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# **Neural Differentiation from Human Embryonal Carcinoma Stem Cells**

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**Chendong Pan**

A Thesis submitted to the University of Durham for the degree of  
Doctor of Philosophy

Department of Biological and Biomedical Sciences

2007

Supervisor: Dr S.A. Przyborski

17 OCT 2007



## **Abstract**

### **Neural differentiation from human embryonal carcinoma stem cells**

It is understood that retinoic acid (RA), sonic hedgehog (Shh) and bone morphogenic proteins (BMPs) play an important role in cell fate determination and the specification of inter-neurons and motor neurons along the dorsal-ventral axis in the neural tube. In this study, we investigated the function of these signalling molecules to instruct the differentiation of human pluripotent stem cells to form specific neuronal subtypes. TERA2.cl.SP12 embryonal carcinoma (EC) cells are a robust caricature of human embryogenesis and an accepted model of neural differentiation. Gene and protein expression analyses using RT-PCR, western blotting and immunocytochemical techniques indicated that human EC cells respond to RA, BMPs and Shh in a conserved manner and regulate neural transcription factors and structural proteins in a predicted way as cells commit toward the motor neuron phenotype. To assess the function of these differentiated neurons, we tested their ability to innervate skeletal muscle myotubes and induce muscle cell contraction. Myotubes contracted only when cocultured with neurons. The number of contractile events increased significantly when cells differentiated into motor neurons were cocultured with myotubes compared to cocultures with cells that formed interneurons. Staining for  $\alpha$ -bungarotoxin showed positive staining in a pattern characteristic of boutons found in neuromuscular junctions. We also showed that muscle contraction could be manipulated pharmacologically: curare and atropine blocked myotube contraction, whereas acetylcholine and carbachol increased the number of contractile events. In other experiments, we have also shown that cells exposed to RA and Shh in conjunction with other growth factors over different time periods, preferentially form oligodendrocytes and/or interneurons. These results indicate it is feasible to control and direct the differentiation of human stem cells and produce specific neuron subtypes in vitro. Furthermore, this system acts as a useful model to investigate the molecular mechanisms and signalling pathways that control neural differentiation in man.

## Declaration

I declare that the work within this thesis, submitted for the degree of Doctor of Philosophy, is my own original work, except where stated, and has not been submitted for another degree at this or any other university.

Signed *Chendong Pan*.....

Date.....14-9-2007.....

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## Acknowledgements

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This thesis is dedicated to my parents and to my daughter Carla.

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## Contents

Abstract	II
Declaration	III
Acknowledgments	IV
Contents	V
List of Figures	XII
List of Tables	XVI
Abbreviations	XVII

### Chapter 1: Introduction

1.1	The five development process of the vertebrate's embryogenesis	1
1.2	Gene expression in the control of embryonic development	4
1.3	DV patterning and neurulation in vertebrate embryo	6
1.4	Specification of the neuron progenitor domain along Dorso-ventral axis	9
1.5	Regulation of neural specification in neural tube	12
1.6	The Shh signalling pathway in DV patterning of neural tube	12
1.6.2	Components of Shh pathway	13
1.6.2.1	Role of Ptc in the Shh receptor complex	14
1.6.2.2	Role of Smo in the Shh receptor complex	14
1.6.2.3	Other Shh binding proteins on the cell surface	16
1.6.3	Cytoplasmic transducers of Shh signalling	17
1.6.4	Transcriptional effectors of Shh pathway	19
1.6.4.1	The interaction of Gli proteins	20
1.6.4.2	Gli factors in neural tube development	21
1.7	Shh signalling and its influence of cell behaviour	23
1.8	Shh signalling and neural differentiation	26
1.8.1	Shh and motor neuron specification	26
1.8.2	Shh and dopaminergic neuron differentiation	30
1.8.3	Shh and oligodendrocyte differentiation	31
1.8.4	Shh and axon guidance	33
1.9	The interaction of Shh and other signalling factors	34
1.10	Retinoic Acid signalling	35

1.10.1	RA source and its signalling pathway	35
1.10.2	Role of RA in neural patterning	37
1.10.3	RA regulates the Shh pathway	39
1.11	Interaction between BMP and Shh signalling	40
1.12	Interaction between FGF and Shh signalling	42
1.13	Interaction between Wnt signalling and Shh signalling	45
1.14	Interaction of other growth factors and Shh signalling	46
1.15	Stem cell models	47
1.15.1	ES cells	47
1.15.2	Adult stem cells	51
1.15.3	Human embryonal carcinoma stem cells	53
1.16	Aims and objectives of the study	56

## **Chapter 2: Materials and General Methods**

2.1	Source of Materials	68
2.2	Instruments and Equipment	74
2.3	Preparation of Standard Solutions	75
2.4	General Methods	58
2.4.1.1	Tera2.cl.sp12 EC cell culture	58
2.4.1.2	Mouse C2C12 Myoblast cell culture	59
2.4.1.3	Neuron-Myoblast co-culture assay	59
2.4.2.1	Cell counting	60
2.4.2.2	Cell proliferation assay	60
2.4.3	Western blot analysis	61
2.4.3.1	Preparation of the SDS-PAGE gel	61
2.4.3.2	Preparation of protein samples and Gel Electrophoresis	61
2.4.3.3	Immunoblotting	62
2.4.3.4	Protein samples extract from Nuclei	63
2.4.4	Reverse Transcription Polymerase Chain Reaction	63
2.4.4.1	Total RNA isolation	63
2.4.4.2	DNA digestion	64
2.4.4.3	Reverse Transcription	64

2.4.4.4	Polymerase Chain Reaction	64
2.4.5.1	Flow cytometry	65
2.4.5.2	Preparation of 2% formaldehyde stock solution	66
2.4.6	Immunocytochemistry staining	66
2.4.7	Transfection of Tera2.cl.SP12 cells with siRNA	67

### **Chapter 3: General Activity of RA and Shh on Tera2.cl.SP12 EC stem cells**

3.1	Introduction	78
3.2	Methods	82
3.2.1	Tera2.cl.SP12 EC cells induction and the channel $\mu$ -slide assay	82
3.2.2	Immunocytochemistry (ICC) staining	83
3.2.3	Flow cytometry	83
3.2.4	RT-PCR	83
3.3	Results	84
3.3.1	The role of RA in Tera2 EC cell differentiation	84
3.3.2	The channel $\mu$ -slide assay of Tera2 EC cells	86
3.3.3	Differentiation of neural precursors by Tera2.cl.SP12 EC cells	99
3.3.4	Patterning genes induced by RA and Shh in Tera2.cl.SP12 EC cells	104
3.3.5	Regulated expression of patterning transcription factors during Tera2.sp12 EC cell differentiation	109
3.3.6	Expression of general neural markers in differentiating Tera2.sp12 EC cells	113
3.3.7	A proposed molecular pathway of motor neuron specification in Tera2	117
3.3.8	Staining of cytokeratin8 a non-neuronal marker during Tera2.sp12 EC cell differentiation	120
3.3.9	Different responses to cytokines after RA induction	125
3.3.10	Effect of RA+Shh on cell proliferation	130
3.4	Discussion	134



## **Chapter 4: Activity of Sonic hedgehog signalling on the differentiation of Tera2.cl.SP12 EC cells**

### **4.1: Shh signalling in Tera2.cl.sp12 EC cell and its interaction with pathways that modify its effects.**

4.1.1	Introduction	140
4.1.2	Methods	142
4.1.2.1	EC cell culture	142
4.1.2.2	Immunocytochemistry (ICC) staining	142
4.1.2.3	RT-PCR	143
4.1.2.4	Flow cytometry	143
4.1.2.5	Western blot analysis	143
4.1.2.6	Statistical Analysis	144
4.1.3	Results	144
4.1.3.1	RA and Shh induce proliferation in Tera2.sp12 EC cells	144
4.1.3.2	RA and Shh induce differentiation in Tera2.sp12 EC cells	145
4.1.3.3	RA and Shh induce neuro-ectodermal differentiation in Tera2.sp12 EC cells	149
4.1.3.4	Regulation of Oct-4 expression in Tera2.cl.SP12 EC cells during exposure to RA and Shh induced	152
4.1.3.5	Regulation of the Shh pathway of 1 week and 2 weeks differentiated Tera2.cl.SP12 EC cells	154
4.1.3.6	RA and Shh regulate neuron differentiation of Tera2.cl.SP12 EC cells	157
4.1.3.7	Ngn-2 expression in RA and Shh induced Tera2.cl.SP12 EC cells	161
4.1.3.8	The cell fate determination in Shh induced Tera2.cl.SP12 EC cells	161
4.1.3.9	BMPs induce proliferation and differentiation in Tera2.cl.SP12 EC cells	164
4.1.4	Discussion	167

### **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	169
4.2.2	Results	171

4.2.2.1	Regulated expression of patterning genes on <i>in vitro</i> model of DV patterning	171
4.2.2.2	Shh pathway in the DV patterning model	175
4.2.2.3	RA acts downstream of Smo in Shh pathway in Tera2.cl.SP12 EC cells	176
4.2.2.4	RA and Shh signalling in motor neuron generation	178
4.2.3	Discussion	181

### **4.3: Using RNAi to study Gli1 function in Tera2.cl.sp12 EC cell model.**

4.3.1	Introduction	182
4.3.2	Results	185
4.3.2.1	RT-PCR of Shh pathway genes in early induced Tera2 EC cells	185
4.3.2.2	Gli-1 and endogenous Shh expression in induced Tera2.cl.SP12 EC cells	187
4.3.2.3	The efficacy of RNAi transfection in Tera2 EC cells	188
4.3.2.4	Gli1 siRNA transfection down-regulates Gli1 and Pax6	188
4.3.2.5	Gli1 transcription factor appears to mediate cell proliferation in Tera2.cl.SP12 EC cells	194
4.3.2.6	Gli1 transcription factor involves in neurogenesis in Tera2 EC cells	195
4.3.3	Discussion	197

## **Chapter 5: Functional assessment of motor neuron phenotypes differentiated from Tera2.cl.SP12 EC cells**

5.1	Introduction	200
5.2	Methods	208
5.2.1	Setup co-culture assay	208
5.2.2	Immunocytochemistry (ICC) staining	208
5.2.3	Quantification of contractile events	209
5.2.4	Pharmacologically manipulation of contractile events	209
5.2.5	Statistical Analysis	209
5.3	Results	210
5.3.1	Myotube differentiation assay	210
5.3.2	Development of the co-culture system to test motor	210

	neuron activity	
5.3.3	The neuronal identity of neuron derived from Tera2 EC cells prepared for co-culture	213
5.3.4	The purity of neuronal like aggregates in the co-culture	213
5.3.5	The motor neuron identity of the differentiated aggregates	216
5.3.6	The interaction between aggregates and myotubes	216
5.3.7	Synapses form between aggregate and myotubes	219
5.3.8	SEM imaging of neurites and neurite endings in the co-culture	219
5.3.9	Possible formation of NMJ in the co-culture between motor neurons and muscle cells	222
5.3.10	RA and RA+Shh induced EC stem cell result in neurons capable of inducing contractile events	224
5.3.11	Non-selective NMJ agonists induce more contractile events	225
5.3.12	Selective AChR agonists induce myotube contractions in the absence of neurons	226
5.3.13	Contractile events in the co-culture can be blocked by AChR antagonists	227
5.4	Discussion	228

## **Chapter 6: Oligodendrocyte and interneuron differentiation from Tera2.cl.sp12 EC cells.**

6.1	Introduction	232
6.2	Methods	239
6.2.1	Oligodendrocyte differentiation protocol	239
6.2.2	Interneuron differentiation protocol	239
6.2.3	Quantification of differentiated oligodendrocytes and interneurons	240
6.3	Results	240
6.3.1	Differentiation of oligodendrocytes from Tera2 EC cells	240
6.3.2	Staining of differentiated oligodendrocytes from Tera2 EC cells	242
6.3.3	Oligodendrocyte precursor differentiation from Tera2 EC cells	246
6.3.4	The possible signalling pathway in oligodendrocyte differentiation	247
6.3.5	Differentiation of interneuron from Tera2 EC cells	249

6.3.6	Quantification of differentiated oligodendrocytes & interneurons	251
6.4	Discussion	253
<b><u>Chapter 7: Summary Discussion and Future Direction</u></b>		<b>257</b>
	References	267

## List of Figures

### Chapter 1

1.1	Development of the pre-implantation blastocyst in human	3
1.2	Schematic diagram shows the derivation of tissues in human embryo	3
1.3	Major derivatives of the ectoderm germ layer	8
1.4	The transcriptional code for each ventral neuron progenitor domain and the corresponding post-mitotic ventral neurons during the murine ventral spinal cord development.	11
1.5	Model of Shh signalling pathway and its interaction with other signalling factors.	13
1.6	Gli proteins specify the patterning of ventral spinal cord	23
1.7	Cross repression of Class I and Class II transcription factors and their regulation by the signalling factors in the developing spinal cord	27
1.8	A) The metabolic pathway for synthesis and degradation of endogenous RA; B) The RAR/RXR heterodimer mediates the effects of RA	36
1.9	Model of BMP signalling pathway	41
1.10	Schematic diagram of a FGF-2 signalling cascade	43
1.11	Models of Wnt signaling pathway in the absence and the presence of Wnt ligand.	46
1.12	The derivation and differentiation of ES cells	49
1.13	Schematic procedures for inducing dopamine neurons (DA) and moter neurons (MN) differentiation.	50

### Chapter 2

2.1	Examples of negative control	67
-----	------------------------------	----

### Chapter 3

3.1	RA induces differentiation of Tera2 EC cells	84
3.2	Schematic showing the model of Dorso-ventral axis using the channel $\mu$ -slide	87
3.2.1	Gli1 staining in the model of Dorso-ventral axis	88

3.2.2	N-Shh staining in the model of Dorso-ventral axis	89
3.2.3	Nestin staining in the model of Dorso-ventral axis	90
3.2.4	Pax6 staining in the model of Dorso-ventral axis	91
3.2.5	Pax7 staining in the model of Dorso-ventral axis	92
3.2.6	Pax6 and Pax7 staining in the model of Dorso-ventral axis	93
3.2.7	Pax6 and Pax7 staining in the model of Dorso-ventral axis	94
3.2.8	Gli1 staining in the model of Dorso-ventral axis	95
3.2.9	N-Shh staining in the model of Dorso-ventral axis	96
3.3	A) Neural precursor marker analysis of induced EC cells	95
	B) Comparison of nestin and $\beta$ III tubulin expression in 1 week and 2 weeks induced EC cells	
3.3.1	Nestin staining in 1 week RA treated EC culture	98
3.3.2	Pax6 staining in 1 week RA treated EC culture	99
3.3.3	Ki67 and $\beta$ III tubulin staining in 1 week RA treated EC culture	100
3.3.4	Comparison of Nestin and $\beta$ III tubulin expression in 1 week and 2 week induced EC cultures	101
3.4	RT-PCR analysis of dorso-vental patterning genes expression in Tera2 EC cells	105
3.4.1	RT-PCR analysis of Pax genes and En-1 expression	105
3.4.2	RT-PCR analysis of motor neuron related patterning genes	106
3.5	The staining of patterning transcription factors	108
3.5.1	Activation of Dlx2 expression in RA and Shh induced cells	108
3.5.2	Investigation of Pax6 expression in RA and Shh induced EC cells	109
3.6.1	Western blotting analysis of neuron markers in induced EC cells	111
3.6.2	The expression of motor neuron marker HB-9 in induced EC cells	112
3.6.3	The expression of ChAT in induced EC cultures	113
3.6	Schematic diagram of molecular pathway involved in motor neuron specification and maturation from human Tera2 EC cells	116
3.7	A) cytokeratin8 and $\beta$ III tubulin staining of induced EC cells	110
	B) comparison of nestin and cytokeratin8 expression of induced EC cells with or without Shh antagonist cyclopamine (CP)	
3.8.1	The expression of cytokeratin8 in RA treated EC cultures	118
3.8.2	The expression of cytokeratin8 in RA+Shh treated EC cultures	119

3.8.3	The expression of cytokeratin8 in Shh treated EC cultures	120
3.8.4	Comparison of nestin and cytokeratin-8 expression of induced EC cells with or without Shh antagonist cyclopamine	121
3.9.1	The staining of $\beta$ III tubulin in 1 week Shh and its antagonists treated rosettes	124
3.9.2	The staining of $\beta$ III tubulin in 1 week BMPs treated rosettes	125
3.9.3	The staining of $\beta$ III tubulin in 1 week various cytokines treated rosettes	126
3.10.1	The staining of Ki67 in 1 week Shh and its antagonists treated rosettes	128
3.10.2	The staining of Ki67 in BMPs treated rosettes for 1 week	129
3.10.3	The staining of Ki67 in various cytokines treated rosettes for 1 week	130
3.11	The expression pattern of transcription factors in channel $\mu$ -slide model matches the <i>in vivo</i> spinal cord model	134

## **Chapter 4.1**

4.1.2.1	Analysis of stem cell surface markers by flow cytometry	142
4.1.2.2	Double staining of nestin and TuJ1 in RA and Shh induced EC cells	143
4.1.3.1	Formation of rosettes in the EC culture	147
4.1.3.2	The morphology of Nestin positive cells in RA and Shh induced EC cultures	148
4.1.4	Western blotting analysis of Oct-4 expression in induced EC cells	150
4.1.5	Regulation of the Shh pathway gene expression	152
4.1.6	Analysis the neural differentiation ability of RA and Shh	155
4.1.6.1	Nestin staining in RA and Shh induced EC cultures	155
4.1.6.2	Neuronal $\beta$ III tubulin staining in RA and Shh induced EC cultures	156
4.1.7	Western blotting analysis of Neurogenin-2 expression in induced EC cells	157
4.1.8	Phase contrast images of EC cells after Shh and RA induction	159
4.1.9	BMPs induce proliferation of EC cells	161

## **Chapter 4.2**

4.2.1	Analysis of Tera2.cl.SP12 EC cells as DV patterning model	170
4.2.2	Analysis Shh pathway genes in the EC cell model of DV patterning	172

4.2.3	Analysis of endogenous Shh and Gli1 expression	173
4.2.4	RA and Shh signalling in motor neuron differentiation	176

### **Chapter 4.3**

4.3.1	RT-PCR analysis of Shh pathway genes in induced EC cells	182
4.3.2	Western blotting analysis of Gli1 expression in induced EC cells	183
4.3.3	The efficacy of Gli1 siRNA transfection assay	185
4.3.4	Gli1 siRNA transfection down-regulates Gli1 and Pax6	186
4.3.5	Gli1 siRNA transfected EC cells have decreased proliferative ability	190
4.3.6	Pax6, nestin and Tuj1 are negative in Gli1 siRNA transfected EC cells after RA and/or Shh induction	192

### **Chapter 5**

5.3.1	Myotube differentiation from C2C12 myoblasts	210
5.3.2	Schematic showing of the development of co-culture system	211
5.3.3	RA induced neuron aggregates are positive for general neuron markers before and after co-culture with myoblasts	213
5.3.4	RA induced neurons in the co-culture system are negative for GFAP and myelin but positive for $\beta$ III tubulin and NF-68	214
5.3.5	The neuron aggregates are positive for motor neuron markers such as HB-9, ISL-1 and LIM-3	215
5.3.6	The interaction between neuron aggregates and differentiated myotubes	217
5.3.7	The neuron aggregates in the co-culture express high level of synapse protein synapsin I	219
5.3.8	Examples of SEM images of neurites in co-culture	220
5.3.9	Neuron aggregates induce AChR clusters on the myotube in the co-culture.	222
5.3.10	Quantification of contractile events stimulated by RA and RA+Shh induced neurons	223
5.3.11	Quantification of contractile events stimulated by AChR antagonists ACh and carbachol	224
5.3.12	AChR agonists induce myotube contractions without neuronal	225



	aggregates	
5.3.13	Pictures of video clips	226

## **Chapter 6**

6.1.1	Schematic showing the change of oligodendrocyte progenitor domain during the mouse spinal cord development	233
6.3.1	The process of oligodendrocyte differentiation from EC cells	241
6.3.2	CNPase and O4 staining of differentiated oligodendrocytes	244
6.3.3	Analysis of oligodendrocyte progenitor markers in RA and RA+Shh induced EC cells	247
6.3.4	Western blot analysis of the signalling pathway in induced EC cells	247
6.3.5	Images of the differentiated interneurons from EC cells	249
6.3.6	GABA staining and the quantification of differentiated oligodendrocyte and interneuron markers	251

## **Chapter 7**

7.1	Schematic procedure for inducing motor neurons, oligodendrocytes and interneurons of Tera2 EC cells.	264
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## **List of Tables**

1.1	Comparison of mouse and human ES, EC cells	56
2.1	RT-PCR primers used in this study	65
2.2	Antibodies from Chemicon	70
2.3	Antibodies from Abcam	70
2.4	Antibodies from Sigma	71
2.5	Antibodies from R&D system	71
2.6	Antibodies from Santa Cruz biotechnology	72
2.7	Antibodies from DSHB	72
2.8	Secondary antibodies	73

## Abbreviations

ADH	Alcohol dehydrogenase
AFP	$\alpha$ -fetoprotein
APS	Ammonium persulphate
bHLH	Basic helix-loop-helix
BMPs	Bone morphogenetic proteins
Boc	bioregional Cdon binding protein
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
Cdon	cell adhesion molecule down regulated by oncogenes
ChAT	Choline acetyltransferase
Chx10	Ceh-10 homeodomain transcription factor expressed in chick V2 neuron
Ci	Cubitus interruptus, homolog of Gli in Drosophila
CNPase	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CNTF	Ciliary neurotrophic growth factor
CO <sub>2</sub>	Carbon dioxide
Cos2	Costal2, the segment polarity gene in Drosophila
CP	Cyclopamine, Shh antagonist
CREB	cAMP response element binding protein
DAT	Dopamine transporter
Dbx	DNA-binding homeobox transcription factor
Dcc	Colorectal cancer suppressor, Netrin1 receptor
dH <sub>2</sub> O	Distilled water
dl1-6	Dorsal interneuron group 1-6
DM-20/PLP	Major myelin proteolipid protein
DMEM	Dulbeccos modified eagles medium
DMEMFG	Dulbeccos modified eagles medium and L-glutamine
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dpc	Days after coitum
DRG	Dorsal root ganglion
dsDNA	Double stranded DNA
DTT	Dithiolthreitol
DV	Dorso-ventral
EC	Embryonal carcinoma stem cell

EDTA	Ethylenediaminetetracetic acid
EGF	Epidermal growth factor
EGTA	Ethylenebis(oxyethylenitrilo)tetracetic acid
ERK	Extracellular signal-regulated kinase
ES	Embryonic stem cell
FCS	Foetal calf serum
FGFR	Fibroblast growth factor receptor
FGFs	Fibroblast growth factors
Fkbp8	Negative regulator of mouse Shh signalling in neural tissue
FORSE	Forebrain surface embryonic marker
FP	Floor plate
Fused	Serine/Threonine protein kinase in <i>Drosophila</i>
Fz-5	Frizzled-5, receptor for Wnt proteins
GABA	$\gamma$ -aminobutyric acid
GAD65/67	Glutamate decarboxylase65/67
GBX	Kodak fixer or developer
Gli	Glioma-associated, Krupper family of Zinc finger transcription factor
GPCRs	GTP-protein coupled receptors
G-protein	Guanine nucleotide binding regulatory protein
GRB2	Growth factor receptor bound protein2
GDP	Guanosine-5'-diphosphate
GTP	Guanosine-5'-triphosphate
SH2	Src homolog 2 domains
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HCL	Hydrochloric acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
Hes1	Hairy and enhancer of split 1, proneural transcription factor
Hip	Hedgehog interacting protein
HNF-3 $\beta$	Winged-Helix family transcription factor, regulates floor plate development
Hom-C	Homolog of Hox in <i>Drosophila</i>
Hox	Homeodomain transcription factor
HRP	Horseradish peroxidase
ICM	Inner cell mass
Ig	Immunoglobulin
IGF	Insulin growth factor
IHC	Immunohistochemistry
IQ	Intelligence
Irx	Iroquois-class homeobox protein, transcription factor
ISL1	Islet1, insulin gene enhancer protein, Lim domain transcription factor

IVF	In vitro fertilization
JNK	c-Jun N terminal kinase
KD/kDa	Kilo daltons
LCAM	E-cadherin 1, cell adhesion molecule
LEF/TCF	Lymphoid enhancer binding factor/T cell factor
Lhx	Chicken Lim domain transcription factor
LIF	Leukaemia inhibitory factor
LIM	Lim domain transcription factor, contain cysteine-rich double zinc fingers
M	Molar
mA	Milli amps
MAPK	Mitogen activated protein kinase
Mash1	Achaete-Scute complex homolog like 1, Proneural transcription factor
Math1	Protein atonal homolog 1, transcription regulation
MBP	Myelin basic protein
mDN	Midbrain dopamine neuron
ml	Milli-litre
mM	Milli-molar
MN	Motor neuron
MNR2	Homeobox gene, specification of motor neuron identity
Mr	Molecular mass
mRNA	Messenger RNA
Msx	Transcription factor
Myf	Myogenic factor
MyoD	Myoblast determination protein 1
NaCl	Sodium chloride
NaHCO <sub>3</sub>	Sodium hydrogen carbonate
NaOH	Sodium Hydroxide
NCAM	Neural cell adhesion molecule
NeuroD	Neurogenic differentiation factor 1, bHLH transcription factor
Ngn2	Neurogenin 2, bHLH transcription factor
Nkx	helix-turn-helix homeodomain transcription factor
nM	Nano Molar
NMJ	Neuro-muscular junction
NSE	Neuron specific enolase
NT-3	Neurotrophic factor-3
O.D.	Optical density
°C	Degrees centigrade
Olig	oligodendrocyte transcription factor, bHLH family
OLP	Oligodendrocyte progenitor

Otx2	Orthodenticle homolog 2, homeobox transcription factor
P/S	Penicillin and streptomycin
p0	Ventral interneuron V0 progenitor
p1	Ventral interneuron V1 progenitor
p2	Ventral interneuron V2 progenitor
p21	p21Clip1, Cyclin dependent kinase interacting protein1
p27	p27Kip1, negative regulator of G1 progression
p3	Ventral interneuron V3 progenitor
p53	phosphoprotein 53 KD, a negative regulator of normal cell growth
PAGE	Polyacrylamide gel electrophoresis
Pax	paired-box transcription factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PFA	Paraformaldehyde
pH	Potential of Hydrogen
PI3K	Phosphoinositol 3 kinase
PKA	Protein kinase A
pMN	Motor neuron progenitor
PNS	Peripheral nervous system
POU	Homeobox domain transcription factor
Ptc	Patched, Shh binding receptor
r1	Rhombomere1
RA	All trans retinoic acid
Rab23	Encoded by open brain gene, Shh pathway regulator in mouse
RALDH	Aldehyde dehydrogenase, RA synthesis enzyem
RAR	RA receptor
RAREs	RA response elements
REN	Shh antagonist, a putative tumor suppressor
RNA	Ribonucleic acid
RPM	Revolutions per minute
RTK	Receptor tyrosine kinase
RXR	Retinoid X receptor
S.D.	Standard deviation
SCNT	Somatic cell nuclear transfer
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
Shh	Sonic hedgehog
Sil	Sonic hedgehog pathway regulator

SMAD	Mothers against decapentaplegic homolog, BMP/TGF $\beta$ signalling mediator
Smo	Smoothened, Shh signalling initiating receptor
SOS	Son of sevenless
Sox	transcription factor involve in neuro-ectoderm specification
SSEA	Stage specific embryonic antigen
Stk36	Fused homolog in mouse
Sufu	The suppressor of Fused in Drosophia
TBS	Tris-buffered saline
TE buffer	Tris-HCL, EDTA buffer
TEMED	NNN'N'-Tetramethylethylenediamine
TGF $\beta$	Transforming growth factor $\beta$
TH	Tyrosine hydroxylase
Tris	Tris(hydroxymethyl)methylamine
U.V	Ultra-violet
V	Volts
v/v	Volume per volume
V0	Ventral interneuron at the dorsal of V1
V1	Ventral interneuron at the dorsal of V2
V2	Ventral interneuron at the dorsal of MN
V3	Ventral interneuron at the dorsal of floor plate
VAD	Vitamin A deficiency syndrome
w/v	Weight per volume
Wnt	Wing less gene in vertebrate?
Xlpou2	Xenopus Pou2 transcription factor
$\mu$ g	Microgram

# Chapter One

## Introduction

### 1.1 The five developmental processes of vertebrate embryogenesis

The development of vertebrates is about the formation of specialized cells, tissues and organs from a single cell to a highly organized adult body. Although it is a continuous delicate process, five main development processes have been identified.

1. First, the fertilization is followed by rapid cell division called cleavage, to produce a number of cells without cell mass increase (the cleavage stages is shown in Figure 1.1). After the third cleavage, the blastomeres form a compact 8 cell ball, and then give rise to 16 cell morula, which is consisted of a small group of internal cells surrounded by a large group of external cells. The external cells will become trophoctoderm and produce no embryonic structures. At 32-cell stage, the small group of internal cells give rise to the inner cell mass (ICM) of about 13 cells, which will become the embryo proper and its associated yolk sac, amnion (Gilbert, 2003). Accordingly, embryonic stem cells derived from the inner cell mass have the ability to form all cell types in the adult body.

From the 8-cell stage, cells start to be organized in a spatial and temporal pattern. The pattern formation initially involves laying down the overall body plan—defining the main axes of the embryo so that the anterior and posterior ends, dorsal and ventral sides of the body are specified.

2. The next stage is the formation of three germ layers—the ectoderm, mesoderm and endoderm. The concept of germ layer is useful to distinguish between regions of the early embryo that give rise to quite distinct types of tissues. The inner cell mass in the blastocyst give rise to three germ layers as shown in Figure 1.2—the derivation of tissues in human



embryos. The inner cell mass proliferates actively and cells are arranged into bilaminars, hypoblast and epiblast. All three germ layers come from epiblast. At the beginning of the gastrulation, primitive streak is present on the epiblast. It is through the primitive streak that cells detach from the epiblast layer and relocate downward to form first the endoderm and then the mesoderm. When endoderm is formed, cells from epiblast layer integrate into the hypoblast, which forms extra-embryonic tissues later. And the newly formed endoderm displaces the hypoblast to the side until the endoderm covers the epiblast layer. In the formation of mesoderm, cells detaching from epiblast insert into the space between epiblast layer and endoderm to form a new cell layer, and cells undergo the transition from an epithelium to mesenchyme. The formation of three germ layers is completed at the end of the gastrulation stage. After the formation of these germ layers, the endoderm gives rise to the gut and its derivatives, such as the liver and the lungs; the mesoderm gives rise to the skeleton-muscular system, connective tissues, and other internal organs such as the kidney and heart; and the ectoderm gives rise to the epidermis and nervous system (Gilbert, 2003). During further pattern formation, cells of these three germ layers acquire different identities so that organized spatial patterns of cell differentiation emerge, such as the arrangement of neurons in the nervous system. In the earliest stages of pattern formation, differences between cells are not easily detected and probably consist of subtle chemical differences caused by a change in activity of only a few key genes.



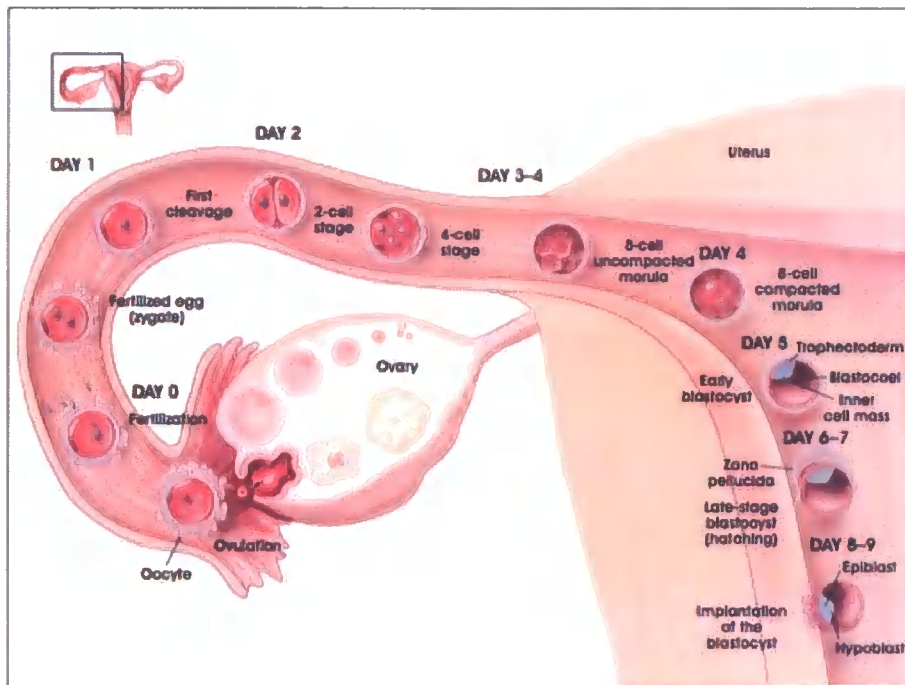


Figure 1.1 Development of the pre-implantation blastocyst in humans. Cleavage is a rapid series of cell divisions that begin with the zygote and end with blastocyst. The blastocyst consists of the inner cell mass and the trophectoderm. The inner cell mass generates three germ layers to become the embryo proper. (After Terese Winslow, 2001, <http://stemcells.nih.gov/info/scireport/appendixA.asp>).

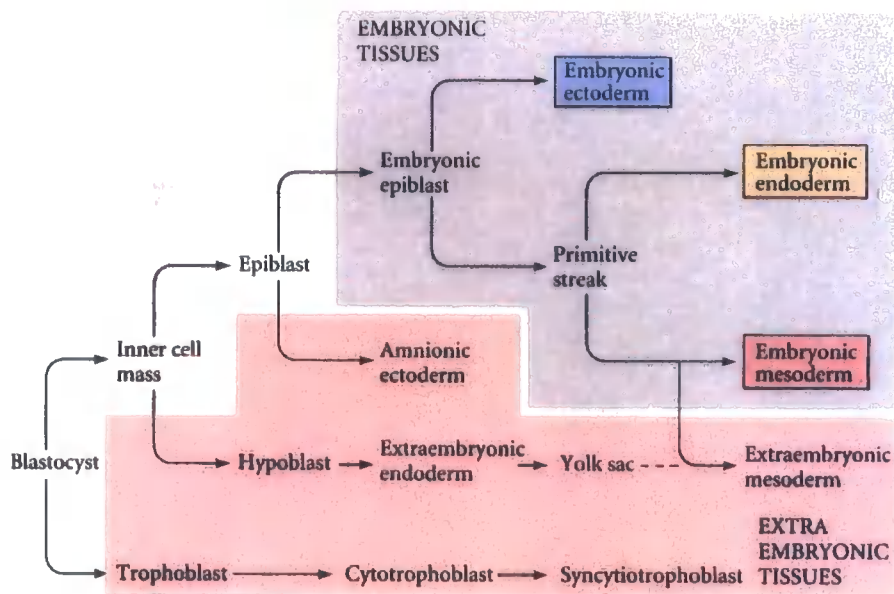


Figure 1.2. Schematic diagram illustrating the derivation of tissues in human embryos. Inner cell mass gives rise to embryonic tissues and part of extra-embryonic tissues, while trophoblast gives rise to only extra-embryonic tissues. (After Luckett 1978; Bianchi *et al.* 1993.)

3. The third important development process is morphogenesis. Embryos undergo dramatic changes in three-dimensional form. The most striking one is gastrulation, during which the gut is formed and the main body plan emerges.

4. The fourth developmental process is cell differentiation, in which cells become structurally and functionally different from each other, ending up as distinct cell types, such as blood, muscle, or skin cells. Differentiation is a gradual process, cells often go through several divisions between the time they start differentiation and the time they are fully differentiated. Cell differentiation starts with determination, which is the commitment of a cell to undergo differentiation. Once a cell is 'determined' it no longer responds to other inducer molecules, and it is not always accompanied by morphological changes.

5. The fifth process is growth. This is increasing the size of cells. In general, there is little growth during early embryonic development and the basic pattern and form of embryos lay down on a small scale. Subsequent growth can be brought about in a variety of ways: cell multiplication, increase in cell size, and deposition of extracellular materials.

These five processes are neither independent of each other nor strictly sequential; indeed, they overlap and influence one another considerably (Gilbert, 2003).

## **1.2 Gene expression in the control of embryonic development**

During the development there are changes in cell state, cell-cell signalling, cell shape, cell movement, cell proliferation and cell death, which are important processes in the formation of an embryo. Changes in the expression of patterning genes during early development are essential for they give cells identities that determine their behaviour and differentiation. To answer how gene expression controls development at the cellular level, it is important to

consider how genes control cell behaviour. Cell behaviour is determined largely by the proteins present within them, which relates to gene expression. The activity of protein is regulated by activation or repression of gene expression. Genes that operate in this way are known as transcription factors.

Transcription factors are proteins that bind to a gene's enhancer or promoter to activate or repress the transcription of the gene (Gilbert, 2003). In general, transcription factors have three major domains. The first is DNA-binding domain that recognizes a particular DNA sequence. The second is a trans-activating domain that activates or suppresses the transcription of the gene whose promoter or enhancer it has bound to. The third is a protein-protein interaction domain that allows the transcription factor's activity to be modulated by other transcription factors or TATA-binding protein associated factors.

There are several families of transcription factors grouped together by their structural similarities and their mechanisms of action. The major transcription factor families that are important in specifying body axes of the patterning include homeodomain transcription factors including HOX, POU, LIM, and PAX; basic helix-loop-helix transcription factors including MyoD, NeuroD and OLIG; zinc finger transcription factors including GLI and nuclear hormone receptors (Gilbert, 2003).

The accumulation of transcription factors in the nucleus will result in tissue-specific expression manner. Usually, genes for transcription factors are activated by other transcription factors; whilst others may be activated by inductive factors from the environment. Transcription factors work in a cascade of sequential events during tissue specification (Shirasaki and Pfaff, 2002).

### 1.3 Dorso-ventral (DV) patterning and neurulation in vertebrate embryo

Pattern formation is the process of induction that leads to the formation of a number of progenitor fields (Stephanie Schmalz, 1999), each of which is committed to specific groups of cells, that is the cells become pluripotent, they lose some of their potency, but still have the ability to differentiate into a restricted numbers of cell types. The first pattern formation in the embryo is anterior-posterior patterning and the second is dorso-ventral patterning (Gilbert, 2003).

The best studied model of developmental patterning has been demonstrated in *Drosophila*. In *Drosophila*, the dorso-ventral polarity is established by the gradient of a transcription factor known as Dorsal (Wang and Ferguson, 2005). Dorsal forms a concentration gradient over a field of cells that established as a consequence of cell-to-cell signalling events. The specification of the dorso-ventral axis in *Drosophila* takes place in several steps. The critical step is the translocation of the Dorsal protein from the cytoplasm into the nuclei of the ventral cells during the 14<sup>th</sup> division cycle (Gilbert, 2003). The effect of Dorsal protein gradient is to activate genes responsible for the ventral cell fate and to repress genes whose proteins specify dorsal cell fates. Since a gradient of Dorsal protein enters the various nuclei along the DV axis, the most ventral cells become mesoderm, and the more lateral become neural ectoderm.

However, dorso-ventral patterning in *Drosophila* is significantly different from vertebrate's dorso-ventral patterning. Very little is known about the mechanisms of dorso-ventral axis formation in human. Most of the knowledge of dorso-ventral patterning in vertebrates is come from the studies of lower species, notably frog, chick and mouse embryos.

During gastrulation, in the frog and chick embryo, cell movement results in three germ layers moving into the correct position, i.e. the ectoderm covers the embryo and the

mesoderm and endoderm move inside the embryo. Prior to that, during the conversion bilaminar embryo to trilaminar embryo, the presence of the primitive streak is a major structural characteristic of an amniotic embryo. At this time, we can identify a basic body plan including the anterior-posterior ends, dorso-ventral sides and left and right sides. In the mammalian embryo, the dorsal axis forms from the ICM cells that are in contact with the trophoblast. The ectoderm receives inductive signals from neighbouring germ layers to give rise to three major domains: the surface ectoderm (primarily epidermis in embryo), the neural crest (peripheral neurons, pigment and facial cartilage), and the neural tube (brain and spinal cord) (Gilbert, 2003). (Figure 3 illustrates the major derivatives of the ectoderm) Inductive tissues like Spemann's organizer in frog, notochord in chick, are derived from mesoderm, and induce secondary tissues from the ectoderm containing central nervous system (CNS) and peripheral nervous system (PNS). The induced tissues have a well organized arrangement along the dorso-ventral and anterior-posterior axes, suggesting that these inductive tissues lead to induction and patterning of the entire ectoderm.

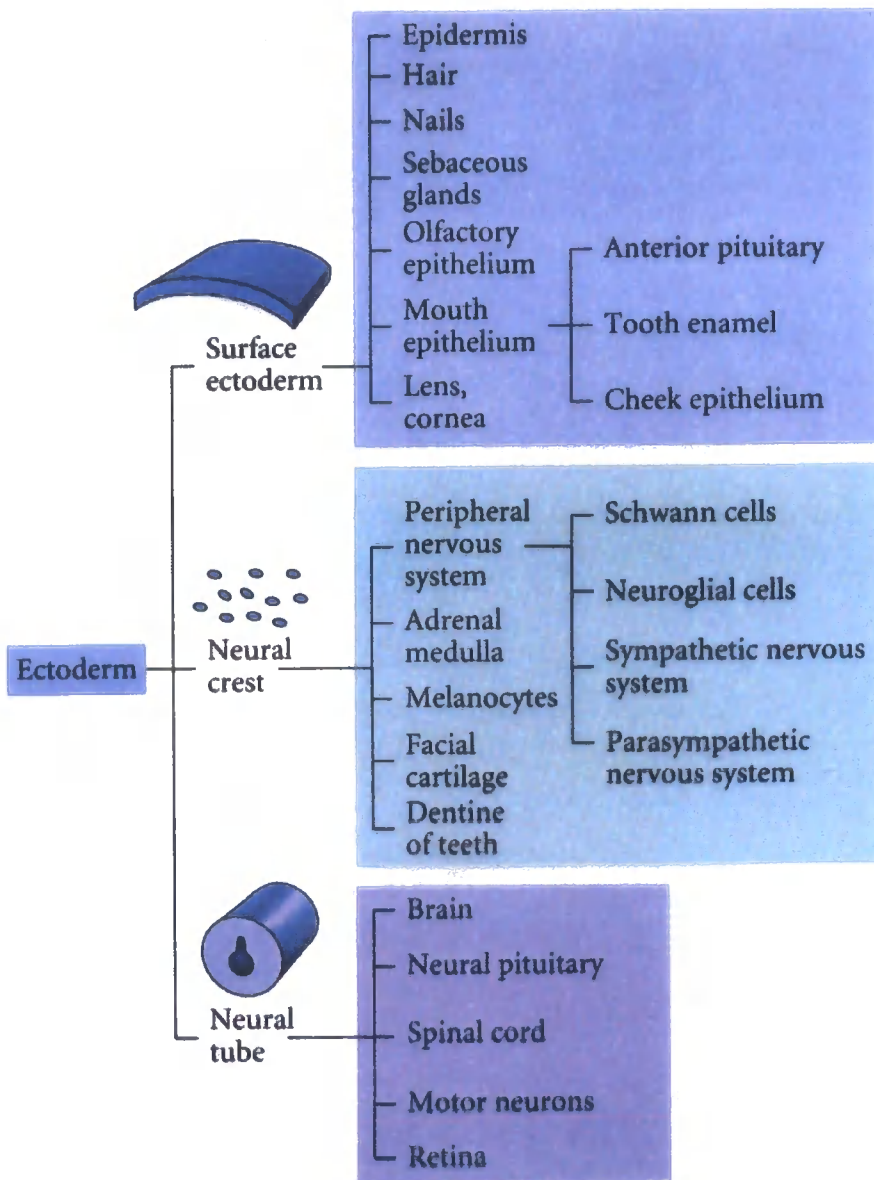


Figure 1.3. Major derivatives of the ectoderm germ layer. The ectoderm is divided into three major domains: the surface ectoderm (primarily epidermis), the neural crest (peripheral neurons, pigment, facial cartilage), and the neural tube (brain and spinal cord). (After Gilbert F Scott, developmental biology, 7<sup>th</sup> Edition.)

