Shh is the predominant factor during the first week, and RA has the predominant effect during the second week. Blocking Shh signalling by cyclopamine in RA+Shh condition, resulted in strong expression of Ptc-1 but no Gli1 expression at the second week, suggesting that Ptc-1 is regulated by RA, but Gli1 is an indicator of effective Shh signalling. Additionally we also observed that Gli2 and Gli3 were not regulated by these factors in EC cells. By using the siRNA to knock down the function of Gli1 in EC cell differentiation, it is confirmed that Gli1 not only mediates a large part of the proliferation effect in EC cells but also activates key patterning transcription factors like Pax6. Blocking Gli1 expression leads to defective expression of nestin and βIII tubulin, suggesting that Gli1 is involved in neural differentiation. Even in RA induced cells, RA can not rescue the entire Gli1 defect, suggesting that RA might stimulate endogenous Shh expression to mediate some of its functions.

RA and Shh have different neural differentiation abilities. After one week, cells induced with either RA or Shh are positive for nestin; after two weeks, RA-induced nestin positive cells form rosettes but this is not observed in Shh induced cells; by five weeks, nestin positive cells are only present in Shh induced cells but not in RA induced cells. Comparing βIII tubulin staining, the number of positive cells increases rapidly in the RA condition, while there are only a few positive cells in Shh condition after 5 weeks induction. This suggests that Shh intends to keep cells at immature progenitor stage and produce no mature neurons, by comparison, RA is a powerful factor capable of inducing terminal differentiation and producing mature neurons after 5 weeks induction. Combining the two factors, we observe mature neurons as seen in the RA condition and extended nestin expression as seen in the Shh condition.

Motor neurons are derived from the ventral spinal cord; this process is well studied *in vivo*, especially in the chick embryo (Novitch et al., 2003; Soundararajan et al., 2006). We

detected HB-9 expression in the RA and RA+Shh induced cells after 3 weeks. HB-9 is a critical transcription factor that plays important role in motor neuron progenitor specification and is persistently expressed in mature motor neurons (Arber et al., 1999). By western blot, RA is shown to induce HB-9 and ChAT expression, the addition of Shh greatly increases the expression level of these two motor neuron markers. We also examine the timing of the addition of Shh: there is no increase in the expression of HB-9 and ChAT if Shh was added after 2 weeks, suggesting that Shh acts only at an early stage of motor neuron differentiation. Increasing the concentration of Shh increases the expression level of motor neuron markers, suggesting that the number of differentiated motor neurons is Shh dose-dependent. The addition of the BMP-4 antagonist chordin also increases the motor neuron expression level, suggesting that BMP signalling may also be involved in this process.

The expression of typical motor neuron markers such as Lim3, Isl-1, HB-9 and ChAT indicates that motor neurons are generated from the RA and RA+Shh induced Tera2.cl.SP12 cells. If the motor neurons are functional, they will form specialized synapses with myotube and induce myotube contraction when depolarized. To test the function of the differentiated motor neurons, we set up a co-culture system. First, cells from mouse muscle cell line C2C12 were differentiated into myotubes, and no spontaneous contraction was observed. We then grew the Tera2.cl.SP12 cells in RA and RA+Shh conditions for about 4 weeks before trypsinizing them and grow as aggregates in the presence of mitotic inhibitors for about 1 week. In the presence of mitotic inhibitors, most of non-neuronal cells undergo apoptosis and the neuronal cells form neuronal aggregates with long extended and well connected neurites. The neuronal aggregates were collected and mixed with the C2C12 cells and maintained in the myotube differentiation medium for about 1 week. The neuronal aggregates extended neurites and made close contacts with

differentiated myotubes. When the motor neurons spontaneously depolarized in the co-culture, myotube contractions were observed.

