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The Role of Bone Morphogenetic Protein 7 in Cortical Neurogenesis

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THE ROLE OF BONE MORPHOGENETIC PROTEIN 7 IN CORTICAL NEUROGENESIS

A Thesis Submitted to the

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ABSTRACT

The cortex is formed through a series of well-organized processes by which neural progenitor cells proliferate, commit to different cell fates and differentiate to neuronal and glial cell types. These complex processes are regulated by a wide range of intrinsic and extrinsic factors, which include members of the bone morphogenetic protein (BMP) family and their receptors. Accumulating evidence suggests that BMP7, a factor clinically used to repair bone fracture and kidney failure, is involved in brain development by contributing to both neural induction and cell differentiation. In this study, the role of BMP7 in cortical neurogenesis was examined during embryonic development in mice. Specifically, neuronal differentiation was enhanced in cortical cultures following treatment with low concentration recombinant human BMP7, whereas higher concentrations enhanced glial differentiation. Comparative analysis of BMP receptor expression during cortical development indicated that activin receptor type IIB (ActRIIB) expression is significantly higher than other BMP receptors during neurogenesis, whereas it is significantly down-regulated following the onset of gliogenesis. Furthermore, the neutralization of ActRIIB in cortical cultures reduced levels of neuronal differentiation, suggesting its involvement in BMP7-induced cortical neurogenesis. Lastly, to further the study of BMP7, its signaling pathway and the downstream nuclear targets, lentiviral systems were developed and optimized to allow BMP7 overexpression and ActRIIB knockdown in the cortex. Similar to the commercially available recombinant BMP7, cortical neurogenesis was enhanced in the presence of lentiviral-derived BMP7.

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LIST OF ABBREVIATIONS
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aa	amino acid
AU	arbitrary units
AAV	adeno-associated virus
ActB	beta-actin
ActRI	activin receptor type I
ActRII	activin receptor type II
BDNF	brain-derived neurotrophic factor
BMP	bone morphogenetic protein
BMPR	bone morphogenetic protein receptor
bp	base pair
BrdU	bromodeoxyuridine
CFDA	carboxy-fluorescein diacetate
CNS	central nervous system
CX	cortex
cPPT	central polypurine tract
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
EPI	external plexiform layer of the olfactory bulb
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FDA	food and drug administration
FGF-2	fibroblast growth factor 2 (basic fibroblast growth factor)
GABA	gamma aminobutyric acid
GDNF	glial cell-line derived neurotrophic factor
GFAP	glial fibrillary acidic protein
hCMV	human cytomegalovirus
hPGK	human phosphoglycerate kinase
HIV	human immunodeficiency virus
kDa	kilo dalton
LTR	long terminal repeat
Lv	lentivirus
mRNA	messenger ribonucleic acid
NFDM	non-fat dry milk
NP	neural progenitor
NSC	neuronal stem cell
PGK	phosphoglycerate kinase
pH	measure of the acidity or basicity of a solution
PI	propidium iodide
PNS	peripheral nervous system
nAb	neutralizing antibody
NCAM	neural cell-adhesion molecule
RCL	replication competent lentivirus
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chainreaction

RT-qPCR	quantitative RT-PCR
SEM	standard error mean
SGZ	subgranular zone
shRNA	short-hairpin RNA
SIN	self-inactivating
SMAD	signaling molecules mothers against decapentaplegic homolog
Sox2	SYR-box-2
SVZ	subventricular zone
TBST	tris-buffered saline Tween-20
TGF-β	transforming growth factor Beta
Trypsin-EDTA	trypsin-ethylenediaminetetraacetic acid
TU	transducing unit
VSV-G	vesicular stomatitis virus envelope
VZ	ventricular zone
WPRE	woodchuck hepatitis posttranscriptional regulatory element

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1. INTRODUCTION

1.1. Cortical Development

1.1.1. The Cortex

The cerebral cortex is the largest division of mammalian brain, derived from the telencephalon, occupying the lateral anterior neural tube [Imayoshi *et al.,* 2008]. It is involved in higher functions such as thinking, sensory perception, movement, spatial reasoning and language. During development, the cortex employs a series of well-organized pathways, leading to the formation of six distinct layers, each of which has a specific composition of neurons varying in size, shape and function. Cortical neurons are in direct contact with glial cells (astrocytes and oligodendrocytes), providing crucial structural and metabolic support in the brain.

1.1.2. Cortical Neurogenesis

Several laboratories, including ours, have shown that that neural stem cells (NSCs) and neural progenitors (NPs) employ a well-orchestrated series of pathways to proliferate, and undergo cell fate commitment and differentiation into neuronal and glial cell types in the cortex [Qian *et al.,* 1998, 2000; Bani-Yaghoub et al., 2006, 2007; Dehay and Kennedy, 2007]. The process through which NSCs and NPs generate neurons is called neurogenesis [Qian et al., 1998; See Figure 1.1]. In the embryonic cortex, neurogenesis initiates from the ventricular zone (VZ), consisting of a thin layer of columnar neuroectoderm cells. As neurogenesis proceeds, the first group of neurons migrates from the VZ toward the pial surface to form the preplate. This structure is later split by migrating cortical plate (CP) neurons, dividing it into a superficial layer and a

deep layer. The cortex eventually forms six layers (I-VI), called marginal zone (MZ), cortical plate, subplate (SP), intermediate zone (IZ), subventricular zone (SVZ) and ventricular zone (VZ) [Temple and Qian, 1995; Kriegstein and Parnavelas, 2006].

In mouse, cortical neurogenesis initiates around embryonic day 10.5 (E10.5) and continues as the primary mode of differentiation up to E17 [Qian *et al.,* 2000]. Cajal-Retzius neurons are among the first neurons created and they migrate to form the preplate at E10.5-E11 [Shen *et al.,* 2006]. At this stage (early neurogenesis), each cortical progenitor can generate on average 10-15 neurons, whereas this number is reduced to 1-2 neurons at E13.5-E16.5. However, despite this age-dependent reduction, neurogenesis peaks at around E13 (mid-neurogenesis) due to the presence of a large number of dividing neural progenitors at this stage [Qian and temple, 2000]. At E16 (late neurogenesis), the neurogenic potential of cortical progenitors decreases, using a well-orchestrated program to initiate the generation of glial cells, called gliogenesis [Qian et al., 1998, 2000]. Thus, there are only a few astrocytes detected in the E16 cortex [Qian *et al.,* 2000]. However, the ratio of astrocytes increases dramatically during late embryonic and early postnatal development.

Together, these processes result in a total of 108.69±16.25 million cells in the mouse brain (170.68±13.86 billion cells in the human brain), of which 70.89±10.41 millions are neurons (86.06±8.12 billion neurons in the human brain) [Herculano-Houzel et al., 2006; Azevedo et al., 2009]. These studies further emphasize the importance of neurogenesis as a critical process in cortical development. Since cortical neurogenesis follows the same chronological order under intrinsic and extrinsic factors *in vitro,* I have used neural progenitors to study the role of BMP in cortical neurogenesis both *in vivo* and *in vitro.*

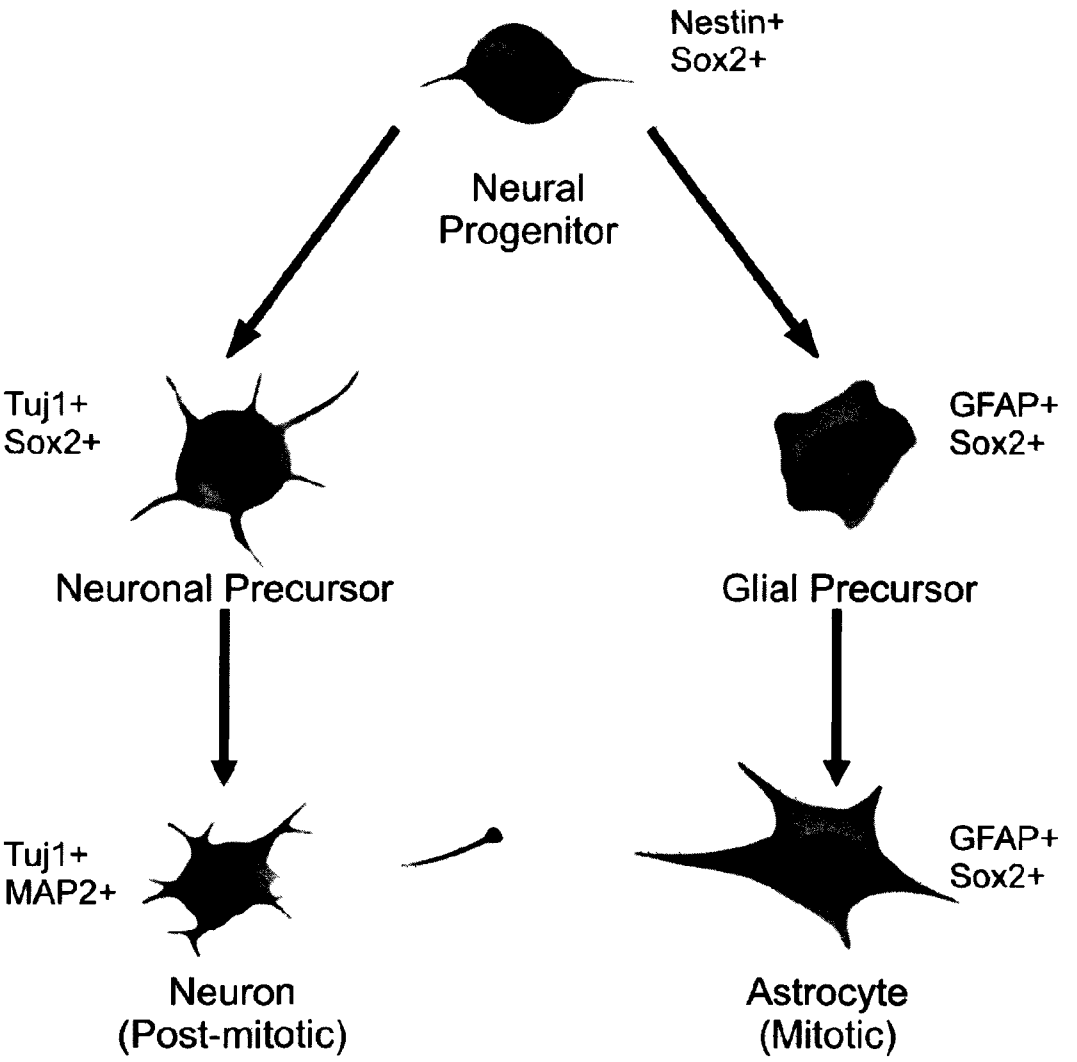

Figure 1.1: Stages of neural cell development. Neural markers change with the different stages of development providing a useful tool to assess cell fate determination.