Neurulation in human embryo includes the formation of the neural plate (day 18-19), neural folds (day 20-21), and the neural tube (day 22-26); the neural tube will develop into the future brain and spinal cord (Stephanie Schmalz, 1999). Neurulation begins as the primitive ectoderm is induced to differentiate into neuro-ectoderm. Inductive factors from the

mesoderm induce the central strip of primitive ectoderm to express NCAM (neural cell adhesion molecule) and elongated filaments and tubules as cell shape changes from cuboidal to columnar epithelial cells. These columnar epithelial cells form neural plate. The cells at the edge of the neural plate are positive for NCAM and LCAM; these cells will later differentiate into neural crest. Signals such as retinoic acid (RA) and sonic hedgehog (Shh) from mesoderm induce cells in the neural plate to express neuro-filaments and neuro-tubules at the apex of the cell, to induce the change in cell shape from columnar to pyramidal epithelial cells. This change causes the neural plate gradually to fold inward to form neural tube. The closure of the neural tube will eventually break free of the surface ectoderm and the specification of neuron progenitor domains along the dorso-ventral axis continues (Stephanie Schmalz, 1999).

#### **1.4 Specification of the neuron progenitor domain along Dorso-ventral axis**

Distinct types of neurons are generated along the dorso-ventral axis of the neural tube. Generally, the sensory neurons reside along the dorsal domain and the motor neurons reside in the ventral region. It has been demonstrated that the extracellular inductive signals instruct the differentiation of the neuron subtypes and numbers along the dorso-ventral axis by mediating the cell-cell interaction and intracellular events (Lee and Jessell, 1999). The intracellular events that relay information to the nucleus of neuro-ectoderm have been identified as transcription factors. And most of these developmental events are conserved from invertebrates to mammals. There are at least four levels of transcriptional regulation in neural tube development, beginning with their operation in progenitor cells and ending with the control of neuron migration, axon pathfindig and neuro-transmitter phenotypes (Lee and Pfaff, 2001).

In the early stage of neural plate, the Pou-domain and Sox transcription factors are expressed as the earliest neural differentiation markers. In *Xenopus*, microinjection of *Xlpou2* mRNA induces the neural differentiation in animal cap (Sasai and De Robertis, 1997). In mice, the Sox proteins are expressed in the neural epithelium at all stages from immature to mature neurons (Chao et al., 2007). In addition, there is interplay between Pou and Sox factors de the regulaton of nestin expression in neural primordial cells (Tanaka et al., 2004). Pou and Sox transcription factors therefore act as effector genes immediately downstream of the neural inductive signalling pathways.

The transcription factors acting downstream of Pou and Sox as proneural proteins are the basic Helix-Loop-Helix (bHLH) transcription factors. The bHLH factors fall into two families: one is related to *Drosophila Achaete-Scute* genes such as *Math1*; the other is related to *Drosophila atonal* genes such as *neurogenin* and *NeuroD*. These factors are critical in the differentiation of neuro-epithelial cells to become neurons (Kintner, 2002). The bHLH transcription factors are not detected in the entire neural plate in vertebrates and they are presumably regulated by neurogenic genes such as those of the Notch/Delta/Serrate/Jagged signalling pathways (Sasai and De Robertis, 1997). It suggested that the main role of these factors *in vivo* during vertebrate neurogenesis is temporal or regional regulation of neuronal differentiation.

Several additional transcription factor families are involved in dorso-ventral specification of the neural tube stage. This includes the winged-Helix family (such as HNF-3 $\beta$ ), the Pax family (e.g. PAX3, PAX6 and PAX7), the Lim family (such as LIM3 and ISL-1), the Msx family (e.g. MSX1) and the Nkx family (such as NKX2.2 and NKX6.1). These transcription factors are expressed by different groups of neuronal progenitors of the neural tube serving as transcriptional codes to mark progenitor domains along the dorso-ventral axis. In the ventral neural tube, five distinct neuronal progenitor domains are generated



from ventral to dorsal neural tube: V3 ventral neuron progenitor domain (p3), motor neuron progenitor domain (pMN), V2 progenitor domain (p2), V2 progenitor domain (p1) and V0 progenitor domain (p0), and later, will give rise to V3, MN, V2, V1 and V0 post-mitotic neurons. The transcription codes for each progenitor domains are summarized here: *Nkx2.2* and *Nkx6* for p3; *Nkx6*, *Olig2*, *Pax6* and *HB9* for pMN; *Irx3*, *Pax6* and *Nkx6* for p2; *Pax6*, *Irx3*, *Dbx2* and *Nkx6* for p1; and *Pax6*, *Irx3*, *Dbx1/2* for p0 (Lee and Pfaff, 2001). After the neurons exit the cell cycle, the code is changed as below: *Isl1* for V3; *Isl1/2* and *HB9* for MN; *Chx10* and *Lhx3/4* for V2; *En1* and *Lhx1* for V1; *Lhx1* for V0 (as shown in Figure 1.4 summary of murine ventral spinal cord development) (Thaler et al., 1999). Dorsal interneurons can be divided into six early born (dIII-6) and two late born (dILA and dILB) neuronal subtypes based on their birthdate, dorso-ventral position and homeodomain protein expression profile (Zhuang and Sockanathan, 2006).

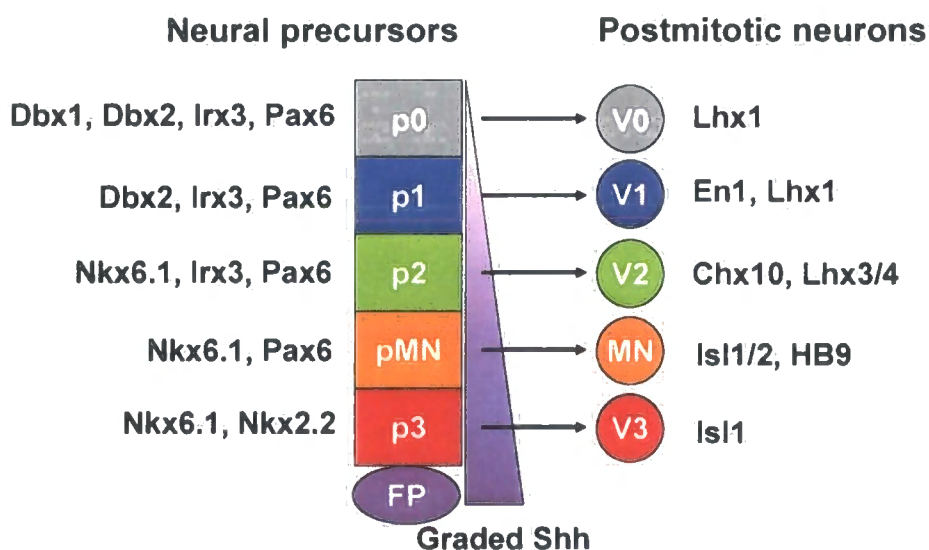


Figure 1.4 The transcriptional code for each ventral neuron progenitor domain and the corresponding post-mitotic ventral neurons during the murine ventral spinal cord development. V0, intermediate zone V0 ventral interneuron; V1, V1 ventral interneuron; V2, V2 ventral interneuron; MN, ventral exiting motor neuron; V3, V3 ventral interneuron; p, progenitor domain.

## 1.5 Regulation of neural specification in neural tube

There are three classes of molecules that are important in neural specification: transcription factors, signalling molecules and cell adhesion molecules. The signalling molecules induce the neuroblasts in neural tube to express transcription factors to initiate patterns of gene expression, and cell adhesion molecules responsible for subsequent cell aggregation and migration. The primary signalling molecules involved in neural tube specification are ventral signalling sonic hedgehog (Shh), dorsal signalling bone morphogenetic proteins (BMPs), and retinoic acid (RA), fibroblast growth factors (FGFs) and Wnts.

## 1.6 The Shh signalling pathway in dorso-ventral patterning of neural tube

The hedgehog proteins in vertebrates are homologues of the *Drosophila* segment polarity gene hedgehog that plays an essential role in the establishment of anterior-posterior polarity of fruit fly (Varjosalo et al., 2006). The vertebrate hedgehog family constitutes at least three members: desert hedgehog, Indian hedgehog and sonic hedgehog. Shh has the greatest number of functions of the three vertebrate homologues. Shh is secreted from notochord and also in the floor plate. Before secretion, Shh is synthesized as approximately 45KD protein precursor, and is proteolytically cleaved to produce a 19KD N-terminal protein that mediates all signalling activities in vertebrates and invertebrates and a 25KD C-terminal protein that possesses protease activity. This 19KD protein was modified by cholesterol at C-terminal, which will play a critical role in Shh signalling by facilitating the regulated secretion and long-range activity of the Shh protein to which it is covalently coupled. The N-terminal of the 19KD protein is modified by palmitic acid, which will increase the inductive potency of Shh (Kohtz et al., 2001).

The most important role of Shh in development is that it acts as a morphogen to control the dorso-ventral patterning of the CNS and somites, the left-right asymmetry, the patterning of the limb, as well as some aspects of organogenesis. In the CNS development, Shh is involved in the determination of ventral neural phenotypes, induction of oligodendrocyte precursors, generation of dopaminergic neurons, proliferation of neuronal progenitor populations and the modulation of growth cone movement (Marti and Bovolenta, 2002).

### 1.6.2 Components of Shh pathway

Shh binds to its receptor complex on the target cell membrane. The receptor complex is composed of Shh binding receptor—Patched-1 (Ptc-1) and signal initiating receptor—Smoothed (Smo) (Murone et al., 1999).

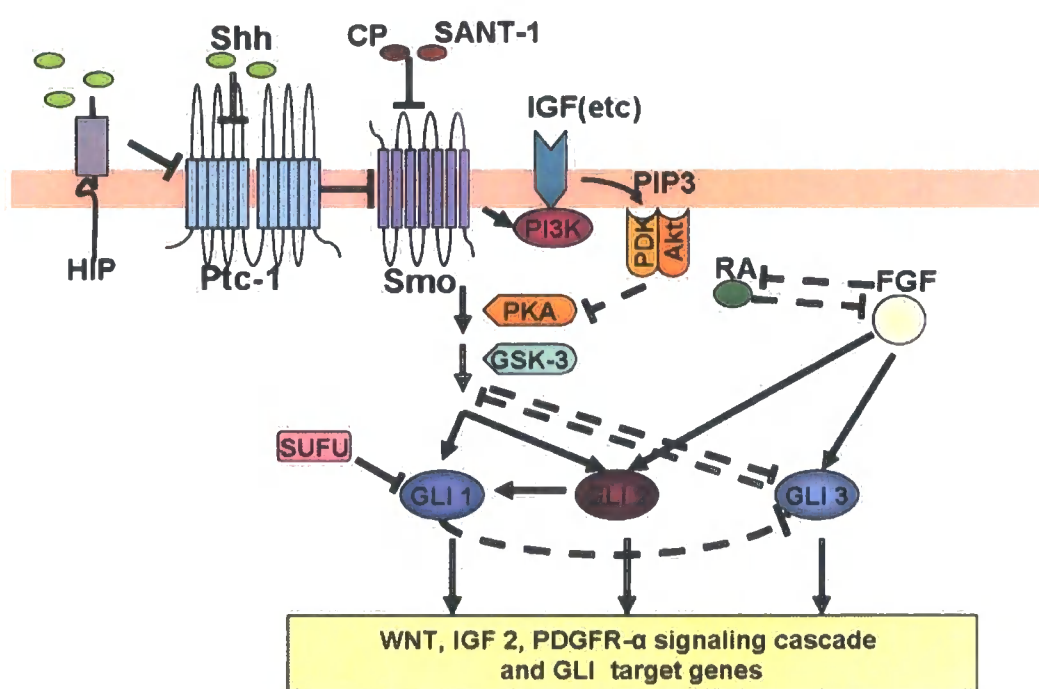


Figure 1.5 Model of Shh signalling pathway and its interaction with other signalling factors. CP, cyclopamine; SANT-1, Shh antagonist; HIP, hedgehog interaction protein.

### 1.6.2.1 Role of Ptc in the Shh receptor complex

Patched (Ptc) is a 12-pass transmembrane protein, that has two isoforms encoded by genes *Ptc-1* and *Ptc-2*. Only *Ptc-1* appears to be active in the CNS (Briscoe et al., 2001). PTC is not only a binding site for Shh, but also a downstream target of Shh signalling, and Shh up-regulating Ptc expression is a conserved response in vertebrates (Chuang et al., 2003). Moreover, Ptc serves as a sponge in the presence of too many Shh molecules, it will prevent Shh from further approaching the target cell and limit the cell response to Shh (Murone et al., 1999). Ptc seems to be a negative regulator of Shh pathway, it always inhibits the phosphorylation of Smo in the absence of Shh. Ptc inhibits Smo activity in a catalytic manner, whereby one molecule of Ptc can regulate about 50 Smo molecules, unlike the normal one molecule to one molecule inhibition model (Taipale et al., 2002).

### 1.6.2.2 Role of Smo in the Shh receptor complex

Smoothed (Smo), the activator of the Shh pathway, is a 7-pass transmembrane protein with homology to G-protein-coupled receptors (GPCRs). It is known to be a proto-oncogene capable of causing cancer-like basal cell carcinoma and medulloblastoma if mutated (Daya-Grosjean and Couve-Privat, 2005). In the presence of Shh, Ptc will release the inhibition on Smo and allow Smo to become phosphorylated, and the phosphorylation of Smo will initiate an activating signal and pass to the downstream components in the cytoplasm. This relationship of Ptc and Smo is evolutionarily conserved in both vertebrates and invertebrates. Specific point mutations in the transmembrane helices of Smo are capable of constitutively stimulating the pathway, effectively bypassing the Ptc inhibition, suggesting that Smo is the signalling component of Shh receptor complex (Xie et al., 1998). However, little is known about the means by which Ptc exerts its inhibitory effect on Smo,

or how Smo communicates with the downstream components in the cytoplasm (Frank-Kamenetsky et al., 2002).

Recent studies revealed that Ptc and Smo function as a receptor complex for Shh signalling transduction (Murone et al., 1999). By Smo deletion mutants and the chimeric receptors between human Smo and Fz-5, one of its closest known homologues, Murone *et al* (1999) demonstrated that the Ptc-Smo interaction occurs mainly through the N-terminal of Smo molecule, with less important contribution provided by the rest of the Smo molecules. Furthermore, they also suggested that the region of Smo required to activate the Gli reporter spans the third intracellular loop (including the adjacent fifth and sixth transmembrane domains) and the seventh transmembrane domain, either of these two domains can lead to a receptor capable of Gli activation.

Corbit and co-workers (2005) conducted the studies in cell culture, zebrafish and mouse embryo to demonstrate that Smo is expressed on the primary cilium of cells in embryo (Corbit et al., 2005). Evidence lead to a model that once the Shh binds to Ptc on the target cell surface, it prompts the cell to move Smo to the primary cilium from the vesicles located round the nucleus of the cell. The removal of the primary cilium from cells leads to defects in neural patterning (Huangfu et al., 2003). It is also demonstrated that Smo ciliary localization depends on Shh signalling, and in reverse, Shh signalling depends on a Smo ciliary localization motif. Thus, Smo acts at the primary cilium to transduce Shh signalling and the Smo ciliary localization is a key regulated step in Shh pathway activation (Corbit et al., 2005). Primary cilium is a specialized cell projection that extends into the extracellular environment, and exists on all vertebrate cells (Singla and Reiter, 2006). Other studies show that primary cilia act as specialized signal transduction organelles required for coupling Smo activity to the biochemical processing of Gli proteins as activator or suppressor (Liu et al., 2005; May et al., 2005).

Smo is a target for cyclopamine in the Shh pathway. Cyclopamine, a plant steroidal alkaloid, is known to induce cyclopia in vertebrate embryos. It has been shown to act by inhibiting the cellular response to the Shh signal. The steroidal nature of cyclopamine and its ability to disrupt cholesterol synthesis or transport indicates that it might affect the action of Ptc, which contains an apparent sterol-sensing domain (Taipale et al., 2000). However, cyclopamine can inhibit Shh pathway activity in the absence of Ptc function and fails to prevent Gli protein over-expression, suggesting that it acts between the Ptc and the Gli (Taipale et al., 2000). Binding assays showed that cyclopamine has a binding site near C-terminal of Smo molecules and it binds directly to Smo to inhibit the pathway (Chen et al., 2002).

### **1.6.2.3 Other Shh binding proteins on the cell surface**

Other protein molecules that act at the surface of cells responding to Shh have been identified. Hedgehog interacting protein (HIP) is a transmembrane protein that attenuates Shh signalling by binding directly to Shh with an affinity similar to that of Ptc-1 (Daya-Grosjean and Couve-Privat, 2005). HIP is transcriptionally activated in response to Shh signalling, loss-of-function mutants for HIP result in an up-regulation of Shh signalling in mouse embryo (Chuang et al., 2003). HIP is specifically expressed in vertebrates, and shares redundancy with Ptc-1. In mouse embryos, HIP is first expressed at 9dpc (days after coitum) later after Shh and Ptc-1, and is activated at the ventral midline of neural tube and in the ventral medial somites next to Shh expressing cells (Chuang and McMahon, 1999). This suggests that HIP serves as a modulator to Shh signalling during development.

Cdon (cell-adhesion-molecule-downregulated by oncogenes) and Boc (bioregional Cdon-binding protein) encode transmembrane type I orphan receptors that consist of 4 or 5 Ig

domain and 2 or 3 fibronectin type III repeats in the extracellular domain and a non-identifiable motif in the intracellular domain. They share high degree of homology in the extracellular domain and are expressed at early stage of development (Okada et al., 2006). It is shown that both of them are targets of the Shh pathway and are negatively regulated by Shh signalling (Zhang et al., 2006a). Cdo and Boc bind to Shh through a high-affinity interaction with a specific fibronectin repeat that is essential for activity (Tenzen et al., 2006). In the neural tube Cdo is expressed within the Shh-dependent floor plate while Boc expression lies within the dorsal limit of Shh signaling. Loss of Cdo results in a Shh dosage-dependent reduction of the floor plate. In contrast, ectopic expression of Boc or Cdo results in a Shh-dependent promotion of ventral cell fates and a ventral expansion of dorsal cell identities consistent with Shh sequestration (Tenzen et al., 2006).

Another protein is Vitronectin, an extracellular matrix glycoprotein, that enhances Shh activity by binding to Shh directly (Pons and Marti, 2000), when added to the culture of neural tube explants, it promotes the generation of motor neurons in the absence of either notochord or exogenous Shh (Martinez-Morales et al., 1997), indicating it is a signalling factor for motor neuron differentiation.

### **1.6.3 Cytoplasmic transducers of Shh signalling**

It remains unclear which molecules function as signal transducers downstream of Smo in the cytoplasm. Or more accurately, the cytoplasmic transducer of the Shh pathway has not been fully recognized. However, the cytoplasmic regulators of the Shh pathway in the target cell are known. In *Drosophila*, Cos2, Fused and Sufu are important in regulating Shh signalling pathway in the cytoplasm, but some of them are not conserved in their role in

vertebrates. For example, *Cos2* does not bridge *Smo* to *Gli* transcription factors in vertebrates like it does in *Drosophila* (Varjosalo et al., 2006).

*Fused* in *Drosophila* is required to activate *Shh* response in high level of *Shh* (Preat et al., 1990). Its homolog in mouse is *Stk36*, which is broadly expressed in the developing embryo. However, mouse mutants that lack all activity of this protein have no apparent defects in hedgehog signalling which suggests that the role of *Fused* is not conserved in mammalian (Huangfu and Anderson, 2006).

*Sufu*, the suppressor of *Fused* in *Drosophila*, is a major repressor of the *Shh* pathway in vertebrates. In the mouse embryo, the mutant shows a strong *Shh* gain-of-function ventralization of cell types in neural tube. There are two spliced isoforms in human, and both are expressed in multiple embryonic and adult tissues (Stone et al., 1999). For both mouse and human isoforms, co-immunoprecipitation of epitope-tagged proteins indicate that they interact with *Gli1*, *Gli2*, and *Gli3*, suggesting that *Sufu* can act by binding to *Gli* and inhibiting *Gli*-mediated transactivation (Stone et al., 1999; Barnfield et al., 2005).

Protein kinase A (PKA) is a conserved negative modulator of the *Shh* pathway. In *Drosophila*, PKA primes the phosphorylation of specific *Ci* (homolog of vertebrate *Gli*) residues, and is required for the proteolytic cleavage of *Ci* to become repressor, therefore, the loss of PKA phosphorylation sites not only prevent the generation of *Ci* repressor but also make it constitutively active (Chen et al., 1999; Price and Kalderon, 1999; Wang et al., 1999). Decreased PKA activity in mouse embryo causes dorsal expansion of *Shh* signal response in some regions of the neural tube, also, decreasing PKA activity results in an increase in cell apoptosis in the abnormal neuroepithelium and dorsal root ganglia, suggesting that PKA activity plays an anti-apoptotic role in the developing neural tube (Huang et al., 2002). In chick embryo, it will cause left-right determination (Rodriguez-Esteban et al., 2001).



Rab23, a vesicle transport protein encoded by *open brain (opb)* gene, is an essential negative regulator of the mouse Shh signalling pathway. At E10.5, Rab23 is present at low levels in most tissues and at high levels in the dorsal spinal cord, somites, limb buds and cranial mesenchyme. The expression pattern of Rab23 is similar to that of Gli3, a transcriptional effector of the Shh pathway. The double mutant of *opb* and *Shh* genes showed that the neural tube was normally patterned independent of Shh signal, and the *opb* mutants lack dorsal cell types (Eggenchwiler et al., 2001). Further experiments show that Rab23 works downstream of Ptc and Smo but upstream of Gli, regulates Shh pathway by promoting the production of Gli3 repressor and preventing the activation of Gli2 (Eggenchwiler et al., 2006).

Several other mouse genes have been identified that may play a role in the cytoplasmic Shh pathway, including genes like *Fkbp8*, *Sil* and *Fused toes*. *Fkbp8* is an antagonist of Shh signalling (Bulgakov et al., 2004), while *Sil* and *Fused toes* sequestration results in the ventral cell type lost in the neural tube (Izraeli et al., 1999; Gotz et al., 2005).

#### 1.6.4 Transcriptional effectors of Shh pathway

In *Drosophila*, the *Cubitus interruptus (Ci)* transcription factor appears to be the obligatory final mediator of hedgehog signalling to activate the expression of hedgehog responsive genes. Ci, a zinc-finger-containing transcription factor, functions as both a repressor and an activator of transcription. In the absence of hedgehog signalling, the C-terminal of Ci is proteolytically cleaved, and the remaining N-terminal and the zinc-finger DNA-binding domain become a transcriptional repressor form of Ci. In the presence of hedgehog signalling, the cleavage is inhibited, the full length of Ci molecule serves as a transcriptional activator (Jacob and Briscoe, 2003). It is reported that the hedgehog gradient

is translating into position-specific gene expression by modulating the ratio of Ci activator and repressor (Aza-Blanc and Kornberg, 1999).

Ci has three homologues in vertebrates: Gli1, Gli2 and Gli3, and its role of transcriptional repressor and activator are also divided into the three Gli factors in a much more complicated manner. The human Gli gene is composed of 12 exons and 11 introns and in the zinc finger coding region which shares a highly conserved splicing pattern with several other Gli family members in vertebrates (Liu et al., 1998). Its subcellular location is the cytoplasm and nucleus, and it is tethered in the cytoplasm by binding to Sufu; when it is activated, its translocation into nucleus is prompted by interaction with STK36 (Pavletich and Pabo, 1993).

In mammals, Gli proteins appear not only to be the mediators of Hh signalling, but also respond to other inputs unrelated to Hh signal like FGF signals (Ruiz i Altaba et al., 2003). The complex interaction between the Gli proteins remains unclear, however, there is evidence of their important role.

#### **1.6.4.1 The interaction of Gli proteins**

Gli1 acts as an indicator for positive Shh signalling, it is strictly dependent on Shh signal for its expression. Gli1 lacks a transcriptional repressor domain and can strongly activate of Shh target genes, including itself (Bai et al., 2002; Bai et al., 2004). When Gli2 is expressed in the absence of Shh signal, it is neither an activator nor a repressor in this situation. If it is activated by Shh, Gli2 will initiate transcription of Shh target genes including Ptc-1 and Gli1 (Bai and Joyner, 2001). Gli3 can be expressed in the absence of Shh signal, while in the presence of Shh, Gli3 is proteolysed to produce transcriptional repressors (Aza-Blanc et al., 2000). Both Gli2 and Gli3 can function as either a transcriptional activator or a

repressor for the Shh target gene transcription, and the role they choose to be is dependent on the cell context (Hatsell and Cowin, 2006).

Research has showed divergent requirements for Gli factors in zebrafish and mouse. In Zebrafish, Gli3 has an early function as an activator of Shh target genes that overlaps with Gli1 activator function in the ventral neural tube, and *in vitro*, Gli3 cooperates with Gli1 to activate transcription in high concentration of Shh; however, Gli3 is required as a repressor at later stages, and shares this repressor activity with Gli2 in the dorsal neural tube (Tyurina et al., 2005). In mouse, Gli2 loss-of-function is associated with diminished Shh-induced target gene expression, while Gli3 loss-of-function was associated with increased Shh target gene expression (Motoyama et al., 2003). The loss of Gli1 alone has no effect on target gene induction (Bai et al., 2002). The *in vitro* experiment of mouse embryonic fibroblasts suggests that Gli2 and Gli3 share common regulatory mechanisms and modulate Shh target gene expressions directly and independently while also regulating Gli1 expression (Sasaki et al., 1999). Gli2 and Gli3 regulate the Gli1 expression by directly binding to Gli1 promoter and induce Gli1 transcription in response to Shh signal (Dai et al., 1999).

#### **1.6.4.2 Gli factors in neural tube development**

In the frog neural plate, Gli1 is expressed transiently in the prospective floor plate during gastrulation and in the cell lateral to the midline during late gastrulation and neurula stages. While Gli2 and Gli3 are absent from the neural plate midline with Gli2 expressed widely and Gli3 in a graded fashion with highest levels in lateral regions (Lee et al., 1997). Consistent with this, in mouse embryos, the three Gli genes have a similar pattern of expression in the early neural plate.

In mouse, Gli1 is expressed in the ventral neural tube, and its expression is dependent on Shh signalling. Both Gli2 and Gli3 are expressed before the neural tube is closed, and the Gli3 expression is confined to intermediate and dorsal spinal cord region, while Gli2 expression is uniformly distributed. However, in the Gli2 loss-of-function mutants, the development of floor plate and adjacent V3 ventral interneurons is severely affected, and the motor neuron region is expanded ventrally (Bai et al., 2002). In the absence of Gli3, Shh signaling is dispensable for the generation and maintenance of oligodendrocyte progenitors (Oh et al., 2005). This suggests that Gli2 contributes to the induction of the most ventral region in the neural tube. Neural tube defects in Gli2 mutants are rescued by Gli1 suggesting that the inductive effects of Gli2 are mediated in the form of transcriptional activator. In contrast, Gli3 functions primarily as transcriptional repressor in mouse neural tube, and this is demonstrated by the fact that the abrogation of Gli3 function will rescue the defects in Shh mutants (Bai et al., 2004).

Efforts have been made to decode the gradient of Shh signal from ventral to dorsal neural tube by Gli proteins. Jacob and Briscoe (2003) have proposed a model of Gli-induced patterning of the ventral spinal cord (see Figure 1.6 Gli proteins specify the patterning of the ventral spinal cord) (Jacob and Briscoe, 2003). According to this model, the ventral to dorsal gradient of Shh activity induces a parallel gradient of Gli activators, with maximum function at the ventral midline and decreases towards the dorsal region; a gradient of Gli repressors activity that antagonizes the Shh gradient. The team led by Briscoe (2006) demonstrated that in chick embryos (Cayuso et al., 2006), by activating Gli constructs, they generated different levels of Gli activity along the dorso-ventral neural tube, and from the highest level of Gli activity to the lowest activity, the well arranged neurons such as the most ventral cell type and motor neurons and intermediate neurons were induced according to the different Gli activity. Their experiments suggest that a gradient of Gli activity is

sufficient to pattern the ventral neural tube and mimic the graded Shh signal. They also demonstrated that small changes in the level of Gli activity are sufficient to regulate the transcription of patterning genes and thus to direct the generation of different ventral neuron subtypes.

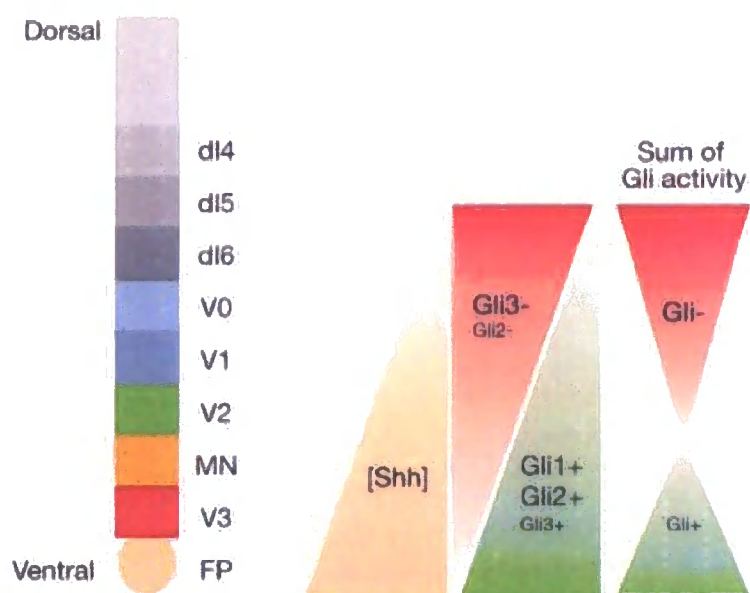


Figure 1.6 Gli proteins specify the patterning of ventral spinal cord. DI4, dI5, and dI6: dorsal interneurons; V0, V1, V2, V3: ventral interneurons; MN, motor neurons; FP, floor plate; Gli+, Gli activator; Gli-, Gli repressor. (After Jacob & Briscoe, 2003.)

### 1.7 Shh signalling and its influence of cell behaviour

It is known that Shh signalling affects cell behaviour such as cell proliferation, cell cycle and cell apoptosis. According Shh plays an important role in the development of vertebrate embryos.

In *Drosophila*, Shh signalling mediator Ci directly promotes the transcription of cyclinD and cyclinE, the two principal regulators of cell cycle during the development. The up-

regulation of cyclinE is accomplished through binding of the Ci to the cyclinE promoter to induce the DNA replication (Duman-Scheel et al., 2002). This machinery is likely conserved in vertebrates. In mouse neuron precursor cells, it is shown that Shh treatment induces cell proliferation accompanied by a rapid response of cyclinD1, D2 and cyclinE mRNA level (Kenney and Rowitch, 2000).

The observations in the chick neural plate and neural tube illustrate these facts: introduction of dominant active form of Ptc-1 inhibits cell cycle progression and/or increases cell death; and Gli3 transcriptional repression mediates the cell cycle arrest at G1 phase via Bcl2 regulation; and Gli3 activator induces neural progenitors over proliferation (Cayuso et al., 2006).

It has been reported that Shh signal regulates cell cycle machinery via the activation of cell cycle progress genes like cyclinD1, cyclinD2, and N-myc. CyclinD2 is preferentially expressed in the posterior neural plate, while the cyclinD1 is expressed in the neural groove. The loss- and gain- function experiments demonstrated that Shh activates the expression of cyclinD1 in the neural groove (Lobjois et al., 2004). And the forced maintaining of cyclinD1 and D2 in neural tube favours the proliferation at the expense of neuronal differentiation.

The aberrant activity of Shh signalling in neural development results in the formation of medulloblastoma, the most common malignant brain tumour in children (Dellovade et al., 2006). Mutations in Shh pathway components like Ptc-1, SUFU and Smo genes contribute to the etiology of medulloblastoma. Medulloblastoma shows high expression of Gli1 and N-myc, C-myc, cyclinD1 and D2, revealing a close connection between activated Shh signalling and impaired cell cycle control (Lee et al., 2003). One of the possible mechanisms is Shh stimulation hampers an exit from cell cycle through blocking cell cycle arrest by inducing the increased activity of the cyclin dependent kinases CDK2 and CDK4

(Fan and Khavari, 1999). Furthermore, the activation of other growth factors may contribute to enhancing medulloblastoma growth (Dellovade et al., 2006). The investigation of the genetically engineered mice showed that activation of Shh/Ptc pathway in neonatal cerebellum is sufficient to induce medulloblastoma in mice and the ectopic expression of Myc oncogene and IGF signalling pathway enhanced the tumour formation in mice (Fults, 2005).

The importance of programmed cell death during vertebrate development has been well established, particularly in the nervous system development. Normally, we generate about three times as many neurons as needed. Programmed cell death occurs at the early stages of neural development to control cell number, the removal of mis-specified cells, and for proper spacing and orientation of neurons (Gilbert, 2003). Although there are reports discussing that Shh signalling does not affect apoptosis gene p53, it is known to negatively regulate p21/cyclin-dependent-kinase-interacting protein1 and p27 through Gli1 to affect the transition of G1 phase to S phase and the cell proliferation (Ohta et al., 2005). By injecting hybridoma cells that secrete anti-Shh antibody to block Shh from the floor plate, notochord and pharyngeal endoderm in chick embryo, cell death was significantly increased in neural tube and neural crest after 1 day of injection which resulted in a much smaller head after 7 days (Ahlgren and Bronner-Fraser, 1999). Therefore, this demonstrates that a role of Shh exists in coordinating the cell survival of neural tube and neural crest. A Shh antagonist, REN, a putative tumor suppressor that is often deleted in medulloblastoma (Di Marcotullio et al., 2004), antagonizes Shh signalling by impairing the Shh dependent Gli transcription and Gli2 mediated gene transcription, and promotes growth arrest by activation of caspase3 (Argenti et al., 2005). This provides further evidence that Shh signalling regulates cell apoptosis during development.

## 1.8 Shh signalling and neural differentiation

Shh acts as a morphogen that promotes the formation of many neural subtypes by regulating the expression of families of transcription factors in cells along the ventral to dorsal axis. Much interest is focused on the specification of motor neuron, dopaminergic neuron and oligodendrocyte. The action of Shh signalling as to how it regulates axon guidance is also considered.

### 1.8.1 Shh and motor neuron specification

Shh is responsible for patterning of the ventral neural tube. The notochord is the initial signalling centre of Shh, and the signalling factor functions in two manners: a short-range signal which acts locally to induce floor plate differentiation along the midline of the neural tube; and a long-range signal which patterns motor neuron and ventral interneuron differentiation (Ericson et al., 1996). In the ventral spinal cord, five distinct classes of neuron subtypes emerge in a precise spatial order from progenitor domains arrayed along the dorso-ventral axis. *In vitro* studies show that two to three incremental fold changes of Shh concentration generates five neuron subtypes similar as seen *in vivo* (Ericson et al., 1997). The gradient model has been established that Shh patterns the ventral neural tube through a concentration gradient mode, with highest Shh concentration at floor plate and decreasing towards the dorsal neural tube (Poh et al., 2002). Each progenitor domain in the ventral neural tube expresses a unique set of molecular markers. And the markers of each progenitor domain is regulated by different thresholds of Shh (Ericson et al., 1997). Most of these molecular markers are belonging to the homeodomain transcription factor family or the basic helix-loop-helix transcription factor family. According to their responses to Shh, these transcription factors are divided into two groups: Class I proteins are repressed by



Shh signalling, which includes transcription factors like Pax6, Irx3, Dbx1/2, Pax3/7; Class II proteins are induced by Shh signalling, including Nkx2.2/2.9, Olig2, Nkx6.1/6.2 (Stamatakis et al., 2005). Class I and Class II transcription factors have a cross-repressive regulatory relationship between them (shown in Figure 1.7): for example, Pax6 from Class I is not compatible with Nkx2.2 from Class II in the same cell, this antagonistic relationship is consistent with the observations that the loss of Pax6 leads to a dorsal expansion of Nkx2.2, while the loss of Nkx2.2 results in a ventral expansion of Pax6 in the neural tube (Lee and Pfaff, 2001). Other pairs that have cross-inhibitory activities are: Irx3/Olig2, Dbx2/Nkx6.1, Dbx1/Nkx6.2. Their possible role in the Shh gradient model is to convert the smooth gradient of Shh into discrete progenitor domains in ventral neural tube (Lee and Pfaff, 2001).

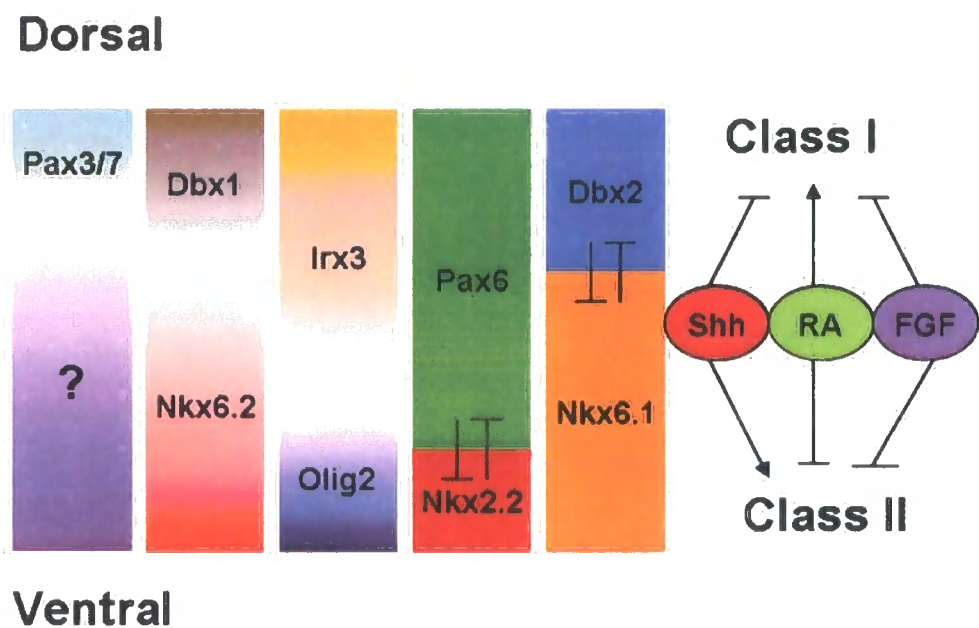


Figure 1.7 Cross repression of Class I and Class II transcription factors and their regulation by the signalling factors in the developing spinal cord. Shh induces Class II and suppresses Class I, while RA induces Class I and suppresses Class II, and FGF suppresses both. (Adapted from Briscoe, J et al, 2001)

In the developing neural tube, each progenitor domain has a unique set of molecular markers that encoded by the patterning transcription factors, suggesting that the transcription factors act in a combinatorial manner to specify distinct cell identities. Motor neuron progenitor domain is characterized with combinatory expression of Pax6, Olig2, Nkx6.1 and Nkx6.2 (Lee and Pfaff, 2001). But the expression of the various molecular markers is a dynamic process, under the developmental control. First, Olig2 is induced by homeodomain genes Pax6 and Nkx6.1, and followed by Olig2 which indirectly induces motor neuron fate through de-repression of motor neuron determinants (Poh et al., 2002). Olig2 appears to be particularly important in motor neuron differentiation. Olig2, a basic helix-loop-helix transcription factor, was first identified as an oligodendrocyte lineage gene, marking oligodendrocyte precursors and progeny (Zhou et al., 2000). But it is first expressed at E8.5 in the mouse spinal cord, a stage that is much earlier than the oligogenesis in the spinal cord. Olig2 has two weak peak expressions at E9.5 and E12.5, which corresponds with the two major periods of motor neuron and oligodendrocyte specification, respectively (Sun et al., 2001). In the loss-of-function assay, the Olig2 mutant results in the complete loss of the motor neuron and oligodendrocyte generation, while giving rise to V2 interneurons and astrocytes (Zhou and Anderson, 2002). The lineage tracing experiment demonstrate that Olig2 positive cells in motor neuron progenitor domain give rise to motor neuron and oligodendrocyte and a subset of astrocytes at the ventral surface of the domain (Masahira et al., 2006). However, if Olig2 positive cells isolated from E9.5 and E13.5 spinal cord were transplanted to chick embryo, motor neurons and oligodendrocytes were generated from E9.5 Olig2 positive cells, whereas E13.5 Olig2 positive cells only generated oligodendrocytes (Mukouyama et al., 2006). It is suggested that Olig2 positive cells have restricted differentiation potential with the progression of development, only the earlier population containing the neurogenic potential. Olig2 also

appears to promote cell cycle exit, there is no self-renewal of Olig2 positive cells observed in the transplantation experiments (Mukoyama et al., 2006). Furthermore, a recent study showed that Olig2, as well as Pax6 and Nkx2.2, has a unique ability to regulate the activity of proneural transcription factors like Neurogenin-1, -2, -3, Mash1 and Hes1, and the dynamic of changes in their co-expression pattern *in vivo* determine the timing of differentiation and the generation of three neural cell lineages (Sugimori et al., 2007).

Unlike Olig2 is a bi-phase transcription factor for progenitor cells, HB-9 is a marker for mature motor neurons and their progenitors. *hb-9*, a homeobox gene, possesses a homeodomain virtually identical to that of *MNR2* (Arber et al., 1999). In chick embryo, the final division of motor neuron progenitors is marked by the onset of expression of two homeodomain proteins: MNR2 and LIM3 (Lhx3) (Sharma et al., 1998; Tanabe et al., 1998). The ectopic expression of *hb-9* mimics *mnr2* in motor neuron-inducing and V2 interneuron-repressing activities in chick, but HB-9 in chick is restricted to post-mitotic motor neurons, suggesting that it has a later role in motor neuron differentiation (Tanabe et al., 1998; Arber et al., 1999). HB-9 deficient mice showed the defective motor neurons inappropriately express V2 interneuron genes and the migration of motor neurons and the axonal projections are perturbed; also the acquisition of motor neuron subtype identities is impaired (Arber et al., 1999). Embryonic stem (ES) cells from *hb-9* promoter transgenic mouse are directed to differentiate into functional motor neurons when treated with Shh agonist and RA (Miles et al., 2004). In adult human olfactory neuroepithelial progenitors, *hb-9* and *Ngn2* co-transfection induces motor neuron fate (Zhang et al., 2006b). In neural stem cells, *hb-9* transfection affects the neurogenesis but can not induce *Islet1* and up-regulate *Lim3*, and fail to respond to Shh and RA, suggesting the transfected neural stem cells did not convert to motor neuron fate (Brejot et al., 2006). It suggests that HB-9 is a

necessary transcription factor in motor neuron differentiation but has a different ability in specifying motor neurons in different stem cells.

### 1.8.2 Shh and dopaminergic neuron differentiation

The dopaminergic neurons are mainly located in two nuclei of the midbrain: the substantia nigra (also called A9 group) and the ventral tegmental area (also called A10 group). Parkinson's disease, which affects more than one percent of the population above the age of 65 years old, is a progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra (Dellovade et al., 2006). The development of midbrain dopaminergic neurons is under the control of a genetic network. Shh is known to be involved in the differentiation of dopaminergic neurons during embryonic development (Hynes et al., 1995; Kim et al., 2003; Roussa and Krieglstein, 2004). The time course of midbrain dopaminergic neurons (mDNs) can be divided into 4 stages: First, progenitors at the ventral ventricular mesencephalic surface that have self-renewing properties and give rise to multiple cell types arise at E7.5. Second, the multipotent progenitors are specified to a dopaminergic neuronal precursor cell fate, and several molecular markers are associated with this population, including *Otx2*, *Lmx1a/b*, *Msx1/2*, *Ngng2*, *Mash1*, *En1/2* and *Raldh1*. The third stage, the dopaminergic precursors exit the cell cycle and begin to display early mDN markers including *Lmx1a/b*, *Ngng2*, *Nurr1*, *En1/2*, *Raldh1* and  $\beta$ III tubulin. Finally, the early mDNs functionally mature, express mature mDN markers, and establish appropriate connectivity (Vernay et al., 2005; Abeliovich and Hammond, 2007).

At the early embryonic midbrain at E7.5 to E9.5, Shh from the ventral floor plate specifies the ventral neuronal subtype, including dopamine neuron progenitors. In Shh null mutant, dopaminergic neurons are missing, and if the Shh pathway activator Smo at E9.0 is

inactivated, the dopamine neurons are significantly reduced (Blaess et al., 2006). The mitotic dopaminergic neuron progenitors show a brief time window sensitive to Shh signal. Shh down-stream mediator Gli1 induce Fox2a expression to stimulate more Shh secretion from the floor plate, together with FGF8 from the midbrain/hindbrain junction, induce mitotic progenitors exit from the cell cycle and expression of early dopamine neuronal markers. Explant cultures show that ectopic expression of Shh and FGF8 are necessary and sufficient for the generation of ectopic dopamine neurons (Ye et al., 1998). At the post-mitotic stage, Shh is also involved in the activation of *Lmx1a*, *Msx1*, *Ngn2* and inhibitory genes *Nkx2.2* and *Nkx6.1*, to further specify the early dopamine neuron identity. And at later stage, Shh induce *Nurr1*, which is required for the maintenance of dopamine neurons and the expression of tyrosine hydroxylase (TH) and dopamine transporter (DAT) (Ang, 2006; Abeliovich and Hammond, 2007). Therefore, Shh is necessary for dopamine neuron induction and differentiation. *Wnt1* is another signalling factor which is also crucial to the induction and specification of dopamine neurons (Salie et al., 2005).