The co-culture was stained with α-bungarotoxin, a snake poison that binds specifically to nicotinic AChR in neuro-muscular junctions; this showed that the differentiated neuronal aggregates induce AChR clusters on the membrane of the myotubes. With higher magnification, many neurite endings form boutons, although there are still many growth cones in the co-cultures, furthermore, where boutons are loacted, there are always AChR clusters co-localized on the myotube membrane. With SEM, we observed the neurite endings form close contacts with myotubes. Although we did not manage to view the neuro-muscular junction structure with TEM, it is plausible that the differentiated neurons and myotubes form effective connection with each other, and this connection is the main cause of the myotube contraction in the co-culture.

Comparing the spontaneous contractions observed in RA and RA+Shh induced neurons, the addition of Shh appears to induce more contractile events in the co-culture. This is consistent with the western blot data that shows that the addition of Shh induces more motor neurons from EC cells. When we manipulated the contractile events with AChR antagonists and agonists, we found that agonists like ACh, carbachol, nicotine and muscarine induce only a few myotube contractions in the absence of neurons; however, the agonists induce more contractile events in the neuron-myotube co-culture conditions. Carbachol is a non-selective AChR agonist, it increases the number of contractile events in the co-culture, indicating that carbachol helps the potential neuro-muscular junction to reach threshold and depolarize. Antagonists, like curare, atropine and α-bungarotoxin can inhibit contractions in the co-culture, indicating that blocking AChR will delete the myotube contractions.

RT-PCR is employed in this study to examine the patterning transcription factors during neural differentiation. The majority of the transcription factors tested show a temporal expression when induced by RA and Shh. The response of transcription factors to RA and Shh is consistent with *in vivo* model. For example, Shh inhibits Class I transcription factors like Pax6, while RA induces Pax6 expression. Shh induces Class II transcription factors like Nkx2.2 and Olig2, while RA represses their expression in EC cells. Furthermore, each transcription factors exhibits a unique response to the RA+Shh condition: in Pax6 and Nkx2.2, RA has the predominant effect, while in Olig2 expression, Shh plays the major role.

Based on these results, we are able to summarize the sequence of transcription factors activation in motor neuron specification in human EC cells. EC cells express high level of Oct4 and low level of patterning transcription factors such as Pax6, Dlx-2, En-1 and Olig2. When EC cells are induced by RA and Shh, additional patterning transcription factors such as Pax3/7 and Nkx2.2, are activated, and the expression level of the transcription factors is elevated. Transcription factors present in the cytoplasm of EC cells are transferred into nucleus and the accumulation of expression in the nucleus is a symbol of EC cell activation. As Oct4, Gli1and Nkx2.2 are down-regulated, Lim3 and MNR2 are activated, indicating that cells become more committed. Of the transcription factors active at this stage, Lim3, MNR2 and Olig2 are all involved in motor neuron specification; furthermore, Olig2 and A2B5 are also involved in oligodendrocyte progenitor specification. With the extinction of Olig2 and A2B5, more motor neuron specific genes are activated. The expression of pan-neuronal transcription factors Ngn2 and NeuroD is then acquired, thus indicating that the generated motor neuron progenitors acquire the general neuronal identity, therefore, the motor neuron progenitor is generated. Later, the progenitor cells will differentiate into different motor neuron subtypes. This transcription factor cascade also builds on the work

from previous *in vivo* experiments which have provided a wealth of information about motor neuron specification and maturation. It suggests that, at least *in vitro*, human motor neuron differentiation follows a similar program to that of lower species.

To further test this activation model of transcription factor expression, we attempted to differentiate oligodendrocytes from EC cells. EC cells were grown with RA or RA+Shh for about 2-3 weeks before replating in oligodendrocyte differentiation medium, which is derived from SATO medium supplemented with growth factors that favour the specification of oligodendrocyte progenitors. After 4 weeks induction, most of the neurons are depleted from the culture and there are oligodendrocyte-like cells present in the culture. At least 25% of cells at this stage are CNPase positive, and half of them are O4 positive. The differentiated oligodendrocytes are A2B5 negative, suggesting that they are not derived from O-2A progenitors, the common progenitor of oligodendrocyte and type II astrocyte. Instead, these oligodendrocytes may share a common progenitor with the differentiated motor neurons.