1.1.3. Neural Stem Cell Maintenance and Differentiation

Despite our current knowledge of cortical development, the cellular and molecular mechanisms of neurogenesis require further investigation. Progress in recent years has identified several intrinsic and extrinsic factors that are involved in the spatiotemporal regulation of neurogenesis, including Sox2, fibroblast growth factor 2 (FGF2), TGF-β and various members of the bone morphogenetic protein (BMP) family. In particular, self renewal of neural stem cells is regulated in part by FGF, TGF-β and BMP signaling, and marked by expression of the homeodomain transcription factors NANOG and OCT4 and the HMG-box transcription factor Sox2. These transcription factors co-occupy and regulate many developmentally important homeodomain genes and collaborate to form an extensive regulatory circuit including autoregulation and feedback loops [Xu *et al.*, 2008]. For instance, through interactions with the minor groove of DNA, Sox proteins are involved in DNA bending, the altering of chromatin structure and the subsequent regulation of transcriptional events. Furthermore, to successfully complete neurogenesis, Sox2 expression must be significantly down-regulated or turned off, as Sox2 overexpression leads to inhibition of neurogenesis in neural stem and progenitor cells [Bani-Yaghoub *et al.,* 2006]. In contrast, Sox2 expression is maintained in glial progenitors and differentiating astrocytes. Only when astrocytes become quiescent, does Sox2 expression become undetectable. Lastly, Sox2 expression can re-initiate in quiescent astrocytes that become activated and undergo proliferation [Bani-Yaghoub *et al.,* 2006].

In turn, members of the Bone Morphogenetic Protein (BMP) family moderately repress Sox2 expression through the activated signaling molecules, mothers against decapentaplegic homolog (SMADs 1/5/8) in human embryonic stem cells (ESCs) [Greber *et al.,* 2008]. The moderate reduction of Sox2 expression in ESC cultures

treated with BMPs induces trophoblast differentiation, whereas, Sox2 is required for neuroectodermal specification and needs to be maintained until the initiation of neurogenesis [Greber *et al.,* 2008]. It remains to be determined how Sox2 expression is regulated by BMP7 and other extrinsic factors during cortical neurogenesis.

1.2. Bone Morphogenetic Proteins (BMPs)

1.2.1. The BMP Family

BMPs, which are members of the transforming growth factor β (TGF β) superfamily, were originally identified in bone and cartilage [Urist *et al.,* 1968]. However, despite their names, BMPs are not limited to skeletal tissues and are involved in a wide range of developmental processes including proliferation, differentiation and apoptosis in a tissue and time dependant manner [Hogan *et al.,* 1996]. For instance, BMP2-mediates the inhibition of neuroectodermal differentiation with an increase in mesoderm and smooth muscle differentiation in ES cells [Gossrau et al., 2007]. It also supports the generation of neural crest phenotypes and promotes distinct subtypes of peripheral neurons, including cholinergic and autonomic phenotypes [Gossrau et al., 2007]. BMP4 is essential in the regulation of retinal progenitor cell differentiation during eye development [Du *et al.,* 2010], and the formation of semicircular canals of the inner ear [Chang *et al.,* 2008] and trophoblast differentiation in human ES cells [Xu *et al.,* 2002]. Despite their distinct functions, BMP2 and BMP4 belong to the same subgroup of BMPs and share similar roles as their amino acid sequence is 92% homologous. In contrast, BMP7, which has emerging developmental significance, belongs to a different subgroup of BMPs, sharing only 60% homology to BMP2 and BMP4.

1.2.2. Bone Morphogenetic Protein 7

BMP7, also known as osteogenic protein-1 (OP-1), is crucial in the development of bones, kidneys and eye in both humans and mice [Dudley *et al.,* 1995; Arkell and Beddington, 1997; Gould *et al.,* 2002; Zouvelou *et al.,* 2009]. In particular, knockout studies show that BMP7 null mice are born with severe skeletal deformities and die shortly after birth, primarily due to severe renal hypoplasia and subsequent kidney failure [Dudley *et al.,* 1995]. Conditional knockout by flanking the first BMP7 coding exon with LoxP sites results in reduction in size, anophthalmia, kidney malformation and skeletal abnormalities such as polydactyl hind limbs [Zouvelou *et al.,* 2008]. In contrast, BMP7 treatment induces bone formation and kidney regeneration [Gould *et al.,* 2002; Gregory *et al.,* 2005], allowing its use in clinics to treat bone fractures and kidney failure. Furthermore, already approved by both Health Canada and the FDA, BMP7 has been used in over 300,000 patients to help repair acute fractures, bone non-unions and spinal fusions [Grgurevic *et al.,* 2007; White *et al.,* 2007]. In addition to its use in the skeletal tissues and kidney repair, it has been suggested that BMP7 applications may be extended to neurological diseases. In support of this notion, BMP7 has been shown to be highly expressed in the dorsomedial cortex during critical stages of embryonic development and in the brain following injury [Zouvelou *et al.,* 2009; Chou *et al.,* 2006]., suggesting possible roles in neurogenesis and neuroprotection.

1.2.3. BMP7 in Cortical Development

There is accumulating evidence that BMP7 is involved in brain development by contributing to neural induction, neuronal differentiation and dendritogenesis [Furuta *et al.,* 1997; Mabie *et al.,* 1999; Liu and Niswander, 2005; Dattatreyamurty *et al.,* 2001]. To mediate its effects in the brain, it is possible that BMP7 uses two separate but complementary modes of action. On the one hand, the presence of BMP7 transcript in the cortex raises the possibility that an autocrine pathway may be involved in this process, although the secretion of mature BMP7 protein by cortical cells has yet to be established [Greber *et al.,* 2008]. Parallel *in vitro* studies show that BMP7 mRNA is expressed during the expansion of neurospheres, but it is down-regulated, as cells differentiate [Deleyrolle *et al.,* 2006]. The lack of evidence supporting BMP7 protein synthesis in neural progenitor cultures raises the possibility that a negative regulatory mechanism exists downstream of transcription. Nonetheless, the activation of the downstream effector proteins of BMP7 signaling, SMAD1/5/8, is observed in only a small percent (<5%) of neural progenitors, suggesting a minor role for autocrine BMP signaling in the cortex [Mathieu *et al.,* 2008]. On the other hand, several laboratories have shown that biologically active BMP7 is secreted by the meninges and the choroids plexus into the cerebral spinal fluid (CSF), where it becomes available to the neural progenitors in a paracrine fashion [Furuta *et al.,* 1997; Liu and Niswander, 2005; Charytoniuk *et al.,* 2000; Krizhanovsky *et al.,* 2006; Dattatreyamurty *et al.,* 2001, Mathieu *et al.,* 2008]. In addition, BMP7 is present in mammalian CSF at relatively low concentrations (0.7 ng/mL in human CSF) [Dattatreyamurty *et al.,* 2001; Gautschi *et al.,* 2007].

Although CSF, and therefore BMP7, is accessible to the cells residing in the subventricular zone (SVZ), other molecules such as noggin are also secreted by ependymal cells to potentiate the formation of new neurons from SVZ and hippocampal-

derived neural progenitors [Lim *et al.,* 2000; Bonaguidi *et al.,* 2008]. The spatio-temporal regulation of BMP7 in neurogenic regions of the brain suggests that the formation of a BMP morphogen gradient may help regulate neural cell fate commitment in a concentration-dependant manner [Yung *et al.,* 2002]. In support of this notion, there is emerging evidence suggesting that low concentrations of recombinant BMP7 may support neuronal differentiation in the late embryonic cortical cultures, whereas high concentrations result in enhanced astroglial differentiation [Chang *et al.,* 2003]. It is also possible that, in addition to its role in cell fate determination, BMP7 can promote both neural cell survival and proliferation in adult hippocampal progenitors [Brederlau *et al.,* 2004]. While these studies are encouraging, the role of BMP7 in cortical neurogenesis still needs to be thoroughly examined while ruling out any indirect roles in cell survival or proliferation. Furthermore, as the BMP receptors display receptor specific functions during development, the BMP receptor(s) involved in this process need to be identified.