### **1.8.3 Shh and oligodendrocyte differentiation**

Oligodendrocytes are myelinating glial cells in the vertebrate nervous system, they ensheath the axon of neurons to support rapid nerve conduction (Butt and Berry, 2000). The oligodendrocyte precursors arise in distinct domain of ventral spinal cord, which coincide with the early established motor neuron progenitor domain. The precursors express basic helix-loop-helix subfamily transcription factors: *Olig1* and *Olig2*. The ventral location of the oligodendrocyte precursors near the floor plate is thought to be under the influence of Shh signalling. The abrogation of Shh signalling, either by chemical inhibitors or Shh blocking antibodies, results in inhibition of oligodendrocyte precursor's specification. In

Shh<sup>-/-</sup>; Gli3<sup>-/-</sup> mutants, Shh is required for the timely emergence of oligodendrocyte precursors and the subsequent differentiation of mature oligodendrocytes (Oh et al., 2005). In mouse development, Gli2 is the primary mediator of Shh signalling. In Gli2<sup>-/-</sup> mutant, the Olig1/2<sup>+</sup> oligodendrogenic domain in the ventral spinal neuroepithelium is markedly reduced, and the initial production of oligodendrocyte progenitor (OLP) cells from the ventral neuroepithelium is much decreased and delayed (Qi et al., 2003). Together, it suggests that Shh signalling is important for the specification and the terminal differentiation of oligodendrocytes. However, the later stage of oligodendrocyte differentiation is not affected, suggesting that other signalling factors such as FGF and RA may also play important role in this process. Shh responsive transcription factors like Olig2 and Nkx2.2 are also important in oligodendrocyte specification.

Mice lacking Olig2 or Olig1/2 function show deficiency of motor neurons and oligodendrocytes while the precursors transform into V2 interneurons and astrocytes, respectively (Lu et al., 2002; Zhou and Anderson, 2002). This suggests that Olig transcription factors not only involve in motor neuron differentiation, but also oligodendrocytes differentiation. Olig1 expression is reported to specify oligodendrocytes at the early stage and later give rise to oligodendrocytes and astrocytes in spinal cord (Liu and Rao, 2004). Olig2 expressing cells undergo sequential dual fates depending on the developmental stage and the other transcription factors being present. Olig2 and Ngn2 co-expression in early spinal cord will result in extended neurogenesis, and in mouse ES cells, induction of Olig2 expression is sufficient for oligodendrocyte specification but not motor neuron (Du et al., 2006). The over-expression of Olig2 in neural stem cells induces mature oligodendrocytes *in vitro*, suggesting that Olig2 is a determinant factor in oligogenesis and can also be an efficient tool to generate oligodendrocytes (Copravay et al., 2006).

Nkx2.2 is a homeodomain transcription factor that contains a DNA binding motif that has a typical helix-turn-helix structure, which is conserved in homeodomain family. Nkx2.2 defines the V3 interneuron progenitor domain in the spinal cord. However, in the Nkx2.2 null mice, a delayed oligodendrocyte differentiation showed that Nkx2.2 promotes the maturation of oligodendrocytes after the progenitors are specified by Olig2 (Sun et al., 2003). It is possible that the interaction between Olig2 and Nkx2.2 determine the V3 and MN domain boundary during early neurogenesis of the spinal cord, before the factors co-express at the later stage to induce Sox10 expression in oligodendrocyte precursors (Sun et al., 2001). Analysis the Olig2 and Nkx2.2 positive cells in the chick and mouse embryos showed that both populations give rise to oligodendrocytes and they eventually co-express in the same cell at a specific stage, which is tightly associated with the expression of myelin associated genes like MBP and DM-20-PLP (Fu et al., 2002). Therefore, Nkx2.2 could be a factor that helps Olig2 positive cells switch to an oligodendrocyte fate at a time cells cease to produce motor neurons.

#### **1.8.4 Shh and axon guidance**

It has been identified that proteins like netrins, Slits, Semphorins, Ephrins and their receptors act as guidance cues for axons to extend to their appropriate targets. However, there is increasing evidence that secreted signalling molecules such as Shh, Wnt, and BMPs, not only dictate the cell fate specification and tissue pattern, but also are used as axon guidance cues (Charron and Tessier-Lavigne, 2005). During the spinal cord development, the dorsal commissural neurons send axons towards and subsequently across the floor plate at the midline to form axon commissures. The axons project towards ventral midline partly because of Netrin1, which is secreted from the floor plate and serves as a

long range chemo-attractant. However, in Netrin1 and its receptor Dcc mutant mice, some of the axons still reach the ventral midline, indicating that another factor from floor plate also have the ability to guide the axons (Charron and Tessier-Lavigne, 2005). And it is highly possible that this factor is Shh, which is secreted from the floor plate. A recent research showed that Cdon and Boc (see Section 1.6.2.3), which bind specifically to Shh, are expressed by commissural axon as an axon guidance ligand for Shh action. The genetic loss of Boc but not Cdon results in commissural axon misguidance without seeming to disrupt spinal cord patterning or the netrin-mediated outgrowth of axons from spinal cord explants (Okada et al., 2006). Shh is not only used as a chemo-attractant for commissural axons (Bourikas et al., 2005), but also a negative chemo-repellent to regulate retinal ganglion cell axon growth (Salie et al., 2005). It is dependent on the cellular context that Shh exerts attraction or repulsion on axon path-finding, the underlying mechanisms are still poorly understood (Charron and Tessier-Lavigne, 2005).

## 1.9 The interaction of Shh and other signalling factors

The development of the vertebrate brain depends on the formation of many neuron cell types in precise locations and at precise times before making functional connections. This process has been showed to rely on complex interactions between morphogenic signalling factors. Understating the interactions between the signalling factors will provide insights into this highly regulated process. Shh has been shown to play an important role in neural specification and differentiation; therefore, further investigation of the interactions between Shh and other signalling factors will help complete our understanding of Shh's function.



## 1.10 Retinoic acid signalling

Retinoic acid (RA) has long been recognized as an important morphogen in mammal development. RA establishes a gradient that is high in the posterior and low in the anterior portion of the embryo. High dose or deficiency of RA disturbs the normal morphogenesis and organogenesis of the embryo. RA is a well known teratogen to human development, its high dose teratogenic effect occurs at 3-5 weeks of pregnancy, resulting in malformations such as: craniofacial alterations, cleft palate, neural tube defects, cardiovascular malformations, thymic aplasia, psychological impairments, and decreased IQ (Gilbert, 2003). The RA deficiency displays a spectrum of malformation affecting the eyes, skeletal system, thyroid and thymus, heart, lung, kidney, etc, also known as VAD (vitamin A deficiency syndrome) (Quadro et al., 2005).

### 1.10.1 RA source and its signalling pathway

RA, a derivative of vitamin A (retinol), is generated by retinol aldehyde dehydrogenase 2 (RALDH2) in the paraxial mesoderm from gastrulation stage onwards. The embryonic tissues do not synthesize retinol, which is delivered from maternal nutrition. RA is derived from retinol and is stored in its precursor form in the mammalian placenta, where the retinol concentration is 8-times higher than the embryo (Satre et al., 1992). Retinol bound to retinol-binding protein is thought to be the most physiologically important retinoid that is transported from mother to fetus and within the fetus. In the embryo, the principal metabolic event occurring for retinol is its conversion into RAs, the active molecules implicated in the molecular control of embryonic morphogenesis and organogenesis (Marceau et al., 2007). The maternal retinol-binding protein does not cross the placenta; the protein used in embryo is of yolk sac origin and can be secreted into the fetal circulation

(Quadro et al., 2005). The bound retinol and retinol binding protein is the substrate of microsomal retinol dehydrogenase or alcohol dehydrogenase (ADH), which will convert the retinol to retinal, the later is oxidized by RALDH2 to form RA (Mey, 2006). RALDH1-3 catalyze the final oxidative step by which retinol is converted to RA, but only RALDH2 is responsible for RA synthesis during early stages of embryogenesis (Chambers et al., 2007).

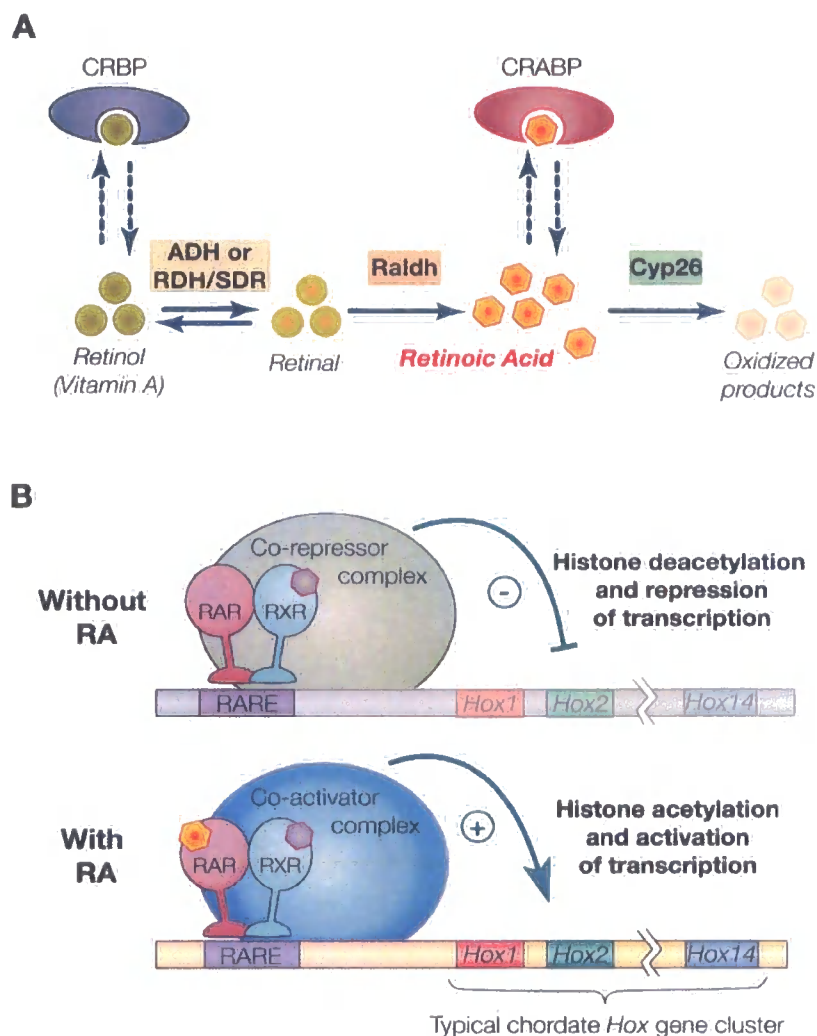


Figure 1.8 A) The metabolic pathway for synthesis and degradation of endogenous RA. B) The RAR/RXR heterodimer mediates the effects of RA. (After F. Marletaz, et al. 2006.)

The biological effects of RA are mediated through ligand-activated transcription factors of the steroid receptor superfamily in the nucleus: the retinoic acid receptors (RARs) and the

retinoid-X receptors (RXRs). RAR and RXR share some functional domains: an N-terminal region containing a transcriptional activation domain, a central region that includes a DNA binding domain and a weak dimerization domain, and an E region that contains a ligand binding domain, a strong dimerization domain, and a transcriptional co-regulator binding domain. In the absence of RA, RAR and RXR form a heterodimer and constitutively bind to RA responsive elements (RAREs) on DNA to silence the transcription by de-acetylating histone associated with the target sequence thereby increasing chromatin condensation. Once RA binds to RAR, the conformation of ligand binding domain will change to release transcriptional co-repressor and bind to co-activator, the co-activator will then mediate the acetylation of histone to activate transcription of target gene (Marletaz et al., 2006). There are three RAR (RAR $\alpha$ , - $\beta$ , - $\gamma$ ) and three RXR (RXR  $\alpha$ , - $\beta$ , - $\gamma$ ). They display specific tissue expression pattern and contribute to tissue-specific functions and developmental programs.

### **1.10.2 Role of RA in neural patterning**

The target genes that RA activates are responsible for carrying out the function of RA. These target genes are directly regulated by RA and include the genes involved in RA metabolism, RARs, Hox genes, and some dorso-ventral transcription factors (Pfahl and Chytil, 1996; Wilson et al., 2004; Goncalves et al., 2005a).

Hox genes are known target genes for RA. When gastrulation begins, the anterior-posterior positional patterning information in all vertebrates is conveyed by the expression of Hox genes. Its homolog in *Drosophila* is called Hom-C. Hom-C genes are arranged in the same order along the anterior-posterior axis, the most 3' gene being required for producing the most anterior structure, and the most 5' gene specifying the posterior abdomen. The human genome has Hox A to D four clusters, each cluster is numbered from 1 to 13, starting from

the gene that is expressed most anterior (Gilbert, 2003). RA signalling controls the anterior-posterior patterning of the developing brain probably acting via Hox genes (Marletaz et al., 2006). The sensitive Hox genes have RA receptor sites in their enhancers, so the exogenous RA will cause certain Hox genes to become expressed in cells that usually do not express them (Maden and Holder, 1992). In the developing central nervous system, homeodomain transcription factor patterning is conserved in vertebrates: generally, Otx expression at forebrain and midbrain, Pax2 expression at r1 (rhombomere1), Hox b1-4 expression in r2 to r7. When RA is over-dosed, Hox b1 and b4 are affected, and this causes hindbrain pattern to become disturbed, the r4 shift anterior to normal r1 position and r7 shift anterior to r6 position to form a big hindbrain, furthermore, forebrain is deleted and midbrain is shrunk. In RA loss-of-function assays, forebrain and midbrain are not affected, but the hindbrain r3 expand posterior to r7 position. It suggests that RA acts as a posteriorizing signal to transform anterior embryonic tissue into a more posterior fate (Schilling and Knight, 2001). RA signalling is also required for dorso-ventral patterning in the vertebrates. At the dorso-ventral axis, RA from adjacent somites can act directly on the generation of certain interneurons and ventral neuron populations. The explant culture showed that RA specified V0 and V1 interneurons by inducing Dbx1/2, Irx3 and En1 expressions (Novitch et al., 2003). How RA regulates dorso-ventral patterning is well demonstrated in RA avian deficiency by the VAD quail model (Wilson et al., 2004). In VAD quail, RA disrupts both the dorsal and ventral patterning genes: the dorsal genes like *Bmp4*, *Bmp7*, *Msx2*, *Pax3*, *Pax7* and *Wnts*; the ventral genes like *Shh*, *Nkx6.1*, *Mnr2*, *Isl1*, *Pax6* and *En1*. The RA deficiency results in a general effect of dorsal expansion of ventral genes, and a reduction of dorsal located genes. However, ventral *Pax6*, a gene is directly regulated by RA, is strongly down-regulated. Comparing the gene expression in normal and VAD quail model, at least 27 novel genes are found to be regulated by RA signalling (Wilson et al., 2004).

The new genes and the found genes might form a genetic network that responds to RA signalling. The network will integrate the anterior-posterior and dorso-ventral information and actually pattern the hindbrain and spinal cord.

RA also regulates the expression of proneural transcription factors like *neuroM* in the caudal neural plate. In the VAD quail model, the expression of *neuroM*, *Delta1*, *Neurogenin1* and *Neurogenin2* are all depleted or absent in the posterior neural tube, which results in a vast reduction in the number of neurons (Wilson and Maden, 2005). This suggests that RA is directly involved in neurogenesis in the spinal cord and it helps progenitors acquire their pan-neural identities.

### 1.10.3 RA regulates the Shh pathway

There is evidence which indicates that RA regulates Shh expression in the vertebrate embryos. From the VAD quail or mouse model, it is revealed that RA regulates Shh expression depending on the tissue context: RA has antagonizing effect on Shh expression in the spinal cord, while RA induces Shh expression in the forebrain development (Halilagic et al., 2007). In the spinal cord, some homeodomain transcription factors are divided into two groups according to their response to Shh. Shh induces Class II factors while represses Class I genes, on the contrary, RA induces most of the Class I genes while inhibits Class II genes (Figure 1.7).

Therefore, the relationship between RA and Shh can be partly shown by the interaction of these two cross-repressive genes along the dorso-ventral axis (Wilson and Maden, 2005). This also shows how RA and Shh regulate the motor neuron differentiation. *Pax6* is induced by RA, as a member of Class I genes. *Pax6* is not directly required for the specification of V1 and motor neuron, but in *Pax6* mutants, the development of these two

neurons is disturbed. It is demonstrated that Pax6 regulates motor neuron specification by establishing the correct progenitor domain (Takahashi and Osumi, 2002). After the boundary is marked by Pax6, the co-expression of Nkx6 and Pax6 can prevent the expression of other transcription factors that are capable of repressing Olig2 expression, therefore, providing a de-repressed context to allow Shh induced Olig2 gene expression. RA acts downstream of Olig2 expression to activate the expression of Mnr2 and Lim3 to confer motor neuron identity and the expression of neuroM/D to promote pan-neuronal identity (Wilson and Maden, 2005).

RA also regulates Shh downstream components like Ptc-1 and Gli transcription factors. Excess RA will up-regulate the expression of Ptc-1 and Gli1 in mouse embryo at apical ectoderm ridge (Shimizu et al., 2007). Evidence shows that Gli3 has used CREB (cAMP-response-element binding protein) as co-activator, and RA has the ability to induce a rapid and sustained phosphorylation of CREB in many cells (Canon et al., 2004), raising the question that Gli3 is a potential target gene for RA. Moreover, it is demonstrated that excess RA attenuates the Gli3 and Gli2 expression in epithelial cells (Goyette et al., 2000).

### **1.11 Interaction between BMP and Shh signalling**

BMP/TGF $\beta$  signalling pathway is one of those best studied pathways because of its importance in embryogenesis. In the early stages of embryogenesis, the high level of BMPs promotes the differentiation of ectoderm into epidermis, while the inhibitors of BMPs are required for neuroectoderm formation (Delaune et al., 2005). BMPs belong to TGF $\beta$  superfamily, BMP ligand binds to its receptor and induces the formation of a complex in which the type II BMP receptor phosphorylates and activates the type I BMP receptor. The type I BMP receptor then propagates its signal by phosphorylating the SMAD1, SMAD5

and SMAD8 proteins. The activated SMADs dimerize with SMAD4 and then the formed SMAD complexes translocate into the nucleus and participate in the regulation of the target genes. BMP signalling determines cell fate in non-neural tissues, including bone, bone marrow, kidney and lungs, and is a critical regulator of neural development.

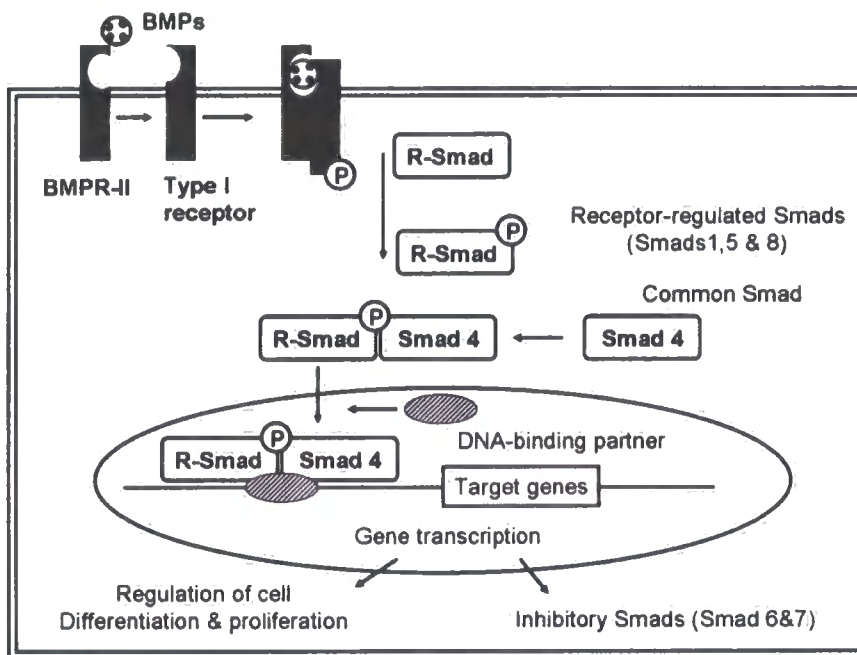


Figure 1.9 Model of BMP signalling pathway. BMP ligands induce heteromeric dimer of type I and type II receptors, receptor-Smad (R-Smad) is phosphorylated and form heterogenic dimer with Common Smad (Smad 4). The resulted complex translocates into the nucleus to bind to specific DNA-binding partner and start gene transcription. (Adapted from J. Strange, et al. 2002.)

BMP signals from ectoderm induce the roof plate at the midline of dorsal spinal cord. The dorsal roof plate becomes a secondary BMP signalling centre. Secreted BMPs diffuse from the dorsal to the ventral spinal cord and establish a BMP gradient to specify the dorsal neuronal subtypes (Nguyen et al., 2000; Timmer et al., 2002). The roof plate is the dorsal equivalent of the floor plate; the secreted proteins form an opposite gradient along the dorso-ventral axis to provide the positional information to the neuron progenitors. The ablation of roof plate results in reduced dorsal Pax7 domain and expanded ventral Pax6

domain, also, dorsal neuron like Math1 positive sensory neurons and Ngn positive interneurons are missing (Lee et al., 2000). *In vitro*, even the neural progenitor cells are exposed to a fixed concentration of Shh, the presence of BMP results in the cells adopting a more dorsal identity (Liem et al., 2000). Furthermore, in the mouse BMP mutant, the reduction of BMP signalling causes ventral cell fate expansion towards dorsal neural tube (Wine-Lee et al., 2004). Therefore, BMPs and Shh have opposing activities in the specification of neuron identity along the dorso-ventral axis. However, BMPs and Shh work together to specify neuron progenitor domains along the dorso-ventral axis. Unlike RA, BMPs and Shh have no function in regulating pan-neuron differentiation such as neuroM/D (Maden, 2006).

### **1.12 Interaction between FGF and Shh signalling**

FGF family has at least 20 known mammalian family members. FGF signalling is critical in many physiological and pathological processes, particularly in embryogenesis, angiogenesis, and tumour growth (Fortin et al., 2005). FGF binds to one of its four receptors (FGFR1-4), which belong to transmembrane receptor tyrosine kinase (RTK) family. The binding of FGF ligand induces receptor dimerization and auto-phosphorylation. The phosphorylated RTK acts as binding site for cytosolic proteins with SH2 domain, like growth factor receptor bound protein 2 (GRB2), once binds to RTK, the SOS (son of sevenless) that bound to GRB2 will be activated. The activated SOS will catalyse GDP-Ras into GTP-Ras. The GTP-Ras is able to initiate several distinct signalling cascades, including MAPK (mitogen activated protein kinase) pathway, PI3K (phosphoinositol 3-kinase) pathway (Klint and Claesson-Welsh, 1999). FGF signalling is long known to be involved in neural plate specification. In the 4-signal neural tube formation model, FGF



signal from the mesoderm actually mark the neural plate, before RA, Shh and BMP specify the neuronal progenitor domains along the dorso-ventral axis (Wilson and Maden, 2005). FGF is now recognized as a morphogen in vertebrate development, by using antibodies, FGF is shown to express at the midbrain and hindbrain joint, developing eyes, somites, branchial arches, limb buds and tail, suggesting that FGF acts as anterior-posterior inducer (Yamaguchi and Rossant, 1995). It regulates anterior-posterior axis partly through Hox genes, for example, Hox c protein distribution in the developing spinal cord show a specific pattern: Hox C5 expresses at somites equal to cervical region, Hox C6 expresses across the cervical and brachial region, Hox C8 across the brachial to thoracic region, the majority of Hox C9 at thoracic region, and the hox C10 marks the lumbar region. FGF induces Hox genes in different concentrations: low level of FGF (5g/mL) induces C6, higher level (25g/mL) induces C6, C8, and C9, higher level (125g/mL) induces all previous plus C10 (Maden, 2006). It suggests that FGF, like RA, establishes a gradient concentration from caudal to rostral neural tube to provide the positional information.

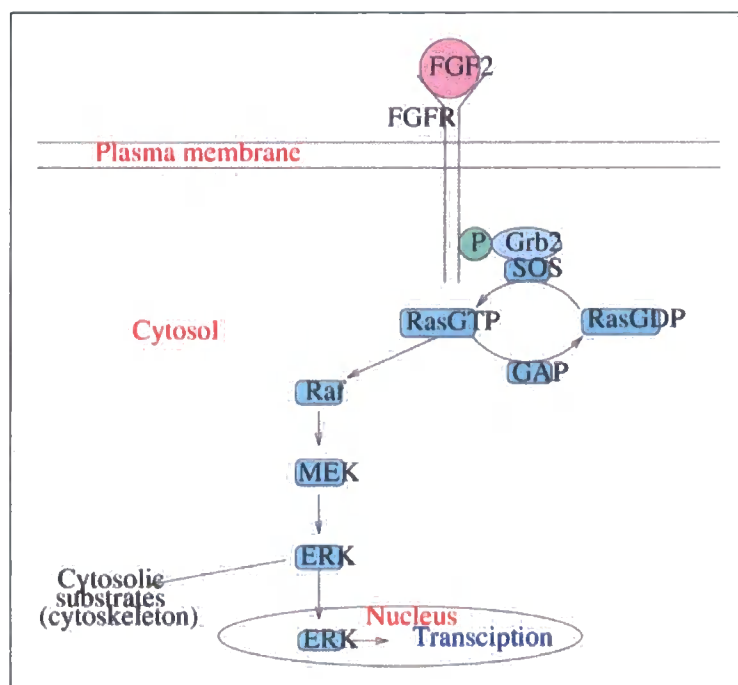


Figure 1.10 Schematic diagram of a FGF-2 signalling cascade. (After C.Lloyd, 1997.)

FGF represses both Class I and Class II genes that are repressed or induced by Shh, suggesting that a complex interaction between these two factors. *In vitro*, spinal cord progenitors from dorsal, when cultured with FGF, express ventral markers like Olig2 and Nkx2.2, while dorsal markers like Pax3/7 will be excluded from the cells, suggesting that FGF can ventralize the spinal cord progenitors. The research also indicates that FGF induces ventralization via Shh-dependent and Shh-independent pathways (Gabay et al., 2003). However, FGFR signalling is not required for Shh specifying ventral progenitors in the ventral spinal cord or forebrain. In granule cell precursors, FGF has opposing effect on Shh's mitogen role; it inhibits Shh-induced cell proliferation and promotes differentiation *in vivo* and *in vitro* through FGFRs and ERK and JNK kinases (Fogarty et al., 2007). There is evidence indicating that some FGF members that act downstream of Shh signalling as a target gene are regulated by Gli3 (Kuschel et al., 2003). Although they have diverse roles in CNS development: they provide different positional information for neural progenitor cells, they co-express in many regions and often regulate similar biological processes: they promote the proliferation of immature progenitor cells, they synergize to induce dopamine neurons in midbrain, they are involved in oligodendrogenesis in the CNS. The cross talk between FGF and Shh also coordinates cell cycle progression, patterning and differentiation of the early spinal cord formation (Lobjois et al., 2004). Further investigation about the interaction of these two factors will improve our understanding of their roles in neural development.

### 1.13 Interaction between Wnt signalling and Shh signalling

Currently the Wnt family has at least 19 members. Wnt signals are transduced through the activation of complexes between  $\beta$ -catenin and lymphoid enhancer-binding factor/T cell factor (LEF/TCF), as shown in the figure below. Some of them, like Wnt1 and Wnt3a, secreted from dorsal midline, have been shown to act as mitogens on neural progenitors and form a dorsal to ventral concentration gradient in the spinal cord (Megason and McMahon, 2002). The dominant model of active  $\beta$ -catenin showed that differentiation of the entire dorsal neurons and VI interneurons and motor neurons is reduced and that apoptosis is promoted along the dorso-ventral axis. Wnt1 and Wnt3a affect proliferation and differentiation indirectly by regulating cell cycle progression and cell cycle exit rather than cell fate specification (Megason and McMahon, 2002). There is also evidence showing that Wnts function with RA and FGF as anterior-posterior inducer (Sasai and De Robertis, 1997).

Both Wnt and Shh signalling have roles in stem cell maintenance, tissue development and tumorigenesis. The interaction of these two factors is not limited in neural tube, but also in epithelial and mesoderm (Iwatsuki et al., 2007). The cross-talk between these two factors takes place at  $\beta$ -catenin level and Gli factors level. The Gli3 repressor can bind to the C-terminal of  $\beta$ -catenin, which contains the C-terminal transactivation domain. This physical interaction inhibits the transcription activity of  $\beta$ -catenin (Ulloa et al., 2007). On the other hand,  $\beta$ -catenin also has a regulatory effect on Shh-Gli pathway.  $\beta$ -catenin has both negative and positive functions in Gli2 and Gli3 regulation, and through Gli2/3 activate Gli1 and Shh expression, suggesting that Wnt signalling control Shh signal transduction and their synergistic action depends on the developmental stage and tissue context (Borycki et al., 2000).

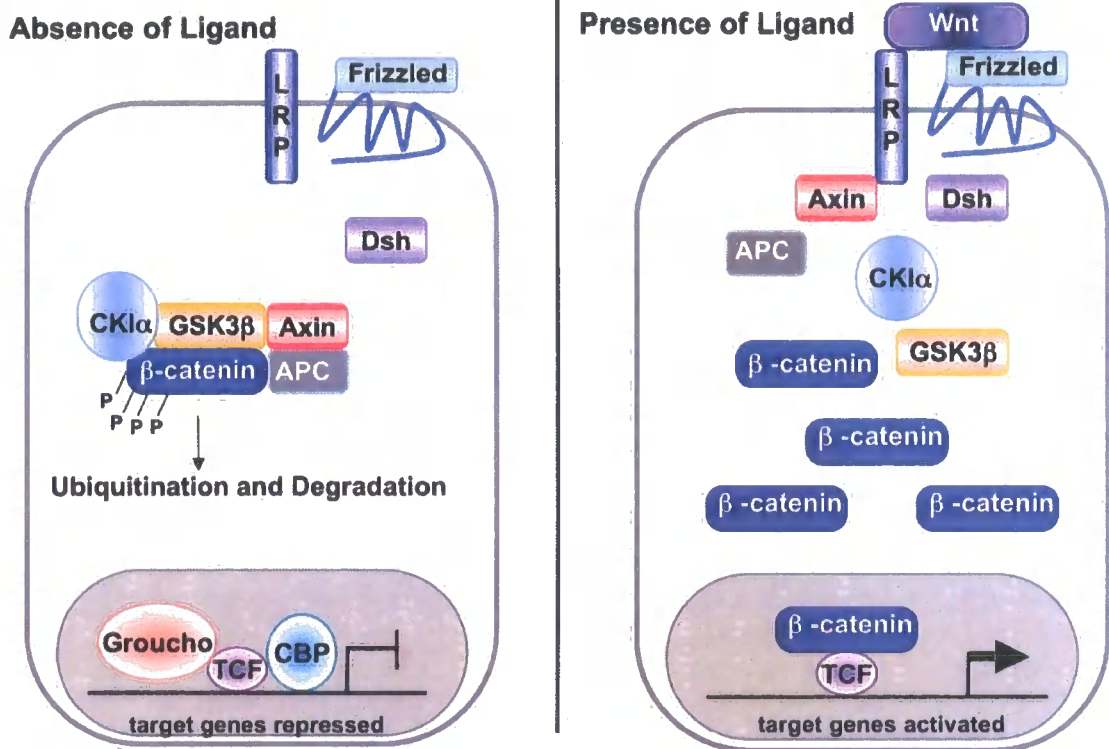


Figure 1.11 The canonical Wnt signaling pathway. In the absence of signal, action of the destruction complex (CK1 $\alpha$ , GSK3 $\beta$ , APC, Axin) creates a hyperphosphorylated  $\beta$ -catenin, which is a target for ubiquitination and degradation by the proteasome. Binding of Wnt ligand to a Frizzled/LRP-5/6 receptor complex leads to stabilization of hypophosphorylated  $\beta$ -catenin, which interacts with TCF/LEF proteins in the nucleus to activate transcription. In a canonical pathway, CK1 $\alpha$ , GSK3 $\beta$ , APC, and Axin act as negative regulators and all other components act positively. (Ater Eisenmann, D.M. 2005, WormBook)

### 1.14 Interaction of other growth factors and Shh signalling

Through Gli transcription factors, Shh activates many other growth factor signalling pathways like IGF, PDGF, and MAPK (Kessarīs et al., 2004). At least part of Shh functions are carried out through these growth factor signalling pathways, including cell growth, cell proliferation, and cell migration. Shh signalling pathway and the interaction between other factors are illustrated in Figure 1.5.

## 1.15 Stem cell models

A stem cell is a cell that has the capability to divide indefinitely, creating more stem cells by self-renewal as well as having the capacity to produce more specialised cellular progeny (Gilbert, 2003). Traditionally, people had used model organisms to study developmental biology. Stem cells now provide an *in vitro* model to study developmental biology at cellular level and molecular level. There are many kinds of stem cells available, including embryonic stem (ES) cells, adult stem cells and tumour derived stem cells. For the purpose of this study, we will focus on embryonic stem cells and adult neural stem cells and human embryonal carcinoma (EC) stem cells and their use as models of neural differentiation.

### 1.15.1 ES cells

Embryonic stem (ES) cells are derived from ICM of embryonic blastocyst, a stage before implantation in the uterus. The formation of ICM and trophectoderm in the blastocyst is the first differentiation event in humans and occurs at approximately five days of development (Stephanie Schmalz, 1999). Cells before this stage have the potential to give rise to any cell of the body. In the blastocyst, only cells of ICM have the potential to generate any cell of the body, cells of trophectoderm will give rise to part of placenta. ICM cells that are cultured *in vitro* under appropriate conditions are ES cells, which are able to proliferate and replicate themselves indefinitely and retain the potential to form any cell of the body. This is demonstrated by ES cell transplantation. When they are injected into a host embryo, each of ES cells can give rise to cells of all tissues except the trophoblast (Asano et al., 2006). The first mouse ES cell line was reported in 1981 (Evans and Kaufman, 1981), but the first human ES cell line is not reported until 1998 (Thomson et al., 1998). Although people attempted to establish human ES cell line as early as the 1980s, the derivation of human ES

cell line was not successful until the optimal culture medium were available and people have experience of growing non-human primate ES cell lines.

Mouse and human ES cells are able to grow indefinitely on the mouse fibroblast feeder cell layer. The activation of LIF/STAT3 and BMP pathways is found to be sufficient for mouse ES cell to proliferate without differentiation in the absence of feeder cell layer (Ying et al., 2003a). However, LIF/STAT3 pathway appears to be inactive in undifferentiated human ES cells (Daheron et al., 2004), and the addition of BMPs to human ES cell cultures causes a rapid differentiation of human ES cells into trophoblast (Xu et al., 2002). Many studies have been focused on human ES cell culture conditions. Growth factor bFGF is best studied, bFGF containing medium allows human ES cells to proliferate in a feeder-free condition (Xu et al., 2001). In addition, Wnt pathway and TGF $\beta$ /activin pathway promote the self-renewal of human ES cells in the absence of feeders (Dravid et al., 2005; Xiao et al., 2006). There are four requirements for developing ideal human ES cell medium: (a), cost-effective and easy to use; (b), composed entirely of defined components not of animal origin; (c), allows cell growth at clonal densities; (d), minimises the genetics and epigenetic changes that accumulate in culture (for more detail <http://stemcells.nih.gov/info/scireport/> ). Mouse ES cells have been used widely in gene targeting experiments (“knockout” model) to study mutations that affect early embryonic development (Matsuda and Aiba, 2004; Porret et al., 2006). This gives us insights to the roles of particular genes during mammalian development. Human ES cells offer an unprecedented access to tissues from the human body; this will support developmental research on the differentiation and function of human tissues. Studies have shown that ES cells undergoing differentiation *in vitro* often recapitulate the program *in vivo* (Zhang et al., 2007). Therefore, human ES stem cells have become invaluable in the study of early events in human development since human embryos are inaccessible due to the ethnic and moral issues.

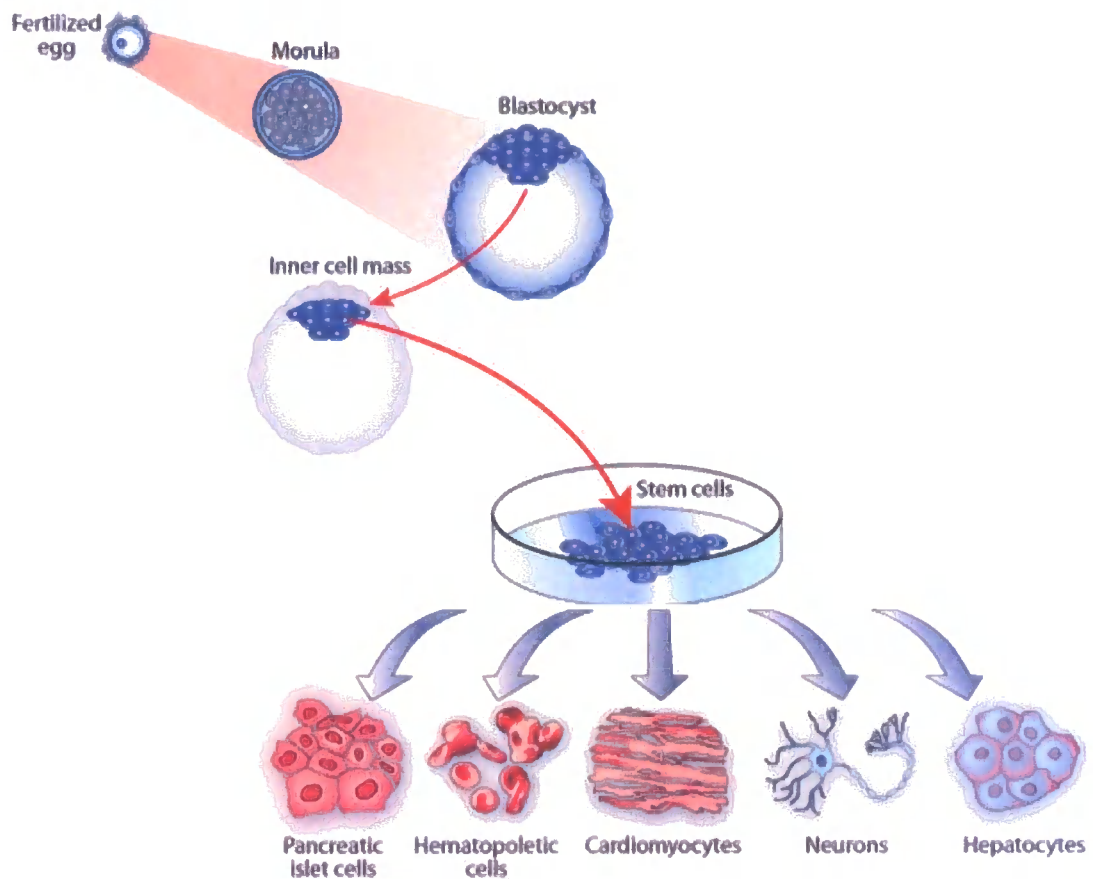


Figure 1.12 The derivation and differentiation of ES cells. ES cell is derived from inner cell mass, and have the ability to differentiate into all derivatives of three germ layers. (Adapted from T.James, 1998, <http://www.kumc.edu/stemcell/early.html>.)

ES cells must first commit to neural lineage before differentiating into neural cells. We know that the inner cell mass forms three germ layers and the progenitor cell of the ectoderm actually undergoes neural differentiation. Similarly, before ES cells become neural stem cells and give rise to neurons and glial cells, the ES cells go through a transit stage called neuro-ectodermal precursors. The endogenous Notch and FGF signalling and exogenous addition of BMP antagonists promote the neural lineage entry from ES cells (Ying et al., 2003b; Lowell et al., 2006; Sonntag et al., 2007). Comparison of the gene profiles of the ES-derived neural stem cells to the fetal neural stem cells have revealed that they share many neural stem cell markers but they also have differences like the key neural

differentiation signalling pathways including FGF, Wnt and LIF (Shin et al., 2007). Data suggest that ES derived neural stem cells retain more plasticity after long term culture *in vitro* but are less specialised. Many studies have been focused on how to direct ES derived neural stem cells differentiation into particular subtypes of neuron, like midbrain dopamine neurons (Sonntag et al., 2007), spinal cord motor neurons (Shin et al., 2005; Singh Roy et al., 2005), and interneurons (Murashov et al., 2004).

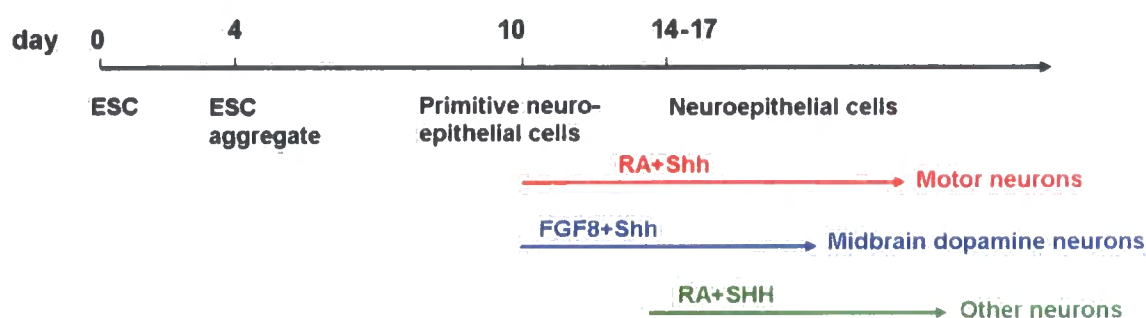


Figure 1.13 Schematic procedures for inducing dopamine neurons (DA) and motor neurons (MN) differentiation. ES cells were differentiated into primitive neuroepithelial cells and then neuroepithelial cells that exhibit neural tube-like rosettes in two weeks differentiation in a chemically defined neural medium (Zhang et al., 2001a) without the presence of morphogens. Treatment with the indicated conditions form resulted dopamine neurons or motor neurons. (Adapted from Zhang et al., 2007.)

Based on the well-defined neuro-epithelial differentiation system from human ES cells and the principles of neural tube development, Zhang *et al* summarize procedures for dopamine neurons and motor neurons (Zhang et al., 2007). Human ES cells grow in a chemical defined neural medium for about 10 days without the presence of morphogens will differentiate into primitive neuro-epithelial cells, if the primitive neuro-epithelial cells are treated with FGF8 and Shh, midbrain dopamine neurons will be induced; if treated with RA and Shh, motor neurons will be generated. However, the same treatments for these neuro-epithelial cells after 14 days, forebrain but not midbrain dopamine neurons and spinal cord interneurons not motor neurons are generated respectively. It suggests that intrinsic



program and extrinsic morphogens coordinate the neuron differentiation from ES cells. Furthermore, the application of developmental insights into correct neuronal subtype production is critical (Novitch et al., 2003). For example, motor neurons, as well as dopamine neurons, are required for sequential activation/inhibition of transcription factors to become mature and acquire proper functions *in vivo* (Li et al., 2005). The incomplete repeat of normal induction program *in vitro* will result in the generation of neurons with a deficit in phenotype and/or functions, and are therefore, not appropriate for cell replacement therapy *in vivo*.

Further investigation is necessary to understand the intrinsic program of ES cells and the timing of addition of proper environmental inductive signals to ES cells so that the correct cell type with appropriate functional neurons can be generated and used in clinical therapy.

### **1.15.2 Adult stem cells**

The adult stem cell is an undifferentiated cell that is found in a differentiated tissue; it can renew itself and become specialized to yield all of the specialized cell types of the tissue from which it originated (Slack, 2000). Most of the adult tissues can not be replaced, but numerous organs contain multipotent stem cells. For example, the haematopoietic stem cells in bone marrow, skin stem cells, and stem cells in intestinal crypts constantly give rise to new cells and replace the large number of dying cells everyday. In fact, adult stem cells can be seen as embryonic cell within adult organs, continuously producing more stem cells as well as cells that can undergo further development and differentiation (Potten and Loeffler, 1990). However, adult stem cells, compared with embryonic stem cells, are often tissue specific, with less plasticity. They are more fragile, they might have nursing cells

around to form a niche to protect them *in vivo*; furthermore, they are often quiet and appear in very low frequency. Therefore, it is difficult to isolate and maintain them *in vitro*.