Next, we examined whether or not Tera2.cl.SP12 cells could give rise to interneurons. At least 50% of differentiated neurons from Tera2 are GABA positive, GABAergic neurona are the inhibitory interneurons that are most frequently observed in the CNS. Further investigation is needed to identify whether these GABA neurons belong to ventral or dorsal groups. It is suggested that GABA interneurons are the most frequent by-products when ES cells are directed to differentiate into motor neurons or dopamine neurons *in vitro*. The specification of GABA neurons depend on the signalling of Shh and FGF (Zhang et al., 2007). The early treatment of FGF8 increases dopamine neurons but later treatment of FGF8 facilitates GABA neuron differentiation (Yan et al., 2005). This suggests that neuro-epithelial cells make cell fate decision depends on the presence of environmental cues such as the amount, sequence and timing of morphogens needed and the additional signals.

In this study, the findings suggest that the neural development mechanisms involving RA and Shh signalling derived from lower species are conserved in man and that Tera2.cl.SP12 EC cells are a useful *in vitro* human model to study neurogenesis along the dorso-ventral axis and the expression profiles during the motor neuron differentiation. In addition, the development of a co-culture model provides a useful tool to study the function of motor neurons and the formation of neuro-muscular junctions or even for use as a drug screen.

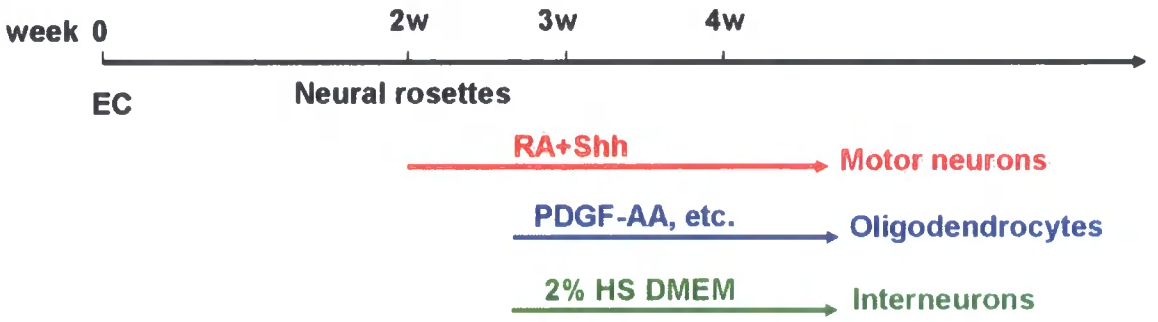

Figure 7.1 Schematic procedure for inducing motor neuorns, oligodendrocytes and interneurons of Tera2 EC cells. EC cells were differentiated into neuro-epithelial cells that exhibit neural tube-like rosettes in two weeks with RA or RA+Shh treatment. Further induction with the indicated conditions results in the differentiation of motor neurons, oligodendrocytes and interneurons.

Future studies are required to improve the efficiency of producing specific neural subtypes. We know that Tera2 EC cells can differentiate into motor neuron, interneuron and glial cells (Figure7.1). When we generate motor neurons from EC cells, the interneuron by-products are also mixed in the motor neurons. The next stage will be to adjust the induction program to produce as pure a populaton of motor neurons as possible, this could be achieved using FACS to select the motor neurons from the neuronal mixture. Application of this pure motor neuron population includes cell transplantation and the assessment of motor neuron function *in vivo*.

This research has enabled a greater understanding of the molecular pathways involved in the specification of particular neural subtypes from human stem cells. It clarifies that RA and Shh interact in gene regulation, cell specification and neuron maturation in a human stem cell model. Furthermore, these findings can be easily applied to human ES cells.

This project presents the first data on the generation of motor neurons and oligodendrocytes from human Tera2 EC cells, and increases an understanding of the molecular mechanisms that regulate neural differentiation in man. This work in turn has provided a platform from which many future studies will arise, since there remain many unanswered questions about the mechanisms of neural development and stem cell biology.

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