1.3. Bone Morphogenetic Protein 7 and its Receptors

1.3.1. BMP7 Protein Structure and Processing

Prior to its maturation, BMP7 is translated as an approximately 50 kDa (431 amino acids) precursor, having an N-terminal signal peptide sequence (29 amino acids), a propeptide domain (263 amino acids), and a mature peptide domain (139 amino acids) (Figure 1.2) [Gregory *et al.,* 2005]. The signal peptide targets immature BMP7 to the endoplasmic reticulum (ER) for post-translational processing. This event occurs via furine-like paired basic proprotein convertase (PACE), cleaving off the propeptide, resulting in a 30 kDa mature BMP7 homodimer [Gregory *et al.,* 2005]. Similar to the

other members of the BMP family, BMP7 forms homo- or heterodimers via C terminal disulfide bonds. Although mature BMP7 is a homodimeric protein consisting of two disulphide-linked 139 amino acid chains, the cleaved propeptide remains non-covalently associated with mature BMP7, guiding it to the extracellular matrix of target cells to initiate BMP signaling [Gregory *et al.,* 2005; Ozkaynak *et al.,* 1990; Swencki-Underwood *et al.,* 2008].

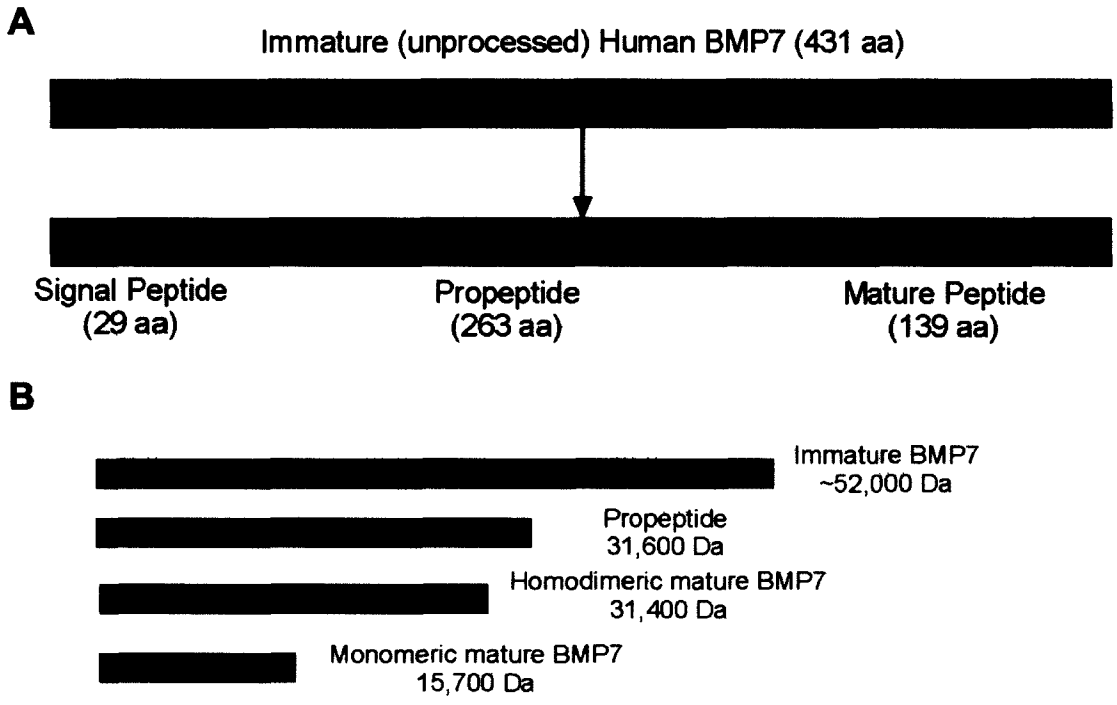

Figure 1.2: BMP7 processing. Mature BMP7 is processed from an immature precursor. (A) Immature BMP7 consists of a signal peptide, propeptide and mature peptide. (B) Mature BMP7 may exist as either a monomer or a homodimer.

1.3.2. The BMP7 Signaling Pathway

To initiate signaling, BMP7 binds to both members of the BMP and activin receptor families. These receptors are collectively referred to as type I and II BMP receptors and include BMP receptor type IA (BMPRIA), receptor type IB (BMPRIB), BMP receptor type II (BMPRII), Activin receptor type I (ActRI), Activin receptor type IIA (ActRIIA) and Activin receptor type IIB (ActRIIB) (Table 1). In the un-activated state, type I and II BMP receptors exist in both preformed type I/II heterotetrameric complexes and as separate type I and II heterodimeric complexes [Attissano and Wrana, 2002]. Separate type I and II receptor complexes signal through cooperative BMP signaling, which is mediated through a sequential binding mechanism: BMPs initially bind to the type II receptors followed by the recruitment of the type I receptors to form heterotetrameric receptor complexes each consisting of two type I and two type II receptors [Attissano and Wrana, 2002].

Table 1.1: BMP7 receptors and synonyms in *Homo sapiens* and *Mus musculus*

Gene	BMP Receptor Type	Common Synonyms
BMPRIA	Type I Receptor	*Alk3, BMPR1a*
BMPRIB		*Alk6, BMPR1b*
ActRI		*Alk2, ActR1a*
BMPRII	Type II Receptor	*BMPR2*
ActRIIA		*ACVR2*
ActRIIB		*ACVR2B*

To initiate signaling, the hydrophobic convex face of BMP7 interacts with the hydrophobic concave face of the highly conserved serine/threonine kinase receptors to

form the binding interface mediated via side chain interactions [Greenwald *et al.*, 2003]. Noteably, unlike BMP2 and 4, BMP7 can bind activin type II receptors as well as ActRI, possibly allowing BMP7 to stimulate other pathways [Zhao *et al.,* 2005; Yu *et al.,* 2005]. Nevertheless, the type II receptors are primarily responsible for ligand binding, whereas type I receptors carry on the signaling cascade following activation [Ten Dijke *et al.,* 1996]. Each BMP receptor consists of the ligand binding domain, a single transmembrane domain, and an intracellular serine kinase domain. Upon BMP7 binding, type II receptors phosphorylate and activate type I receptors, which then phosphorylate the associated receptor-regulated proteins, SMADs [Motazed *et al.,* 2008]. Once activated, SMAD1/5/8 dissociates from the receptor complex and binds to SMAD4, allowing the SMAD complex to translocate to the nucleus (Figure 1.3). Evidence indicates that SMAD1 can be detected in the nucleus within 20 minutes following BMP7 treatment [Guo *et al.,* 2001].

In the nucleus, the SMAD1/5/8 and 4 complex associates with DNA binding partners and transcription factors to regulate gene expression. In particular, muscle segment homeobox 1 (Msx1), a gene known to repress neuronal differentiation in early stage chick embryos contains several SMAD binding sites, indicating its role as a target of BMP signaling [Furuta *et al* 1997; Ramos and Robert, 2005; Ramos and Robert, 2005]. Interestingly, Msx genes are highly expressed in the embryonic dorsomedial cortex where BMP7 is abundantly expressed [Zouvelou 2009]. In addition to the *Msx* genes, BMP4-induced SMAD1/5/8 activation has been shown to moderately repress *SRY (sex determining region Y)-box 2 (SOX2)* in human embryonic stem cells [Greber *et al.,* 2008; Kobayashi *et al.,* 2008]. These results have been further supported by the data from our laboratory showing that Sox2 down-regulation is required for neuronal differentiation in neural stem and progenitor cells [Bani-Yaghoub et al., 2006].

Although SMADs appear to be the most popular intermediate molecules to conduct BMP signaling, other signaling molecules such as TGFβ activated kinase 1 (TAK1) and TAK1 binding protein 1 (TAB1) seem to contribute to this pathway as well [Yamaguchi *et al.*, 1995; Attisano and Wrana, 2002]. In this study, SMAD-mediated BMP signaling was examined during embryonic cortical neurogenesis.

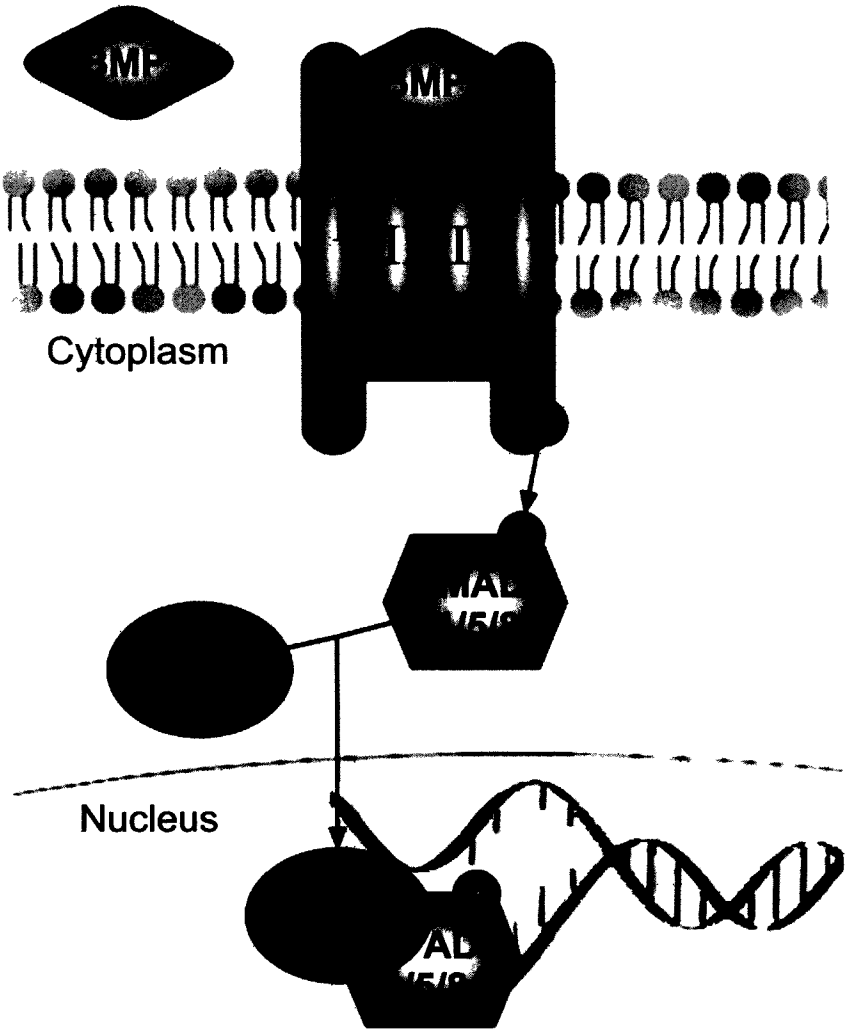

Figure 1.3: BMP signaling pathway. BMP7 signals through BMP receptors to activate SMAD signaling. BMP7 binds to Type II receptors which activate the Type I receptors to phosphorylate SMAD1/5/8 (pSMAD1/5/8). The phosphorylated SMAD1/5/8 complex associates with SMAD 4 and translocates to the nucleus to regulate gene expression.