There are two adult stem cells which are promising in neural induction. One is mesenchymal stem cells, and the other is neural stem cells (Zhao et al., 2004) (Altmann and Brivanlou, 2001; Xu et al., 2006). Mesenchymal stem cells were first isolated from bone marrow, however, it is later reported that they exist in many adult tissues (Jiang et al., 2002a). Although C.M. Verfaillie's group showed that the bone marrow derived mesenchymal stem cells gave rise to neural cells and endodermal cells when injected into adult mice (Jiang et al., 2002b; Schwartz et al., 2002), there are controversial debates about the neural differentiation of these cells, because they apparently have to reprogram themselves and differentiate trans-lineage to adopt a completely different cell fate, this is not likely to happen spontaneously. Some of their work can not be repeated by other groups (Holden, 2007). Furthermore, the neural cells derived from mesenchymal stem cells are not mature, fully functional cells that can restore lost function *in vivo* (Mezey et al., 2000). It is quite exciting to find out that the hippocampus and subventricular zone retain self-renewal proliferating cells that capable of differentiating into new neurons and glia even in adult (McKay, 1997). Neural stem cells can be generated from these areas or by immortalizing neural progenitors from the neural tube. Unlike the ES cells, neural stem cells have already been programmed to become neural cells. For example, the hippocampal stem cells *in vivo* progress through a defined six-step sequence of differentiation before ultimately turning into functional mature neurons (Scheffler et al., 2006). Additional environmental factors such as Shh, RA and FGF will help the differentiation of neural stem cells. Shh not only plays a role in neural stem cell proliferation, but also provide differentiation information to direct the neural stem cells to become hypothalamic dopamine neurons (Ohyama et al., 2005). However, neural stem cells obviously have less plasticity, which means that a stem

cell from one adult tissue can not generate the differentiated cell types of another tissue (Brazelton et al., 2000). They can not differentiate into motor neurons after gene modification like forced expression of HB-9 even in the presence of Shh (Brejot et al., 2006). Addition of appropriate signals to the *in vitro* hippocampal stem cells will facilitate this developmental progression. More information about the intrinsic program and the function of environmental signals during neural stem cell differentiation will enable us to find out how the pluripotent ES cells recapitulate the developmental differentiation cascades to generate various neural cell types and to examine the incomplete differentiation instructions during ES cell differentiation. Neural stem cells are perfect for studying neurogenesis *in vitro* and generation of disease models, but using them for cell replacement therapy, there is still a long way to go (Rothstein and Snyder, 2004).

### 1.15.3 Human embryonal carcinoma stem cells

Human embryonal carcinoma stem cells (EC) are derived from teratocarcinomas. Some EC cell lines like ES cells have the capability to differentiate into derivatives of all three germ layers. Because of their tumour property, EC cells can not be used in clinical medicine, but they are very useful models of developmental systems in the laboratory.

The Tera2 EC cell line was established in culture during the 1970s, which was originally derived from a metastasis of a human testicular teratocarcinoma. EC cells share many cell surface markers and cell properties with ES cells, as shown in the Table1—the comparison of mouse and human ES, EC cells. They all express high level of SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 and Oct-4 (Stewart et al., 2003; Schopperle and DeWolf, 2007). The EC cell line NTERA-2 is derived by Andrews et al. after passage of Tera2 to nude mice to form

a teratoma like xenograft. Single-cell clones of NTERA-2 were isolated from the xenograft, and NTERA-2 clone D1 became a widely used standard cell line (Andrews et al., 1985).

NTERA-2 cells have the capability to differentiate into a variety of cell types, including mesodermal myocytes and endodermal HNF-4 $\alpha$  positive cells (Pal and Ravindran, 2006).

When NTERA-2 cells were exposed to RA and other agents in culture, they lose their stem cell surface markers such as SSEA-3 and TRA-1-60. If NTERA-2 cells were exposed to BMP, hexamethylene bisacetamide (HMBA) or RA in low density, cells resembling extraembryonic endoderm and cells with neural properties were yielded (Andrews, 2001).

Now the differentiation of well-developed neurons from NTERA-2 has been extensively studied (Przyborski et al., 2000). The differentiation of NTERA-2 into neurons appears in many ways to recapitulate the steps that occur during the embryonic development of the nervous system (Przyborski et al., 2000; Alexander Przyborski et al., 2003; Przyborski et al., 2003). Transcriptional profiling of neuron differentiation of NTERA-2 indicates that the induced neurons share the ventral neuronal characteristics, which is consistent with ES cells (Przyborski et al., 2003). Neurons derived from NTERA-2 cells after RA treatment comprise only 2-5% of all the differentiated cells. However, the differentiated neurons possess a typical neuronal morphology and express neural markers like neurofilaments, furthermore, they express tetrodotoxin-sensitive sodium channels, glutamate receptors and voltage-gated calcium channels, suggesting that they are fully functional neurons (Rendt et al., 1989; Younkin et al., 1993; Squires et al., 1996). By differential trypsinization and treatment with mitotic inhibitors, neurons can be highly enriched from the cultures (Pleasure et al., 1992). This technique is also used in this study to generate pure postmitotic neuronal aggregates.

By using immunomagnetic isolation, SSEA-3 positive cells were selected from Tera2 cells and the single cell of SSEA-3 positive cells was cloned to establish new cell lines

(Przyborski, 2001). Tera2.cl.SP12 is one of these cell lines with characteristics in common with other EC cell lines such as NTERA-2: it is expressed high levels of ES cell markers such as Oct-4, SSEA-3 and TRA-1-60; it responds well to RA, 2 to 3 days exposure is sufficient to commit cells to differentiate. It is well demonstrated that these cells can differentiate into neurons and glia, and the transplantation of these cells into nude mice results in the formation of epidermal and neural tissues, suggesting that the plasticity of these cells is limited to ectoderm (Stewart et al., 2005). Therefore, Tera2.cl.SP12 provides a useful model of to study derivatives of primitive ectoderm. Tera2.cl.SP12 cells are grown as monolayer cultures without feeder cells in 10% DMEM which makes it very easy to handle the cells. The cultures respond to retinoic acid to induce neurons, making it an ideal model to study neurogenesis *in vitro*. In this study, Tera2.cl.SP12 cells were used as an *in vitro* model to study the neurogenesis along the dorso-ventral axis in embryo, and to investigate the role of Shh signalling during this process.

**Table1 Comparison of mouse and human ES, EC cells**

(Adapted from <http://stemcells.nih.gov/info/scireport/appendixA.asp>)

Marker name	Mouse ES/EC cell	Human ES cell	Human EC cell
SSEA-1	+	-	-
SSEA-3	-	+	+
SSEA-4	-	+	+
TRA-1-60	-	+	+
TRA-1-81	-	+	+
Oct-4	+	+	+
Telomerase activity	+	+	+
Feeder cell dependent	Yes	Yes	Some
Factors that aid in stem cell self-renewal	LIF and other factors that act through gp130 receptor and can substitute for feeder layer	Feeder +serum; Feeder + serum-free medium + bFGF	Serum; Feeder + serum Depend on cell line
Teratoma formation in vivo	+	+	+
Growth characteristics in vitro	Form tight, rounded multi-layer clumps; can form EB	Form flat, loose aggregates; can form EB	Aggregates or monolayer, depend on cell line

### 1.16 Aims and objectives of the study

Currently, efforts are being made to generate different neural subtypes from all varieties of stem cells. However, it is still a big challenge that the generated neurons are still functional when used for cell replacement therapy *in vivo*. At least it is not realistic at this stage. We know the most of the differentiation *in vitro* will recapitulate the developmental program *in vivo*, therefore, using stem cells as *in vitro* models to study the molecular pathways of embryonic neurogenesis will be useful for human developmental studies. The study of the signalling factors that function during the neurogenesis also provides us with insights to this process, and the availability of *in vitro* human stem cell models will facilitate this work, making it more applicable. Understanding the mechanisms of the cell fate

commitment, neural specification to functional neuron generation will enable us to guide the stem cells to differentiate into the specific neurons we need.

The main focus of this research project is to evaluate the function of the signalling molecules such as Shh, RA and BMPs to instruct the fate of human pluripotent stem cells to form specific neuron subtypes. Tera2.cl.SP12 embryonal carcinoma stem cells are a robust caricature of human embryogenesis and an accepted model of neural differentiation. It provides us a platform to test the hypotheses that the mechanisms of neural development derived from lower species are conserved in man. Specially, the project will:

- Examine the general expression profile of Tera2.cl.SP12 EC cells, especially the expression of transcription factors known to be involved in vertebrate cell fate determination during the induction of neural development.
- Examine the expression of components of Shh signalling pathway, and investigate its role in cell fate determination and its regulation by RA during the differentiation into motor neurons. Furthermore, by using siRNA technique, we gain understanding of how Gli1, the Shh signalling indicator, functions in the regulation of cell proliferation and neural development.
- Investigate the function of motor neurons differentiated from Tera2.cl.SP12 EC cells. Neuron-myoblast co-culture assay will be developed to test the ability of the motor neurons to innervate skeletal muscle myotubes and analyse the contractile events.
- Develop methods for oligodendrocytes and interneurons differentiation from Tera2.cl.SP12 EC cells.

# Chapter Two

## Materials and General Methods

### 2.4 General Methods

#### 2.4.1.1 Tera2.cl. SP12 EC cell culture

Tera2.cl.SP12 EC cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Ltd; Paisley, Scotland) supplemented with 10% fetal calf serum and 2mM L-glutamine (DMEMFG) at 37°C in 5% CO<sub>2</sub>. In preparation for differentiation, cultures of confluent Tera2.cl.sp12 were briefly treated with 0.25% trypsin (Life Technologies) / 2mM EDTA in PBS for 2-3 min to produce a suspension of single cells. Differentiation of Tera2.cl.SP12 was performed as either monolayer or as suspension aggregates.

Monolayer: suspended Tera2.cl.sp12 cells were plated at  $2 \times 10^4$  cells/cm<sup>2</sup> in DMEMFG, containing  $10^{-5}$ M RA (Sigma) and/or 25-100ng/ml Shh-N (R&D Systems). Incubation media were changed every 2-3 days.

Aggregates: Tera2.cl.SP12 EC cells suspended in DMEMFG were seeded at  $5 \times 10^5$  cells/dish into sterile 90-mm-diameter Petri dishes normally used for bacterial cultures. Suspension cultures were maintained for 24 hours before the addition of inductive factors. Induced suspension cultures were maintained for 2 weeks or as indicated during which the incubation medium was replaced every 2-3 days.

To produce neurons, 28-day RA and/or Shh treated cultures were treated with 0.25% trypsin/2mM EDTA/PBS for approximately 5 min at 37°C. The resulting cell suspension were split 1:2, transferred to fresh culture dish, and cultured without RA or N-Shh in 10% DMEM with mitotic inhibitors (1μM cytosine arabinoside, 10μM fluorodeoxyuridine, and 10 μM uridine; all from sigma). After 4-5 days, replated cells were briefly treated with 0.1% trypsin/2mM EDTA/PBS for approximately 2 minutes at room temperature and neuronal cells were dislodged by sharp lateral blows to the side of the culture flask. Dislodged cells were collected for later use.



#### **2.4.1.2 Mouse C2C12 Myoblast cell culture**

Mouse C2C12 muscle cell line (provided by Dr. Rumaisa Bashir of Durham University) were maintained in a nutrient-rich growth medium containing DMEM supplement with 10% fetal calf serum, 2mM L-glutamine, 100U/ml penicillin and 100mg/ml streptomycin at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% humidity. Cells were kept under 70% confluent. To induce differentiation, myoblasts at 70-90% confluent were switched to the myotube differentiation medium, which is DMEM supplemented with 2% horse serum, 2mM L-glutamine, 1mM sodium pyruvate, 100U/ml penicillin and 100mg/ml streptomycin. Medium is changed every other day until the myotube is formed and ready for analysis.

#### **2.4.1.3 Neuron-Myoblast Co-culture assay**

Neurons were produced from Tera2.cl.SP12 EC cells as above. Mouse C2C12 muscle cell line is maintained in DMEM supplemented with 10% fetal calf serum and 2mM L-glutamine. When set up the co-culture assay, neurons and C2C12 cells were mixed together and seeded on Laminin (10µg/ml) / poly-D-lysine (1mg/ml) coated cell culture dish or coverslips in C2C12 growth medium, allowed neurons and C2C12 cells attach the coated culture dish or coverslips overnight. Check the co-cultures were well attached on the dish and the cells reach more than 90% confluent before replace growth medium with myotube differentiation medium: 2% horse serum, 2mM L-glutamine and 1mM sodium pyruvate. The co-cultures were kept in this myotube differentiation medium for 4 or 5 days and change medium every other day until the neurons stretch out with long neurites and C2C12 cells merged to form myotubes. The co-culture show myotubes twitching when observed under the microscope. Curare (10µM, Sigma) and Atropine sulphate (10µM, Sigma) were used to block the contracting myotubes, carbachol (2µM, Sigma), Acetyl choline (2µM, Sigma), Nicotine (2µM, Sigma), Muscarine (2µM, Sigma), Pilocarpine (2µM, Sigma) were used to stimulate the potential myotubes contracting. Results were recorded by video clips.

#### **2.4.2.1 Cell counting**

Cells were washed by calcium-free phosphate-buffered saline (PBS) and then dissociated by 0.25% trypsin / 2 mM EDTA for approximately 5min at 37°C. The trypsin was neutralized by foetal calf serum. The resulting single cell suspension was counted by a Neubauer hemocytometer. A ten fold dilution was needed if the culture saturated or very dense. A clean coverslip was placed at the center of the hemocytometer, a drop of well mixed cell suspension was added at each notch of the hemocytometer to introduce cell sample. If the hemocytometer has no notches, a drop of cell mixture is placed at the centre of gridded area of the hemocytometer, cover with a coverslip on top, before count it under the microscope. Count the four corner squares on the grids on the hemocytometer, calculate the average, multiple by  $1 \times 10^4$  to determine the number of cells per millilitre.

#### **2.4.2.2 Cell proliferation assay**

Method I. Appropriate number of cells was used to set up cultures for inductive factors induced cell proliferation. Cells were grown as previous described, and cells were collected and counted on selected time points. Cell number was determined as described in Section 2.4.2.1 and the results were graphed to show the increase of the cell number on those selected time points.

##### **Method II. MTT assay**

Cell were seeded in 96-well plate in a triplicate set of wells, inductive factors were added as required, change medium as usual. The reaction was followed the user's manual (Promega) with minor modification. In brief, the cell samples in the 96-well plate were washed with PBS twice, before replaced with 100µl culture medium, 15 µl of Dye Solution was added to each well and plate was incubated at 37°C for up to 4 hours with 5% CO<sub>2</sub>. After incubation, add 100 µl of the Solubilization Solution/Stop Mix to each well. One hour after the addition of the Solubilization Solution/Stop Mix, using the multi-channel pipette to mix the content of the well to get a uniformly color solution. And care should be taken to avoid bubble formation. Record the absorbance at 570nm wavelength using a 96-well plate reader. Plot the absorbance to show the cell proliferatin trend or compare to the standard line of the cell

number and absorbance to calculate the actual cell number in each well and then plot the cell proliferation curve.

### **2.4.3 Western Blot Analysis**

#### **2.4.3.1 Preparation of the SDS-PAGE Gel**

The resolving gel (10%) was prepared by mixing 5ml distilled water with 2.5ml 1.5M Tris buffer (Section 2.3.5), 2.5ml 40% acrylamide (Section 2.3.6), 100 $\mu$ l 10% SDS buffer, 100 $\mu$ l APS (10% w/v ammonium persulphate) and 10 $\mu$ l TEMED. The polyacrylamide solution was immediately poured into a BioRad gel caster holding 2 gels, using gel plates of 10 x 8 cm and spacers of 1mm width. Analysis pure Isopropanol (100 $\mu$ l) was added over the top of each gel to straighten the level of the gel. The gels were covered with parafilm and were allowed to polymerise for 30 minutes at room temperature. Pour the stacking gel after clean the isopropanol on the top of the resolving gels, the stacking gel (4%) was prepared by mixing 2.4ml distilled water, 500 $\mu$ l 40% acrylamide, 1ml 0.5M Tris buffer, 50 $\mu$ l 10% SDS buffer, 50 $\mu$ l APS and 10 $\mu$ l TEMED. The stacking gel solution was immediately poured into the mini-slab gel above the resolving gel. A welled comb was inserted into the stacking gel. The gel was allowed to polymerise for 30 minutes after which the comb was carefully removed and the wells were washed with distilled water before use. Or gels with combs were individually wrapped in tissue and stored in 1x running buffer (Section 2.3.7) at 4°C until use.

#### **2.4.3.2 Preparation of Protein Samples and Gel Electrophoresis**

Protein samples were prepared from Tera2.cl.SP12 EC cells and their induced counterparts in protein isolation buffer (1% NP40, 50mM Tris-HCl [pH8.0], 150mM NaCl, 1mM MgCl<sub>2</sub>, and protease inhibitors). Small pellet add 50 $\mu$ l protein isolation buffer and big pellet add 100 $\mu$ l, leave on ice for 30min, vortex once or twice, then centrifuge at maximum desktop centrifuger for 4min. Protein concentration was determined using a Bradford based assay and sample volumes were adjusted appropriately (BSA was used as a standard). Prior to sample loading, 1 $\mu$ g of protein was denatured in sample loading buffer (0.5M Tris-HCl, pH6.8, 10% glycerol, 70mM lauryl sulphate, 5% 2-mercaptoethanol, 15 $\mu$ l bromophenol

blue) for 3min at 95°C. Samples were electrophoresed for 45min at 200 volts (V), until the appropriate pre-stained molecular weight marker (15 kDa) was at the bottom of the gel.

#### **2.4.3.3 Immunoblotting**

After SDS-PAGE (Section 2.4.6), the proteins from the gel were immediately transferred to PVDF membranes. A transfer cassette sandwich was constructed with the following order of components each of which had been pre-equilibrated in transfer buffer (Section 2.3.10), sponge, two sheets of blotting paper, PVDF membrane, the SDS-PAGE gel, another two sheets of blotting paper and a final piece of sponge. On addition of each component to the transfer cassette air bubbles were carefully removed by pressing each layer flat with a test tube. Proteins were transferred at a constant voltage of 100 V for 1 hours using a BioRad mini-gel transferring apparatus. Confirmation of protein transfer was achieved by staining blots with 0.2% Ponceau S (w/v) in 5% acetic acid.

Following the transfer of the proteins, the PVDF membrane was briefly rinsed with TBS (Section 2.3.12) and incubated with blocking buffer (PBS pH 7.4 containing 5% (w/v) dried milk, 0.02% (v/v) Tween-20) 15ml for 1 hour at room temperature or 4°C overnight with gentle shaking. After blocking of the non-specific antibody sites the PVDF membranes were washed with ~10ml of TBS. The appropriate affinity-purified primary antibodies (Section 3.4.2.1) were diluted in incubation buffer (PBS pH 7.4 containing 2.5% (w/v) dried milk) to working concentrations. The PVDF membranes were incubated with the diluted primary antibody solution (5ml) for 1 hour at room temperature or over night at 4°C with gentle shaking.

After incubation in primary antibody, the PVDF membranes were washed four times in wash buffer (TBS pH 7.4 with 0.2% (v/v) Tween-20) 10ml at 10 minute intervals with gentle shaking at room temperature. PVDF membranes were then incubated with horseradish peroxidase (HRP) labelled secondary antibody, depending on what the primary antibody was raised in, at a dilution of 1/1000 in incubation buffer (5ml). The membrane was incubated for an hour with gentle shaking at room temperature. The unbound secondary antibody was removed by washing the membrane as described above. Immunoreactive bands on the PVDF membranes were developed by processing in a 10ml solution containing 68mM p-coumaric acid (40µl), 1.25mM luminal (100µl) and 30% H<sub>2</sub>O<sub>2</sub>

(6 $\mu$ l) for 1 minute at room temperature. After removal of the reagents the immunoblot was wrapped in cling film and placed in a film cassette. The immunoblot was exposed to Hyperfilm™ for various lengths of time (1-5 minutes). The film was then developed in Kodak machine.

#### **2.4.3.4 Protein samples extract from Nuclei**

Prepare 5ml Buffer A (10mM HEPES, 10mM KCl, 0.1mM EDTA, 1mM DTT, 0.5mM PMSF) with 50 $\mu$ l protease inhibitors cocktail and 200 $\mu$ l IGEPAL, 0.5ml Buffer A was added to each plate and let it stand 10min at room temperature. Scrape the cells and collect in a 1.5ml tube, place on ice before centrifuging at 15, 000g for 3min at 4°C. The tube was kept on ice before discard the supernatant. Prepare 1ml Buffer B (20mM HEPES, 0.4M NaCl, 1mM EDTA, 10% Glycerol, 1mM DTT, 0.5mM PMSF) in a 1.5ml tube with 10 $\mu$ l protease inhibitors cocktail. Add 50 $\mu$ l of this buffer to each tube after discarding the supernatant. Vortex the sample every 5min for 30min, then shake the tube vigorously on a shaker in cold room overnight. Centrifuge the sample at 15, 000g for 5min at 4°C, collect the supernatant and measure its Bradford concentration before aliquot the sample. The aliquots were stored at -80°C.

#### **2.4.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)**

##### **2.4.4.1 Total RNA isolation**

Remove all the medium before add 1ml TRI reagent for 10cm<sup>2</sup> tissue culture dish, let it stand at room temperature for 10min, collect the TRI reagent mixture into a 1.5ml ependorf tube, do not allow any visible cell clusters in the tube. Shake the tube vigorously for 15 seconds after adding 200 $\mu$ l chloroform into the tube, allow it stand at room temperature for 10min before centrifuging at 12, 000g for 15min at 4°C. Aspirate out aqueous phase to a new 1.5ml ependorf tube and then add 0.5ml isopropanol, freeze the samples in -20°C for 3 hours or overnight before centrifuging at 7,500g for 10min at 4°C. Remove all the supernatant and add 1ml 75% ethanol and vortex and centrifuge at 7,500g for 10min at 4°C. Remove supernatant and air dry the pellet for 5min, reconstitute in RNase free water. The extracted total RNA samples were stored at -80°C. Make sure all the reagents and materials were RNase-free.

#### **2.4.4.2 DNA digestion**

The DNA contaminant in the Total RNA was digested by DNA-free™ (DNase Treatment and Removal Reagent) from Ambion. In detail, total RNA was dissolved in 45µl RNase free water, 5µl 10x DNase I Buffer and 1µl rDNase I (2U/ul) were added to the tube before the tube was incubated for 30min at 37°C in water bath. To stop the reaction, 2µl of DNase Inactivation Reagent was added to the tube and incubate 2min at room temperature with occasional mixing, spin down at 10,000g for 2min at 4°C. Total RNA can be used immediately or stored at -80°C until use.

#### **2.4.4.3 Reverse Transcription**

The reverse transcription reaction was carried out in a 25µl system. All the reagents were purchased from Invitrogen. The reaction was followed the user's manual with minor modification. In brief, in a 0.5ml tube, combined 50µM Oligo(dT)<sub>20</sub> primer, 2µg of RNA and dNTP mix and adjust volume to 12µl with RNase-free water. Denature RNA and primer by incubating at 65°C for 5min and then place on ice. Prior to use, Vortex the 5× cDNA synthesis buffer for 5 second. Prepare a master reaction mix on ice by mixing the 5× cDNA synthesis buffer, 0.1M DTT, 40U/µl RNaseOUT™, RNase-free water and 15U/µl AMV Reverse Transcriptase, according to the ratio of 4: 1:1:1:1 respectively. Pipette 8µl of the master reaction mix to each reaction tube on ice, mix the content gently and incubate at 45°C for 50-60min in a thermal cycler. Terminate the reaction by incubating at 85°C for 5min, add 1µl RNase H and incubate at 37°C for 20min to digest the RNA in the tube. The synthesized the cDNA can be stored at -20°C or used for PCR immediately.

#### **2.4.4.4 Polymerase Chain Reaction**

Gently mix 1µl of synthesized cDNA with 24µl of a master reaction mix, which contains 10× PCR buffer, 50mM MgCl<sub>2</sub>, 10mM dNTP mix, 10mM sense primer, 10mM antisense primer, 5U/µl Taq DNA polymerase and RNase-free water, with the ratio of 50:15:10:10:10:4:381. Heat the reaction at 94°C for 2min and then perform 30-40 cycles of PCR with optimized condition for the sample. Always do a reaction with RNA without

reverse transcription as a negative control. The target gene name, sense primer, antisense primer, annealing temperature, product size and source of primers were listed as followed:

Table 2.1 RT-PCR primers used in this study

<i>Gene</i>	Sense	Anti-sense	Annealing temperature (°C)	Product size (bp)	reference
<i>En-1</i>	GACAAGCGGCCGC GGACCGCGTTACAC	TGGTTGTACAGTC CCTGGGCCATGAG	63	233	(Roy et al., 2004)
<i>Gapdh</i>	ACCACAGTCCATGC CATCAC	TCCACCACCCTGT TGCTGTA	57	450	(Li et al., 2005)
<i>Gli-1</i>	CAGAGAATGGAGC ATCCTCC	TTCTGGCTCTTCC TGTAGCC	58	413	(Dahmane et al., 1997)
<i>Gli-2</i>	TGGCCGCTTCAGTG ACAGATGTTG	CGTTAGCCGAAT GTCAGCCGTGAAG	60	200	(Dahmane et al., 1997)
<i>Gli-3</i>	GTGGAAATGGTTGA GTCCATG	GCCCAGAGGCTC ATGTCACC	59	370	(Wild et al., 1997)
<i>Hb-9</i>	CGAGGACGACGAG GACCATT	CGGTTCTTCTCAC ACGCACTC	55	447	(Roy et al., 2004)
<i>Isl-1</i>	GCAGCATCGGCTTC AGCAAG	GTAGCAGGTCCG CAAGGTG	61	356	(Roy et al., 2004)
<i>Nkx2.2</i>	TGCCTCTCCTTCTG AACCTTGG	GCGAAATCTGCC ACCAGTTG	59.4	337	(Li et al., 2005)
<i>Oligo2</i>	AGGAGGCAGTGGC TTCAAGTC	CGCTCACCAGTCG CTTCATC	61.4	314	(Li et al., 2005)
<i>Pax-3</i>	CACCAGGCATGGAT TTTCC	TTGTCAGGAGTCC CATTACCT	60	137	(Okada et al., 2004)
<i>Pax-6</i>	CGGCAGAAGATTGT AGAG	GATGACACGCTT GGTATG	56	289&331	(Zhang et al., 2001b)
<i>Pax-7</i>	CCACAGCTTCTCCA GCTACTCTG	GGGTTGCCCAAG ATGCTC	60	113	(Okada et al., 2004)
<i>Patched-1</i>	GAATCCAGGCATCA CCCACC	CCACGTCCTGCAG CTCAATG	57	490	(Dahmane et al., 1997)

#### 2.4.5.1 Flow Cytometry

Cells were trypsinized to produce a single cell suspension as described above for flow cytometry analysis. Cells were transferred to a round bottomed 96-well plate at a density of  $5 \times 10^5$  cells per well and rinsed with wash buffer, which is PBS with 0.1% BSA and 0.1% sodium azide. Cells were subsequently incubated for 30min on ice with the primary antibody diluted in appropriate amount of wash buffer. After three washes, cells were incubated with mouse secondary antibody conjugated to FITC for a further 30min on ice

with foil protect from light. Following three washes, cells were resuspended in 500 $\mu$ l wash buffer and analysed by a Coulter XL flow cytometer.

For staining of intracellular antigens,  $1 \times 10^6$  PBS-washed cells of the single cell suspension were resuspended in 0.875ml of cold PBS, then 0.125ml of cold 2% formaldehyde solution was added and the mixture was vortexed briefly. The cell suspension was incubated for up to an hour at 4°C, before centrifuged for 5min at 250g. Cells were resuspended in cold sterilised PBS and can be stored at 4°C for several weeks before use. Or cells were gently resuspended in 1ml of room temperature PBS with 0.2% Tween 20. The cell suspension were incubated in a 37°C water bath for 15min, before centrifuging, add 1ml PBS with 0.2% Tween 20 to the tube. The supernatant was removed and the primary and secondary antibody staining procedure was preceded as above.

#### **2.4.5.2 Preparation of 2% Formaldehyde Stock Solution**

Weigh 2g paraformaldehyde powder and add to 100ml protein-free PBS, then heat the solution to 70 °C in a fume hood. Be sure that do not exceed this temperature until the paraformaldehyde goes into solution. Allow the solution to cool to room temperature before adjust it's pH to 7.4 using 0.1M NaOH or 0.1M HCl, if needed. The solution was filtered and stored at 4 °C protected from light.

#### **2.4.6 Immunocytochemistry Staining**

Cells were cultured on 16-mm glass coverslips or tissue culture plates for different time periods before fixed in 4% PFA in PBS for 30min on ice. Cells were incubated for 1hour at room temperature or in cold room overnight in PBS with 10% horse serum, 1% Triton-X-100 prior to further 1hour incubation with primary antibody. Cells were washed three times with PBS before incubating for 30min in the dark with secondary antibody conjugated to fluorescence dye. Following three washes in PBS, cells were examined under a Nikon diaphot 300 fluorescence microscope. For double staining, cells were incubated in the mixture of two primary antibodies at proper dilution in primary antibody dilution buffer (1% BSA, 0.05% sodium azide in PBS) for 1hour at room temperature or 4°C overnight. After three 2min rinses, cells were incubated in the mixture of two fluorescence conjugated secondary antibodies in PBS for 30min at room temperature and then followed by three



2min rinses in PBS with 0.1% BSA. Cells were counterstained with Hoechst 33342 for 10min at room temperature before three washes of PBS and examined under the fluorescence microscope. Cells without primary primary antibody incubation serve as negative control.

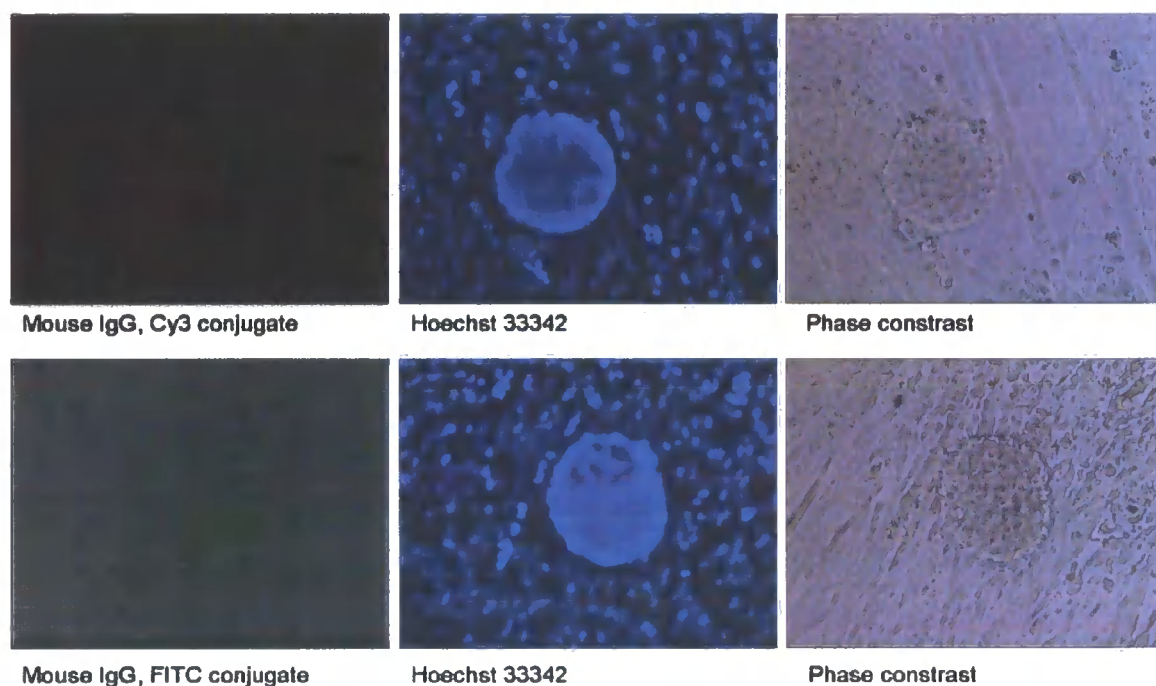


Figure 2.1 Examples of negative control. C2C12 myoblasts and differentiated neuron aggregates were mixed and differentiated in myotube differentiation medium for about 4 to 5 days before fixed. The fixed samples were stained as indicated secondary antibodies but without primary antibody incubation.

#### 2.4.7 Transfection of Tera2.cl.SP12 cells with siRNA

In a single well of 24-well plate,  $10^5$  cells were seeded in 0.5ml growth medium shortly before transfection. Cells were kept in incubator before the transfection. 37.5ng siRNA was diluted in 100 $\mu$ l culture medium without serum to give a final siRNA concentration of 5nM. 3 $\mu$ l of HiperFect Transfection Reagent was added to the diluted siRNA and mix by vortexing. Before transfection, the sample was incubated at room temperature for 7min to allow the formation of transfection complexes. Add the complexes drop by drop onto the cells before gently swirl the plate to make sure uniform distribution of the transfection

complexes. Cells were grown with transfection complexes under the normal growth conditions. And for fluorescently labeled siRNA, microscopic analysis was performed at 24h, 48h and 72h after transfection. The protein of positive control of siRNA silencing samples was collected at 72h after transfection. The inductive factors were applied to the siRNA transfected cells for different time period after 72h transfection. Before transfection, make sure all work is carried out in an RNase-free environment; if it is first time, the 5nmol lyophilized siRNA should be dissolved in 250µl siRNA Suspension Buffer and heat at 90°C for 1min then 37°C for 60min. The siRNA solution can be frozen at -20°C and freeze-thawed numerous times. The 21nt double stranded siRNA used in this study is target human Gli1 gene. The target sequence of Gli1 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. Gli1 siRNA duplex was purchased from QIAGEN, and the sequences were provided above.

## **2.1 Source of Materials**

### **2.1.1 Sigma-Aldrich Chemical Company (Poole, Dorset, UK)**

Kodak D-19 Developer

Kodak Unifix

Ampicillin

Agar

Dulbeccos modified Eagles medium

Sodium Bicarbonate

Sodium Hydroxide

Trypsin EDTA

Ethylenediaminetetracetic acid (EDTA)

DTT (Dithiothreitol)

EDTA (Ethylenediaminetetracetic acid)

Foetal calf serum.

Folin-ciocalteau phenol reagent.

Hydrogen peroxide (30% v/v)

Luminol.

N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES)

p-coumaric acid.

Pre-stained molecular weight markers (molecular weight range 205-31.5kDa)

SDS (Sodium dodecyl sulphate)

Sodium azide

Sodium hydroxide.

Sodium phosphate

Tris

Tween-20.

B-actin

B-mercaptoethanol

### **2.1.2 BDH Laboratory Supplies (Leicestershire, UK)**

TEMED (N,N,N',N'-Tetramethylethylenediamine)

Acrylamide

Ammonium persulphate

Methanol

Glycerol

Ethanol

DMSO (Dimethyl sulphoxide)

Sodium hydrogen carbonate

HCL (Hydrochloric acid)

Sodium chloride

Potassium chloride

### **2.1.3 Promega Ltd (Southampton, UK)**

DNA molecular weight markers

Cell proliferation kit

### **2.1.4 Amersham Biosciences (Aylesbury, Bucks, UK)**

Blotting paper

PDFV Hi-bond P membrane

Hyperfilm

HRP linked secondary antibody-Rabbit

HRP linked secondary antibody-Mouse

HRP linked secondary antibody-Rat

ECL and ECL-PLEX kit

**2.1.5 QIAGEN Ltd (Dorking, Surrey, UK)**

QIAGEN<sup>®</sup> RNAi Human/Mouse Starter kit

Gli-1 RNAi duplexes

## 2.1.6 Primary Antibodies

Table 2.2 antibodies from Chemicon

Antibody	Source	Antibody subtype	Dilution	Product size (kD)
Goat anti-choline acetyltransferase (ChAT)	CHEMICON, AB144P-200UL	polyclonal antibody	WB 1:1000	68-70
Rabbit anti-Hb9/HLX9	CHEMICON, AB5963	polyclonal antibody	ICC 1:400, WB 1:1000	40.6
Rabbit anti-PAX6	CHEMICON, AB5409	polyclonal antibody	ICC 1:500; WB 1:1000	50
Rabbit anti-Dlx2	CHEMICON, AB5726	polyclonal antibody	ICC 1:1000, WB 1:1000	34
Rabbit anti-glutamate decarboxylase65/67 (GAD65/67)	CHEMICON, AB1511	polyclonal antibody	ICC 1:1000, WB 1:5000	65 and 67
Mouse anti-choline acetyltransferase (ChAT)	CHEMICON, MAB305	IgG	ICC 1:100	
Mouse anti-CNPase 2',3'-cyclic nucleotide 3'-phosphodiesterase	CHEMICON, MAB326	IgG	ICC 1:500	
Mouse anti-nestin	CHEMICON, MAB353	IgG	ICC 1:200	
Mouse anti-neuron specific enolase	CHEMICON, MAB324	IgG	WB 1:1000	45
Mouse anti-keratin, epithelial	CHEMICON, MAB1612	IgG	ICC 1:400	
Mouse anti-myelin/oligodendrocyte specific protein	CHEMICON, MAB328	IgM	ICC 1:1000	
Rabbit anti-Gli1	CHEMICON, AB3444	polyclonal antibody	ICC 1:400, WB 1:5000	118
Rabbit anti-NG2 chondroitin sulphate proteoglycan	CHEMICON, AB5320	polyclonal antibody	ICC 1:200	

Table 2.3 antibodies from Abcam

Antibody	Source	Antibody subtype	Dilution	Product size (kD)
Mouse anti-Ki67 monoclonal antibody	Abcam, cat. no. ab10913,	Ms IgG1	ICC 1:50	Product size (kD)
Rabbit anti-Gli1	Abcam, cat. No. ab7523	polyclonal antibody	ICC 1: 300, WB 1: 5000	Product size (kD)
Rabbit anti-cytokeratin 8	Abcam, ab15465	polyclonal antibody	ICC 1:100	Product size (kD)
Rabbit anti-synapsin I	Calbiochem, cat. No. 574778	polyclonal antibody	ICC 1:500	Product size (kD)
Neuronal class III $\beta$ -Tubulin	COVANCE, catalog number: PRB-435P	polyclonal antibody	ICC 1:2000, WB 1:5000	50kD

Table 2.4 antibodies from Sigma

Antibody	Source	Antibody subtype	Dilution	Product size (kD)
anti-neurofilament 160 clone RMO44	SIGMA, N 2787	IgG	WB 1:1000	160
anti- $\beta$ -tubulin isotype III clone SDL.3D.10	SIGMA, T 8660	IgG	ICC 1:500, WB 1:1000	46
anti-neurofilament 68 clone NR4	SIGMA, N 5139	IgG	ICC 1:400	68
anti-MAP2 clone HM-2	SIGMA, M 9942	IgG	ICC 1:400	
anti-synaptophysin clone SVP-38	SIGMA, S 5768	IgG	ICC 1:400	
anti-cytokeratin peptide 8 clone M20	SIGMA, C 5301	IgG	ICC 1:200	
anti-tyrosine hydroxylase clone TH-2	SIGMA, T 1299	IgG	ICC 1:1000, WB 1:5000	60-68
anti- $\alpha$ -fetoprotein (AFP) clone C3	SIGMA, A 8452	IgG	ICC 1:200	
anti- $\gamma$ -aminobutyric acid (GABA) clone GB-69	SIGMA, A 0310	IgG	ICC 1:50	
anti- $\beta$ -actin clone AB-15	SIGMA, A 5441	Ms IgG	WB 1:5000	
anti-galactocerebroside	SIGMA, G 9152	polyclonal	ICC 1:50	
Anti-skeletal myosin	SIGMA, M 7523	Rb polyclonal,	ICC 1:20	

Table 2.5 antibodies from R&D system

Antibody	Source	Antibody subtype	Dilution
Monoclonal Anti-oligodendrocyte marker O4 antibody clone O4	R&D SYSTEM, MAB1326	Ms IgM	ICC 1:1000
Monoclonal anti-human/mouse/rat/chicken Pax7 antibody clone PAX7	R&D SYSTEM, MAB1675	Ms IgG1	ICC 1:1000
Monoclonal anti-human/mouse Oct-3/4 antibody clone 240408	R&D SYSTEM, MAB1759	rat IgG2b	WB 1-2 $\mu$ g/ml
Monoclonal anti-human/mouse Shh N-terminal peptide antibody clone 171018	R&D SYSTEM	rat IgG2a	ICC 1-3 $\mu$ g/ml, WB 1-2 $\mu$ g/ml

**Table 2.6 antibodies from Santa Cruz biotechnology**

Antibody	Source	Antibody subtype	Dilution	Product size (kD)
Goat anti-NCAM (C-20)	SANTA CRUZ BIOTECHNOLOGY, INC. cat. No. sc-1507	polyclonal antibody	ICC 1:200, WB 1:1000	140-250kD
Mouse anti-Smad1 (A-4)	SANTA CRUZ BIOTECHNOLOGY, INC., cat. No. sc-7965	monoclonal antibody	ICC 1:200, WB 1:1000	52-56kD
Goat anti-p-Smad1 (Ser 463/Ser 465)	SANTA CRUZ BIOTECHNOLOGY, INC., CAT. NO. sc-12353	polyclonal antibody	ICC 1:200, WB 1:1000	52-56kD
Goat anti-NeuroD (N-19)	SANTA CRUZ BIOTECHNOLOGY, INC., CAT. NO. sc-1084	polyclonal antibody	ICC 1:200, WB 1:1000	50kD
Mouse anti-p-ERK (E-4)	SANTA CRUZ BIOTECHNOLOGY, INC., CAT. NO. sc-7383	monoclonal antibody	WB 1:1000	42 and 44
Goat anti-ERK-1 (C-16)	SANTA CRUZ BIOTECHNOLOGY, INC., CAT. NO. sc-93	polyclonal antibody	WB 1:1000	
Goat anti-Neurogenin 2 (C-16)	SANTA CRUZ BIOTECHNOLOGY, INC., CAT. NO. sc-19233	polyclonal antibody	WB 1:1000	

**Table 2.7 antibodies from DSHB**

Antibody	Source	Antibody subtype	Dilution
Islet-1 clone 40.2D6	Developmental Studies Hybridoma Bank	Ms IgG1	ICC 1:50
Lim3 clone 67.4E12	Developmental Studies Hybridoma Bank	Ms IgG1	ICC 1:50
TRA-1-60	Developmental Studies Hybridoma Bank	Ms IgG	ICC 1:100
SSEA-3	Developmental Studies Hybridoma Bank	Ms IgG	ICC 1:100
A2B5	Developmental Studies Hybridoma Bank	Ms IgG	ICC 1:100

### 2.1.7 Secondary Antibodies

Table 2.8 secondary antibodies

Antibody	Source	Dilution
Anti-goat IgG (whole molecule) FITC conjugate	SIGMA, F 7367	1:400
Anti-rabbit IgG (whole molecule) FITC conjugate	SIGMA, F 7512	1: 160
Anti-mouse IgG (whole molecule) FITC conjugate	SIGMA, F2012	1:400
Anti-mouse IgG (whole molecule) TRITC conjugate	SIGMA, T5393	1:400
Anti-mouse IgG (H+L chain) Allophycocyanin-conjugated	Jackson ImmunoResearch, 715-136-151	1:400

### 2.1.8 Chemicals and recombinant proteins

Cyclopamine, *V. californicum* (Calbiochem, catalog number: 239803, stock solution in DMSO 10mg/ml)

SANT-1 (TOCRIS, catalog number: 1974, solubility: DMSO to 50mM)

Hoechst 33342 (Molecular Probes, H-3570, 10mg/ml)

A-Bungarotoxin (Molecular Probes)

Acetyl choline chloride (SIGMA, A-2661-25G)

Carbamoylcholine chloride (SIGMA, C4382-1G)

Atropine sulphate salt monohydrate (SIGMA, 11330-5G)

(+)-Tubocurarine chloride hydrate (SIGMA, T2379-100MG)

Nicotine (SIGMA, N0267-100MG)

Muscarine chloride (SIGMA, M104-5MG)

Pilocarpine hydrochloride (SIGMA, P6503-5G)

N-Acetyl-L-cysteine (SIGMA, A9165-5G)

Forskolin (SIGMA, F-6886-10MG)

Progesterone (SIGMA, P-8783-5G)

Putrescine (SIGMA, P-5780-5G)

Sodium azide (SIGMA, S-2002-100G)

Laminin from engelbreth-holm-swarm murine sarcoma basement membrane (SIGMA, L-2020-1MG, 1mg/ml in Tris buffered NaCl, cell culture tested)



Collagen (SIGMA,

Recombinant human Sonic Hedgehog, amino terminal peptide (R&D SYSTEM, CAT NO. 1314-SH/CF)

Recombinant human BMP-2 (E.coli-expressed) (R&D SYSTEM, CAT NO. 355-BEC, reconstitution in 4mM HCl containing 0.1% human BSA, and stock solution no less than 10µg/ml)

Recombinant mouse Chordin (R&D SYSTEM, catalog number: 758-CN/CF, reconstitution in sterile PBS with 0.1% BSA, stock solution no less than 100µg/ml)

Recombinant human BMP-4 (R&D SYSTEM, catalog number: 314-BP/CF, reconstitution in sterile 4mM HCl with 0.1% BSA, stock solution no less than 100µg/ml)

Recombinant human BMP-6 (R&D SYSTEM, catalog number: 507-BP/CF)

Recombinant human BMP-7 (R&D SYSTEM, catalog number: 354-BP/CF, reconstitution in 4mM HCl with 0.1% BSA, stock solution no less than 100µg/ml)

Human Ciliary Neurotrophic Factor (rHu CNTF) (First Link (UK) Ltd. Catalog number: 65-05-206)

Human Epidermal Growth Factor (rHu EGF) (First Link (UK) Ltd. Catalog number: 62-00-206)

Human Neurotrophic Factor-3 (rHu NT-3) (First Link (UK) Ltd. Catalog number: 65-20-206)

Human Platelet Derived Growth Factor-AA (rHu PDGF-AA) (First Link (UK) Ltd. Catalog number: 62-61-206)

Human Fibroblast Growth Factor-basic (rHu FGF-basic) (First Link (UK) Ltd. Catalog number: 62-04-209)

Recombinant human insulin (SIGMA, 12643-250MG, solubility: 10mg at 0.5ml 0.01M HCl)

## **2.2 Instruments and Equipments**

**Spectrophotometry:** Heλios β ThermoSpectronic

**Centrifuges:** eppendorf centrifuge 5810R, Beckmann Avanti 30 Centrifuge

**Incubators:** SANYO CO<sub>2</sub> Incubators

**Orbital shaker:** Stuart Scientific

**Heating Block:** Grant

**Magnetic Stirrer:** Bibby Sterilin, Heated stirrer FALC.

**Water bath:** Grant

**pH meter:** HI9321 Microprocessor pH Meter, Hanna Instruments

**Balances:** CP423S, Sartorius

**Electrophoresis equipment:** Pharmacia, Amersham Biosciences

**Microscopes:** Nikon Eclipse TS100 microscope

**Photography:** Nikon digital camera DXM1200

**PCR machine:** Mastercycler gradient, eppendorf

**Flow cytometer:** Coulter XL

**Other equipment:** Scanner for films, Power packs Amersham Biosciences. Vortexer  
Scientific Laboratory Supplies

**Glassware, plastics and disposables:** Bibby Sterilin, Greiner bio-one Cellstar, Sarstedt

## 2.3 Preparation of Standard Solutions

### **Stacking gel buffer:**

0.5M Tris-glycine, pH 6.8, containing 8mM EDTA and 0.4% (w/v) SDS.