1.3.4. Regulation of BMP7 Signaling

The BMP7 half-life has been reported as only 30 minutes in serum [Grgurevic *et al.,* 2007]. Additionally, BMP-receptor complex formation and turnover is extremely fast, as the half-life of the BMP7-tetrameric receptor complex appears to be as short as 10-100 ms [Mueller *et al.,* 2003]. Despite the short half-life for BMP7 and the BMP7-receptor complex, other ligands contribute in a spatio-temporal manner to ensure efficient signaling throughout development. For example, BMP antagonists such as Noggin [Chmielnicki *et al.,* 2004] and Gremlin [Greber *et al.,* 2008] bind to various BMP ligands and receptors, thereby regulating receptor-ligand association and preventing downstream signaling. Other mechanisms of regulating BMP signaling occur through Smurf1 (SMAD ubiquitin regulatory factor-1)-mediated SMAD1/5/8 (and possibly BMP receptor) degradation. Furthermore, binding of SMAD 6 to BMP type I receptors is involved in the intracellular regulation of SMAD1/5/8 activation [Chen, 2004].

BMP receptor affinities may also contribute to BMP ligand-specific roles. BMP7, in particular, has a higher affinity for type II receptors (with the exception of the type I receptor ActRI) relative to other BMPs such as BMP2 and 4, which preferentially bind the type I receptors. Furthermore, BMP7, TGFβ and activin all share preference for BMP type II receptors, however, the binding interface between BMP7 and type II receptors is distinct from that of TGF-β and activin [Greenwald *et al.*, 2003]. In support of this, discrete BMP receptor expression repertoires in association with various ligands have been suggested to fine-tune cell proliferation and differentiation during embryonic development [Deleyrolle et al., 2005; Xu *et al.,* 2008]. Therefore, the expression and regulation of particular BMP receptors may cooperate in regulating BMP7 signaling during cortical development and require further investigation.

1.3.5. BMP Receptors in Cortex

Previous studies have shown that BMP receptors (BMPR1A, BMPRIB, BMPRII, ActRI and ActRII) are present in the brain [Söderström *et al* 1996; Funaba *et al.,* 1997; Mehler *et al.,* 1997; Zhang *et al.,* 1998]. However, it has yet to be determined which BMP receptors are expressed during the various stages of cortical development. To this end, a few studies have revealed functional significance of certain BMP receptors in the cortex, using gene manipulation techniques such as overexpression and dominant-negative or null mutants. For instance, BMPRIA overexpression has been shown to inhibit neurogenesis and promote gliogenesis in the SVZ [Lim *et al.,* 2000]. In contrast, genetically manipulating the BMPRIA gene by cre/LoxP reduces the number of immature oligodendrocytes at birth, whereas, the calbindin positive interneurons are increased in the dorsomedial cortex [Samanta *et al.*, 2007]. In addition to its role in the SVZ, disruption of BMPRIA prevents the choroids plexus specification, further supporting the role of this receptor during cortical development [Hebert *et al.,* 2002].

BMP7 preferentially binds ActRI, but its affinity for BMPRIA and BMPRIB is increased when complexed with BMPRII [Chen *et al.,* 2004]. This differential binding between type I receptors and BMPRII appears to have no effect on neurogenesis, but may regulate cell survival and the generation of oligodentrocytes from glial progenitors [Brederlau *et al.,* 2004; Lim *et al.,* 2000]. In addition, BMPRII is involved in actin reorganization and dendritogenesis via LIM domain kinase-1 (LIMK1) activation [29]. Therefore, type I and type II receptors may serve complementary functions.

1.4. The Application of BMP7 in the Brain

In North America alone, over 2.5 million individuals suffer from traumatic brain injury (TBI) or stroke annually [American Heart Association]. Cortical injury resulting from TBI and stroke often leads to long-term disability and a significant reduction in quality of life. Current therapies, which include special diets and extensive rehabilitation programs are limited in their recovery potential, highlighting the need for new strategies in regenerative medicine. Thus, advancing our understanding of the role of BMP7 in cortical neurogenesis may lead to the development of new therapies in brain repair to treat cortical injury. In addition, as BMP7 has recently been shown to have a neuroprotective role in the brain, its clinical suitability is becoming more apparent [Chou *et al.,* 2006; Tsai *et al.,* 2007].

1.4.1 BMP7 Delivery

Direct BMP7 delivery methods into the brain via intraventricular or intracranial administration, can often be ineffective due to the short half-life of BMP7, non-specific tissue interactions and the requirement for multiple injections [Emerich *et al.,* 2004]. As a result, the reduced effectiveness in association with added costs plague direct delivery methods, highlighting the necessity for a more effective BMP7 delivery system [Song *et al.,* 2008]. Therefore, the development of a BMP7 gene delivery system may provide a more efficient and effective delivery system for future studies *in vivo.*

1.4.2. BMP7 Delivery via a Viral Vector

Due to recent advancements in the safety and efficiency of viral vectors, viral-mediated gene delivery may provide a valuable tool to study BMP7 in the brain. Viral-

mediated gene delivery is highly efficient, as long-term expression of a factor is possible following a single infection. Furthermore, depending on the type of viral vector used, genomic integration of a transgene can lead to the creation of stable BMP7-producer cell lines, eliminating the need for further genetic manipulation. Rather than infecting host tissue, such a system may be used for cell-mediated BMP7 delivery *in vivo*.

1.4.3. Lentiviral Vectors

Currently, lentiviral vectors (lentivectors) are amongst the most promising gene delivery tools into mammalian cells [Debyser, 2003; Dull *et al.,* 1998; Mitta *et al.,* 2005; Hioki *et al.,* 2007]. Lentivectors are derived from the human immunodeficiency virus (HIV-1) genome and enable the production of replication-incompetent lentivirus which can deliver genes up to 10kb in size to both mitotic and post-mitotic cells [Trono, 2000]. The ability of lentiviruses to transduce post-mitotic cells is of great importance and particularly useful in delivering genes to neurons. Another exciting property of lentiviral vectors is that transduction leads to stable integration of the gene of interest into the host's genome, therefore supporting consistent and long-term expression of a particular gene without selective pressure.

In terms of safety and their potential for *in vivo* studies, the third generation lentivectors are currently the most advanced and suitable lentiviral-based gene delivery system [Mitta *et al.,* 2005; Hioki *et al.,* 2007]. In contrast to the first generation lentivectors, the second and third generation vectors are devoid of several other genes that are crucial for viral pathogenicity but not essential for the production of functional lentivirus. Both the second and third generation lentivectors lack the accessory factors *nef, vif, vpr* and *vpu*, however, third generation lentivectors are unique in that they also lack the functionally important HIV regulatory gene, *tat*. The *tat* gene encodes for a

powerful transcriptional activator, which is required for efficient viral replication and viral gene expression. Particularly, in the third generation lentivectors, the replication function of Tat is replaced through the introduction of a constitutively-active cytomegalovirus (CMV) immediate-early promoter in the upstream LTR of the transfer plasmid. Replacing the gene encoding the undesirable Tat protein with the CMV promoter helps maintain sufficient transcription of the vector genomic RNA in Tat-deleted packaging systems [Debyser, 2003; Dull *et al.,* 1998]. Therefore, third generation vectors only require the viral proteins *gag*, *pol*, *rev* and *env* for the production of functional lentivirus significantly reducing the risk of forming replication-competent retrovirus (RCR) from a random recombination event.

1.4.4. Lentiviral Production

A variety of stable packaging cell lines have been generated to improve the safety and facilitate efficient large-scale viral production. In addition to improved efficiency, limiting the number of plasmids carrying the different viral proteins during transfection helps eliminate the risk of plasmid recombination events and the production of an RCR. Therefore, human embryonic kidney 293 (HEK293) cells have been engineered to stably express all or some of the necessary proteins (*gag*, *pol, rev* and/or *env*) for lentiviral production [Debyser, 2003; Broussau *et al.,* 2008]. Stable packaging cell lines that constitutively express *gag* and *pol* and conditionally express the cytotoxic viral proteins *rev* and VSV-G are among the most advanced packaging cell lines with the highest safety rating since co-transfection of multiple plasmids encoding for the different viral proteins is not required in this system. Thus, lentiviral production in these cell lines requires the transfection of only the unified transfer plasmid containing the LTRs, packaging signal (ψ), RRE and the transgene-encoding expression cassette [Broussau *et al.,* 2008].

1.5. Hypotheses and Objectives

1.5.1. Hypotheses

- BMP7 enhances the differentiation of cortical progenitors into neurons.
- BMP7-induced neurogenesis is mediated in a receptor-specific manner.

1.5.2. Objectives

- To characterize the role of BMP7 in cortical neurogenesis *in vitro*.
- To determine the expression level of the BMP receptors over different stages of cortical development both *in vitro* and *in vivo*.
- To examine the mechanism of BMP7-induced neurogenesis in a receptor-specific manner.
- To prepare and test a consistent and functional BMP7 delivery system.

2. METHODS

2.1. Cell Culture

2.1.1. Preparation of Primary Cortical Cultures

Cortical tissues were obtained from time-pregnant CD1 mice (Charles River, St. Constant, QC). Time-pregnant mice were sacrificed via CO_2 inhalation at gestational day 10-17 in accordance to CCAC standards (Protocol 2008.19, National Research Council Canada, Institute of Biological Sciences (NRC-IBS), Ottawa, ON). Embryos were dissected out of the amniotic sacs into Hank's Buffered Saline Solution (HBSS; Invitrogen, Burlington, ON) and placed on ice. The heads and the dorsal telencephalons were sequentially isolated under a dissection microscope. The dorsal telencephalons were freed of meninges and dissected further to isolate the embryonic cortex. Embryonic day 13 cortical neural progenitors (E13CX) were cultured on poly-*L*-lysine coated 6- or 12-well tissue culture plates (Nunc, Thermo Fischer Scientific, Rochester, NY, USA) in DMEM containing 10% FBS. Approximately 18 hours following plating, the FBS concentration was reduced to 1% FBS and after an additional 24 hours the cells were placed in serum-free conditions (DMEM + N2 supplement; Invitrogen) for the remainder of the experiment to limit astrocytes differentiation and indirect effects of serum proteins on progenitor cells.

2.1.2. BMP7 Treatment

Primary cortical cultures were treated with 1, 10, 20 or 30 ng/mL of commercially-available rhBMP7 (growth factor domain; R&D Systems, Minneapolis, MN, USA) or lentivirally-transduced 293 cell produced BMP7 (LvBMP7), which were added to the culture plates once every 24 hours. In the case of BMP7 treatment and lentiviral

infections, BMP7 treatment was withheld until 24 hours following the first lentiviral infection. Medium was replenished every two days during the course of the experiment and cells were examined up to 14 days *in vitro* (DIV).

2.2. Cellular and Molecular Assays

2.2.1. Immunocytochemistry

Cultured cells were fixed in 65% ethanol containing 0.15 M NaCl for 10 min at room temperature and stained according to Bani-Yaghoub *et al.,* (2006). Briefly, following fixation and prior to immunostaining cells were washed in PBS (3 x 5 min). Immunohistochemistry was performed on prepared slides containing 8 µm sections of paraffin-embedded tissue. The sections were initially de-waxed in 3 consecutive xylene baths for 5 min each and subsequently rehydrated with a graded ethanol treatment of 100%, 95% and 70% ethanol for 5 min each. Following rehydration, the sections were washed in PBS (3 x 5 min). Both fixed cultures and deparaffinized sections were then incubated for 30 min in protein block solution (Dako Cytomation, Inc, Mississauga, ON). Primary antibodies were diluted in Dako Antibody Diluent and approximately 100 uL were applied directly on top of the sections or fixed cells and allowed to incubate in the well or in a humidity chamber for 1 hour at room temperature. Primary antibodies included: Nestin (1:10; Chemicon-Millipore, Billerica, MA USA, USA), Sox2 (1:100; NRC-IBS, Ottawa, ON), βIII-Tubulin (1:1; Sigma-Aldrich, St. Louis, MO, USA), MAP2(a+b) (1:200; Sigma-Aldrich,), GFAP (1:200; DakoCytomation, Copenhagen, Denmark), BrdU (1:1000; Sigma-Aldrich). Followed by another wash step in PBS, samples were incubated for 1 hour with fluorescently-tagged secondary antibodies, washed in PBS (3 x 10 min) and rinsed in ddH_2O prior to application of Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA) and coverslips. Mounting medium was

prepared with the addition of 1 ng/mL of Hoechst solution (Sigma-Aldrich). Immunostained cells either on plates or slides were viewed under an Axiovert 200 M fluorescent microscope (Carl Zeiss, Toronto, ON) and subsequently stored at 4° C. For neuronal and glial differentiation studies, five independent studies were performed with at least 10,000 cells counted per culture condition.

2.2.2 Cell Survival and Proliferation Assays

E13CX cells grown in dissociated culture in the absence or presence of BMP7 were assayed for cell survival and proliferation, using 5-carboxyfluorescein diacetate (CFDA; Sigma-Aldrich), propidium iodide (PI; Sigma-Aldrich) and BrdU (Sigma-Aldrich), respectively. In addition to 1 ng/mL of BMP7 treatment, a higher dose (20 ng/mL) was added to a subset of cultures to examine possible roles in cell survival and differentiation at higher concentrations. Cell survival studies were performed 1DIV and 5DIV with the addition of 1 μM CFDA and 1 μg/mL PI. Approximately 20 min following the addition of CFDA and PI, surviving (CFDA-positive) cells were counted relative to dead (PI-positive) cells via fluorescence microscopy. To assay DNA synthesis (cell proliferation), E13CX cells were treated with or without BMP7 (1 ng/mL), and BrdU (10 μM) was supplemented to the culture media for 17 hours at 37° C. Cultures were fixed 7 hours following the removal of BrdU and washed in PBS (3 x 5 min). DNA was denatured with a 20 min incubation in 4N HCl and neutralized for 15 min with 100 mM sodium tetraborate (pH 9.0). Following an additional wash step with PBS (3 x 5 min), cultures were stained with anti-BrdU, as previously described. Cell proliferation over a 24 h period (spanning 1DIV to 2DIV) was examined, using fluorescence microscopy. Cell proliferation was assessed in over 2000 cells for each condition.

2.3. Gene Expression Analysis

2.3.1. RNA Isolation

RNA was isolated from mouse embryonic stem (mES) cells, E13 cortical cultures (0, 1, 3, 5, 9 DIV) and tissues derived from Embryonic day 10 brain (E10 brain) and E13, E16, post-natal day 10 (PN10) and adult neocortices using TriReagent and DNase I, as previously described in Bani-Yaghoub *et al.*, 2006. Briefly, 50mg of fresh tissue sample were ground or homogenized in 1 mL TRI Reagent solution. Cultured cells (>$1x10^6$ cells) were resuspended and lysed in Tri Reagent (1 mL per $5x10^6$ cells). Extracted total RNA was treated with Dnase I (Ambion/Applied Biosystems, Streetsville, ON) for 30 min at 37° C and quality was ensured via visualization of the 18S and 28S rRNA bands following denaturing agarose gel electrophoresis.

2.3.2. First-Strand cDNA Synthesis

Following the isolation of pure RNA, cDNA synthesis was performed using Superscript III reverse transcriptase, according to manufacturer's Protocol. The resulting cDNA was purified with a polymerase chain reaction (PCR) purification kit (Qiagen, Mississauga, ON) and cDNA concentrations were determined with an Oligreen assay kit (Invitrogen) and a SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.3.3. RT-PCR

Reverse transcriptase PCR (RT-PCR) was performed to detect the presence of BMP7 and the known BMP receptors in the developing brain and cortex. Primers were designed to function in both RT-PCR and RT-qPCR applications corresponding to the 3' portion of mRNA encoding BMPRIA, BMPRIB, BMPRII, ActRI, ActRIIA, and ActRIIB. Primer sequences and amplicon sizes are listed in Table 2. PCR was conducted with the

addition of 2 ng of cDNA in 25 uL reaction volumes under the following conditions: 1 activating step at 95° C for 2.5 min, followed by 35 cycles of denaturing at 95° C for 30 seconds, annealing (at primer-specific temp) for 30 s, and elongation at 72° C for 30 s. An additional elongation step was performed for 5 min at 72° C subsequent to the final cycle. The resulting amplicons were visualized on 1.5-2% agarose gels containing 30 ng/mL Ethidium Bromide. Densitometric analysis of the DNA bands was used to determine relative expression patterns based on the size (the sum of all the pixel values after background correction) and the intensity of a band (in pixels), using an Alpha-Innotech Fluor Chem 8900 imager and AlphaEase software.

2.3.4. Real-time RT-qPCR

Real-time RT quantitative PCR (RT-qPCR) was performed as a more accurate and sensitive method to determine relative BMP receptor expression throughout cortical development *in vivo* and *in vitro*. Three independent samples (2 ng of DNA each) were amplified in separate reactions in triplicate, using a Fast SYBR Green Master Mix (Applied Biosystems) on a 7500 Real-Time PCR System (Applied Biosystems). Analysis of the results was performed, using comparative quantitative methods ($2^{-\Delta\Delta Ct}$) where gene expression was normalized to the mean levels of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-Actin expression per sample. PCR efficiencies for all primers were determined using Real-Time PCR Miner [Zhao and Russell, 2005]. Fold-differences were shown relative to the least expressed BMP receptor. Primer efficiencies were also determined (post-hoc analysis) and summarized in Table 2.1.