### **Resolving gel buffer:**

50mM Tris, 384mM glycine, 1.8mM EDTA and 0.1% (w/v) SDS, pH 8.8.

### **Stock acrylamide:**

30% (v/v) Acrylamide and N.N'-methylenebisacrylamide..

### **Sample buffer:**

30mM Sodium hydrogen phosphate, 30% (v/v) glycerol, 0.05% (v/v) bromophenol blue and 7.5% (w/v) SDS, pH 7.0. (DTT added later)

### **Pre-stained molecular weight markers:**

Pre-stained standards (protein molecular weight range 205-31.5 kDa, Sigma) stored in sample buffer, see above.

### **Transfer buffer:**

25mM Tris, 192mM glycine and 20% (v/v) methanol, pH 8.4.

### **Phosphate buffered saline (PBS):**

4mM Sodium hydrogen phosphate, 1.7mM potassium hydrogen phosphate, 137mM sodium chloride, 107mM potassium chloride, pH 7.4.

**Tris buffered saline (TBS):**

50mM Tris-HCL, 0.9% sodium chloride, pH 7.4.

**TE Buffer:**

10mM Tris pH 8.0 containing 1mM EDTA

**HBS buffer (Hepes buffered saline):**

280mM Sodium chloride and 1M sodium hydrogen phosphate pH 7.12.

**Buffer A:**

10mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA

Add just before use: 1mM DTT, 0.5 mM PMSF, 5 ul of 10 ug/ul of aprotinin, leupeptin, and pepstatin A to 5 ml of buffer.

**Buffer B:**

20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% Glycerol

Add just before use: DTT, PMSF, aprotinin, leupeptin, pepstatin A as above.

## Chapter Three

### General Activity of RA and Shh on Tera2.cl.SP12 EC stem cells

#### 3.1 Introduction

Stem cells are cells that have two unique properties, the capacity for long term self-renewal without senescence and the potential to differentiate into at least one specialized cell types (Rippon and Bishop, 2004). The embryo produces many transient stem cells to provide sufficient specialized cells for the development of all tissue and organs during embryogenesis. EC cells, the first established pluripotent stem cell lines, derived from teratocarcinomas. Some EC cells are capable of continuous expansion in culture and can be induced to differentiate into cells of three germ layers. Because of their cancer derived nature, EC cells are not suitable for medical uses; however, they are proven to be a useful model system in the lab.

Although knowledge from the developmental biology reveals that the mechanism underlying development are conserved from lower system to higher systems, it also emphasizes that each species has unique features and that human development, presently the most complicated system, differs greatly from other vertebrate models like mouse and chick. The *in vivo* model of human embryogenesis is relatively inaccessible to experimental analysis and manipulation due to ethnical and moral reasons, hence *in vitro* cell model as an alternative system will provide useful information.

Human ES cells were first isolated by Bongso et al in 1994, and later in 1998, the isolated cells were established as a cell line and maintained undifferentiated until present (Bongso et al., 1994; Thomson et al., 1998). The ES cells are characterised by the expression of cell surface markers SSEA-3, SSEA-4 and TRA-1-60, formation of teratocarcinoma upon transplantation, high telomerase activity and the capability to differentiate into derivatives of all three germ layers. These cells were first grown on fibroblast feeder layer in specific ES cell growth medium, and they are expensive to maintain in the laboratory. In contrast to this, human EC cells can be maintained conveniently with 10% DMEM.

Tera2.cl.SP12 EC cells are derived from parent Tera2 lineage. There is evidence to suggest that Tera2 cell line can only produce ectodermal cell types like neural cells, epidermal cells and neural crest derived-cells (Andrews, 1984; Andrews et al., 1984; Andrews et al., 1985; Przyborski et al., 1998; Przyborski et al., 2000; Horrocks et al., 2003; Stewart et al., 2003; Przyborski et al., 2004). These data indicate that Tera2-derived cultures may represent stem cells of the primitive ectoderm and thus provide a useful model system to study the molecular mechanisms involved in cell fate determination in human embryonic ectoderm (Stewart et al., 2005). RA, Shh and BMPs are natural inductive factors involved in neurogenesis in embryonic neural tube formation, which is derived from ectoderm; accordingly, Tera2.cl.SP12 EC cells would provide an appropriate platform to study the function of these inductive factors during neurogenesis.

As a morphogen in neural tube, RA is secreted from mesodermal somites and exerts potent effects on growth, differentiation, embryonic patterning and axis formation. The biological effects of retinoids are mediated by two nuclear receptors: the retinoic acid receptors (RARs) and the retinoid-X receptors (RXRs). Each group has  $\alpha$ -,  $\beta$ -,  $\gamma$ - three subtypes, as a consequence of alternative splicing and dual promoter usage and share the common C-terminal sequences (Luo and Ross, 2006). Evidence has shown that Tera2 cells express

RAR $\alpha$ , RAR $\gamma$  and RXR $\alpha$ , but not RAR $\beta$ , RXR $\beta$ , or RXR $\gamma$  at the total cellular Northern blot (Moasser et al., 1995). RA treatment results in the induction of RAR $\beta$ , as well as the up-regulation of RAR $\alpha$  and RAR $\beta$  expression within 24h of treatment (Moasser et al., 1996). These RA receptors act as inducible transcription factors, after activation by RA, they associate with retinoic acid response elements (RAREs) to regulate RA target genes. Like other patterning transcription factors, RA receptors also have specific tissue expression patterns and are thought to contribute to tissue-specific functions and developmental programs (Ruberte et al., 1993). For example, astrocytes and oligodendrocytes are formed in the presence of activated RAR $\alpha$ , whereas motor neurons are formed when RAR $\beta$  is activated, furthermore, the RAR $\alpha$  signalling is one of the final step required for a mature motor neuron phenotype (Goncalves et al., 2005b).

It is known that RA induces EC stem cell differentiation (Przyborski et al., 2000), but the precise mechanisms of how RA affects neural differentiation of EC cells are still unclear. Further studies need to be done on which molecular pathways that activated by RA during the neurogenesis. Since Tera2.cl.SP12 EC cells provide us an *in vitro* embryonic ectodermal model, we can use it to study RA signalling in neurogenesis along the DV axis. Shh is one of the neural tube morphogens secreted from notochord and the ventral floor plate, and is the only hedgehog family member that is reported to be expressed in the normal CNS (Echelard et al., 1993). Shh induces the proliferation activity in neural precursors and in other tissues, however, in the neural tube, Shh shows a spatially restricted pattern of expression in cells located at the ventral midline, yet governs the differentiation of diverse cell types throughout the ventral half of the neural tube (Patten and Placzek, 2000). In contrast to RA which exerts its effect through nuclear receptors, Shh binds to its receptor complex on the membrane of target cells. The 12-pass trans-membrane protein Ptc1 is the binding site of the receptor complex; it inhibits a 7-pass trans-membrane protein

Smo, which is the initial signalling activator of the Shh receptor complex. The effects of Shh are mediated by zinc-finger family transcription factors Gli-1, -2 and -3, all these three Gli proteins are expressed in the developing neural tube, and recent studies indicated that Shh signalling is transduced, without amplification, into a gradient of Gli activity that orchestrates patterning of the ventral neural tube (Stamatakis et al., 2005).

There are few reports about the Shh pathway in embryonal carcinoma stem cells, except that it is demonstrated that Shh-Gli pathway contributed to the tumor formation and cell proliferation. In ES cells, Shh is involved in the differentiation into neuroectoderm (Maye et al., 2004), specification and differentiation of motor neurons (Maye et al., 2004), serotonergic neurons (Goridis and Rohrer, 2002; Alenina et al., 2006), dopaminergic neurons (Ahn et al., 2004), peripheral neurons and floor plate cells (Mizuseki et al., 2003). Because of the similarity between the EC cells and ES cells, we believe EC cells also have the ability to respond to Shh, therefore, we propose it is possible to modulate the cell proliferation, cell fate commitment and differentiation in human EC cells.

In our study, we show that Tera2.cl.SP12 EC cells expressed the Shh pathway receptor component Ptcl and mediator Gli genes by RT-PCR and that the expression level of these genes are inducible by Shh and the Shh pathway regulators. In the channel  $\mu$ -slide assay, Gli1 showed a corresponding expression level to Shh gradient concentration, suggesting the Shh-Gli pathway is conserved in this human EC cell line, and therefore, Tera2.cl.SP12 EC cells can be used as a tool to investigate the regulation of cell fate determination during the early embryogenesis by Shh.

The interaction of RA and Shh plays important role in ventral neurogenesis along the DV axis, especially the specification of motor neuron progenitors, however, the transcription factor network that governs this process can only be deduced from lower species *in vivo* models. Our studies summarise a series of transcription factors activated by RA and Shh

interaction that leads to motor neuron progenitor generation, this will provide useful information about how to direct human stem cells to differentiate into specific neural cell types and how to develop methods to generate them efficiently.

## 3.2 Methods

### 3.2.1 Tera2.cl.SP12 EC cells induction and the channel $\mu$ -slide assay

Tera2.cl.SP12 EC cells were induced by RA and/or Shh or BMPs as described in Chapter 2. The induced time points were shown in the text. The concentration of inductive factors is shown as below: RA 10 $\mu$ M, Shh 25ng/ml, BMPs 10ng/ml, otherwise indicated in the text.

The 6 channel  $\mu$ -slides were provided by Ibidi Integrated BioDiagnostics. Cells were prepared at 4x10<sup>4</sup> cells/ml with or without RA and 30 $\mu$ l of the cell mixtures were first seeded in each  $\mu$  channel, and then 100 $\mu$ l of 25ng/ml Shh and 10ng/ml BMPs were added to the column of the  $\mu$  channel respectively. Cells in the channel  $\mu$ -slide were allowed to grow for 3 days, 5 days and 7 days before fixed and analysed by ICC. Images were taken at BMPs end, Shh end and at the middle of the channel respectively.

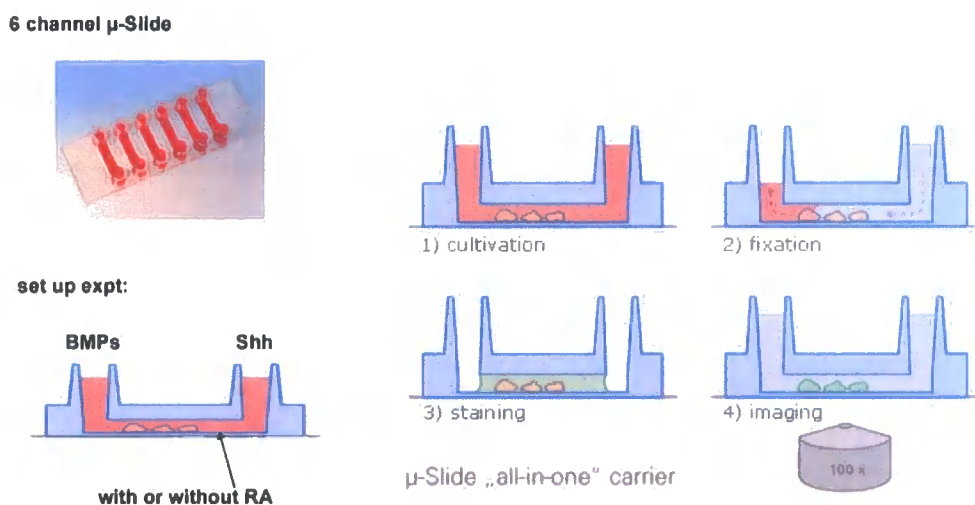


Figure 3.2A schematic showing of the channel  $\mu$ -slide assay. The 6 channel  $\mu$ -slide as shown above, the capacity of the channel is 30 $\mu$ l, and the capacity of each column is 100 $\mu$ l.



### **3.2.2 Immunocytochemistry (ICC) staining**

The fixed cultures were washed with PBS before applying to immunocytochemical staining. Primary antibodies used in this study included  $\beta$  III tubulin (Covance 1:1000), Dlx2 (Chemicon, 1:1000), Pax6 (Chemicon, 1:500), Pax7 (R&D system, 1:1000), nestin (Chemicon, 1:200), SSEA-3, TRA-1-60, A2B5 TRA-1-60 (Developmental Studies Hybridoma Bank, 1:100), Ki-67 (Abcam, 1:50), ChAT(Chemicon, 1:400), HB-9 (Chemicon, 1:400). Images were collected using a Nikon fluorescence microscope or a confocal microscope (Zeiss LSM 510).

### **3.2.3 Flow cytometry**

The procedure for cell surface marker and intracellular marker staining is described in Chapter2. The primary antibodies in this study: SSEA-3, TRA-1-60, A2B5 TRA-1-60 (Developmental Studies Hybridoma Bank, 1:100), nestin (chemicon, 1:200),  $\beta$  III tubulin (Covance 1:1000).

### **3.2.4 RT-PCR**

The procedure and primers are described in Chapter2. Before analysis, the manipulation of cells like the induced time and factors are shown in the text.

### 3.3 Results

#### **3.3.1 The role of RA in Tera2.cl.SP12 EC cells differentiation**

Tera2.cl.SP12 EC cells are pluripotent progenitor cells with characteristic cell surface markers that are present in embryonic stem cells, for example, over 98% of EC cells are TRA-1-60 positive, 75% SSEA-3 positive and less than 3% of EC cells are A2B5 positive (Figure3.1A). Tera2.sp12 EC cells are very sensitive to RA induction, 24hours treatment with RA results in approximately 20% of EC cells losing stem cells marker SSEA-3, and after 3days RA treatment, only 15% of EC cells remain SSEA-3 positive and about 8% of cells are A2B5 positive when examined by flow cytometry (Figure3.1A). Furthermore, the induced cells also accumulated the lineage specific intracellular marker when extended the exposure time to RA. For example, neuron specific enolase (NSE) is an enzyme expressed primarily in neurons and serves as a general neuronal marker. The result from western blot confirmed that induced EC cells show a time-dependent increase of NSE expression when extended the exposure time to RA, and the addition of Shh further increased the NSE expression from day1 compared to RA induction (Figure3.1B). Examination the Shh pathway signalling mediator Gli1 and BMP pathway signalling mediator Smad-1 in 3 days and 1 week RA induced cells, showed no significant change in the protein level of these signalling pathway mediators suggesting that RA regulates EC cells by its own signalling pathway, but RA may have cross talk in regulating target genes between the two factors.

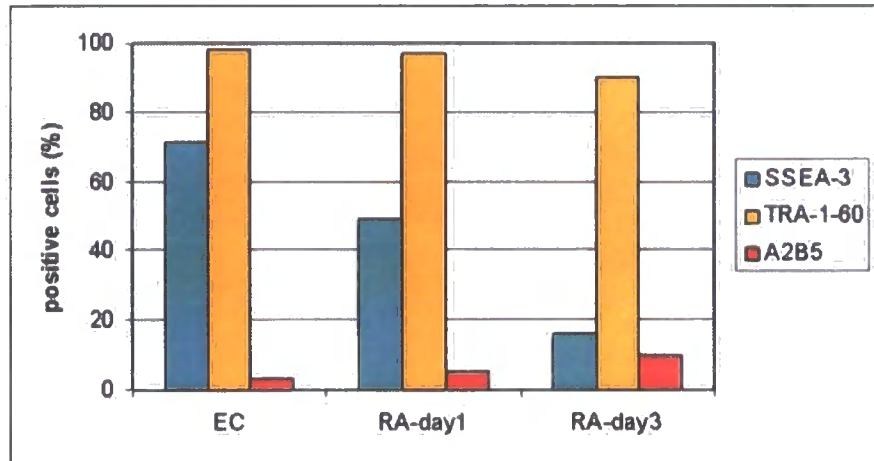
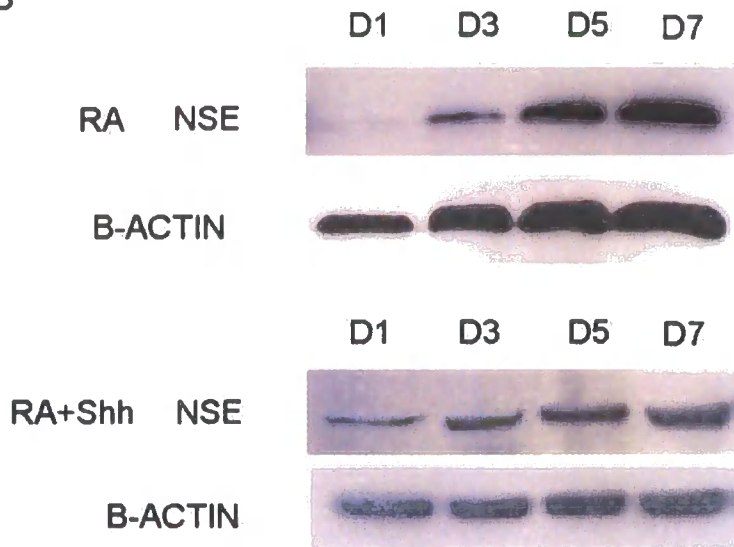
**A****B**

Figure 3.1 RA induces differentiation of EC cells. A) RA induces a reduction of stem cell surface markers detected by flow cytometer; EC cells were induced by RA (10 $\mu$ M) for 1 day and 3 days before collected and analysed the stem cell surface markers SSEA-3 and TRA-1-60, and differentiation marker A2B5 by flow cytometry. B) RA induces the neuronal marker NSE expression in a time-dependent manner as shown by western blot analysis. EC cells treated with RA from day1 to day7 and collected cytoplasmic protein for NSE analysis. The detected NSE band is about 45KD, and  $\beta$ -actin is 45KD.

### **3.3.2 The channel $\mu$ -slide assay of Tera2.cl.SP12 EC cells**

We used the  $\mu$  shape channel slide from Ibidi Company to investigate the effects of Shh and BMPs on Tera2.cl.SP12 EC cells with or without RA present. The cell growing channel was first filled with 30 $\mu$ l growth medium with EC cells with or without 10 $\mu$ M RA, then one column of channel is filled with 25ng/ml Shh and the other with 10ng/ml BMP-4, -6 and -7, so that a gradient is established toward the opposite end at each column of the channel. At 3 days time, EC cells were examined in the channels. The number of cells at the Shh end and BMPs end of the channel was not equivalent. More cells were present at Shh end. Furthermore, the morphology of cells also showed some differences, cells at Shh end still possessed an EC cell morphology, whereas cell at the BMP end appeared more morphologically differentiated. Induced cells were subsequently examined by immunostaining of nestin and Pax-6. Images were taken from the adjacent area at both ends and the midline of the channels. For nestin, the neural progenitor marker, no significant change of the staining intensity was seen at either ends or at the midline (Figure 3.2.3). However, cells showed different staining patterns at the BMP end and a 'rounder' staining pattern at the Shh end. For Pax6, a neural patterning transcription factor, the staining was confined from the midline to Shh end, while the staining at the BMPs end was much weaker. This somewhat mimics *in vivo* expression of Pax6 which is a ventral patterning factor (Figure 3.2.4). With RA treatment, Pax6 is more confined to the Shh end. For dorsal transcription factor Pax7, it is weakly expressed, and RA did not show predominant effect in regulating Pax7 expression (Figure 3.2.5). We then examined the effect of Shh on Shh staining. Shh staining was also confined from the midline to Shh end (Figure 3.2.2). Consistent with this, one of the Shh signalling mediators, Gli1 showed stronger staining from the Shh end to the midline of the channel (Figure 3.2.1). We detected BMP signalling mediator Smad-1 but

not its activated form p-Smad-1. Smad-1 was not expressed at the BMP end but was at the Shh end (data not shown).

At the 5 day time point, we again examined the cells in the channels. The cell number at the BMPs end was significantly less than the Shh end, also the morphology of cells was more differentiated than cells at the Shh end. In the presence of BMP and Shh but without RA, cells lost proliferation ability, cells were also more differentiated (Figure 3.2.6-- 3.2.10). The nestin staining at the Shh end was intense with the RA treated cells, and cells acquired a spindle shape compared to the cells at the BMPs end, suggesting that RA and Shh play important role in neural differentiation, while the co-ordination of RA and BMPs promote nestin cells to differentiate and reduce the nestin expression (Figure 3.2.10). Exposure to RA also maintained Shh (figure 3.2.9) and Gli1 (figure 3.2.8) and Pax6 (figure 3.2.6) staining confined at the Shh end compared to non-treated cells, suggesting that RA has important role in regulating patterning transcription factors.

Shh, BMPs and RA are the natural inductive factors in neural tube. In the neural tube, Shh and BMPs work in antagonizing manner, with Shh secreted from the notochord and ventral floor plate producing a gradient towards dorsal neural tube, and BMPs secreted by ectoderm and roof plate producing an opposite gradient toward ventral neural tube. Therefore, the channel slide assay can be used to model this *in vitro* and study the expression of patterning transcription factors. Considering that most of the transcriptional profiles are from chick and mouse embryo, the EC cell model provided some useful information about how these factors regulate the expression of human neural tube patterning genes.

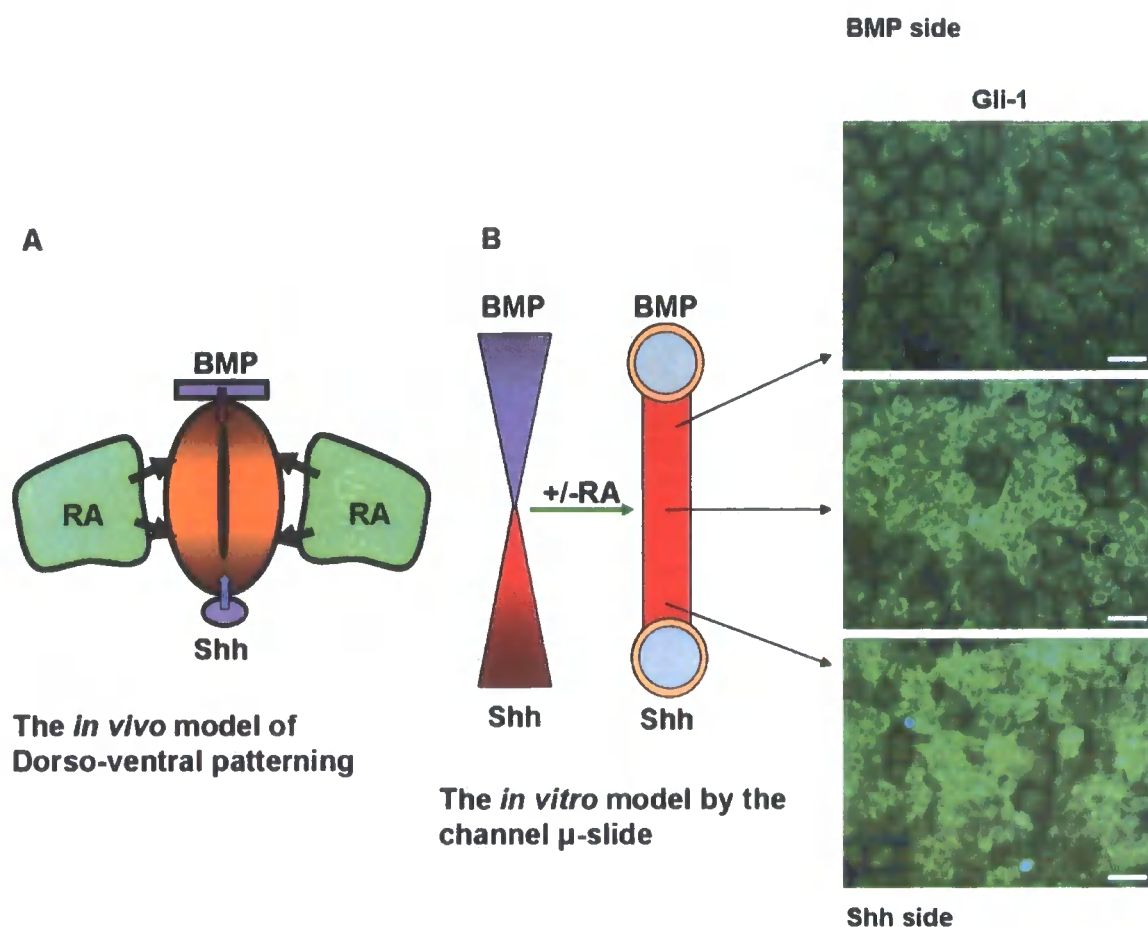


Figure 3.2 Schematic showing the model of Dorso-ventral axis using the channel  $\mu$ -slide. A, Schematic showing the *in vivo* model of dorsoventral patterning. B, Schematic showing the *in vitro* model using the channel  $\mu$ -slide. Graded BMP and Shh were established from the column of the channel after cells were seeded in the channel supplemented with or without RA. Images were taken at the position in the channel marked by the start of the long black arrows. Cells were fixed with 4% PFA and immuno-labelled with antibodies specific to neural progenitor marker Nestin and the Dorso-ventral patterning transcription factors such as Pax6, Pax7, N-Shh and Gli1. Scale bars = 50 $\mu$ m.

**BMP side**

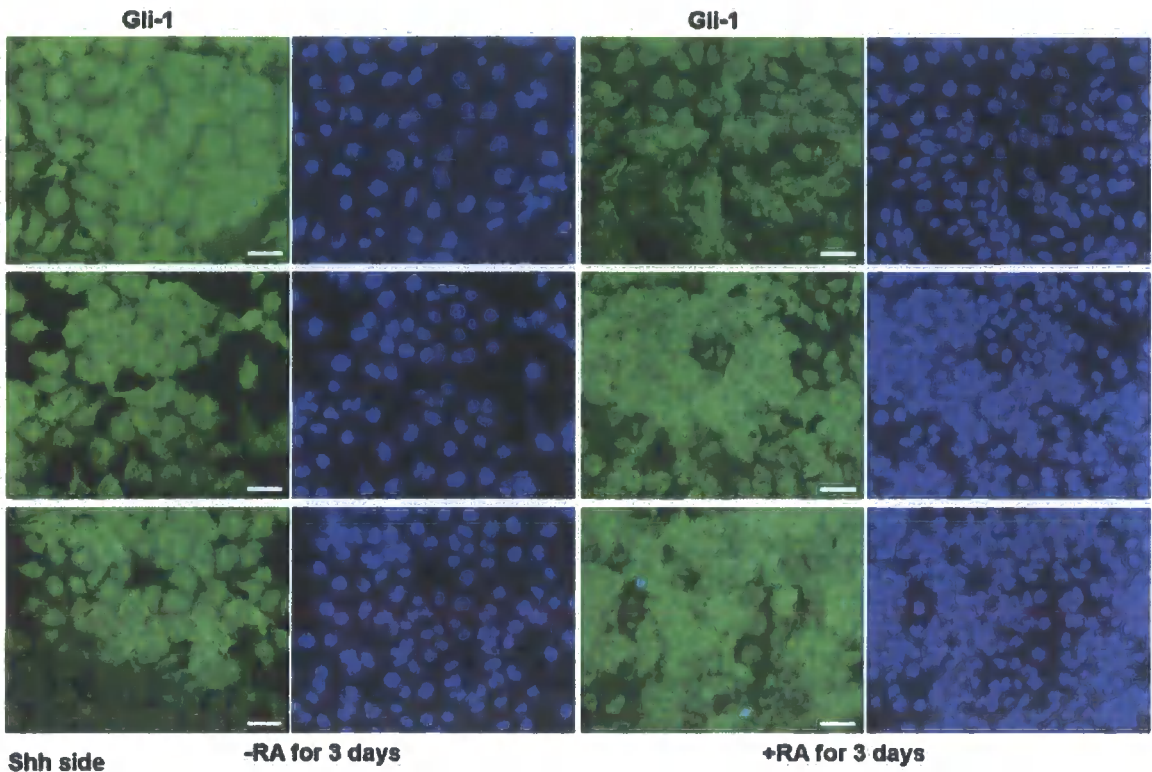


Figure 3.2.1 Gli1 staining in the model of Dorso-ventral axis using the channel  $\mu$ -slide. EC cells were seeded in the channel at 30,000 cells/channel supplemented with or without RA ( $10\mu\text{M}$ ) in  $30\mu\text{l}$  growth medium before  $100\mu\text{l}$  Shh ( $25\text{ng/ml}$ ) or BMPs ( $10\text{ng/ml}$ ) were added to the column of the channel to setup the gradient concentration respectively. EC cells were maintained for 3 days prior to fixation with 4% PFA and labelling with antibodies specific to Gli1. The nuclei were counterstained with hoechst. Images were taken at the BMP side, Shh side and at the middle of the channel. Note that Gli1 staining is stronger at the Shh side in the presence of RA. Scale bars =  $50\mu\text{m}$ .

**BMP side**

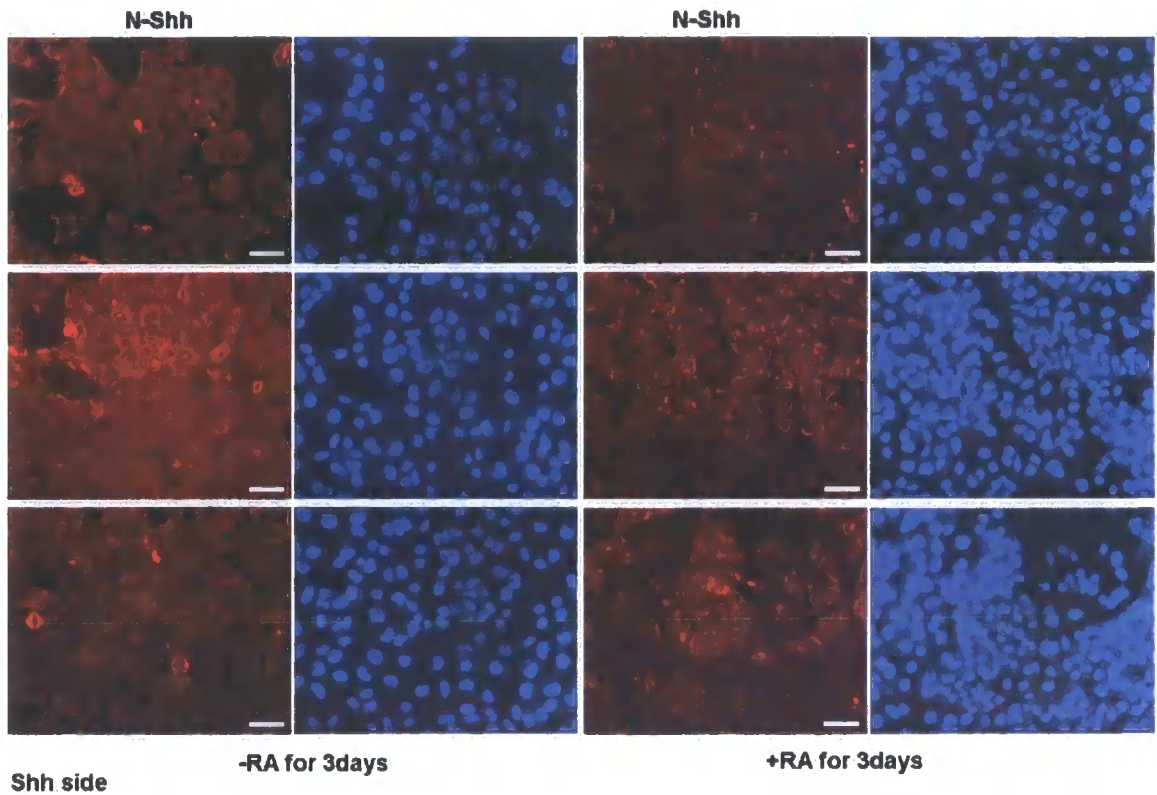


Figure 3.2.2 N-Shh staining in the model of Dorso-ventral axis using the channel  $\mu$ -slide. EC cells were seeded in the channel at 30,000 cells/channel supplemented with or without RA (10 $\mu$ M) in 30 $\mu$ l growth medium before 100 $\mu$ l Shh (25ng/ml) or BMPs (10ng/ml) were added to the column of the channel to setup the gradient concentration respectively. EC cells were maintained for 3 days prior to fixation with 4% PFA and labelling with antibodies specific to N-Shh. The nuclei were counterstained with hoechst. Images were taken at the BMP side, Shh side and at the middle of the channel. Note that N-Shh staining is stronger at the Shh side in the presence of RA. In the hoechst staining, more nuclei were observed at the Shh side compare to the BMP side. Scale bars = 50 $\mu$ m.



**BMP side**

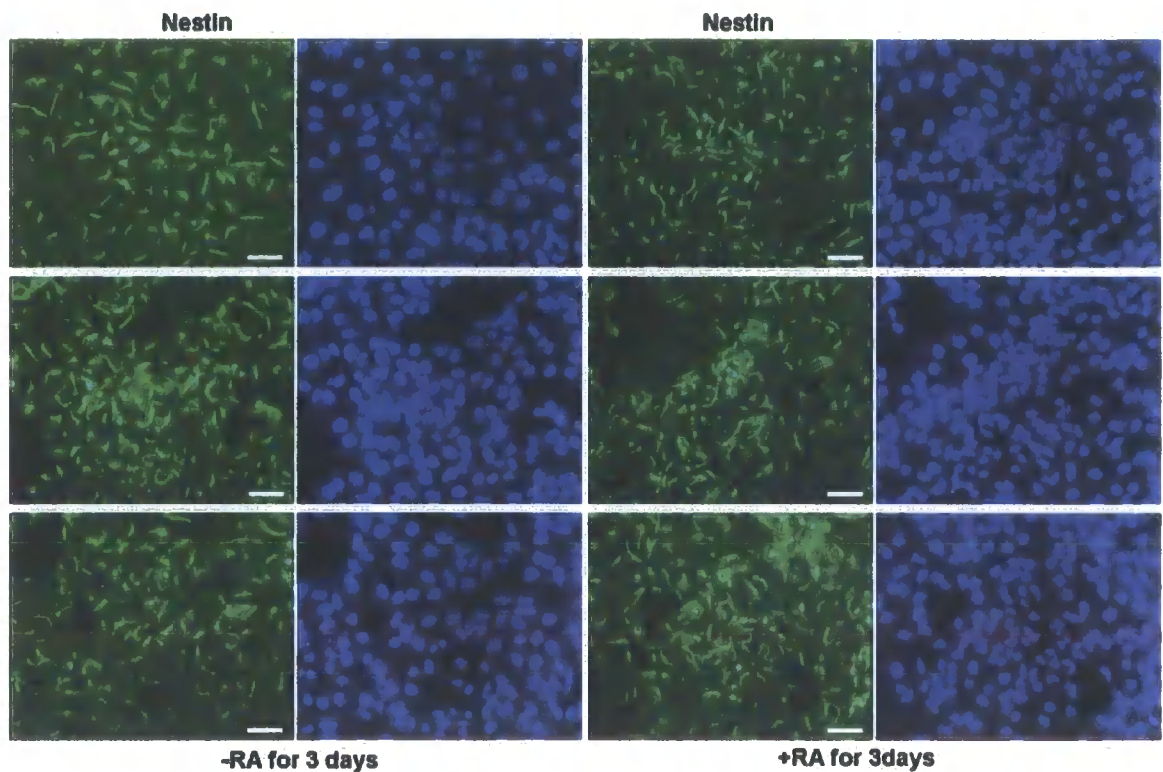


Figure 3.2.3 Nestin staining in the model of Dorso-ventral axis using the channel  $\mu$ -slide. EC cells were seeded in the channel at 30,000 cells/channel supplemented with or without RA (10 $\mu$ M) in 30 $\mu$ l growth medium before 100 $\mu$ l Shh (25ng/ml) or BMPs (10ng/ml) were added to the column of the channel to setup the gradient concentration respectively. EC cells were maintained for 3 days prior to fixation with 4% PFA and labelling with antibodies specific to Nestin. The nuclei were counterstained with hoechst. Images were taken at the BMP side, Shh side and at the middle of the channel. Note that Nestin staining is equal at the both sides in the absence or presence of RA, while cell morphology at the Shh side is 'rounder' compare to the BMP side. Scale bars = 50 $\mu$ m.

**BMP side**

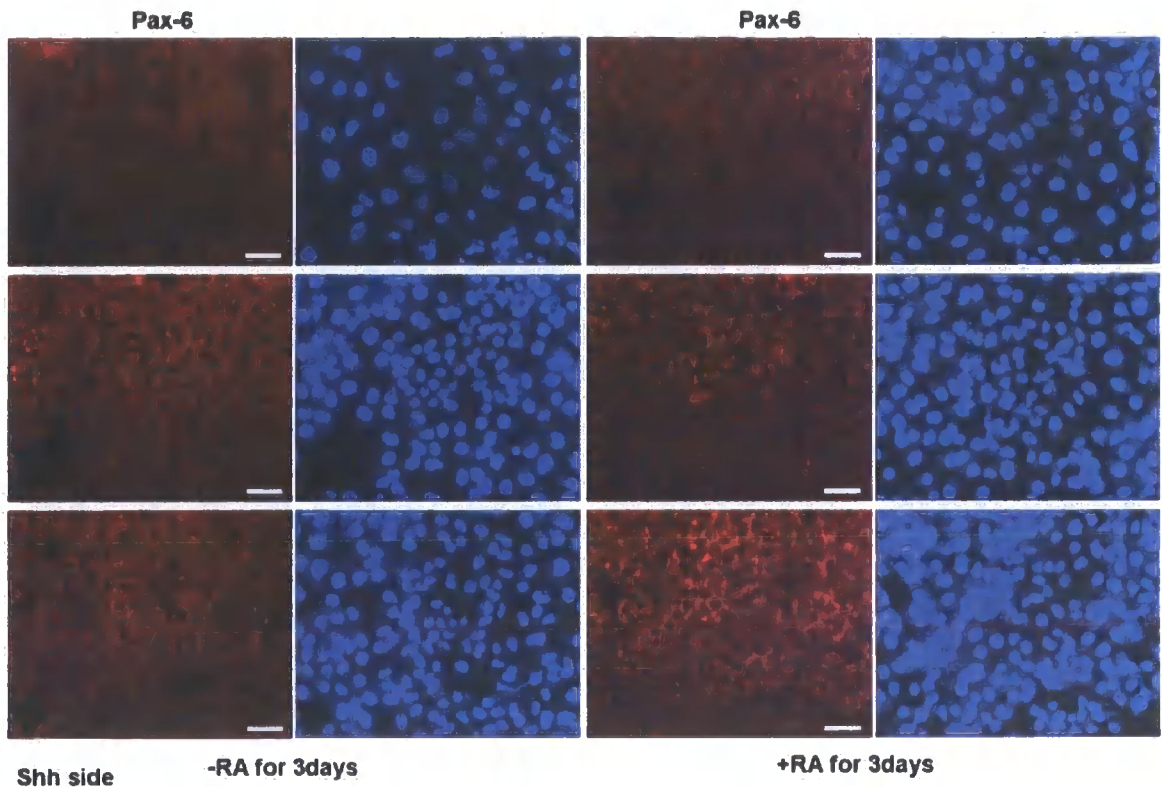


Figure 3.2.4 Pax6 staining in the model of Dorso-ventral axis using the channel  $\mu$ -slide. EC cells were seeded in the channel at 30,000 cells/channel supplemented with or without RA (10 $\mu$ M) in 30 $\mu$ l growth medium before 100 $\mu$ l Shh (25ng/ml) or BMPs (10ng/ml) were added to the column of the channel to setup the gradient concentration respectively. EC cells were maintained for 3 days prior to fixation with 4% PFA and labelling with antibodies specific to Pax6. The nuclei were counterstained with hoechst. Images were taken at the BMP side, Shh side and at the middle of the channel. Note that Pax6 staining is stronger at the Shh side in the presence of RA. In the Hoechst staining, there are more nuclei at the Shh side compare to the BMP side. Scale bars = 50 $\mu$ m.

**BMP side**

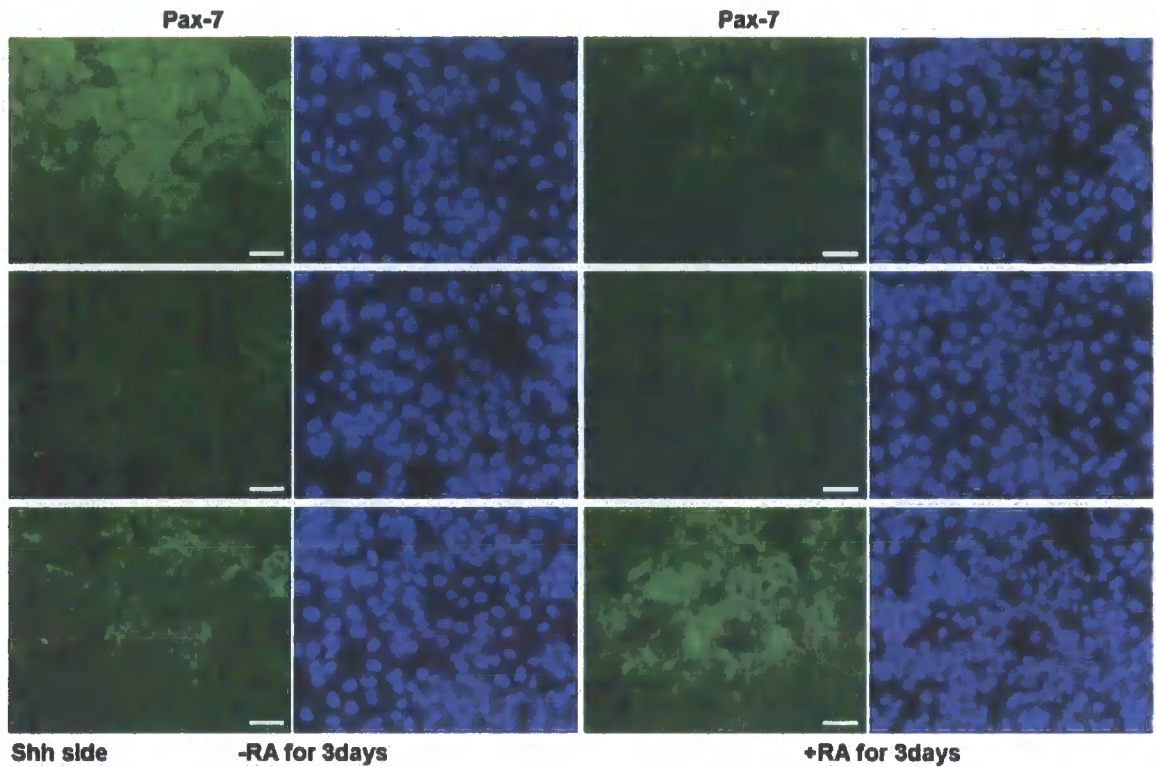


Figure 3.2.5 Pax7 staining in the model of Dorso-ventral axis using the channel  $\mu$ -slide. EC cells were seeded in the channel at 30,000 cells/channel supplemented with or without RA (10 $\mu$ M) in 30 $\mu$ l growth medium before 100 $\mu$ l Shh (25ng/ml) or BMPs (10ng/ml) were added to the column of the channel to setup the gradient concentration respectively. EC cells were maintained for 3 days prior to fixation with 4% PFA and labelling with antibodies specific to Pax7. The nuclei were counterstained with hoechst. Images were taken at the BMP side, Shh side and at the middle of the channel. Note that Pax7 staining is stronger at the Shh side in the presence of RA. In the hoechst staining, more nuclei were observed at the Shh side compare to the BMP side. Scale bars = 50 $\mu$ m.