Table 2.1: Primer sequences, properties and conditions used for qPCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Task	T_A	Produ
BMP7	GAGCTGTACGTCAGC	TGAACCAGTGTCTGGA	qPC	60	152
BMPRI	GGGAAATGGCTCGTC	CAACCTCACGCATGTC	qPC	60	108
BMPRI	AGCAATCGCCCATCG	TTCAAGCTCTCGTCCA	qPC	60	186
BMPRI	TCGATGTCCAGCACA	GGGCTAGGGATTCGAG	qPC	60	92
ActRIA	CACATTACCATGAAAT	CTCTTCAGATCTCGAT	qPC	60	169
ActRII	CTGCAGATGGACCCG	CACAGAGCATTGCCAT	qPC	60	159
ActRII	GACGGGCCTGTCGAT	GTGATCCTTAATCGTG	qPC	60	120
GAPD	ACCACAGTCCATGCC	TCCACCACCCTGTTGC	PCR	60	452
GAPD	TTGTCTCCTGCGACT	GGTCCAGGGTTTCTTA	qPC	60	180
ActB	GTACTCTGTGTGGAT	GCTCAGTAACAGTCCG	qPC	60	138

2.4. Lentiviral Vectors and Molecular Cloning

2.4.1. Construction of pLv-BMP7-GFP

A third generation lentiviral transfer vector (pTet07CSII-CMV-GFPq; Broussau et al., 2008) and a commercially-available plasmid containing the full-length cDNA sequence of mouse BMP7 (pCMV-SPORT6-BMP7; OpenBiosystems, Huntsville, AL, USA) were utilized to clone a BMP7 lentivector. Initially, a 2051 base pair (bp) DNA sequence of mouse BMP7 was cut from pCMV-SPORT6-BMP7 with the restriction endonucleases AgeI and XhoI. The vector pTet07CSII-CMV-GFPq was linearized with AgeI and XhoI to form compatible ends for ligation. The cut BMP7 DNA fragment was ligated into pTet07CSII-CMV-GFPq upstream of an Internal Ribosomal Entry Site (IRES) and the gene encoding for Green Fluorescent Protein (GFP), using T4 DNA ligase (NEB). The resulting plasmid, pLv-BMP7-GFP, represents a third generation transfer lentivector with the transgenes BMP7 and GFP separated by IRES under the control of a single CMV promoter and flanked only by the HIV-1 Long Terminal Repeats (LTRs). All

vectors were subsequently sequenced to verify sequence integrity. The resulting lentivector DNA was further prepared as previously described.

2.4.2. ActRIIB shRNA Lentiviral Vectors

Third-generation lentiviral vectors (7,084 bp) encoding unique short hairpin RNA (shRNA) sequences targeting mouse ActRIIB were obtained from the RNAi Consortium (TRCN0000022639-TRCN0000022643; OpenBiosystems). Each dual-promoter vector regulated shRNA expression via the human U6 promoter and puromycin resistance (PuroR) via the human phosphoglycerate kinase (hPGK) promoter. The backbone vector, pLKO.1 (7050 bp), contained 18 bps of non-hairpin DNA (5'-CCGCAGGTATGCACGCGT-3') in place of shRNA inserts [Maffat *et al.,* 2006]. To facilitate identification the ActRIIB shRNA vectors were renamed to pActRIIB-shRNA-A through E, in which the only difference between the vectors were their unique shRNA sequence (Sequence A: 5'- GCTGGCTAGATGACTTCAATT-3', B: 5'-GCAGAGTGAACGGGAAATCTT-3', C: 5'- CGATGAGTACATGCTGCCCTT-3', D: 5'-GCTGTGAAGATCTTCCCACTT-3', E: 5'- CTCTCATACCTGCATGAGGAT-3').

2.4.3. ActRIIB Lentivector Knockdown Efficiency

The five vectors were tested for knockdown efficiency in mouse neural progenitor cultures. Initial knockdown efficiency tests were performed via transfection into an immortalized striatal progenitor cell line [Kiyomi *et al.,* 2006]. Transfections were performed with Lipofectamine 2000 (Invitrogen) in the ratio of 1 µL Lipofectamine to 1 µg of DNA. Additional tests were performed in E13CX cultures, using ActRIIB-shRNA lentivirus produced in HEK 293SF cells individually transfected with vectors pActRIIB-shRNA-A, B, C, D or E, as previously described. Approximately 48 hours following either

transfection or infection, total RNA was collected and RT-qPCR was performed, as previously described. The level of ActRIIB mRNA present in neural progenitor cultures transfected/infected with either pActRIIB-shRNA-A, B, C, D or E was compared relative to a non-transfected/non-infected control and a pLKO.1 transfected/infected control. Comparative quantitation was performed using the internal controls GAPDH and β-actin.

2.4.4. Construction of pLKO.1-EGFP and pActRIIB-shRNA-EGFP

In an effort to track both transfection and infection, the puromycin resistance gene was replaced with a fluorescent marker. Therefore, the gene encoding for enhanced green fluorescent protein (EGFP) was cloned into the vector pActRIIB-shRNA-D, since it was determined to be the most effective at knocking down ActRIIB in cortical progenitors. Additionally, EGFP was also cloned into the control vector, pLKO.1. This was achieved through a multiple-step cloning procedure involving the vectors pEGFP-N1 (4,733 bp; Clontech, Mountain View, CA, USA), pUC19 (2,686 bp; Invitrogen), pLKO.1 and pActRIIB-shRNA-D. With the exception of pEGFP-N1, all other plasmids were inoculated on Lauria-Bertani (LB) ampicillin selective plates directly from glycerol stocks or bacterial stabs. Individual colonies were selected and grown in 2 mL of LB media containing 100 µg/mL carbenicillin (ampicillin analog; Invitrogen). Plasmids were isolated the following day, using a Qiagen plasmid miniprep kit (Qiagen).

Initially the vectors pLKO.1 and pActRIIB-shRNA-D were digested with BamHI and KpnI to both linearize the vectors and remove the puromycin resistance genes downstream of their hPGK promoter. Next, the plasmid pEGFP-N1 was transformed into methyltransferase deficient (*dam*$^-$/*dcm*$^-$) chemically competent *Escherichia coli* (New England Biolabs (NEB), Ipswich, MA, USA) to remove methylation at the XbaI restriction site. Colonies were selected from LB agar plates containing 100 ug/mL kanamycin and

grown in 2mL of LB media also containing 100 ug/mL kanamycin. EGFP was cut from pEGFP-N1, using the restriction enzymes BamHI and XbaI (NEB). The resulting EGFP fragment was excised from a 1% agarose gel and purified using a Gene-Clean® gel extraction kit according to the manufacturer's protocol (Qbiogene, Irvine, CA, USA). The extracted EGFP fragment was then blunted (5' overhang fill-in and 3' overhang excision) with a Quick Blunting Kit (NEB).

The vector pUC19 was then cut and linearized with SmaI (NEB), a blunt-end forming endonuclease. The SmaI site, which was near the center of the multiple cloning site (MCS) of pUC19 was flanked by BamHI and KpnI. BamHI and KpnI were required to flank the EGFP fragment (in the 5' to 3' or (+) orientation) for insertion into the BamHI/KpnI digested lentivectors pLKO.1 and pActRIIB-shRNA-D. Therefore, the blunt-end EGFP fragment was inserted into SmaI digested pUC19, using a Quick Ligation Kit (NEB), forming both vectors pUC19-EGFP(+) and pUC19-EGFP(-). Following restriction gel screening via NotI digestion, the correctly oriented EGFP gene was cut from pUC19-EGFP(+) with BamHI and KpnI and gel purified. The initial BamHI/KpnI digested lentivectors pLKO.1 and pActRIIB-shRNA-D were gel purified to remove the cut puromycin resistance gene fragment. The BamHI/KpnI digested EGFP fragment was then ligated into both BamHI/KpnI digested pLKO.1 and pActRIIB-shRNA-D in separate reactions to vector the EGFP-containing lentivectors pLKO.1-EGF and pActRIIB-shRNA-EGFP. The lentivectors were prepared from transformed *E. coli* DH5α chemi-competent cultures, using a Qiagen maxiprep kit, according to the manufacturer's protocols (Qiagen). Both pLKO.1-EGFP and pActRIIB-shRNA-EGF were sequenced to verify cloned products (NRC-IBS DNA sequencing service, Ottawa, ON).

2.5. Lentivirus Production, Titration and Infection

2.5.1. Transfection and Lentivirus Production

The Human Embryonic Kidney packaging cell line HEK293SF-PacLV [Broussau *et al.,* 2008] was used to produce third generation lentivirus. The 293SF-PacLV cell line constitutively expresses the lentiviral proteins *gag/pol* and *rev*, while expressing *VSV-G* under the control of the transcriptional regulators CymR (Cumate-inducible) and rtTA2s-M2 (doxycycline-inducible) [Broussau *et al.,* 2008]. A total of $1.0x10^7$ 293SF-PacLV cells were seeded in a 10cm tissue culture dish (BD Falcon, VWR, Mississauga, ON), transfected with 30 µL of Lipofectamine 2000 (Invitrogen) and 15µg of lentivector DNA in 6 mL of Freestyle EX media (Invitrogen) and incubated at 37°C in 5% CO_2. Six hours following transfection, the medium was replaced with 6mL of fresh Dubecco's Modified Eagles Medium (DMEM) supplemented with 1 µg/mL doxycycline and 50 µg/mL cumate (4-Isopropylbenzoic acid) for the induction of *VSV-G*. The lentivirus-containing medium was harvested (~6 mL per 10 cm dish) both 48 and 72 hours post-transfection and filtered to remove cell debris with a 10 mL syringe and a 0.45 µm low protein binding filter (Millipore). In parallel experiments, the lentivirus-containing medium was concentrated via centrifugation at 2,000x g for 20 minutes in sterilized Amicon Ultra-15 centrifugal protein concentrators (Millipore). Both unconcentrated and concentrated viruses were either used immediately for infection or stored at -80° C in 10% FBS for further use.