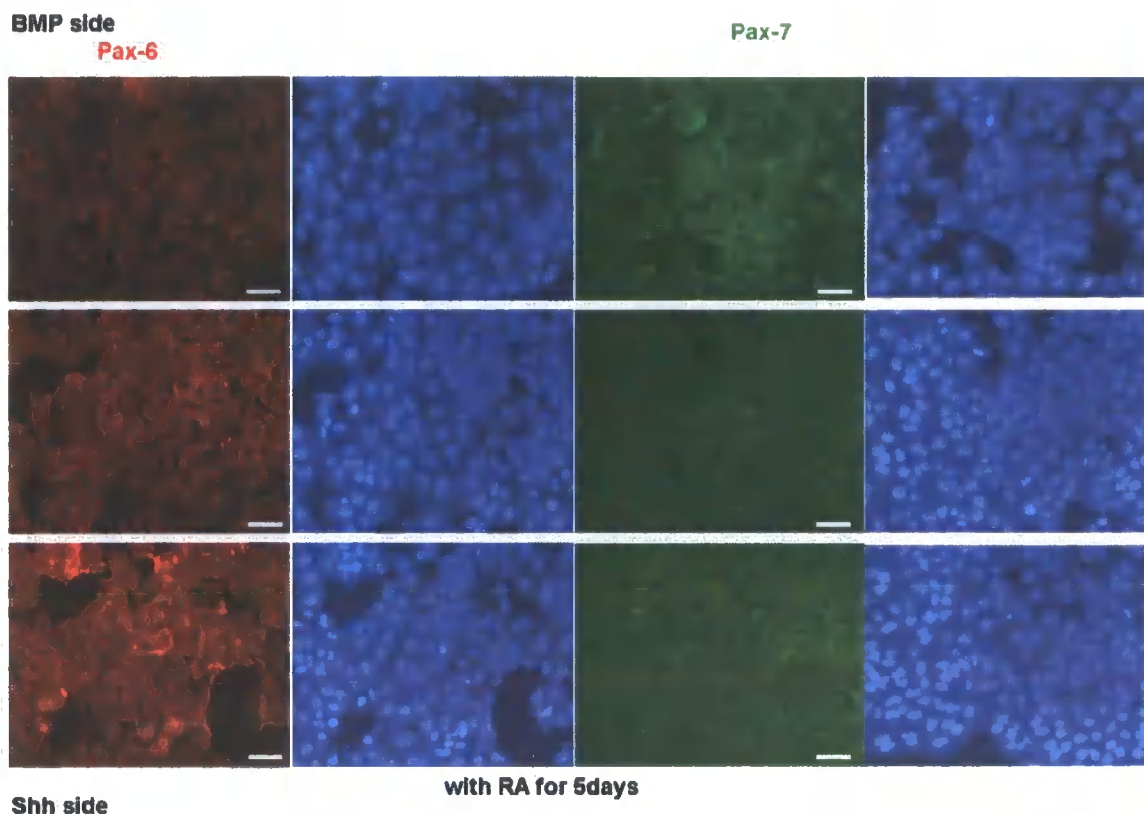


Figure 3.2.6 Pax6 and Pax7 staining in the model of Dorso-ventral axis using the channel  $\mu$ -slide. EC cells were seeded in the channel at 30,000 cells/channel supplemented with RA ( $10\mu\text{M}$ ) in  $30\mu\text{l}$  growth medium before  $100\mu\text{l}$  Shh ( $25\text{ng/ml}$ ) or BMPs ( $10\text{ng/ml}$ ) were added to the column of the channel to setup the gradient concentration respectively. EC cells were maintained for 5 days prior to fixation with 4% PFA and labelling with antibodies specific to Pax6 and Pax7. The nuclei were counterstained with hoechst. Images were taken at the BMP side, Shh side and at the middle of the channel. Note that Pax6 staining is stronger at the Shh side in the presence of RA, while Pax7 staining is weak. In the hoechst staining, more nuclei were observed at the Shh side compare to the BMP side. Scale bars =  $50\mu\text{m}$ .

**BMP side**

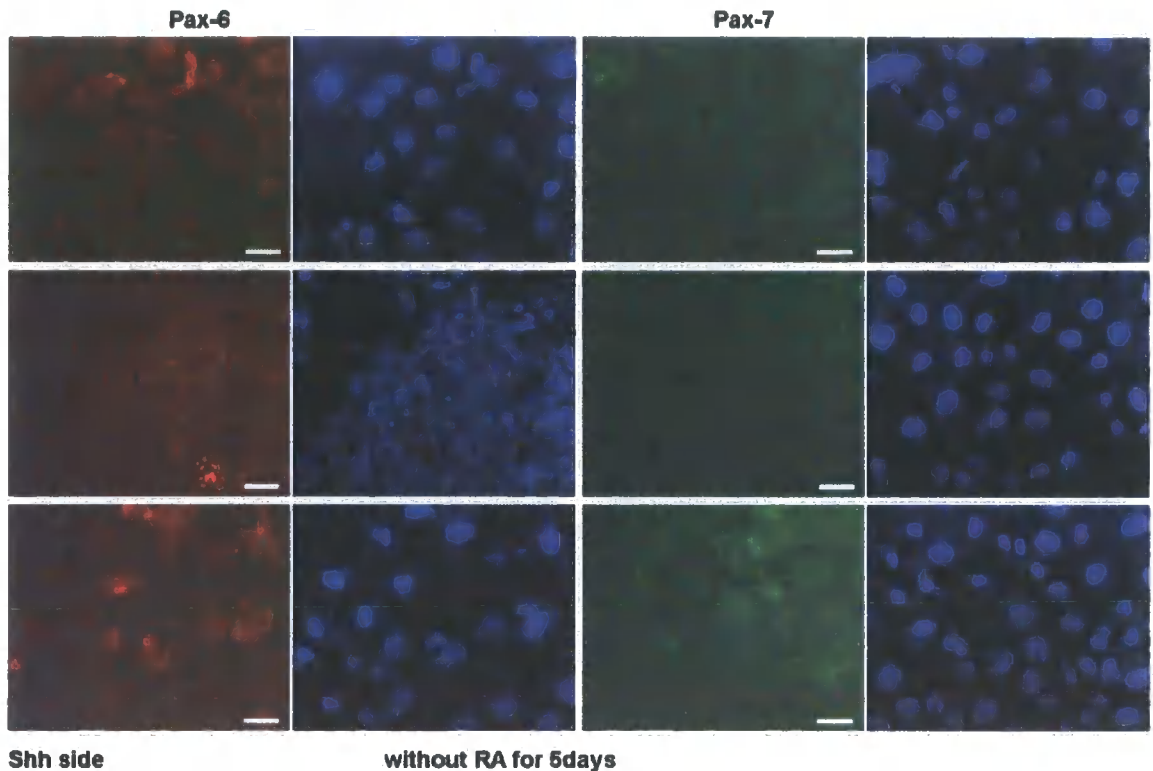


Figure 3.2.7 Pax6 and Pax7 staining in the model of Dorso-ventral axis using the channel  $\mu$ -slide. EC cells were seeded in the channel at 30,000 cells/channel supplemented without RA ( $10\mu\text{M}$ ) in  $30\mu\text{l}$  growth medium before  $100\mu\text{l}$  Shh ( $25\text{ng/ml}$ ) or BMPs ( $10\text{ng/ml}$ ) were added to the column of the channel to setup the gradient concentration respectively. EC cells were maintained for 5 days prior to fixation with 4% PFA and labelling with antibodies specific to Pax6 and Pax7. The nuclei were counterstained with hoechst. Images were taken at the BMP side, Shh side and at the middle of the channel. Note that Pax6 and Pax7 staining are weak at the channel in the absence of RA. In the hoechst staining, nuclei observed at the Shh side and the BMP side have no difference. Scale bars =  $50\mu\text{m}$ .



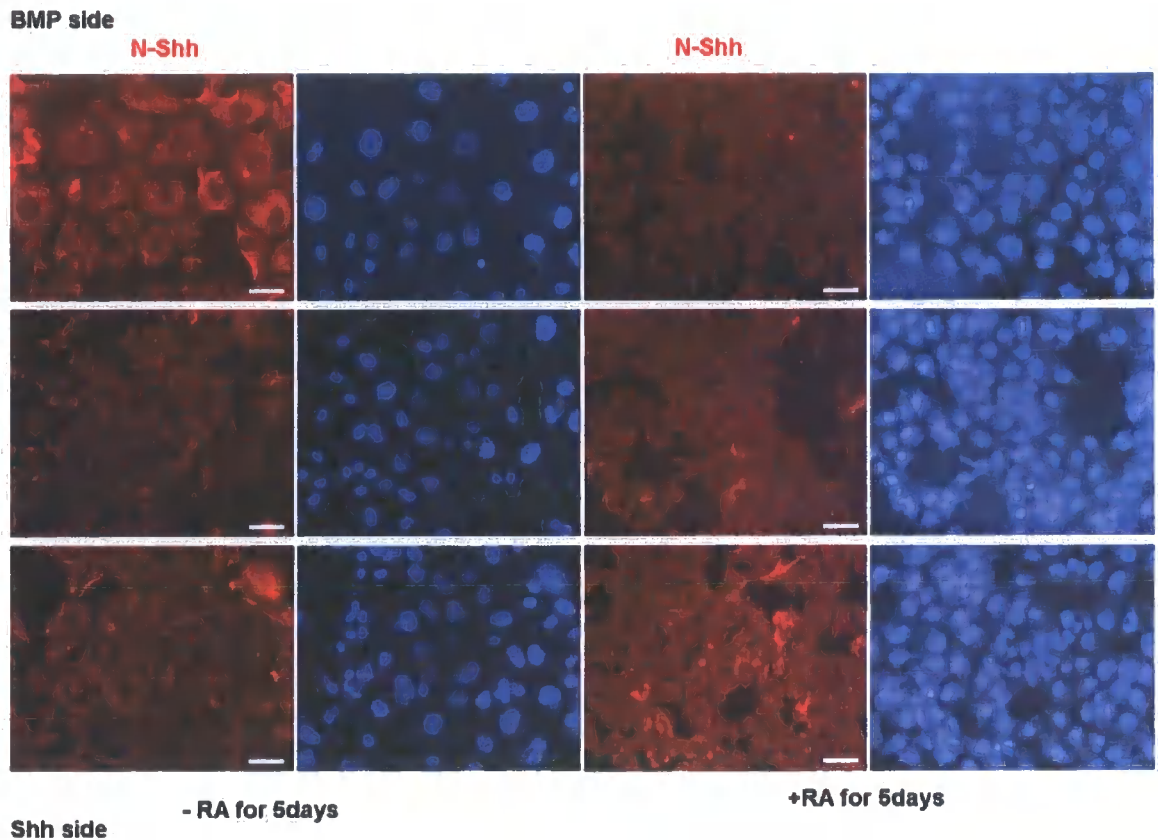


Figure 3.2.9 N-Shh staining in the model of Dorso-ventral axis using the channel  $\mu$ -slide. EC cells were seeded in the channel at 30,000 cells/channel supplemented with or without RA ( $10\mu\text{M}$ ) in  $30\mu\text{l}$  growth medium before  $100\mu\text{l}$  Shh ( $25\text{ng/ml}$ ) or BMPs ( $10\text{ng/ml}$ ) were added to the column of the channel to setup the gradient concentration respectively. EC cells were maintained for 5 days prior to fixation with 4% PFA and labelling with antibodies specific to N-Shh. The nuclei were counterstained with hoechst. Images were taken at the BMP side, Shh side and at the middle of the channel. Note that N-Shh staining is stronger at the Shh side in the presence of RA. In the hoechst staining, more nuclei were observed at the Shh side compare to the BMP side. Scale bars =  $50\mu\text{m}$ .

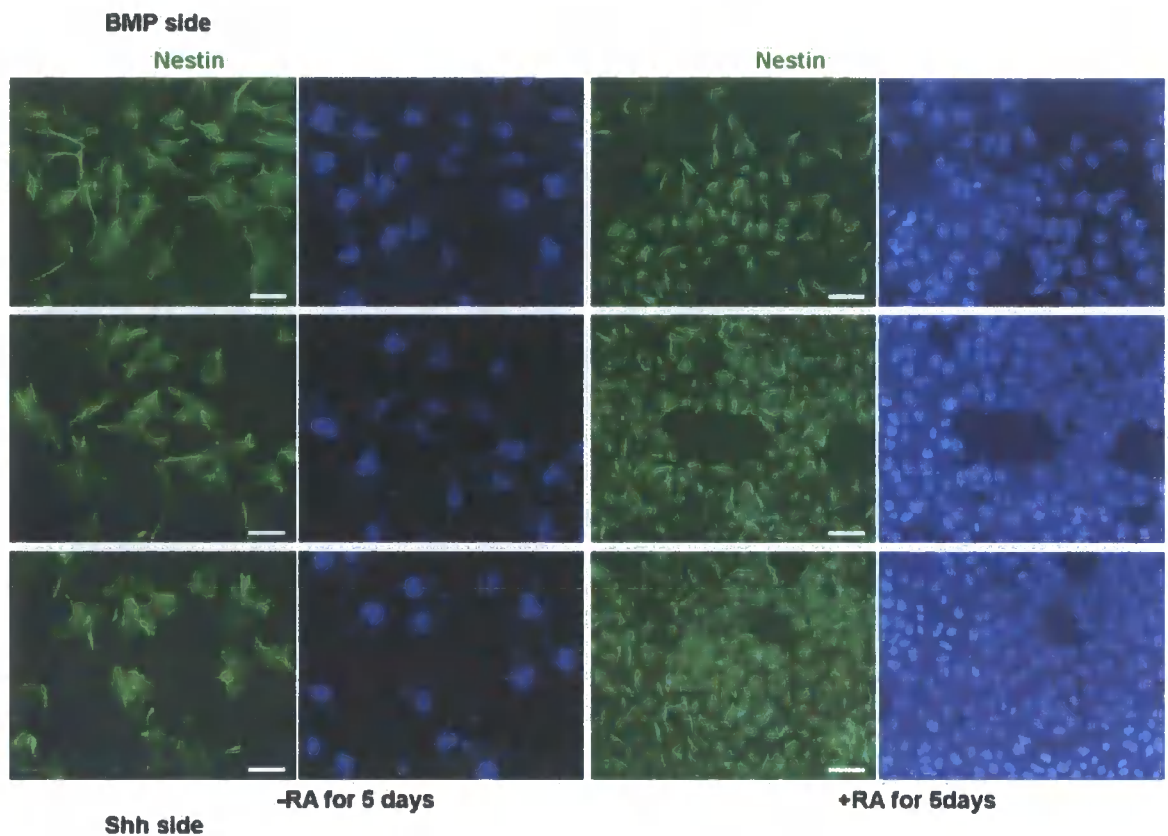


Figure 3.2.10 Nestin staining in the model of Dorso-ventral axis using the channel  $\mu$ -slide. EC cells were seeded in the channel at 30,000 cells/channel supplemented with or without RA (10 $\mu$ M) in 30 $\mu$ l growth medium before 100 $\mu$ l Shh (25ng/ml) or BMPs (10ng/ml) were added to the column of the channel to setup the gradient concentration respectively. EC cells were maintained for 5 days prior to fixation with 4% PFA and labelling with antibodies specific to Nestin. The nuclei were counterstained with hoechst. Images were taken at the BMP side, Shh side and at the middle of the channel. Note that Nestin staining is stronger at the Shh side in the presence of RA. In the hoechst staining, more nuclei were observed at the Shh side compare to the BMP side. Scale bars = 50 $\mu$ m.



### **3.3.3 Differentiation of neural precursors by Tera2.cl.SP12 EC cells**

We further cultured the Tera2.cl.SP12 EC cells with RA, RA+Shh and Shh alone for 1 week to examine how the EC cells respond to these conditions. We detected intense staining of nestin in all three conditions. Nestin positive cells in RA treated EC cells showed narrow bipolar neural precursor morphology and started to form rosette like structures (Figure 3.3.1A-C). The epithelial-like EC cell elongated the cell body in response to RA before the nestin fiber was stained at the apex of the cell. The polarity of nestin staining disappeared when cells formed rosettes, which are composed of bipolar nestin positive cells (Figure 3.3.1C). We also observed non-neural Nestin positive cells, which have a pale stained large cytoplasm and highly positive nucleus (Figure 3.3.1D). These cells will differentiate into cells other than neurons. However, we detected some scattered bright  $\beta$ III tubulin cells in RA and RA+Shh conditions, but not in Shh induced cells (Figure 3.3.3). Pax6 staining was positive in all three conditions as well, but the RA and RA+Shh induced cells were positive in the cell nuclei, while the Shh induced cells were cytoplasm positive, suggesting that Pax6 is activated in RA and RA+Shh conditions at the time of examination (Figure 3.3.2). In contrast, non-induced EC cells and Shh induced cells possessed more Ki67 (a marker of proliferation) positive cells compared to RA and RA+Shh induced cells (Figure 3.3.3). After 2 weeks induction, nestin cells form well arranged rosettes in RA and RA+Shh conditions, while no rosettes formed in Shh induced condition, furthermore, more  $\beta$ III tubulin positive cells were present in RA and RA+Shh conditions, whereas only one or two positive cells in Shh condition (Figure 3.3.4). The observation suggested that EC cells induced by these conditions might acquire neural properties and become neural precursors. Interestingly, there were only patches of positive Pax6 stained in nuclei in RA and RA+Shh conditions, most of Pax6 staining was still in the cytoplasm of Shh induced cells (Figure 3.5B). This suggests that the temporal Pax6

activation may be required by EC stem cell to express  $\beta$ III tubulin to become a neuronal progenitor.

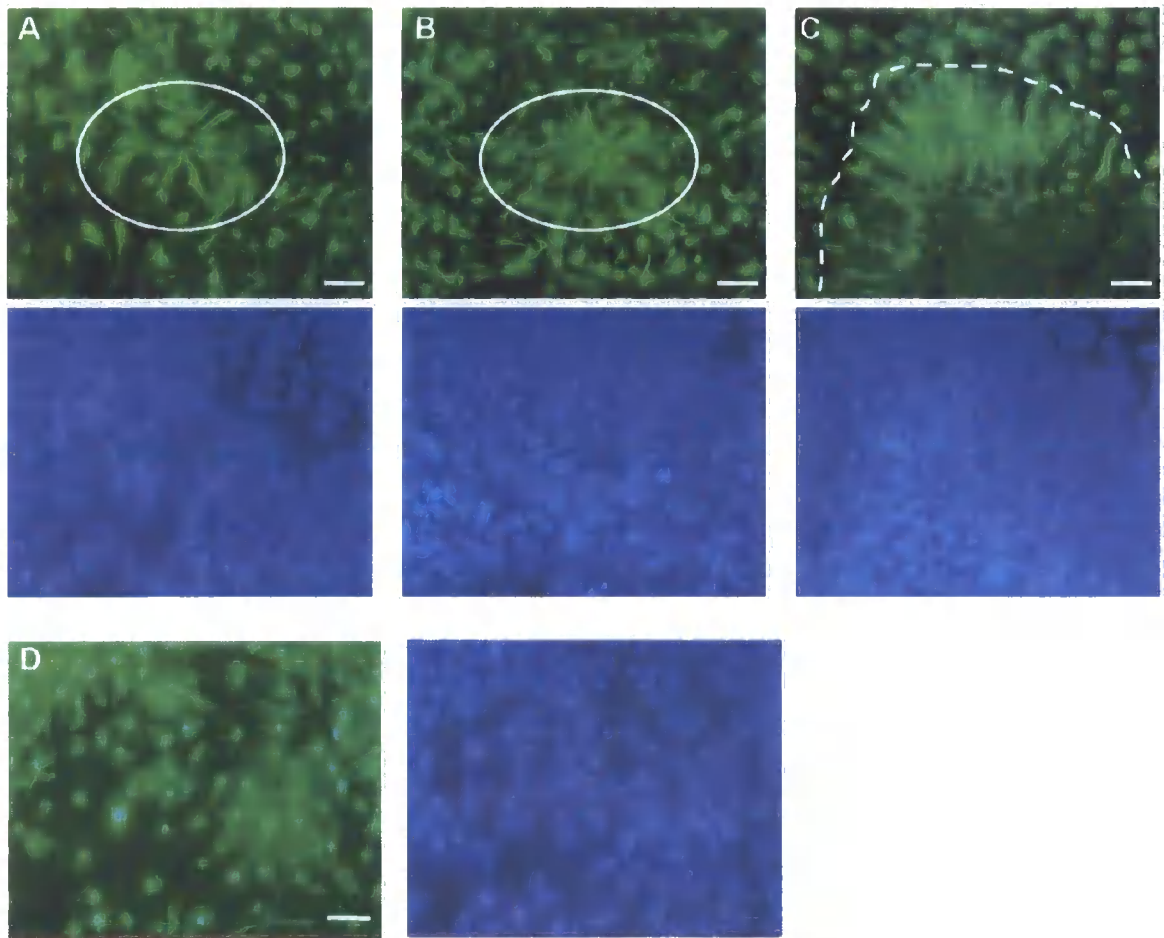


Figure 3.3.1 Nestin staining in 1 week RA treated EC culture. EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10 $\mu$ M) for 1 week prior to fixation with 4% PFA and labelling with antibodies specific to Nestin. The nuclei were counterstained with hoechst. Note that Nestin staining showed polarity in the cells before rosette formation (A and B), and rosette is composed of bipolar Nestin positive cells (C). Nestin staining is highly positive in the nuclei while pale in cytoplasm of the cells which formed no neurons (D). Scale bar = 20 $\mu$ m.

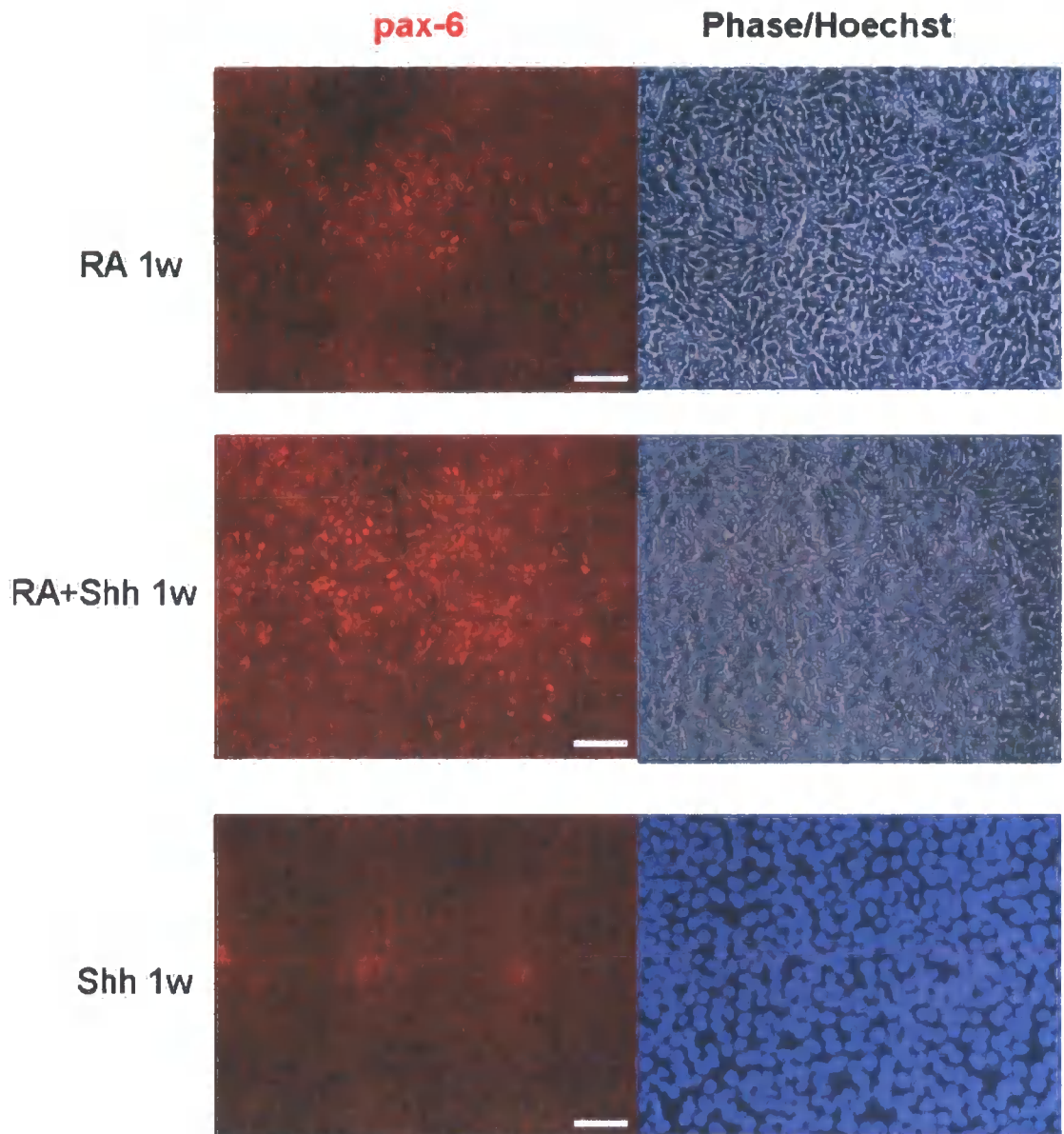


Figure 3.3.2 Pax6 staining in 1 week RA treated EC culture. EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10μM) or Shh (25ng/ml) for 1 week prior to fixation with 4% PFA and labelling with antibodies specific to Pax6. The nuclei were counterstained with Hoechst or a phase contrast image is shown instead. Note that Pax6 staining is confined in the nuclei of the cells in RA and RA+Shh conditions while Pax6 staining is extinguished in the nuclei of the cells of Shh treated culture. Scale bar = 50μm.



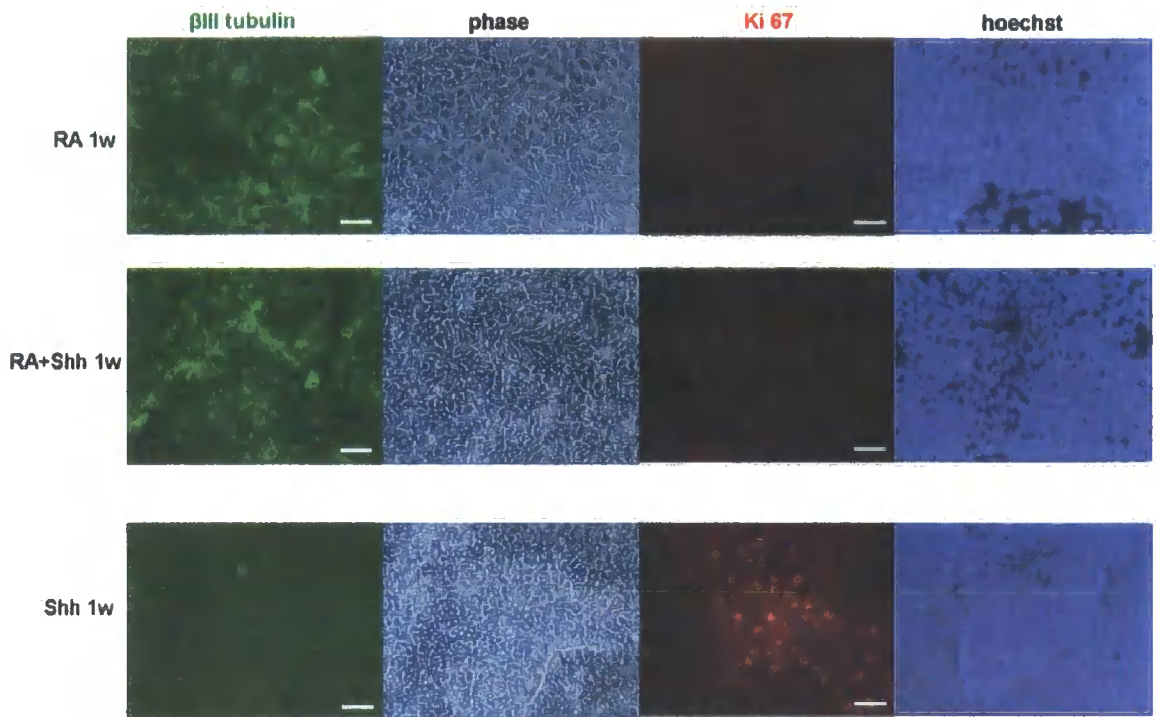


Figure 3.3.3 Ki67 and  $\beta$ III tubulin staining in 1 week RA and Shh treated EC culture. EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10 $\mu$ M) or Shh (25ng/ml) for 1 week prior to fixation with 4% PFA and labelling with antibodies specific to Ki67 (red) and  $\beta$ III tubulin (green). The nuclei were counterstained with Hoechst or a phase contrast image is shown instead. Note that  $\beta$ III tubulin staining is positive in RA and RA+Shh conditions while no staining in the cells of Shh treated culture. Scale bar = 50 $\mu$ m.

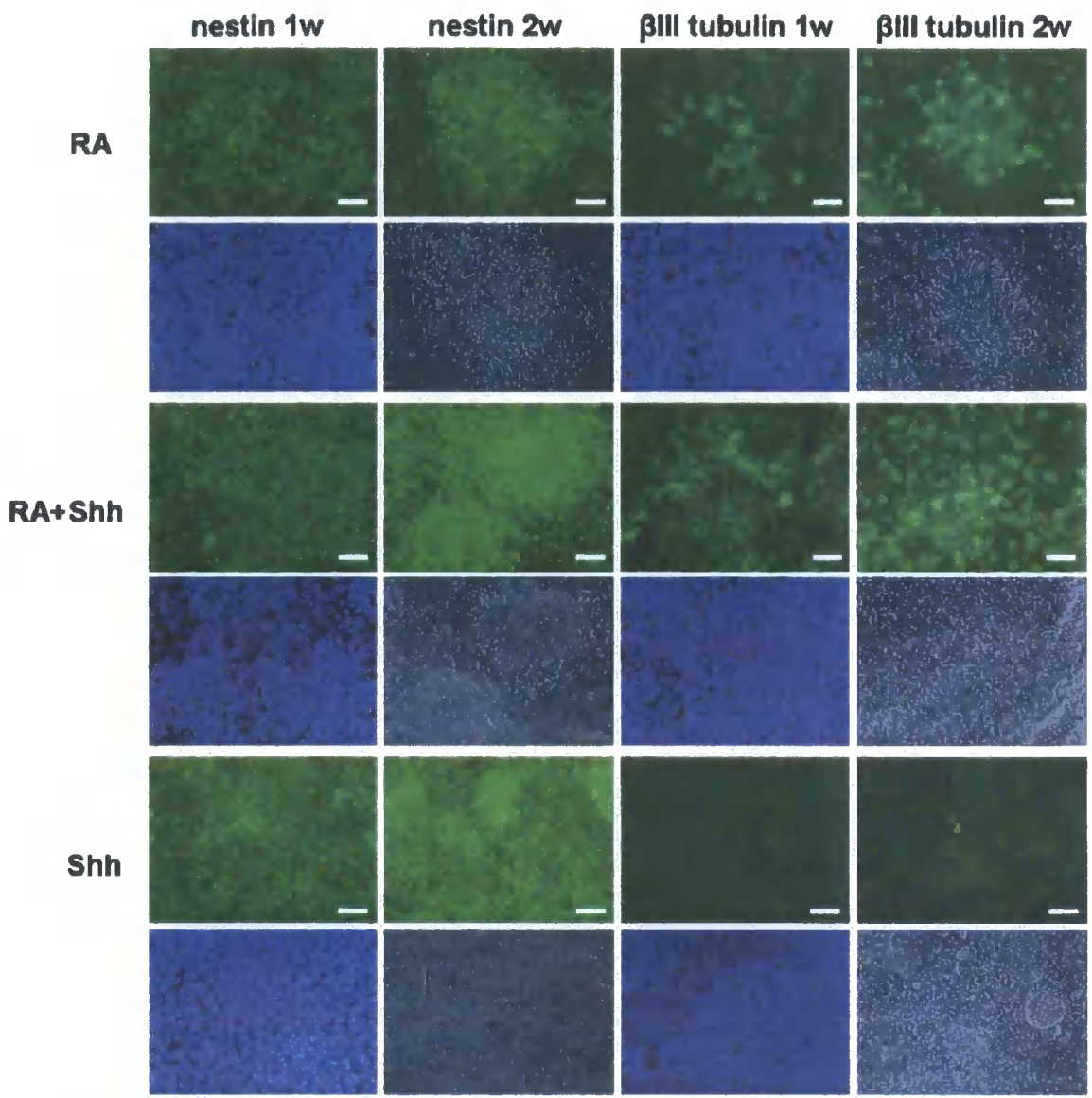


Figure 3.3.4 Comparison of Nestin and  $\beta$ III tubulin expression in 1 week and 2 week induced EC cultures. EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10 $\mu$ M) or Shh (25ng/ml) for 1 week prior to fixation with 4% PFA and labelling with antibodies specific to Nestin and  $\beta$ III tubulin. The nuclei were counterstained with Hoechst or a phase contrast image is shown instead. Note that  $\beta$ III tubulin staining is positive in RA and RA+Shh conditions while no staining in the Shh treated culture. Scale bar = 50 $\mu$ m.

### **3.3.4 Patterning genes induced by RA and Shh in Tera2.cl.SP12 EC cells**

Research based on *in vivo* models has suggested that there are two kinds of genes defined by their responses to Shh signal: Class I genes, which are repressed by Shh, includes *Pax-3*, *-6* and *-7*, etc; Class II genes, which are induced by Shh, includes *Nkx2.2*, *Olig2*, etc (Lee and Pfaff, 2001). From experiments using the channel  $\mu$ -slide assay, we observed that patterning transcription factors such as Pax6 and Gli1 had reduced fluorescence intensity from the Shh end to the BMPs end, suggesting that patterning genes respond to different Shh concentrations. To address this question, we performed RT-PCR to examine the patterning gene expression profile induced by different concentration of Shh. EC cells were also grown as suspension cultures to check if there is a significant difference in the expression of transcription factors between the suspension culture and monolayer culture.

*Pax* genes were first investigated. *Pax3* and *Pax7* are dorsally expressed patterning genes, but they are activated by all the Shh concentrations tested (Figure3.4.1). The level of expression increased over time and Shh concentration, which is different from the previous reports that *Pax3* and *Pax7* are only activated by low concentrations of Shh (Incardona et al., 1998). Compared to the monolayer culture, cells in suspension culture expressed low level of *Pax3* and *Pax7* in response to Shh signal. *Pax6* is a ventral patterning gene, which is repressed by Shh *in vivo* (Schnapp et al., 2005). Our results were consistent with this, with only very weak expression was detected by our RT-PCR assay. Because RA is another important signal for ventral neural tube progenitors, we also investigated how it regulates Pax gene expression in EC cells (Figure3.4.1). RA induced *Pax-3*, *-6* and *-7* expression and their expression levels increased with RA exposure time, which is consistent with conclusion from *in vivo* models that RA induced all these three Pax genes expression (Wilson and Maden, 2005). When we exposed the EC cells to 25ng/ml Shh and 10 $\mu$ M RA simultaneously, we also detected the expression of all three Pax genes, however, the

expression level of *Pax6* and *Pax7* was not increased by time, suggesting that Shh modifies the RA signal in regulating the RA induced patterning genes expression.

We also examined *Nkx2.2* and *Olig2*, which are Shh induced genes (Afonso and Catala, 2005). *Olig2* expression is activated in response to Shh as we expected, but its expression level is not regulated by Shh at the concentrations tested (Figure3.4.2-*Olig2*). Despite that we observed some expression of *Nkx2.2* (Figure3.4.2-*Nkx2.2*); its expression level is regulated neither by Shh concentration nor by Shh exposure time. Furthermore, our results show that RA repressed the expression of these two genes, which is consistent with the previous report that RA represses Class II genes (Wilson et al., 2004). The effect of RA+Shh also activated the expression of *Nkx2.2* and *Olig2*, but *Nkx2.2* is not expressed at all the time points until day7 in monolayer culture, suggesting that in *Olig2* regulation, Shh plays the dominant effect while in *Nkx2.2*, RA takes control.

*Isl1* and *HB-9* are two patterning transcription factors that play important role in motor neuron progenitor specification (Arber et al., 1999; Thaler et al., 2004); these two genes were also activated by Shh. *Isl1* is Shh and RA inducible, but it showed a down-regulated effect in RA+Shh condition (Figure3.4.2-*Isl1*). Unlike *Isl1*, *HB-9* is not activated until EC cells were exposed to Shh for 1 week, and RA induced no *HB-9* expression in EC cells, which is different from the reports that RA activated *HB-9* expression in some *in vivo* models (Wilson and Maden, 2005), a possible reason is RA has no effect in *HB-9* early regulation, it works from 2 weeks induction.

*En-1* is expressed at the midline of neural tube at the DV axis (Simon et al., 2005). It is said that it is also a marker for mid-brain cells and patterning the developing ventral limb. EC cells express low level of *En-1*, and Shh and/or RA induced or maintained the *En-1* expression in EC cells.

Taken as a whole, we summarised that EC cells can serve as *in vitro* model for DV patterning, although some gene expression is not perfectly consistent with the conclusions drawn from *in vivo* models. The reason for this inconsistency could be the difference between *in vivo* and *in vitro* or the difference between species, most of the results are from chick and mouse models not human. Although there are some differences in gene expression profile in suspension culture compared to monolayer cultures, we choose the monolayer system as our model for DV patterning. This was primarily because the differences are less and, the monolayer culture system is easier to work with and observe the cell morphology and cell status.



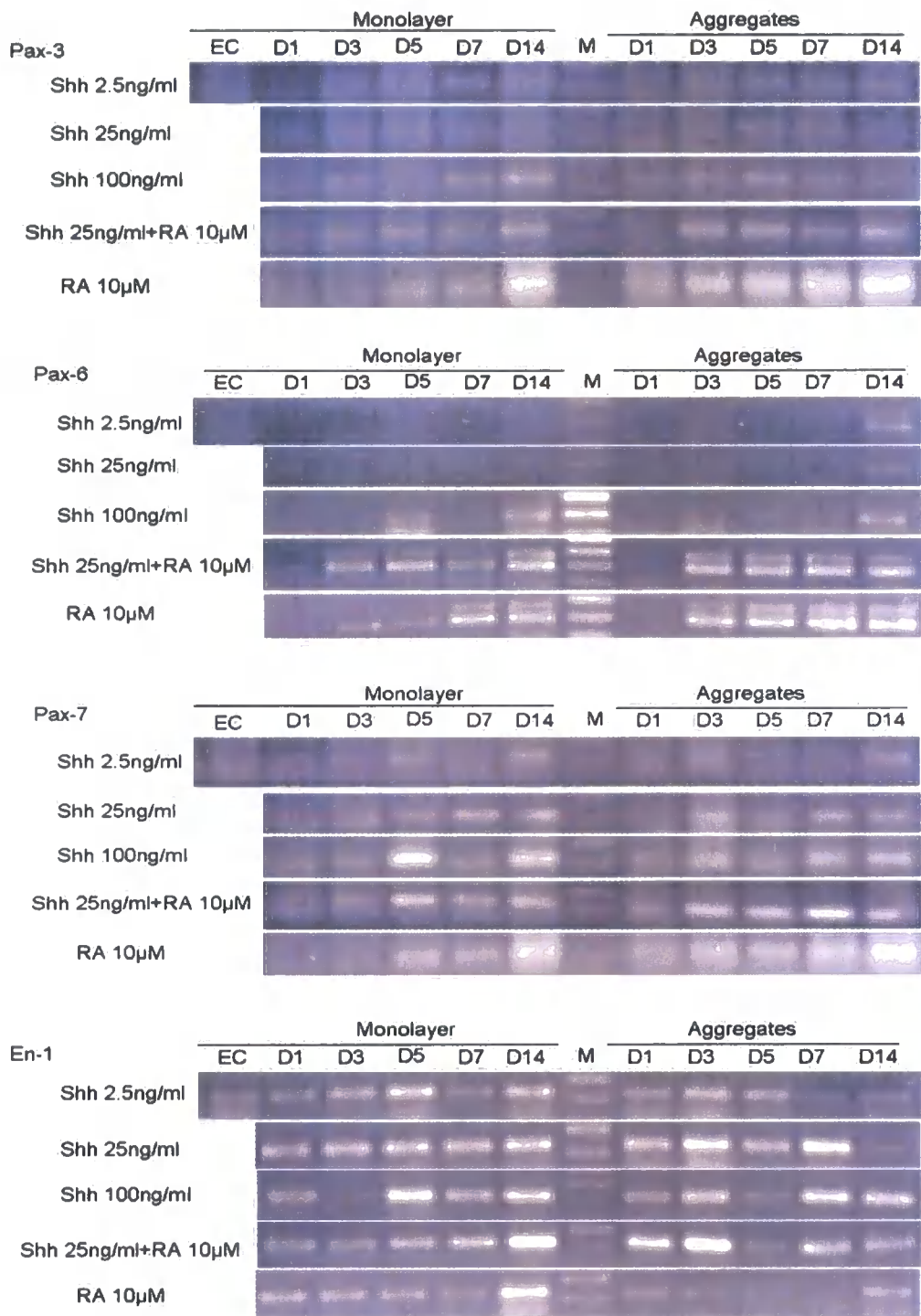


Figure 3.4.1 RT-PCR analysis of Pax genes and En-1 expression in EC cells. EC cells were seeded in the 6 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10µM) or Shh (25ng/ml) for up to 2 weeks prior to isolation of total RNA. GAPDH, which is served as internal control, is shown in Figure3.4.2.