2.5.2. FACS-based Titration and Lentiviral Infection

The human embryonic kidney cell line, HEK293GPG (designated HEK293 from here on) in conjunction with fluorescence-activated cell sorting (FACS) analysis were

used to determine the infection titers (transducing units (TU)/ mL). Briefly, HEK293 cells were seeded in six-well plates at a density of $1.0x10^6$ cells/well and incubated at 37°C in 5% CO_2 for 24 hrs or until cells were approximately 80-90% confluent ($2.0x10^6$ cells/well). To remove potential cell debris prior to infection, the medium was replaced with 1.7 mL/well of fresh DMEM with 1% FBS. Serial dilutions were prepared with DMEM in the ratios 1:1, 1:10 and 1:100 from 30x concentrated lentivirus-containing medium. Each HEK293-containing well was transduced with 300 µL of the lentivirus serial dilution. Polybrene (hexadimethrine bromide; Millipore) was added to a final concentration of 8 µg/mL to each well and the plates were subsequently incubated at 37°C in 5% CO_2. Following a 48 hr incubation period, the transduction efficiency was evaluated with fluorescence microscopy to determine GFP expression. Infected HEK293 cultures were passed weekly and maintained in HEK293 media (DMEM + 10% FBS + 1µg/mL doxycycline).

HEK293 cultures were harvested 48 hours post-infection for FACS analysis. Briefly, medium was removed and cells were washed with 1x phosphate-buffered saline (PBS). Next, 200 µL of 0.25% Trypsin (Invitrogen) was added to each well and following a short incubation period (1 min) at RT, the cells were resuspended in 1 mL/well of PBS containing 10% FBS, briefly vortexed to dissociate the cells and stored on ice. An aliquot of the sample was counted with a haemocytometer to determine the approximate cell density per well. The samples were immediately analyzed on a MoFlo flow cytometer (DakoCytomation), using Summit software (Ottawa Health Research Institute FACS Service, Ottawa, ON). For each sample at least 40,000 events were collected. The titer of the unconcentrated virus was determined by applying the following formula: transducing units/ml = (% Infected Cells x Total Cell Number in Well x Dilution Factor)/ (Volume of Inoculum Added to Cells x Fold Concentration).

2.5.3. Lentiviral Production and Infection of Primary Cortical Cells

Lentiviral production for infection of primary cultures was performed as previously described with a few modifications. Most importantly, all media conditions during both transfection and lentiviral production were serum-free to help minimize variables during concentration and infection of primary cortical progenitors. In addition, concentration of the pLKO.1-EGFP and pActRIIB-shRNA-EGFP-derived lentivirus (Control-Lv and ActRIIB-shRNA-Lv, respectively) was performed using Lenti-X concentrator according to the manufacturer's protocol (Clontech). Briefly, the Lenti-X concentrator was added to the 0.45 μm filtered lentiviral-containing media in the ratio 1:4, respectively. After mixing by inversion, the mixture was incubated at 4° C for at least 30 min. The lentivirus was then pelleted out from the mixture via centrifugation for 1 hour at 1,500 x g and 4°C. The pellet was resuspended in DMEM (serum-free) to a 50x stock. Concentrated lentivirus was kept at 4° C for up to 5 days, or frozen at -80° C for long-term storage.

Cortical cultures were infected with control-Lv or ActRIIB-shRNA-Lv both at a multiplicity of infection (MOI) of 100 lentiviral particles per cell. The cationic polymer, polybrene, was omitted from the lentiviral infections of cortical progenitors. Infection efficiencies were assessed 48-hours post-infection via EGFP expression and fluorescence microscopy. Upon infection, cortical progenitors were cultured in the presence or absence of 1 ng/mL rhBMP7. After 5DIV, cultures were fixed for immunostaining or mRNA collection to assess neuronal differentiation and ActRIIB knockdown efficiency.

2.6. Protein Assays

2.6.1. Whole Cell Lysates and Conditioned Media Collection

Protein assays including Western Blotting and ELISA, were performed to evaluate BMP7 production in the lentivirally-transduced cultures and to examine the Lv-BMP7 biological activity in E13 cortical cultures. Samples used to evaluate Lv-BMP7 processing included total cell lysates (50 µg/well), 5x or 10x concentrated media (50 µL/well) collected from GFP lentivirus and BMP7 lentivirus infected HEK293 cultures as well as adult mouse cerebral spinal fluid (CSF) (2 µL/well) and rhBMP7 (R&D Systems). CSF was collected from CD1 adult mice (2-10 µL/mouse) according to Barten *et al.,* (2005). Total cell lysates were extracted with complete extraction buffer (10mM Hepes, 10mM KCl, 1.5 mM $MgCl_2$, 1X Roche Complete protease inhibitor, 1% CHAPSO, 0.2mM EDTA, pH 7.4) and media was concentrated using Amicon Ultra-15 centrifugal protein concentrators (Millipore), according to manufacturer's protocol in a Sorvall Legend benchtop centrifuge (Thermo Scientific).

Complementary Western Blots were performed to analyze the relative protein expression of the BMP receptor, ActRIIB, in cortical development. Samples included both whole brains and cortices from E15, E18, PN7 and adult mice. ActRIIB protein was detected following incubation with the ActRIIB neutralizing antibody overnight (1:200; R&D Systems). In addition, Western Blot analysis was performed to evaluate SMAD1/5/8 phosphorylation in cultures treated with ActRIIB neutralizing antibody (nAb) (R&D Systems) to assess the contribution of ActRIIB to BMP signaling in E13 cortical cultures. Phospho-SMAD1/5/8 accumulation in the nucleus was evaluated in E13 cortical cultures grown under the following conditions: no treatment, 1 ng/mL rhBMP7 alone, 3 µg/mL ActRIIB nAb alone and lastly co-treatment of 1 ng/mL rhBMP7 with 3 µg/mL ActRIIB nAb. All BMP7 treatments were performed up to 1 h. In the case of co-treatment with BMP7 and ActRIIB nAb, BMP7 was added to the cultures following a 15 min pre-treatment with ActRIIB nAb.

2.6.2. Nuclear Isolation and Protein Extraction

Phospho-SMAD1/5/8 accumulation in the nucleus was evaluated, using nuclear lysates collected from E13 cortical cultures treated with Lv-BMP7 or rhBMP7 (1 and 30 ng/mL). Briefly, E13 cortical cultures that were treated for 1 hour with BMP7 were initially washed with cold 1xPBS and then incubated in hypotonic lysis buffer (1mM $NaHCO_3$, 5mM $MgCl_2$, 1X Roche Complete protease inhibitor, pH 7.5) for 5 min. Centrifugation was performed at 1000 x g for 5 min to isolate intact nuclei. Isolated nuclei were resuspended and washed in CE buffer (10mM Hepes, 10mM KCL, 1.5 mM $MgCl_2$, 0.1% CHAPSO, 1X Complete protease inhibitor (Roche Diagnostics Canada, Laval, QC), 25% glycerol, pH 7.4) and incubated for 10 minutes on ice. Nuclei were pelleted (1000 x g, 5 min) and lysates were extracted with complete extraction buffer.

2.6.3. Western Blotting

Western Blots were performed as previously described by Bani-Yaghoub *et al.,* 2006. Briefly, samples were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to "Hybond C" nitrocellulose (GE Healthcare, Piscataway, NJ, USA). Transferred blots were blocked for 1 hour in NFDM/TBST (5% non-fat dry milk / 50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 0.05% Tween-20) followed by incubation with primary antibodies either against the BMP7 propeptide (See Figure 2.1; 1:1000; Cell Signaling, Boston, MA, USA), mature BMP7 (0.1 µg/mL; R&D Systems), phospho-SMAD1/5/8 (1:1000; Millipore, Billerica, MA, USA) and/or β-actin (1:1000; Sigma-Aldrich). Blots were washed in TBST (3 x 5 min) and incubated with anti-rabbit or anti-mouse HRP-conjugated secondary antibodies and examined with a FluorChem 8900 imager (Cell Biosciences, Santa Clara, CA, USA) following 5 minutes incubation in Immuno-Star WesternC solution (BioRad, Hercules,

CA, USA). Protein molecular weights were estimated with a Full-Range Protein Marker (GE Healthcare). Densitometric analysis was performed, using the FluorChem 8900 software (Cell Biosciences).