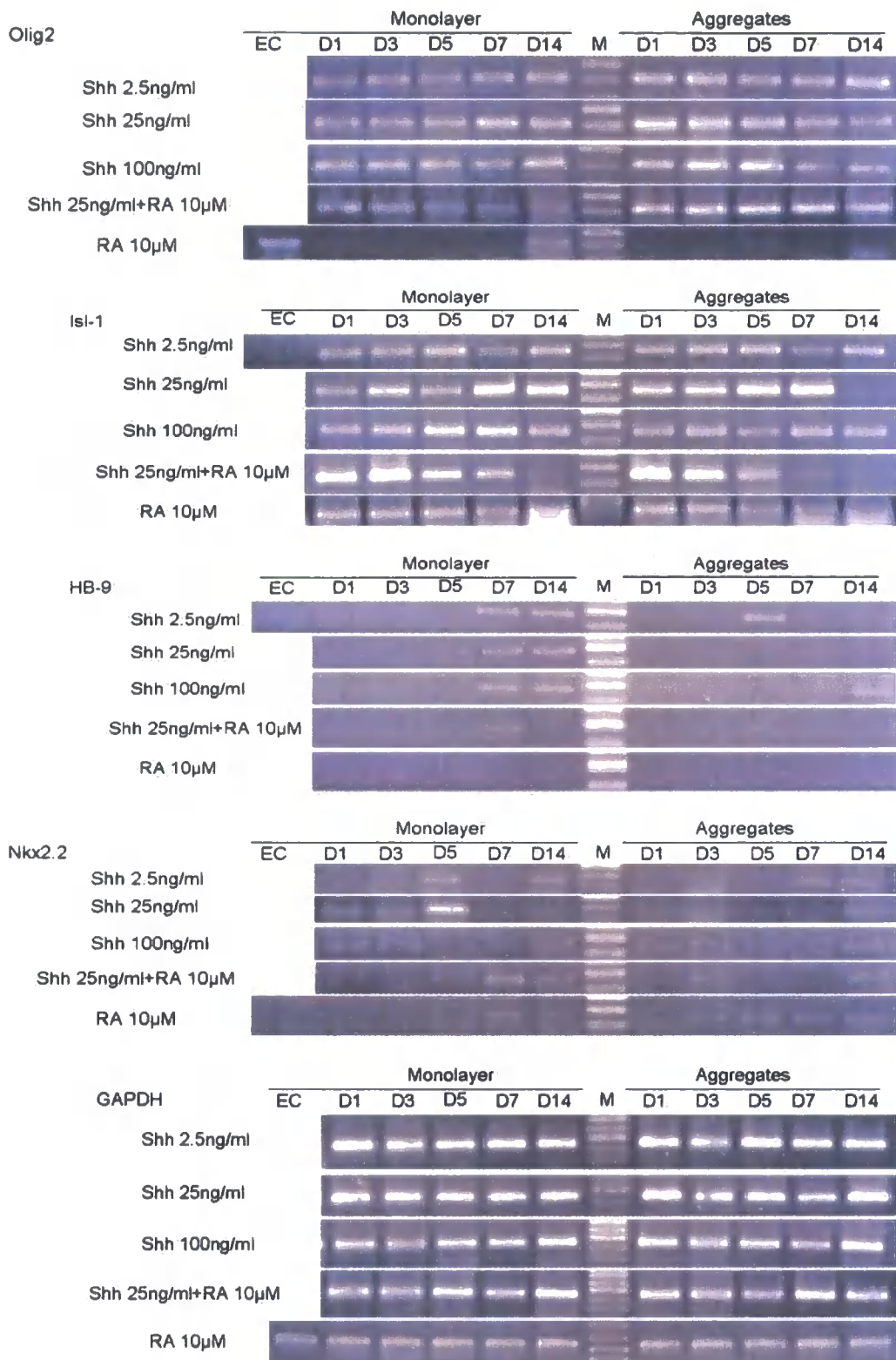


Figure 3.4.2 RT-PCR analysis of motor neuron related patterning genes. EC cells were seeded in the 6 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10μM) or Shh (25ng/ml) for up to 2 weeks prior to isolation of total RNA. GAPDH is served as internal control.

### **3.3.5 Regulated expression of patterning transcription factors during Tera2.sp12 EC cell differentiation**

To confirm our results from RT-PCR, we conducted immunocytochemistry staining using antibodies specific to the patterning transcription factors.

Dlx2 is an early patterning transcription factor expressed in ventral neural progenitors (Rallu et al., 2002). Non-induced EC cells express low level of the Dlx2 gene (Figure3.5.1). If cells were over-grown, Dlx2 was only present in the cytoplasm, suggesting that Dlx2 does not activate other downstream transcription factor genes in these cells. In contrast to non-induced EC stem cells, cells induced by RA expressed high levels of Dlx2, and the staining was confined to the nucleus of cells, suggesting that Dlx2 is playing its role as a transcription factor in the nuclei to activate other downstream transcription factors. Shh is not a powerful inducer for Dlx2 expression from our staining, Dlx2 staining induced by RA could be abolished by low concentration of Shh (2.5ng/ml), while 25ng/ml Shh could not abrogate the expression of Dlx2 induced by RA but rather lower the expression level. This suggests that RA is the critical regulator for Dlx2 activation, and Shh could be a modulator for Dlx2 activation. Overall Pax7 staining was weak in RA and Shh induced EC cells, which is consistent with the channel  $\mu$ -slide assay (data not shown). For Pax6 staining, it is induced by RA at the early stage of differentiation, the expression was activated in nuclei after two days exposure to RA and the expression level became greater after seven days of treatment (Figure3.5.2). Subsequently, the expression level diminished and, only small patches of positive cells remaining after 2 weeks. RA+Shh elicited the similar temporal expression pattern of Pax6 staining, suggesting RA has the predominant effect on Pax6 expression. However, in Shh-induced and non-induced EC cells, Pax6 staining was confined at cytoplasm, suggesting that Pax6 activated transcription factor expression cascade did not occur in these cells. Although Pax6 is not induced by Shh, the staining

pattern of Pax6 in Shh and RA+Shh induced cells suggested that Shh did not abolish the Pax6 activation and may play a role in maintaining the Pax6 expression.

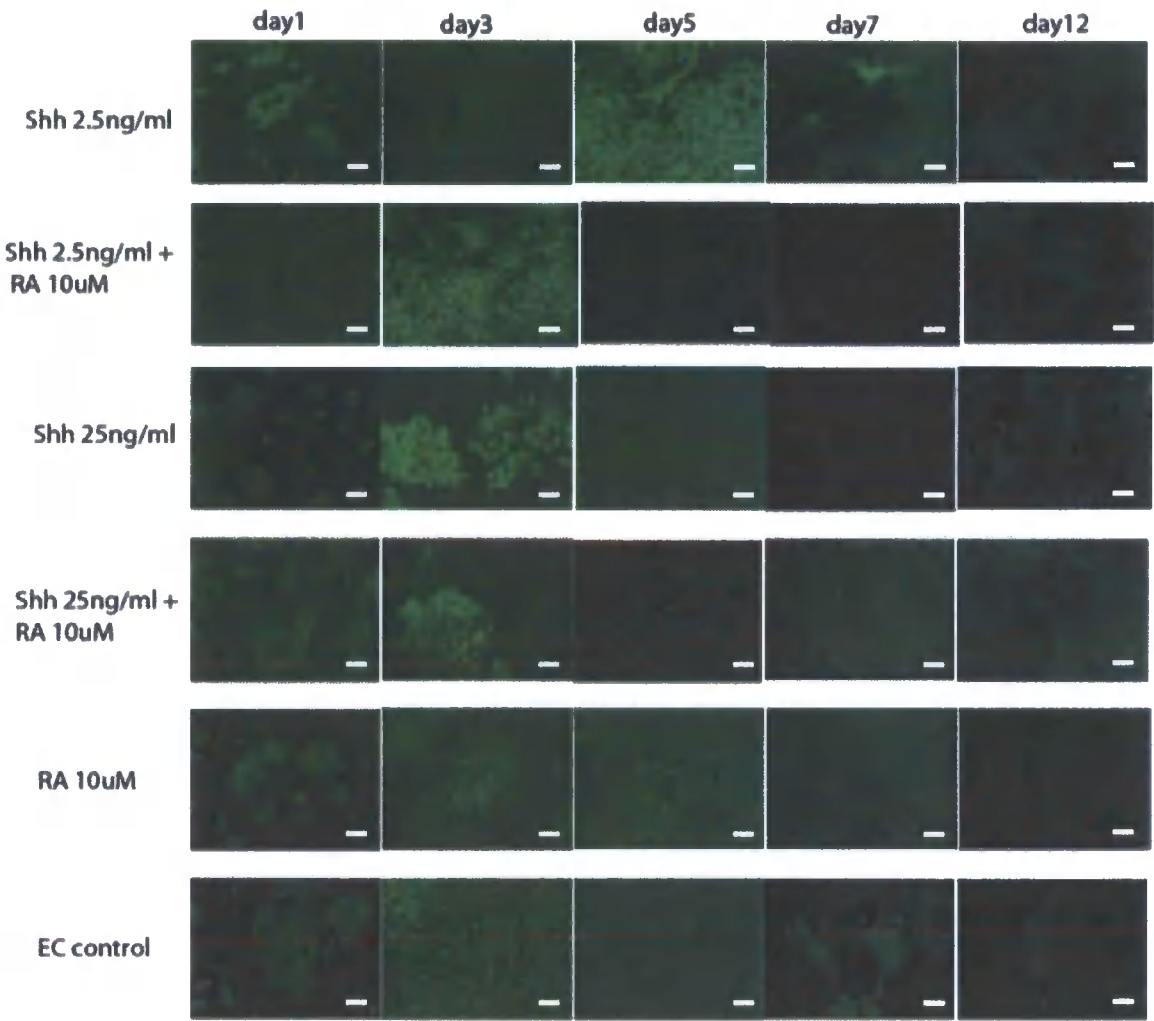


Figure 3.5.1 Activation of Dlx2 expression in RA and Shh induced cells. EC cells were induced by 2.5ng/ml and 25ng/ml Shh and 10µM RA for the indicated time points. 2.5ng/ml Shh can not activate intra-nuclear Dlx2 staining, Dlx2 can be activated by 25ng/ml Shh and 10µM RA, and staining in RA condition last until day5, suggesting that Shh has the predominant effect in Dlx2 expression. Scale bars = 50µm.

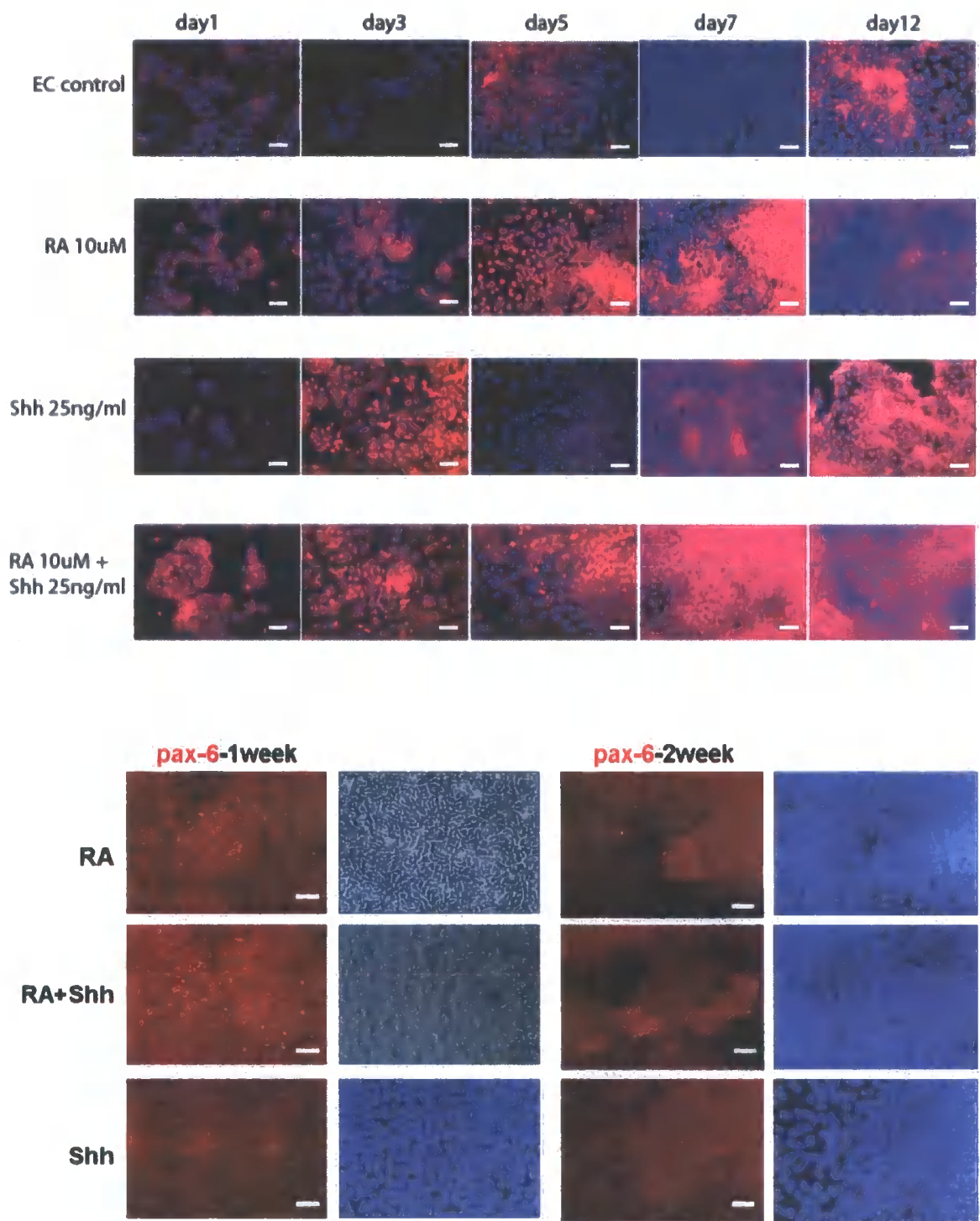


Figure 3.5.2 Investigation of Pax6 expression in RA and Shh induced EC cells. EC cells were induced by 25ng/ml Shh and 10 $\mu$ M RA for the indicated time points. The induced cultures were fixed with 4% PFA and immuno-labelled with Pax6. The nuclei were counterstained with Hoechst or a phase contrast image is shown. Note that Pax6 were activated by 10 $\mu$ M RA and the staining is located at nuclei. Scale bars = 50 $\mu$ m.

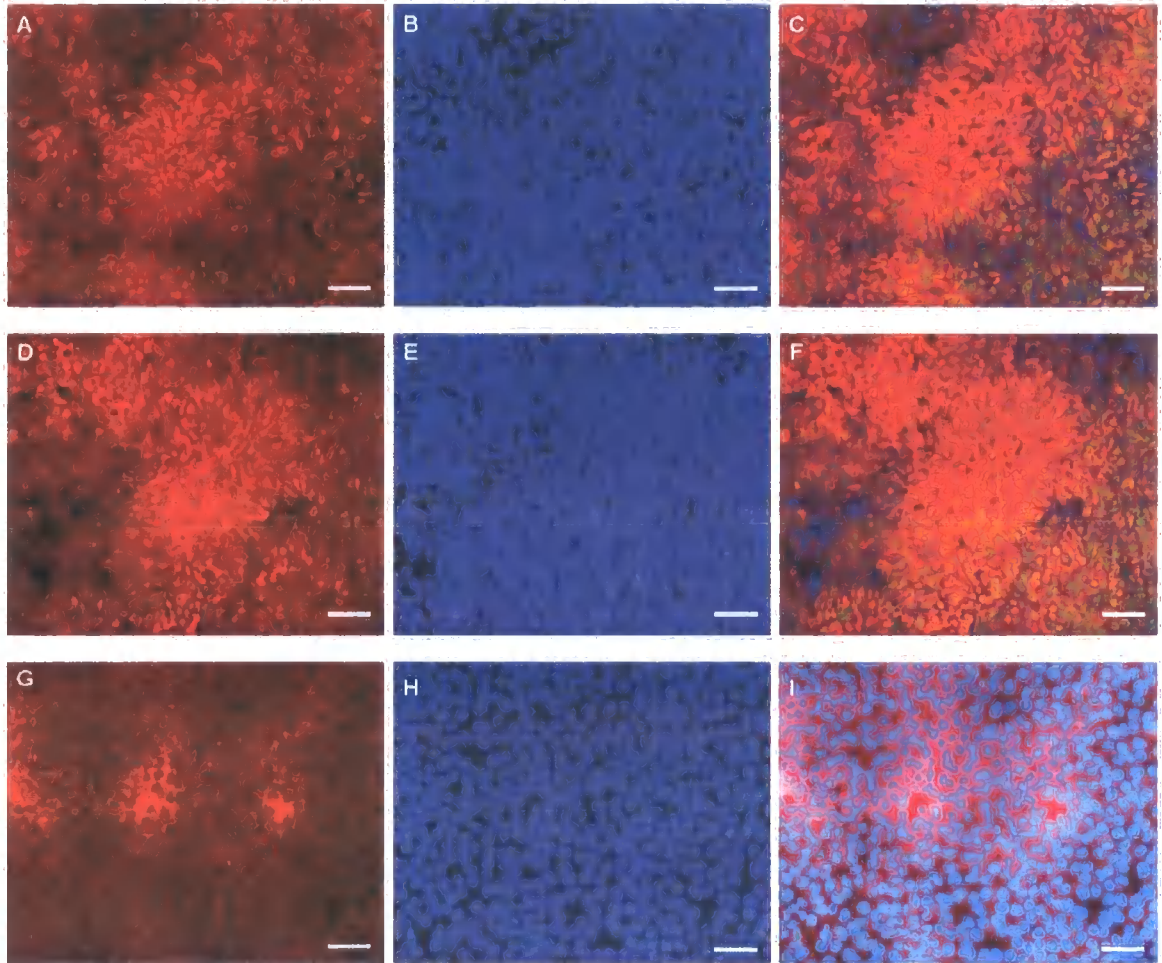


Figure 3.5.3 Investigation of Pax6 localisation in RA and Shh induced EC cells. EC cells were induced by 10µM RA (A-C) or by 25ng/ml Shh and 10µM RA (D-F) or by 25ng/ml Shh (G-I) for the 1 week. The induced cultures were fixed with 4% PFA and immuno-labelled with Pax6. The nuclei were counterstained with Hoechst and a merged image is shown. Note that Pax6 were activated by 10µM RA and the staining is located at nuclei, while Pax6 staining is in the cytoplasm of Shh induced cells. Scale bars = 50µm.

### **3.3.6 Expression of neural markers in differentiating Tera2.sp12 EC cells**

After induction by RA and RA+Shh for 2 weeks, protein samples were collected from the induced EC cells and analysed by western blotting (Figure3.6.1). In both conditions, we detected expression of NeuroD1, which is one of the transcription factors that regulates neurogenesis (Hallam et al., 2000). NeuroD1 induces the formation of ectopic neurons and conversion of neuronal precursor cells into neurons (McCormick et al., 1996). We detected no NeuroD1 staining in EC cells after 3 weeks treatment, suggesting that NeuroD1 is expressed transiently in EC cell differentiation (data not shown).

NCAM, the neural cell adhesion molecule, has been implicated as having a role in cell-cell adhesion, neurite outgrowth, synaptic plasticity, and learning and memory, most importantly, NCAM expression in ectoderm marks the formation of neural plate, and suggesting the NCAM positive cells are neuronal and glial bi-potent (Seidenfaden et al., 2006). In our assay, NCAM expression was also detected in RA and RA+Shh induced cells, suggesting that these cells had the potential to differentiate into neuron or glial cells. Furthermore, the detection of Tuj1 and NSE expression in the induced cells indicates that neurons exist in these cultures. In addition, we detected endogenous Shh present in cells induced by these two conditions. Shh signal mediator Gli1 was also detected by RT-PCR during the EC cell differentiation under the same conditions (figure 4.1.5), these data suggest that the Shh pathway may be involved in the differentiation even when RA alone is used to induce cell differentiation.

In order to identify the subtype of neurons forming under these conditions, we did double staining of HB-9 and  $\beta$ III tubulin on the induced EC cells after three weeks treatment of RA and RA+Shh (Figure3.6.2). From the staining pattern, the  $\beta$ III tubulin positive cells

exhibited long neurites and distinct round cell bodies. The nuclei of these cells were also positive for HB-9, suggesting that the neurons may have acquired a motor neuron identity. After one more week differentiation, we did another double staining of ChAT and  $\beta$ III tubulin on the cells treated with the same conditions and found that some cell aggregates with extended long processes were positive for  $\beta$ III tubulin and ChAT (Figure3.6.3). Because ChAT is a critical enzyme for Ach synthesis, we postulated that these  $\beta$ III tubulin positive cells have the ability to produce motor neuron neurotransmitter, which is a basic function of motor neuron. Overall these data indicated that RA and RA+Shh can induce the formation of motor neurons from Tera2.cl.SPI2 EC stem cells.

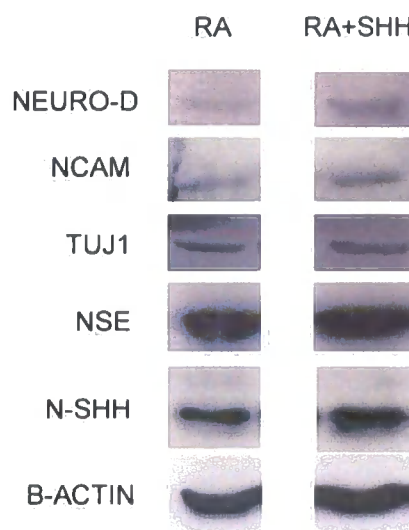


Figure 3.6.1 Western blotting analysis of neuron markers in induced EC cells. EC cells were seeded in the 6 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10 $\mu$ M) or Shh (25ng/ml) for 2 weeks prior to isolation of cytoplasmic protein and western blot analysis. B-actin is served as internal control. The band size of the bands that are shown above: NeuroD, 50KD; NCAM, 140KD; TUJ1, 50KD; NSE, 45KD; N-SHH, 20KD;  $\beta$ -actin, 45DK.



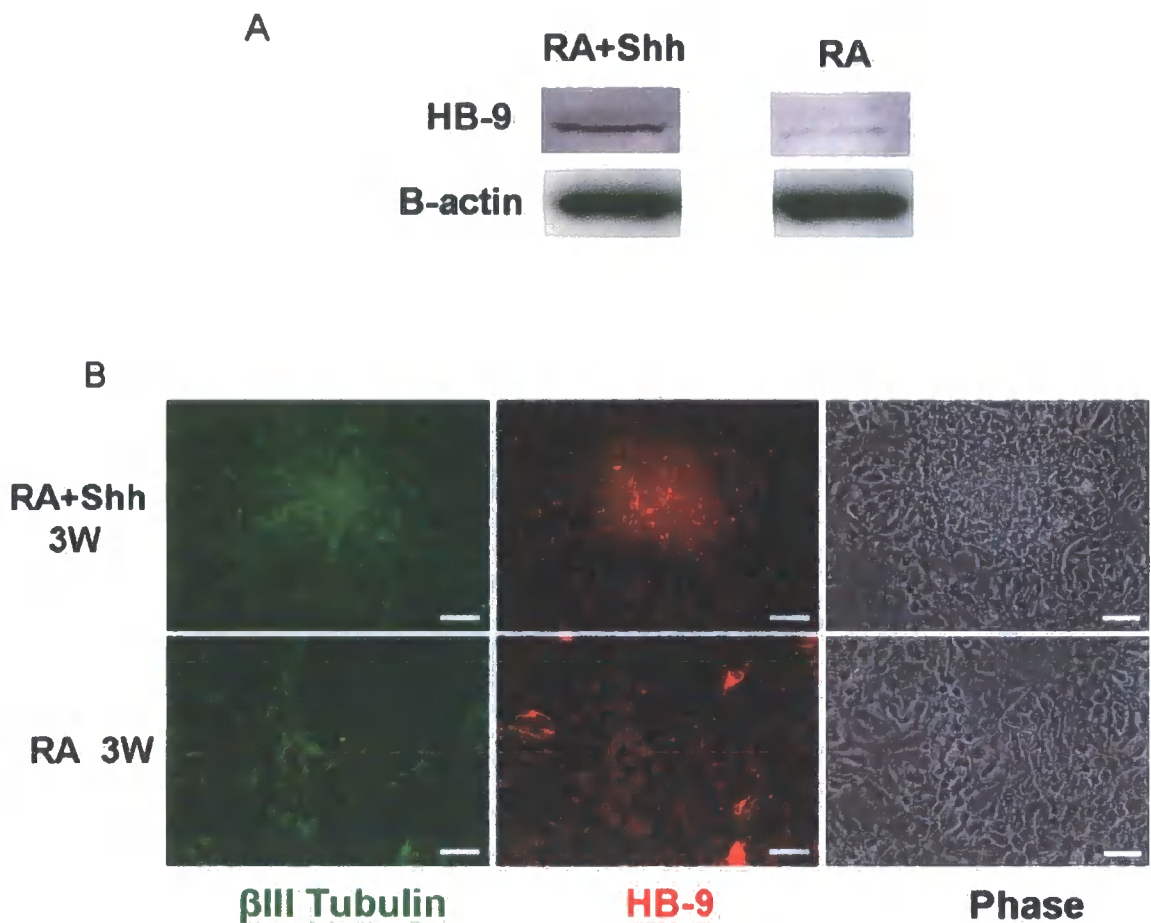


Figure 3.6.2 The expression of motor neuron marker HB-9 in induced EC cells. A, EC cells were seeded in the 6 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10 $\mu$ M) or Shh (25ng/ml) for 3 weeks prior to isolation of cytoplasmic protein and analysis HB-9 expression by western blot. B, EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10 $\mu$ M) or Shh (25ng/ml) for 3 weeks prior to fixation with 4% PFA and labelling with antibodies specific to HB-9 (red) and  $\beta$ III tubulin (green). The phase contrast images are shown. Note that  $\beta$ III tubulin staining is positive in RA and RA+Shh conditions while HB-9 staining is positive in the nuclei in both cultures. Cells with HB-9 staining in cytoplasm are not  $\beta$ III tubulin positive neurons. Scale bars = 50 $\mu$ m.

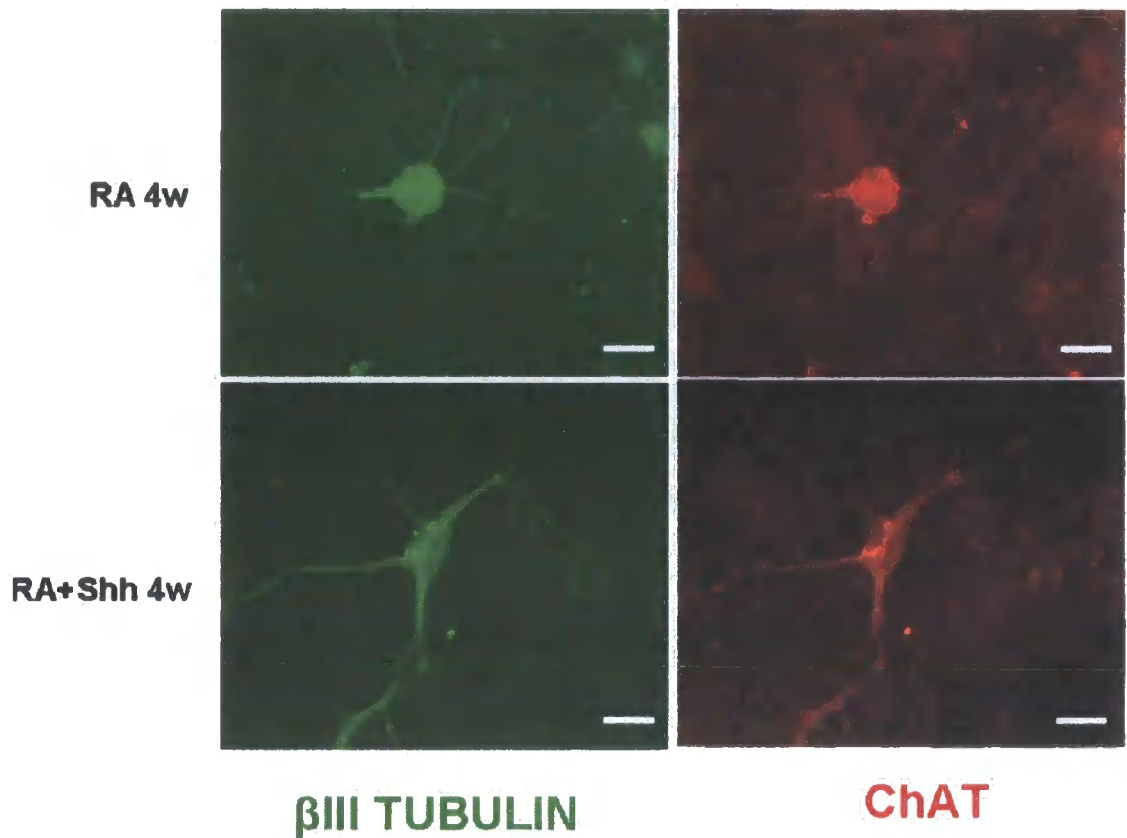


Figure 3.6.3 The expression of ChAT in induced EC cultures. EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10 $\mu$ M) or Shh (25ng/ml) for 4 weeks prior to fixation with 4% PFA and labelling with antibodies specific to ChAT (red) and  $\beta$ III tubulin (green). Note that the neuronal aggregates are positive for both  $\beta$ III tubulin staining and ChAT staining in the cultures. Scale bars = 50 $\mu$ m.

### **3.3.7 A proposed molecular pathway of motor neuron specification in Tera2 EC cells**

From the previous expression data as determined by RT-PCR (Figure3.4.1-3.4.2), western blotting (Figure3.6.1 and Figure3.6.2A) and immunocytochemistry (Figure3.2.1-3.2.9, Figure3.3.1-3.3.3, Figure3.5.1-3.5.3 and Figure3.6.2B), a summary figure was constructed to explain this regulation in regard to motor neuron specification of human EC stem cells.

We can define the EC cells by the following characteristics: these cells express very weak Pax6, Dlx2, En-1 and Olig2, while Oct-4 expression (Figure 4.1.4.2) is abundant. These cells are not committed to the neural lineage. When inductive factors like RA and RA+Shh were added to these cells, more genes were activated like the dorsal patterning genes *Pax3* and *Pax7*, the ventral most patterning genes *Nkx2.2*. The weak expression of Dlx2 was activated rapidly and exclusively in the nuclei of the induced cells after 2 days exposure to the inductive signals, although this diminished within 1 week. The Patterning factor Pax6 was more slowly activated. Its most abundant expression is 1 week after the induction of cell differentiation. The expression was also confined to the nuclei of the induced cells. During this time, the stem cell marker Oct-4 was down regulated within 2 weeks (Figure 4.1.4). The expression of Gli1 and endogenous Shh were also down regulated after 2 weeks (Figure 4.3.2 and Figure 4.2.3).

From previous *in vivo* data, we know that Nkx2.2 must give way to Nkx6.1 before the motor neuron progenitor domain is defined (Vallstedt et al., 2001). Therefore, Nkx2.2 is transiently activated in induced EC cells, as detected by RT-PCR (Figure 3.4.2-Nkx2.2).

After 2 weeks induction, A2B5 positive cells were detected by flow cytometry, and there were over 70% of A2B5 positive cells in the culture (Figure 4.1.2.1). After three weeks treatment, the cells lose A2B5 antigen, while HB-9 and Isl1 staining were positive, therefore we proposed that there exist such a group of cells that express Lim3 (Figure5.3.5) and MNR2, which are the upstream transcription factor of HB-9 and Isl1. Because MNR2

is a transcription factor that its sequence is virtually identical to HB-9 and the ectopic expression of HB-9 mimics MNR2 expression *in vivo* but HB-9 is restricted to post-mitotic motor neuron, suggesting it has a later role in motor neuron differentiation (Tanabe et al., 1998; Arber et al., 1999). Although we did not examine the expression of MNR2 in EC culture, we proposed MNR2 is expressed upstream of HB-9 in EC cultures like it did *in vivo*. These cells are also Olig2 positive, the expression of *Olig2* was earlier than HB-9 by RT-PCR (Figure 3.4.2-Olig2 and 3.4.2-HB-9). This group of cells express A2B5, Olig2, MNR2 and Lim3, indicated that the cells have the ability to differentiate into motor neuron progenitors or oligodendrocyte progenitors.

It is difficult to 'catch' the cells and analyse them at this stage, however, we can test their differentiation ability by growing the cells in motor neuron favourable conditions and oligodendrocyte favourable conditions to test whether there are motor neurons and oligodendrocytes. Forming a motor neuron progenitor, the activation of Neurogenin2 (Figure 4.1.7) and the extinction of Olig2 will help the cells acquire general neuron identity and promote cell cycle exit (Mizuguchi et al., 2001; Lee et al., 2005), at the same time the expression of MNR2 and Lim3 will activate their downstream transcription factors to give the cells motor neuron identity (William et al., 2003). At motor neuron maturation, NeuroD, which is activated by Neurogenin2, is diminishing to allow motor neuron progenitors convert to mature motor neuron (Figure 3.6.1) (Bang and Goulding, 1996); with the help of RA, different subtype of motor neuron is specified (Goncalves et al., 2005a).

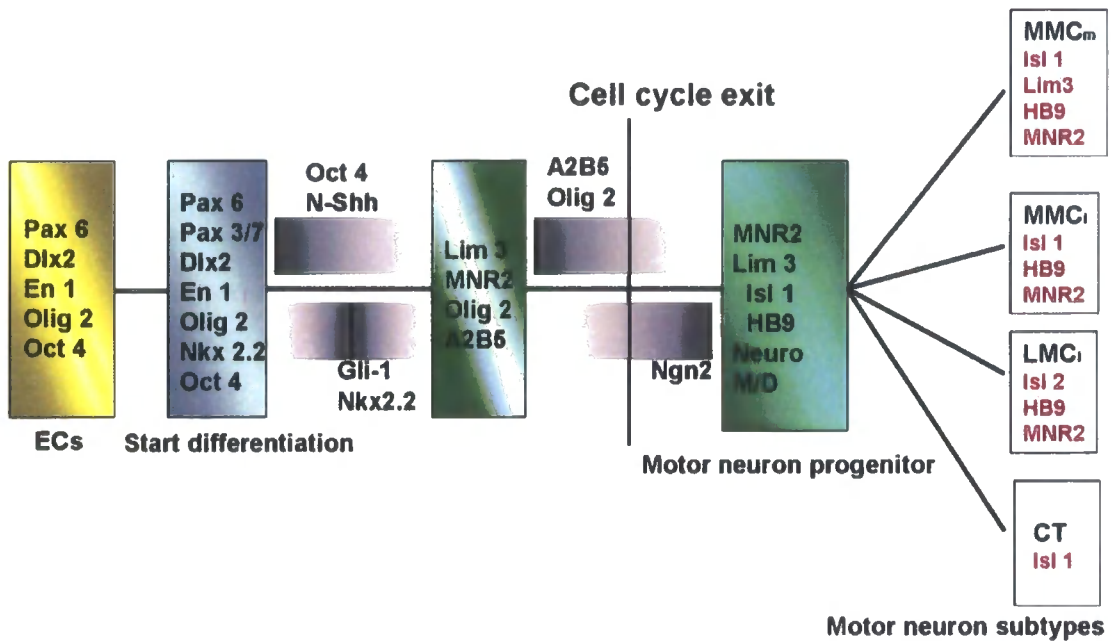


Figure 3.7 From expression profiling data a schematic diagram of molecular pathway involved in motor neuron specification and maturation from human EC cells was constructed. EC stem cells expressed low level of Pax6, Dlx2, En1, Olig2, but high level of Oct4. When EC stem cells are induced by RA and Shh, more patterning genes are expressed (Pax3, Pax7 and Nkx2.2) and the expression level of gene are up-regulated (Pax6, Dlx2, Olig2 and Gli1) and Oct4 is down regulated. Transient expressions of Ngn2 and NeuroD before motor neurons acquire their pan-neuronal identity. The distinction of A2B5 and the acquisition of HB-9 mark the establishment of the motor neuron identity. MMC<sub>m</sub>: Median Motor Column<sub>median</sub>; MMC<sub>i</sub>: Median Motor Column<sub>lateral</sub>; LMC<sub>i</sub>: Lateral Motor Column<sub>lateral</sub>; CT: Column of Terni.

### **3.3.8 Staining of cytokeratin8 a non-neuronal marker during Tera2.sp12 EC cell differentiation**

EC cells were induced by RA and /or Shh for 2 weeks, and then cells were trypsinized and replated and grown in low cell density for another 2 weeks under the same conditions, in order to allow cells to exit the cell cycle and mature within 2 weeks. We performed a double staining of  $\beta$ III tubulin and cytokeratin8 to examine the induced cells. In RA induced cells, we detected well differentiated  $\beta$ III tubulin positive cells and a small fraction of cytokeratin8 positive cells (Figure3.8.1). These two types of cells are not usually co-expressed in one cell based on the information by merging the staining of the antibodies. In RA+Shh induced cells, we observed the similar situation in the culture (Figure3.8.2). However, in Shh induced cells, we observed neither many well differentiated  $\beta$ III tubulin positive cells, nor any bright positive cytokeratin8 positive cells (Figure3.8.3). Furthermore, the morphology of many cells was very different from the RA and RA+Shh induced cells, which was still epithelial like without long processes. Nestin was checked in these induced cells, and only weak level of expression in RA and RA+Shh induced cells was noted. Whereas in Shh induced cells, there were still many nestin positive cells, and these nestin positive cells show no co-expression of cytokeratin8. If we blocked the Shh signalling by Shh antagonist cyclopamine, there were no nestin positive cells in the culture (Figure3.8.4). These data suggest that Shh signalling tends to keep cells at the progenitor stage, while RA appears to induce cells to terminal differentiation.

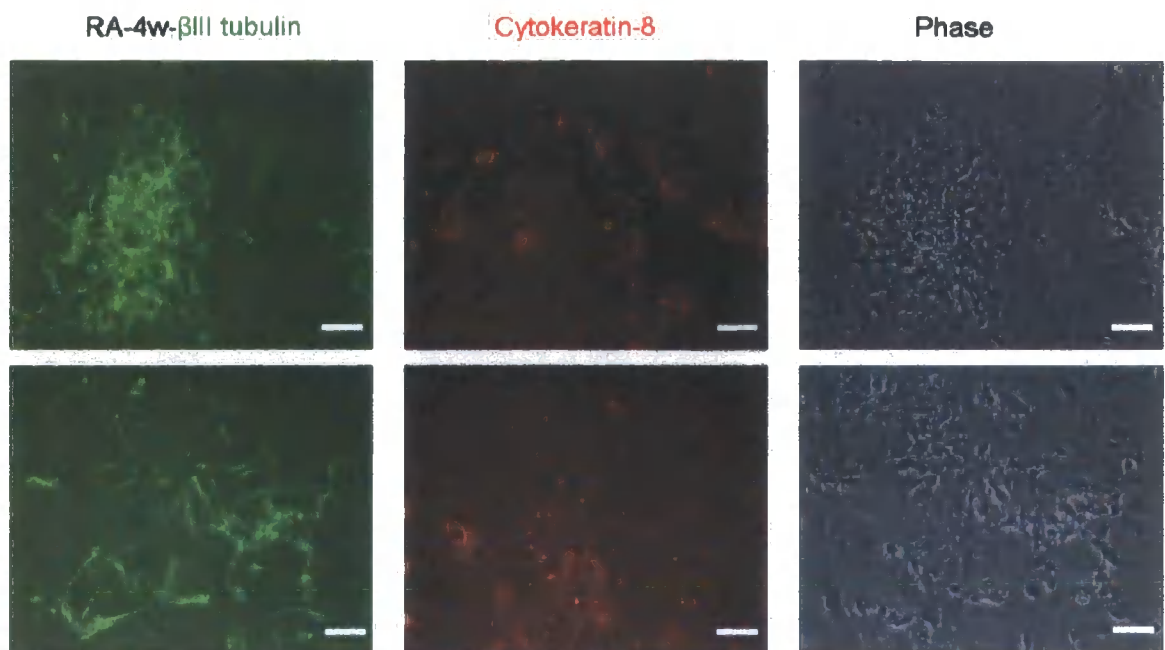


Figure 3.8.1 The expression of cytokeratin8 in RA treated EC cultures. EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10μM) for 2 weeks and then were replated and grown in low density (100,000cells/well) under the same condition for another 2 weeks prior to fixation with 4% PFA and labelling with antibodies specific to cytokeratin8 (red) and βIII tubulin (green). Note that the cells are positive for either βIII tubulin or cytokeratin8 but usually not co-expressed in the cultures. Scale bars = 50μm.

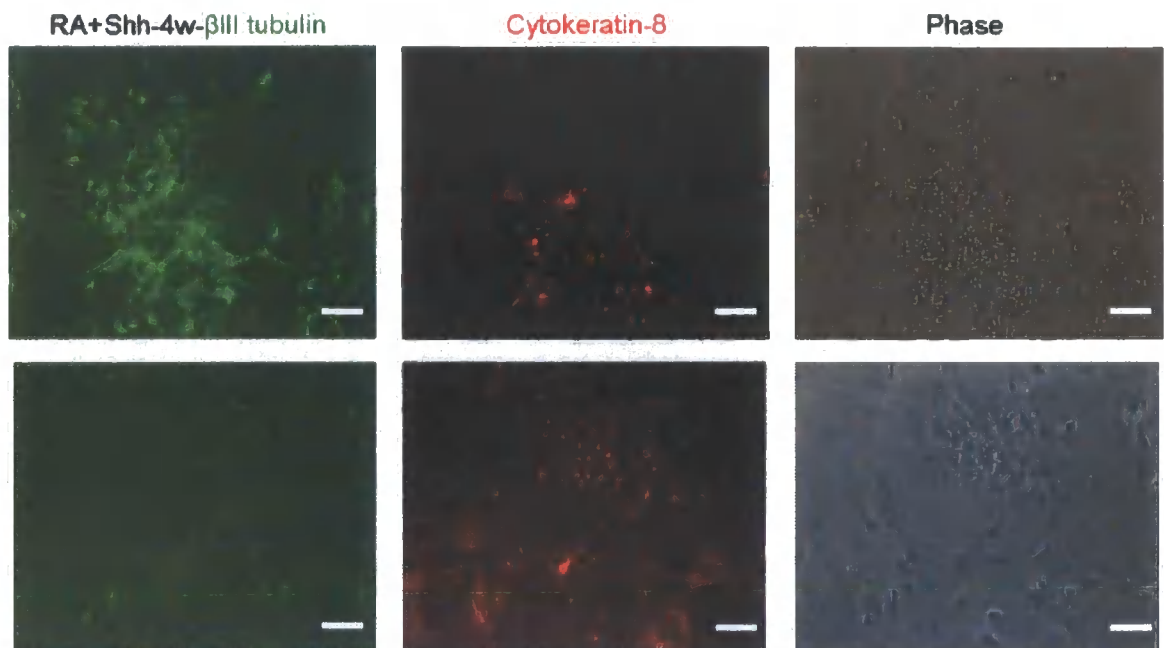


Figure 3.8.2 The expression of cytokeratin8 in RA+Shh treated EC cultures. EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10μM) and Shh (25ng/ml) for 2 weeks and then were replated and grown in low density (100,000cells/well) under the same condition for another 2 weeks prior to fixation with 4% PFA and labelling with antibodies specific to cytokeratin8 (red) and βIII tubulin (green). Note that the cells are positive for either βIII tubulin or cytokeratin8 but usually not co-expressed in the cultures. Scale bars = 50μm.



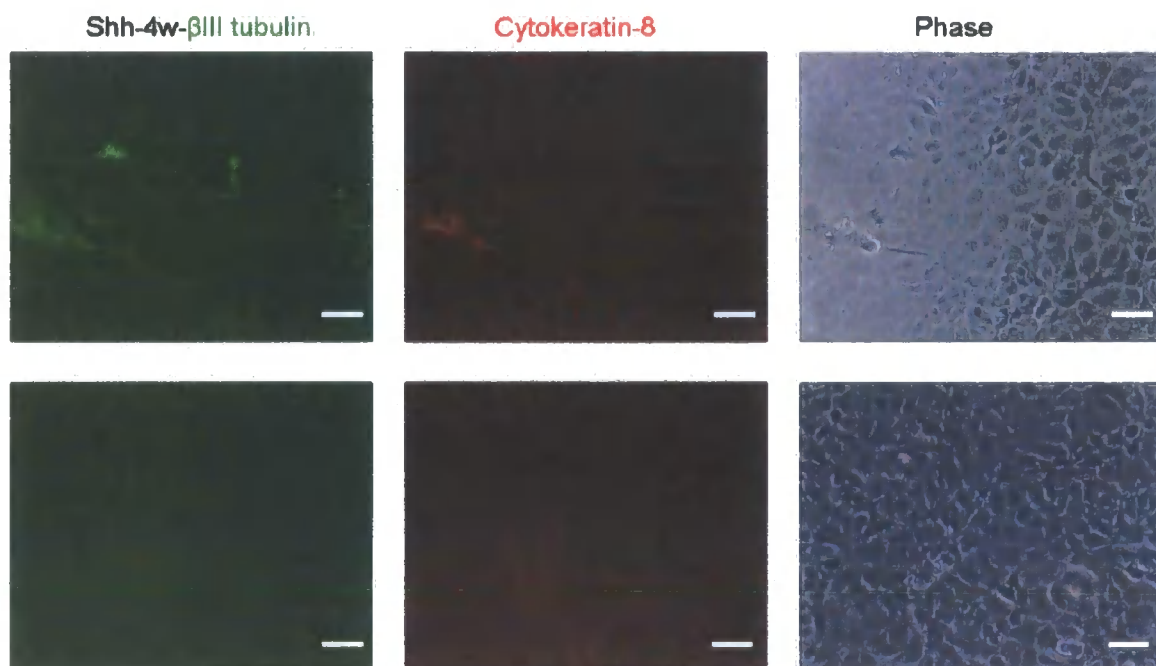


Figure 3.8.3 The expression of cytokeratin8 in Shh treated EC cultures. EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with Shh (25ng/ml) for 2 weeks and then were replated and grown in low density (100,000cells/well) under the same condition for another 2 weeks prior to fixation with 4% PFA and labelling with antibodies specific to cytokeratin8 (red) and βIII tubulin (green). Note that only a few cells are positive for βIII tubulin and cytokeratin8 staining in the cultures. Scale bars = 50μm.

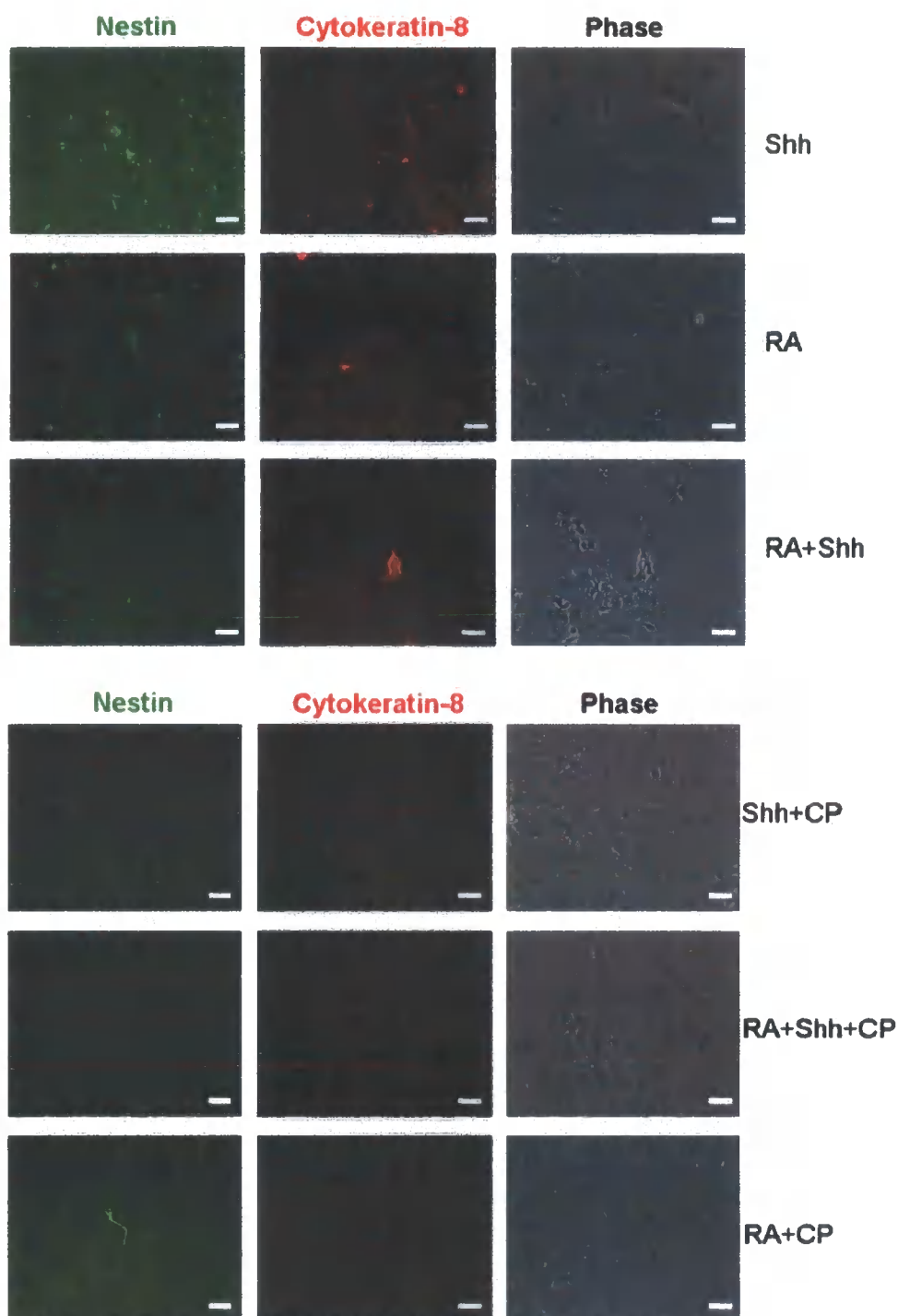


Figure 3.8.4 Comparison of nestin and cytoke­ratin-8 expression of induced EC cells with or without Shh antagonist cyclopamine (CP). EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10µM), Shh (25ng/ml) and CP (1µM) for 2 weeks and then were replated and grown in low density (100,000cells/well) under the same condition for another 2 weeks prior to fixation with 4% PFA and labelling with antibodies specific to cytoke­ratin8 (red) and nestin (green). Note that a few cells are positive for nestin and cytoke­ratin8 staining in the Shh cultures, while no nestin positive cells in the cultures supplemented with CP. Scale bars = 50µm

### **3.3.9 Different responses to cytokines after RA induction**

After induction by RA for 2 weeks, EC cells formed neural rosettes, round structures resembling neuroepithelium differentiated from ES cells (Zhang et al., 2007). In the developing spinal cord, neurons and Glia are differentiated from neuroepithelial cells, when neuroepithelial cells perceive additional environmental factors (Kalyani et al., 1997; Chandran et al., 1998). To generate neurons efficiently, information of cytokines and their ability to induce neurons from neuroepithelial cells will help to design novel neural induction strategies. After 2 weeks, different cytokines were added to the formed neural rosettes to replace RA signal. After 1 week, cells were fixed and assessed for their ability to induce Tuj1 positive cells (Figure 3.9.1-3). In RA induced cells, abundant Tuj1 positive cells with extended neurites were seen. The effect of Shh signalling was then investigated. A few Tuj1 positive cells were noted when Shh was added to the culture. In contrast, more Tuj1 positive cells with very long neurites were present in the cultures, if cyclopamine or SANT-1, the Shh pathway inhibitor, was added to the culture, suggesting that inhibition of Shh signalling allows the neuroepithelial cells to differentiate into mature neurons and also the neurites outgrow from neurons.

We next examined the effect of different BMP proteins on neural rosettes (Figure 3.9.2). Only weak expression of Tuj1 was observed in BMP2 induced cells and a few strong Tuj1 positive cells, suggesting that BMP2 induced no significant neurogenesis of neuroepithelial cells. In BMP4 induced rosettes, neuron-like cells with short neurites were observed. Slightly more neuron-like cells were observed in BMP6 or BMP7 induced rosettes, these cells were characterized by their small round cell bodies with very long extended neurites. The Tuj1 positive cells BMPs induced are different from those RA induced cells. This data that different BMP proteins have different ability in inducing neurons, especially BMP2

induces the least neurons; furthermore, BMP2 is a critical factor to induce dorsal interneuron from ES cells (Murashov et al., 2004).

No strong positive Tuj1 cells were observed, when FGF2 was added to the rosettes, but the long processes of the cells suggesting their neuronal identity (Figure3.9.3). In PDGF induced cells, we detected no obvious neuronal like cells and no positive Tuj1 staining. In EGF induced cells, only a few Tuj1 positive cells observed. However, in CNTF induced cells, we observed many Tuj1 positive cells with very long neurites and small cell body, suggesting that CNTF has the ability to induce neurons.

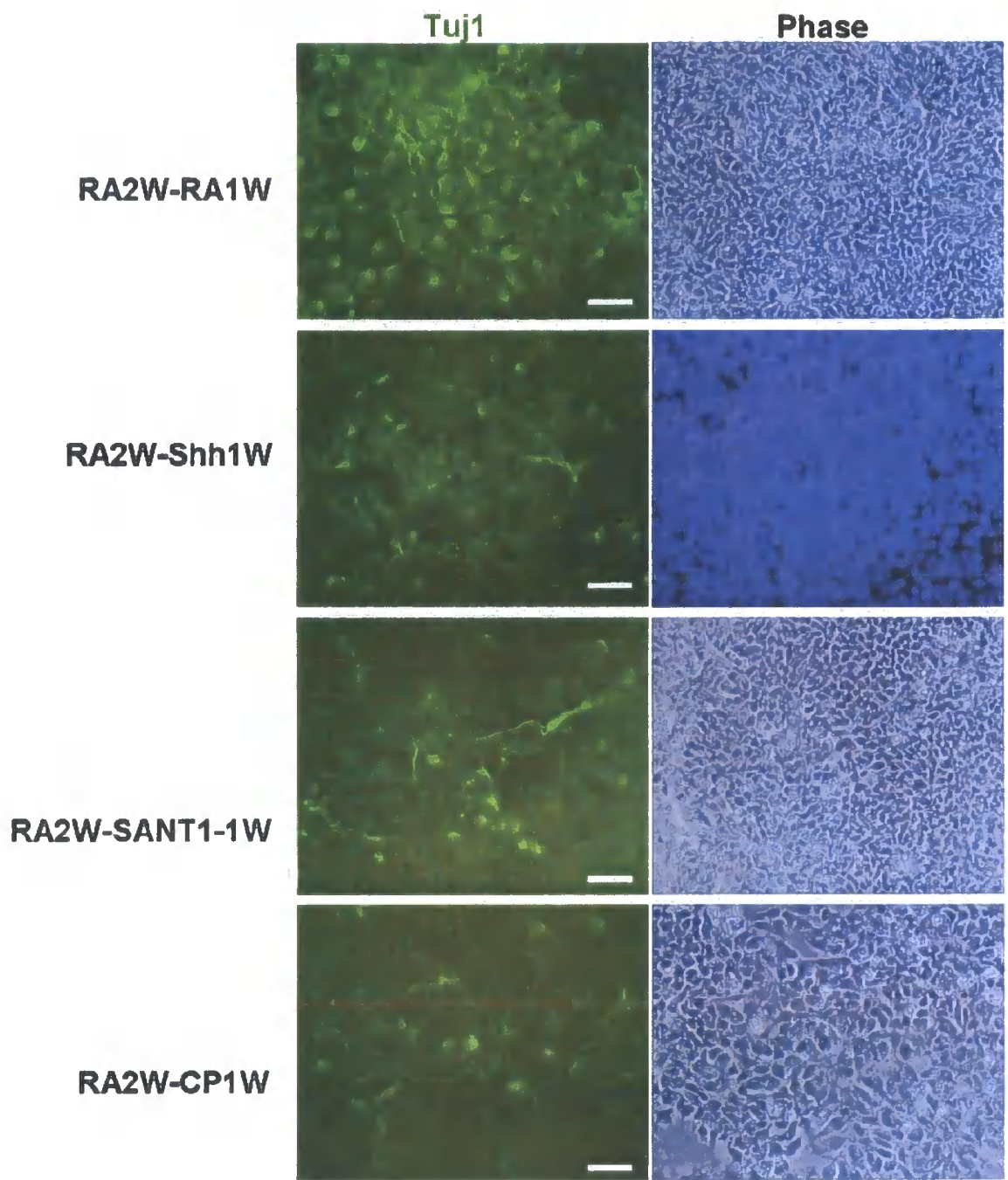


Figure 3.9.1 The staining of  $\beta$ III tubulin in 1 week Shh and its antagonists treated rosettes. EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10 $\mu$ M) for 2 week before Shh (25ng/ml) or CP (1 $\mu$ M) or SANT-1 (30nM) was added for 1 week. The nuclei were counterstained with Hoechst or a phase contrast image is shown instead. Note that  $\beta$ III tubulin staining is positive in RA, while much less positive cells in the Shh treated culture. Scale bars = 50 $\mu$ m

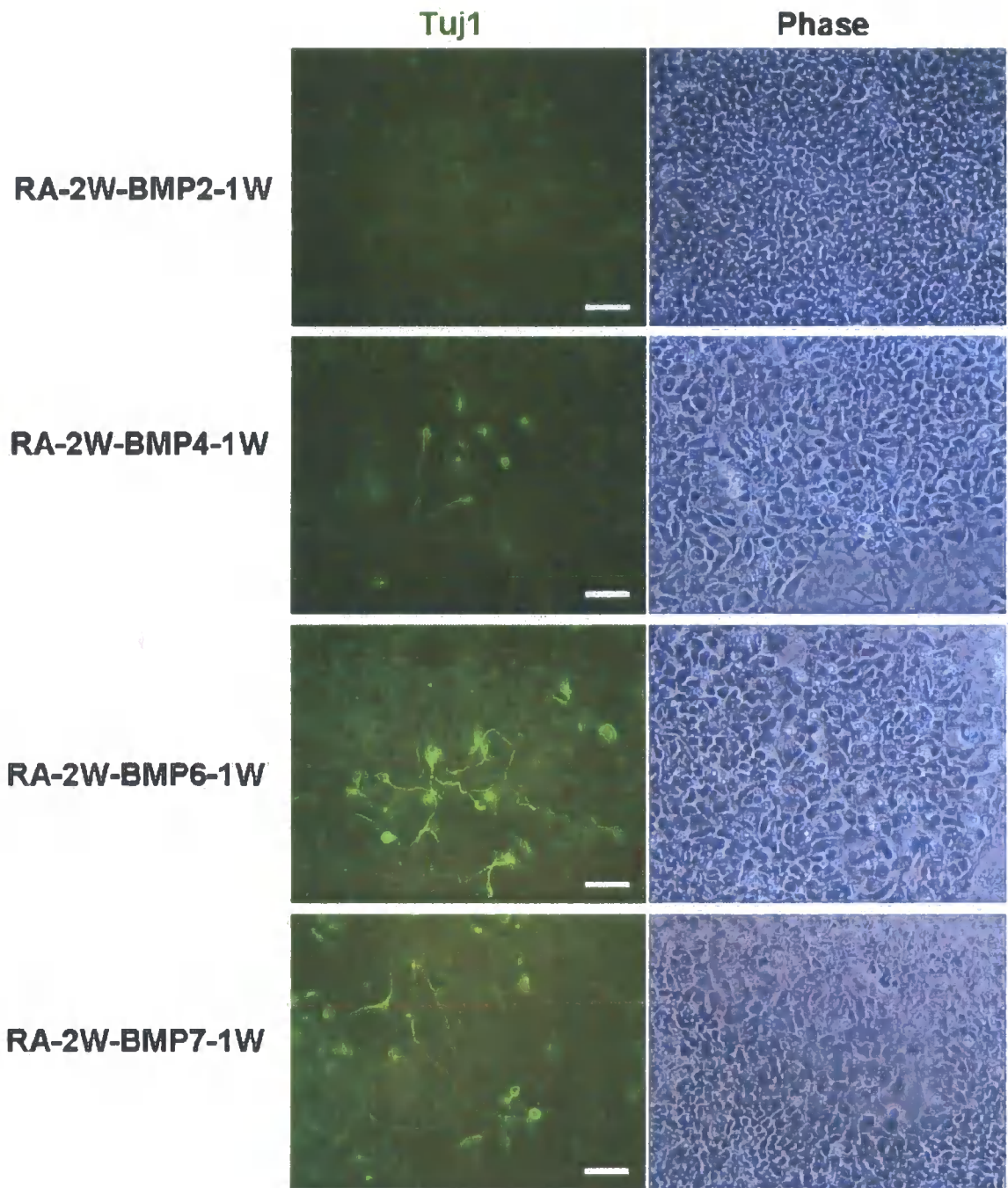


Figure 3.9.2 The staining of  $\beta$ III tubulin in 1 week BMPs treated rosettes. EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10 $\mu$ M) for 2 week before BMPs (10ng/ml) was added for 1 week. The phase contrast images are shown. Note that  $\beta$ III tubulin staining is weak in BMP2, while more positive cells in the BMP-4, -6 and -7 treated cultures. Scale bars = 50 $\mu$ m

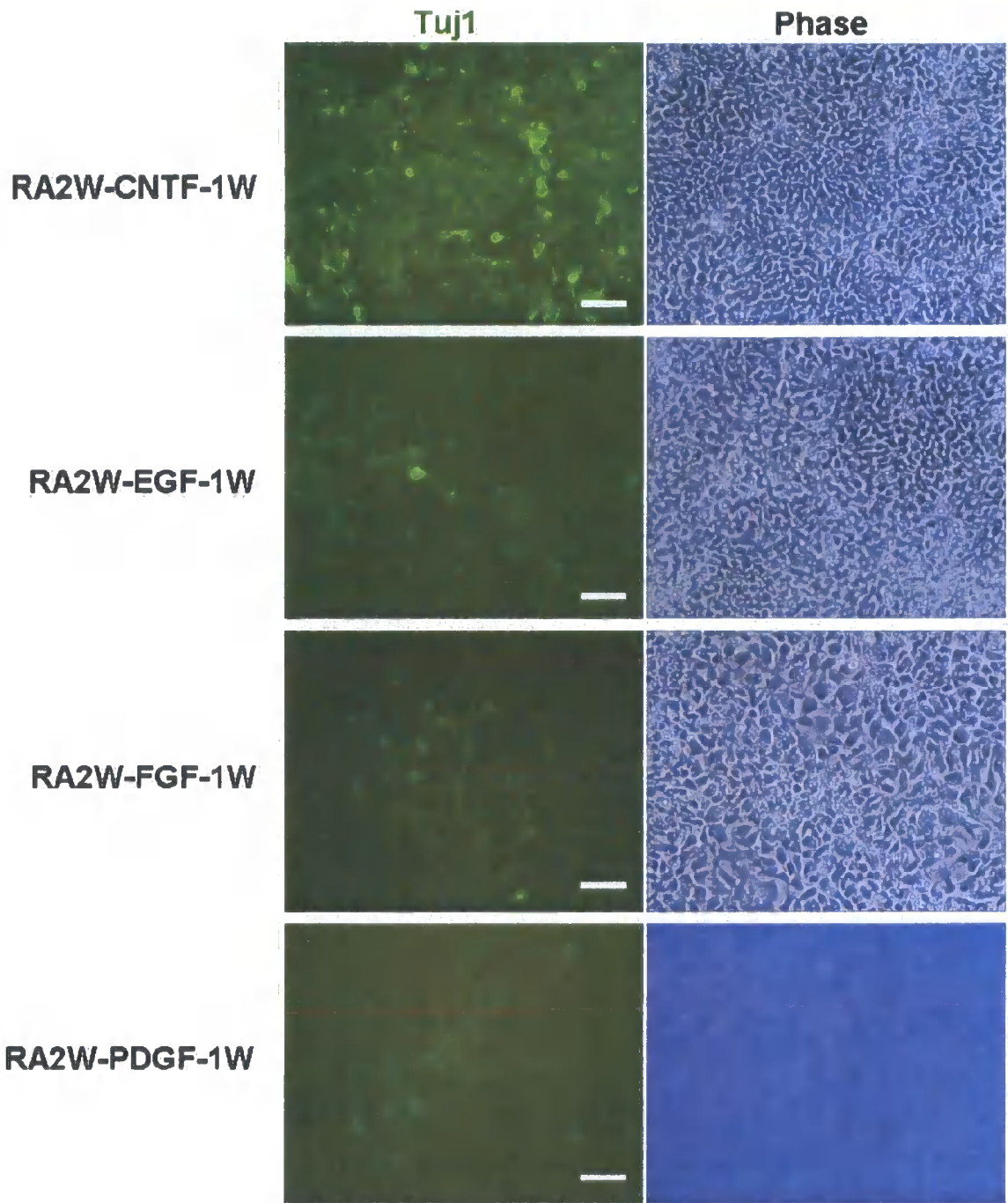


Figure 3.9.3 The staining of  $\beta$ III tubulin in 1 week various cytokines treated rosettes. EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10 $\mu$ M) for 2 week before CNTF (10ng/ml) or EGF (10ng/ml) or FGF (10ng/ml) or PDGF (10ng/ml) was added to the cultures for 1 week. The nuclei were counterstained with Hoechst or a phase contrast image is shown instead. Note that  $\beta$ III tubulin staining is positive in CNTF induced culture, while much less positive cells in the PDGF treated culture. Scale bars = 50 $\mu$ m

### **3.3.10 Effect of RA+Shh on cell proliferation**

From previous results, it is evident that RA and RA+Shh induce similar neural rosettes from EC cells. Neural rosettes were formed when EC stem cells were induced by RA and RA+Shh for 2 weeks. The RA induced rosettes and the RA+Shh induced rosettes were grown with different cytokines for another week before assessing their proliferation ability by Ki67 staining (Figure 3.10.1-3.10.3). Ki67 positive cells were detected when RA, Shh, RA+Shh or FGF-2 was added to the RA+Shh induced rosettes. In contrast, these cytokines induced no Ki67 positive cells when they were added to the RA induced rosettes. This suggests that RA+Shh induced rosettes have more proliferative cells, and Shh is preserving cell proliferation ability in this combination. This provides us a tool to generate greater number of neurons.



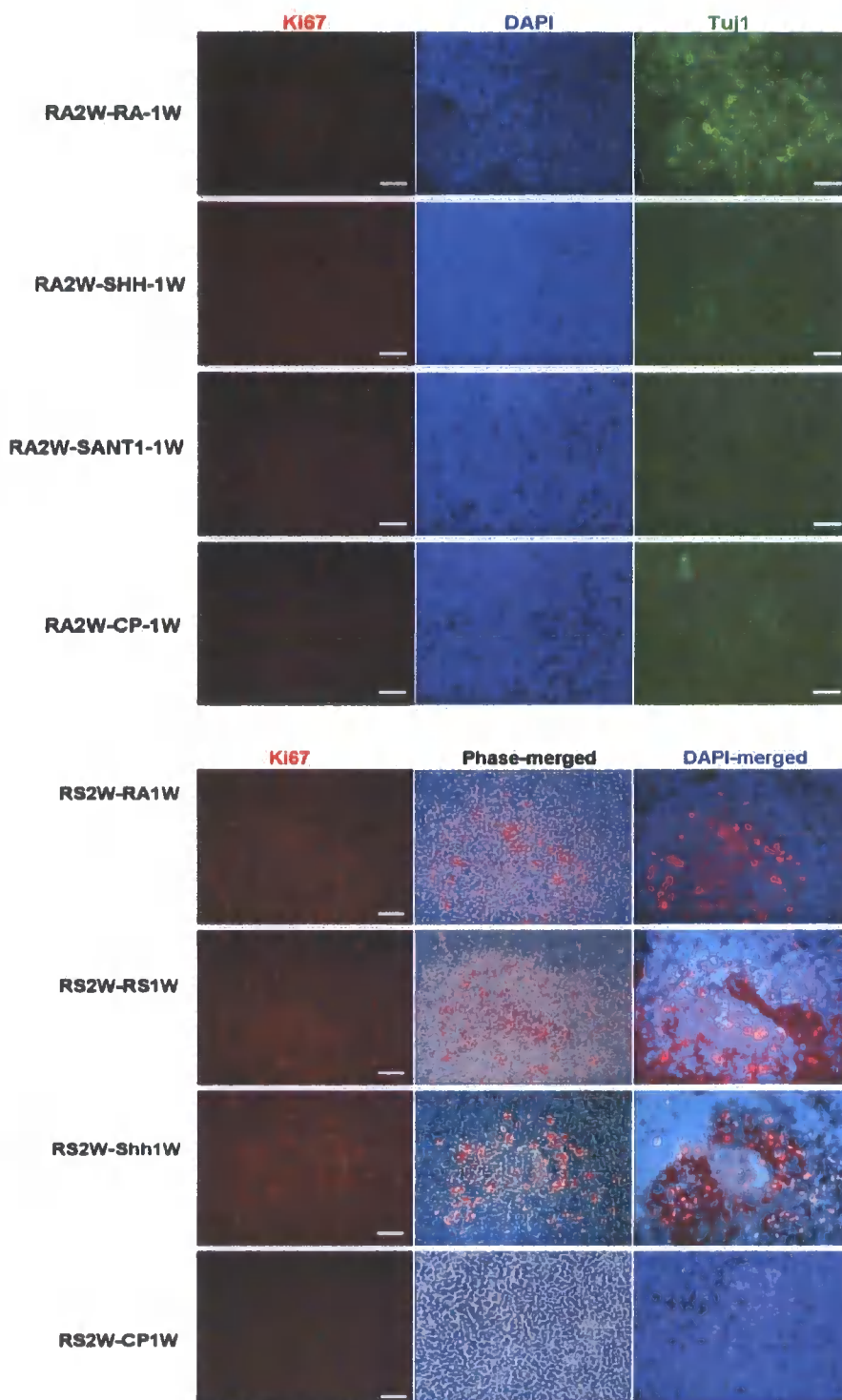


Figure 3.10.1 The staining of Ki67 in 1 week Shh and its antagonists treated rosettes. EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10µM) and/or Shh (25ng/ml) for 2 week to allow rosettes formation before Shh (25ng/ml) or CP (1µM) or SANT-1 (30nM) was added to the RA induced rosettes (upper panel) or RA+Shh induced rosettes (lower panel) for 1 week. The nuclei were counterstained with Hoechst or a phase contrast image is shown instead. Note that Ki67 staining is negative in RA induced rosettes, while more positive cells in the RA+Shh induced rosettes. Scale bars = 50µm

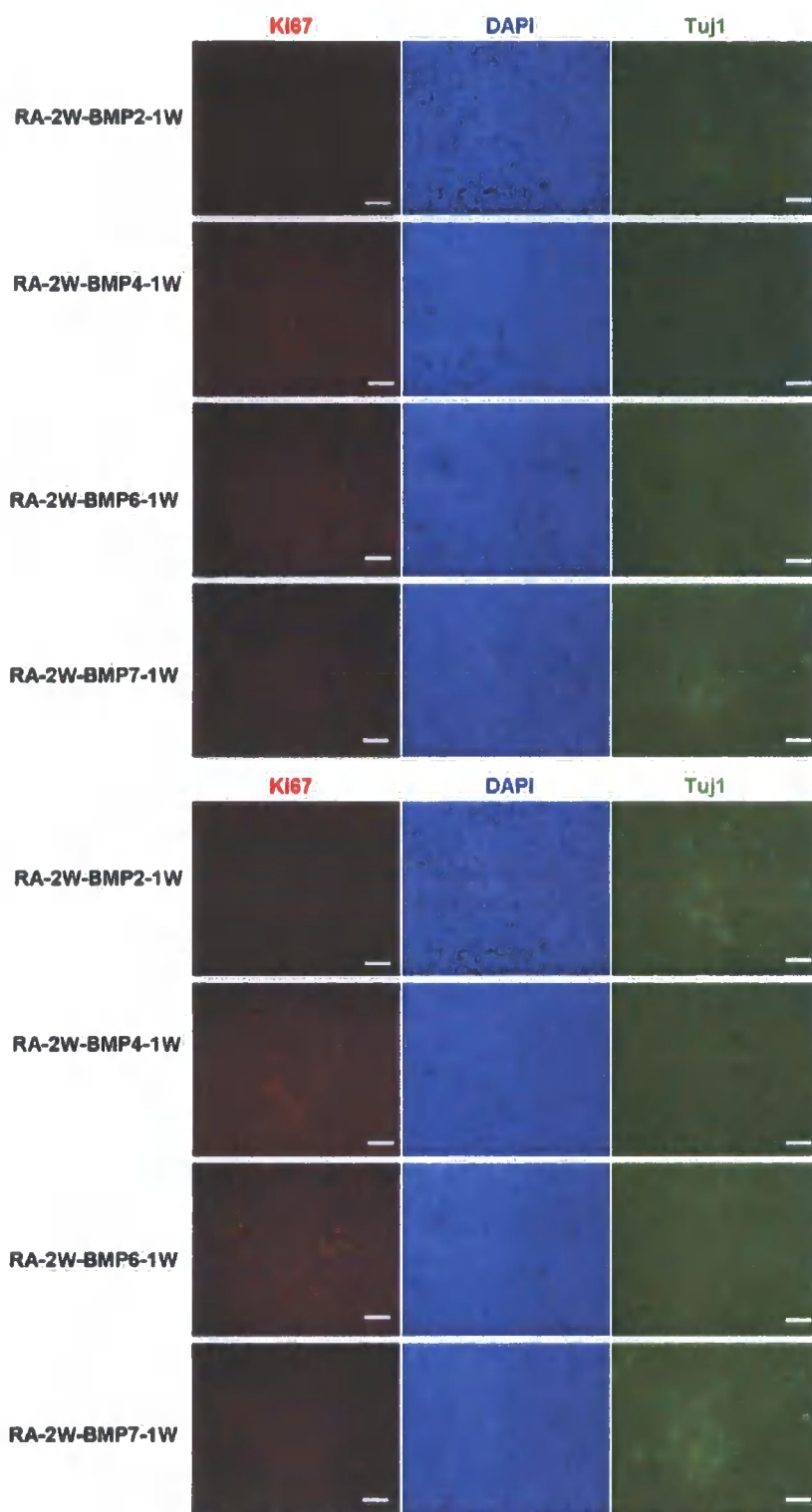


Figure 3.10.2 The staining of Ki67 in BMPs treated rosettes for 1 week. EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10 $\mu$ M) and/or Shh (25ng/ml) for 2 week before BMPs (10ng/ml) was added to the RA induced rosettes (upper panel) or RA+Shh induced rosettes (lower panel) for 1 week. Note that Ki67 staining is weak in all the treated rosettes. Scale bars = 50 $\mu$ m

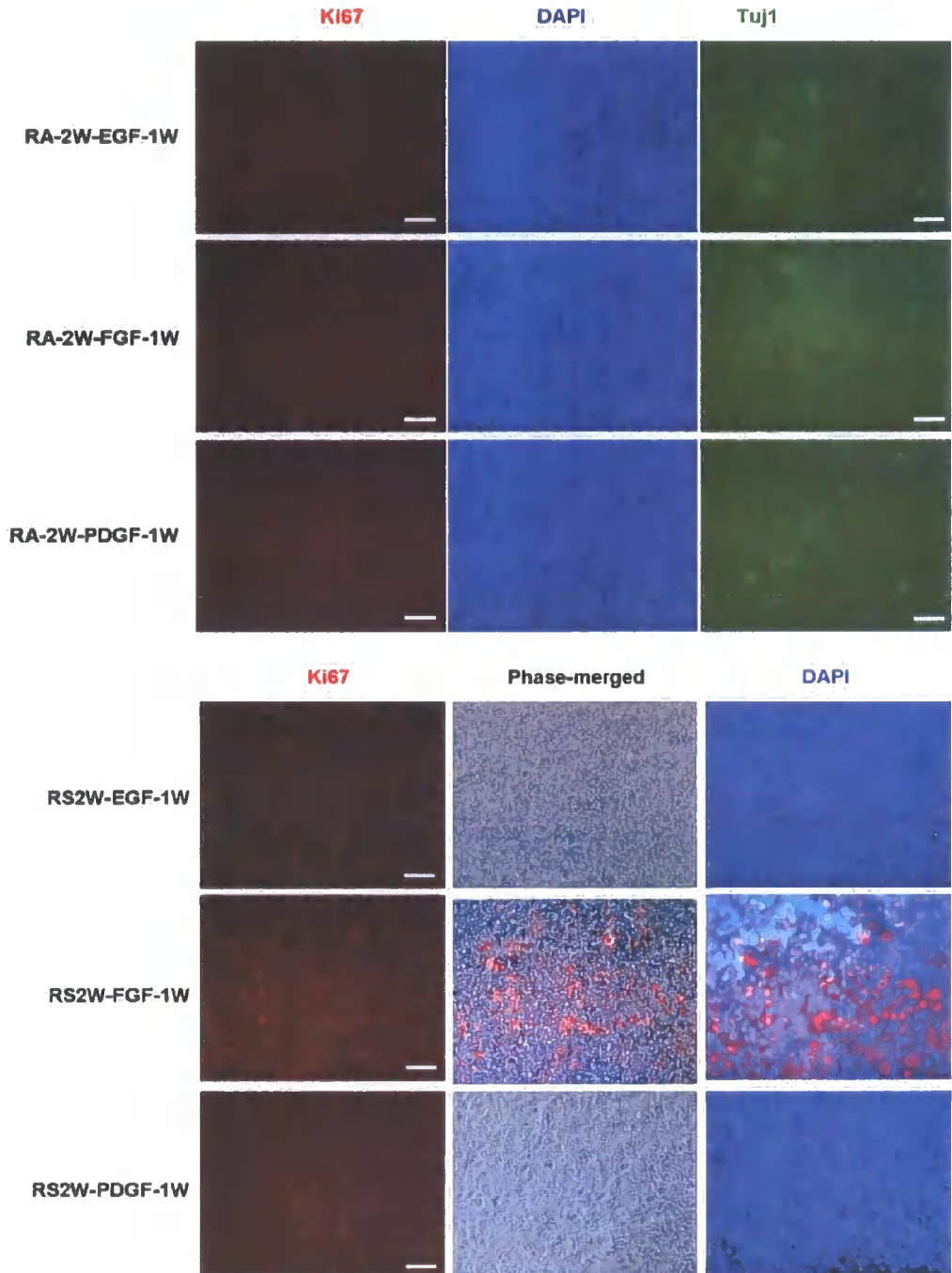


Figure 3.10.3 The staining of Ki67 in various cytokines treated rosettes for 1 week. EC cells were seeded in the 12 well plate at 20,000 cells/cm<sup>2</sup> supplemented with RA (10μM) and/or Shh (25ng/ml) for 2 week to allow the rosettes formation before CNTF (10ng/ml) or EGF (10ng/ml) or FGF (10ng/ml) or PDGF (10ng/ml) was added to the RA induced rosettes (upper panel) or RA+Shh induced rosettes (lower panel) for 1 week. The nuclei were counterstained with Hoechst or a phase contrast image is shown instead. Note that Ki67 staining is positive in FGF induced RA+Shh rosettes, while no positive cells in the RA induced rosettes. Scale bars = 50μm

### 3.4 Discussion

Previous work in the laboratory has demonstrated that RA is a potent inductive factor in Tera2.cl.SP12 EC cell differentiation (Przyborski, 2001). Following the procedure described in methods and materials about EC cell differentiation, we were able to produce large, almost entirely pure population of neuronal aggregates from Tera2.cl.SP12 EC cells (Horrocks et al., 2003; Stewart et al., 2003). The change of cell surface marker is a sensitive way to monitor the differentiation of stem cells and it can be measured conveniently by flow cytometry. In our study, Tera2.cl.SP12 EC cells expressed high levels of the pluripotent stem cell markers SSEA-3 and TRA-1-60. RA exerts its effects within 24h of treatment, during this time Tera2.sp12 EC cells loss 20% of SSEA-3 positive cells, and lose more than 50% of positive cells after 3 days of treatment. It suggested that RA affect the EC stem cell surface marker expression and induced differentiation within 24 hours, which is consistent with the finding that RA induces or up-regulates its receptors expression in EC cells after 24 hours (Moasser et al., 1995). There is increased NSE expression in RA induced EC cells, indicating that RA signalling was mediated to its target gene and differentiation was progressing.

The data show that RA induced EC cells form a rosette like structures within 2 weeks differentiation. Neuro-epithelial rosettes are also observed human ES cells at 14days after removed from feeder layer for differentiation (Li et al., 2005). The cells in rosettes expressed the neural tube and radial-glia marker 3CB2 in their centre part and the neuronal marker Tuj1 at their edges. They also expressed the neural precursor markers nestin, neuro-ectodermal markers Pax6 and Sox1 and the midbrain marker Otx2, while a few cell co-expressed Pax2 and En1. Rosettes also expressed the forebrain marker BF-1 and co-expressed with the forebrain surface-embryonic marker-1 (FORSE-1) (Sonntag et al.,

2007). The neural rosettes derived from EC cells have a similar formation time with rosettes from ES cells. This structure is also found in teratoma after transplantation of Tera2.cl.SP12 EC cells into nude mice (Przyborski et al., 2004). EC derived rosettes expressed Tuj1 at the rosette edge and were Pax6 and nestin positive like rosettes derived from ES cells. We therefore conclude that neuro-epithelial rosettes formation is the necessary stage for neural differentiation in both EC and ES cells. Recently researchers have developed methods to differentiate ES cells into neuro-ectodermal cells. Given the dose relationship between EC and ES cells, we speculate that human EC and ES cell may use the same molecular mechanisms during differentiation to form rosette derived neuro-epithelial cells and subsequent specialized neural cells.

We have known that EC cells treated with RA for 2 weeks will develop rosettes and the cells in rosettes represent neural tube-like cells. We used these cells as a platform to study other growth factors that are present in central nervous system to explore their ability to generate neurons. We first study the inductive factors in the neural tube. If the cells are continuously induced by RA, cells will produce well differentiated  $\beta$ III tubulin positive neurons. However, if cells are induced by Shh, only a few less-differentiated neurons observed in the culture, suggesting that Shh signalling did not contribute to neuron maturation. It is demonstrated that BMPs are required in vivo for the generation of sympathetic neurons (Streit and Stern, 1999). In our study, BMP2 inhibits the neurogenesis of neural rosettes, whereas BMP-4, -6 and -7 induced a few neurons with very long neurites and small round cell body, suggesting that BMP signalling plays a role in specifying the neuronal subtype from these neural rosette cells. For other growth factors, CNTF, FGF and EGF induced neurons, while PDGF inhibited neurogenesis. This suggests that each growth factor has different effect on neuron differentiation, some may help in neurite subtype specification, and some may help in neurite extension, or even switch off the neuron fate.

To further explore how the growth factors regulate these neural tube-like cells will provide useful information about neural fate regulation in embryonic neural tube, and also will help in generating specific neuron subtype cells *in vitro*.

From the cell morphology and cell specific marker staining, we know that RA and RA+Shh have similar effects on EC cell differentiation. Both of them induced rosettes at 2 weeks time, and produced mature neurons after 4-5 weeks induction. Furthermore, if we use RA+Shh 2week induced rosettes as platform to study the growth factors such as FGF, there is no significant differences in neurogenesis between RA induced rosettes and RA+Shh induced rosettes. However, when we checked the proliferation association marker Ki67 in the induced rosettes, there are more proliferating cells in RA+Shh induced rosettes. In contrast, in the cultures of RA induced rosettes, no proliferative cells were observed. This suggests that Shh affect the cell proliferation by keeping cells in the cell cycle. Consistent with the observation *in vivo* in chick embryos, Shh pathway promotes the proliferation of neuroepithelial cells (Cayuso et al., 2006).

We can differentiate Tera2.cl.SP12 EC cells into neural tube-like cells by RA signalling, suggesting that these EC stem cells represent the cells in primitive ectoderm. The channel  $\mu$ -slide provide us a possibility to grow these ectodermal cells inside the channel on the slide and setup Shh and BMPs gradient concentration from the column at one end of the channel towards the other end. In this way, we study the interaction between Shh and BMPs in regulating the growth of EC cells. It is demonstrated that Shh opposes cell cycle arrest in epithelial cells (Fan and Khavari, 1999). More cells are at the Shh side and the cells are less differentiated at this side of the channel, suggesting that Shh induces EC cell proliferation like it does in epithelial cells. BMPs induced EC cells differentiation but not into neurons, the expression pattern of nestin in the channel also demonstrated that Shh induced progenitor cells proliferation. RA plays a critical role in regulating the transcription

factor expression. This is demonstrated in the RA deficiency model in quail. The VAD quail model provides *in vivo* information of how RA regulates dorso-ventral patterning transcription factors: the deficiency of RA results in a general effect of dorsal expansion of ventral genes and a reduction of dorsal located genes (Wilson et al., 2004). The staining intensity of Pax6 and Shh is summarised in the figure below. Comparing the two models, the expression pattern of the transcription factors in the channel  $\mu$ -slide model matched their expression pattern *in vivo*. This suggests that we can mimic the dorso-ventral patterning model by using channel  $\mu$ -slide. The expression pattern in channel  $\mu$ -slide suggested that RA also helps in maintaining ventral identity, and without RA signal, the expression domain will move dorsally.

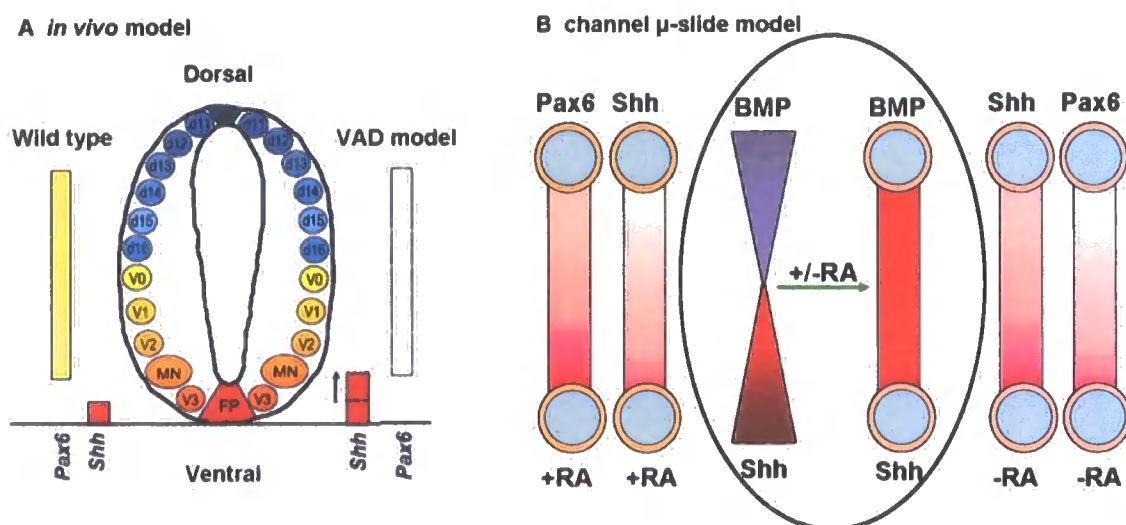


Figure 3.11 The expression pattern of transcription factors in channel  $\mu$ -slide model matches the *in vivo* spinal cord model. The BMP column of the channel mimics the roof plate of the spinal cord while the Shh column mimics the floor plate. The *in vivo* model is adapted after Wilson et al., 2004. VAD model is a model of RA deficiency.

These experiments also showed how RA regulates transcription factor expression. Pax6 is a ventral patterning gene, expressed in early neural tube precursor cells. In Shh induced cells and non-induced EC cells, cells express low levels of Pax6 and the Pax6 staining is

localized in the cytoplasm (Figure 3.5.3). When EC cells are induced by RA or RA+Shh, Pax6 is activated and moves into the cell nucleus, and Pax6 staining in the cytoplasm disappears. After 2 weeks induction, Pax6 expression is switched off in both cytoplasm and nucleus. We know that Pax6 is induced by RA signal and suppressed by Shh signal, but RA shows predominant effect in RA+Shh induced cells, and Shh appears not to prevent RA from activating the Pax6 expression. It remains unclear how Shh signal keeps Pax6 within the cytoplasm without entering the nucleus to activate the downstream transcription factors. Other transcription factors like Dlx2 also showed a similar pattern of expression, but it is activated within 3 days after the induction and the expression is switched off after 7 days induction. This data suggest that the patterning transcription factors are activated in EC cells by inductive factors in a sequential manner, and the transcription factors in the cytoplasm will not be able to activate the down-stream transcription factors, unless shuttled into the nucleus.

RA and Shh play important roles in motor neuron specification and maturation. In embryo, it is difficult to separate the RA and Shh effects during motor neuron generation. They work synergistically to specify motor neurons, and work sequentially. Shh operates during early differentiation and helps produce enough cells whereas RA works at later stages especially to mature the cells. In our *in vitro* cell model, we attempted to examine the effects of RA and Shh independently and in combination. EC stem cells are differentiated into neurons by RA not Shh, suggesting that RA is the key factor in EC cells acquisition of pan-neuronal identity, while Shh is the major regulator of EC cell proliferation. The interaction between these two will be addressed in Chapter 4.

Human ES cells have the ability to differentiate into all cell types of the body. It has been demonstrated that human ES cells can be differentiated into motor neurons (Li et al., 2005; Shin et al., 2005). Human ES cells from embryonic bodies were collected and grown as cell



aggregates in the ES cell growth medium for 4-5 days. After the addition of FGF2 for another 4-5 days, the induced cell aggregates were differentiated into neuroectodermal cells (rosettes) before further differentiated into motor neurons. In the presence of Shh and RA, the induced neuroectodermal cells adopt a motor neuron fate. The differentiated motor neurons induce the myotube twitching if co-culture with myotubes. Note that human ES cell-produced neuroepithelial cells are critical in neural differentiation. Addition of RA at the primitive stage (10days after ES cell differentiation) of these cells, a spinal cord motor neuron identity will be adopted. At later stages, the neuroepithelial cells can differentiate into oligodendrocytes and astrocytes but not readily produce motor neurons (Zhang et al., 2007).

EC stem cells differentiate into motor neurons have not been reported yet. It is known that EC cells respond to RA and differentiate into neurons (Przyborski et al., 2003), but not many information about specific neuron subtypes, only dopamine neurons are reported (Cheung et al., 1999; Sasai, 2002). In this study, we characterised the human EC cell-produced neural rosette stage. The cells of neural rosette adopted a motor neuron cell fate like they did in human ES cell cultures, suggesting that human EC and ES cells share the same molecular mechanisms during the motor neuron differentiation. The generation of motor neurons from human ES cells requires activation of a set of transcription factors and inhibition of another set at the same time (Li et al., 2005). In human EC cells, the sequential activation/inhibition of transcription factors forms a molecular pathway during the specification of motor neurons. It is appropriate to believe that the detected molecular pathway from human EC cells also direct the specification of motor neuron from human ES cells.