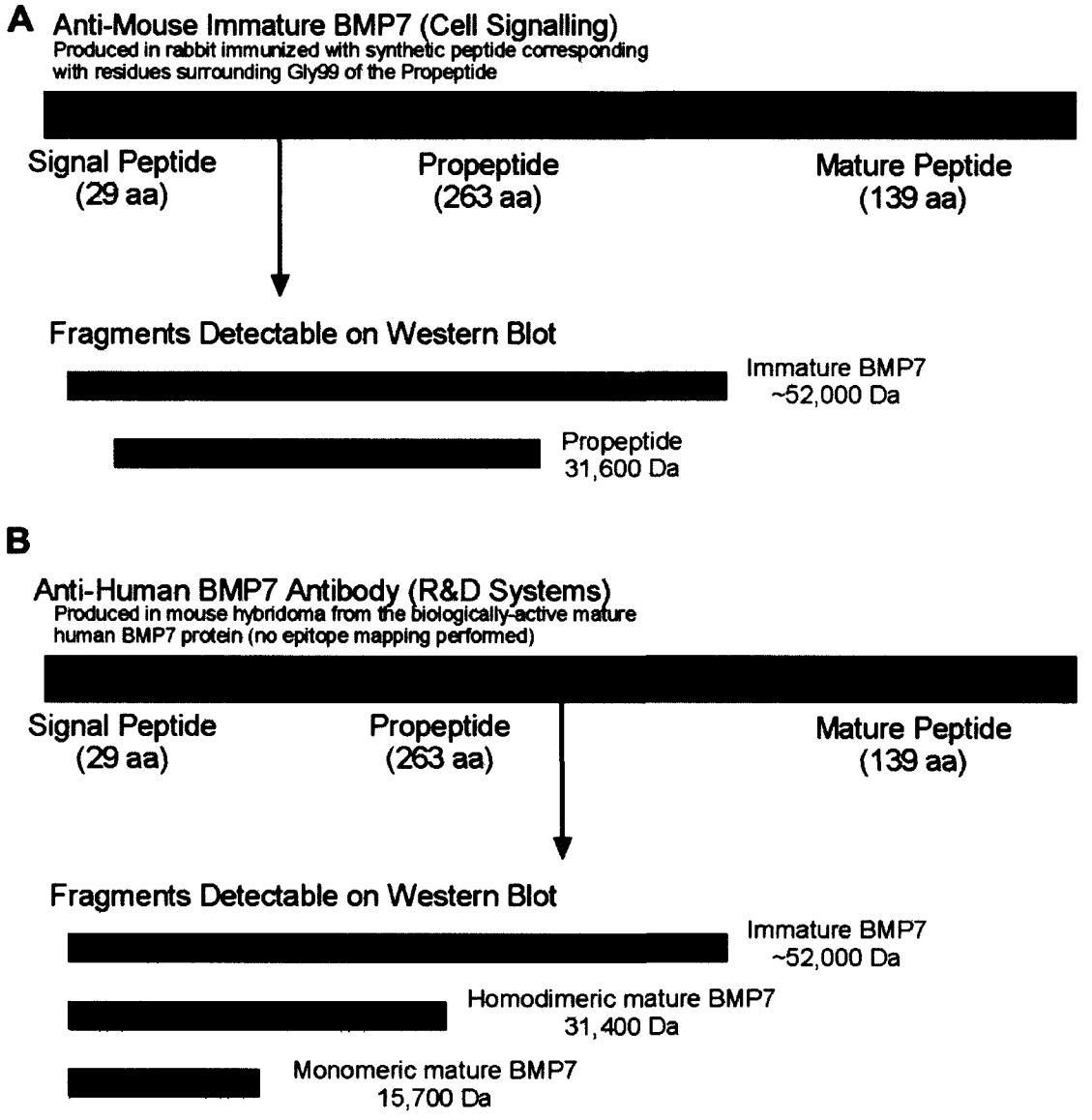

Figure 2.1: BMP7 Antibodies. (A) Anti-mouse immature BMP7 (Cell Signaling) specifically targets the propeptide of BMP7 either alone or in unprocessed immature BMP7, whereas (B) anti-human BMP7 targets the mature BMP7 peptide in the immature or mature monomeric or dimeric BMP7 protein.

2.6.4. Enzyme-Linked Immunosorbent Assay (ELISA)

BMP7 protein levels were measured in the conditioned media (collected over 24 hrs) of primary neural cultures and in their lysates. Samples included E13 cortical progenitors (1DIV), neurons (5DIV) and meninges (5DIV), E15 cortical astrocytes (7WIV), adult mouse cerebrospinal fluid (CSF) and adult kidney. In addition, cultures of HEK293 or human amniotic fluid (HAF; courtesy of Dr. Andrée Gruslin) cells infected with BMP7 containing an IRES-GFP or the control GFP lentivirus were plated at $1x10^6$ HEK293 cells or $2.5x10^5$ HAF cells per mL of media in a BD Falcon 6-well plate (BD Biosciences, Mississauga, ON, Canada). Therefore, each well contained approximately $2.0x10^6$ HEK293 cells or $5.0x10^5$ HAF cells in 2 mL of media. Following two washes with PBS and the addition of fresh medium, samples were collected at the time-points 0, 1, 2, 4, 6, 12, 15, 18 and 24 hours. The samples were taken from infected cultures at 72 hours (HEK293), 5 days (HAF) and up to 4 weeks post-infection. Cells were counted both prior to and following media collection over a 24 hour period and were averaged to determine the level of BMP7 released per $1x10^6$ cells in 1 mL of media via ELISA.

BMP7 protein levels were quantified down to 100 pg/mL (lower limit), using a BMP7 enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol (R&D Systems). Briefly, a 96-well Nunc Maxisorp plate was incubated overnight at room temperature in BMP7 capture antibody (2 µg/mL in PBS). Standards were prepared in the range of 50 to 8000 pg/mL in reagent diluent (1%BSA in PBS, pH 7.2-7.4). Samples were prepared 1:1, 1:10 and 1:100 in triplicate with reagent diluent. Following a 1 hour incubation period in reagent diluent, 200 µL of each standard and sample were added to the coated-plate and incubated at room temperature for 2 hours. The plate was then washed five times with 200 µL of wash buffer (0.05% Tween-20 in PBS, pH 7.2-7.4) per well. Approximately 200 µL of BMP7 detection antibody (0.5 µg/mL

in reagent diluent + 2% heat-inactivated goat serum) was added to the plate and incubated for another 2 hours at room temperature. Following another wash step, 100 µL of horseradish peroxidase (HRP)-conjugated antibody (1:180 in reagent diluent) was added to the plate and incubated for 20 min at room temperature. The wells were washed again and 100 µL of Tetramethylbenzidine (TMB) substrate solution was added to each well and incubated at room temperature. Following 20 min, 50 µL of 2N H_2SO_4 was added to each well and absorbance was measured for each well, using a SpectraMax 340 microplate reader (Molecular Devices) at 450 nm and the amount of BMP7 was calculated from the standard curve in the detection limit range.

2.7. Statistical Analysis

In the neuronal differentiation assays involving BMP7 or LvBMP7 treatment, results were obtained from at least three independent biological samples, each in triplicate. The ratio of MAP2 positive Hoechst positive cells was obtained over the total number of Hoechst positive cells. Similar method was used to determine the ratio of Nestin, Sox2 or βIII tubulin positive cells over the total number of cells, using Hoechst as a counter-stain. Cell survival, proliferation and receptor neutralization assays were performed in triplicates for at least two independent experiments. Additionally, LvBMP7 production assays were performed using two independent biological samples, each in triplicate. All results were presented as mean ± standard error mean (SEM), applied to one-way ANOVA or student t-test (GraphPad Prism 5) and analyzed for at least a 95% confidence level, using the Bonferroni test.

3. RESULTS

3.1. BMP7 in Cortical Neurogenesis

In this study, the role of BMP7 in neurogenesis was examined using mouse cortex as a model. The endogenous expression levels of BMP7 and the BMP receptors were examined during differentiation in cortical cultures, using RT-PCR and RT-qPCR. Complementary *in vivo* experiments were performed to assess the expression of BMP7 and the BMP7 receptors in the developing cortex. These experiments were followed by the evaluation of neural progenitors with respect to survival, proliferation and differentiation in the absence or presence of BMP7 at the physiological level (1 ng/mL), under serum-free conditions. Finally, receptor knockdown and BMP7 lentiviral vectors were designed, constructed and tested to further enhance our understanding of the role of BMP7 in the cortex.

3.1.1. BMP7 and BMP Receptor Expression

Initially, the expression of BMP7 in the developing cortex was examined using RT-PCR. BMP7 mRNA was determined to be present in mouse embryonic stem cells (mES), E9.5 and E10 brains, and E12.5, E14, E16, E17 and adult cortices (Figure 3.1A). The intensity of bands in RT-PCR suggested that BMP7 expression was the lowest in mES cells, whereas a stronger intensity was observed from E9.5 to E14, when cortical neurogenesis occurs actively. The expression of BMP7 transcript in the brain tissue (free of meninges) suggested that BMP7 protein could be secreted by neural tissue in the brain. To evaluate this possibility, ELISA was performed to determine whether BMP7 protein is secreted by cortical cells at various stages of differentiation. Using ELISA to the lower limit of 0.05 ng/mL, BMP7 protein was not detected in either the cell lysates or

the media (up to 10x concentration) obtained from E13 cortical cultures (Table 3.1). Further analysis of neural, neuronal and astroglial cultures showed that only the cell lysates obtained from pure astrocytes express BMP7 protein. However, there was no BMP7 detected in the media collected from these cultures (Table 3.1).

Table 3.1: Summary of BMP7 expression in different cortical cultures, using ELISA

Sample	**Detection of BMP7 Protein**	
	Cell Lysates	Conditioned Media (10x)
Positive Control 1: Adult Kidney	+	not examined
BMP7-Lv infected HEK293 cells	+	+
E13 cortical progenitors (1DIV)	-	-
E13 cortical neurons (5DIV)	-	-
E15 Astrocytes (7WIV)	+	-

*DIV: days *in vitro*; WIV: weeks *in vitro*

Since several claims have been made that the choroids plexus and meninges serve as main source of BMP7, parallel experiments were designed to distinguish the expression level between neural and meningeal tissues, using E13 and E16 cortices. In agreement with the previous reports, RT-qPCR demonstrated abundant levels of BMP7 in the meninges at E16 (and to a lesser degree at E13), compared with those of neural tissue (Figure 3.1.B). In both cases, the level of BMP7 was much higher relative to that of neural progenitors and neurons ($p<0.01$; Figure 3.1B).

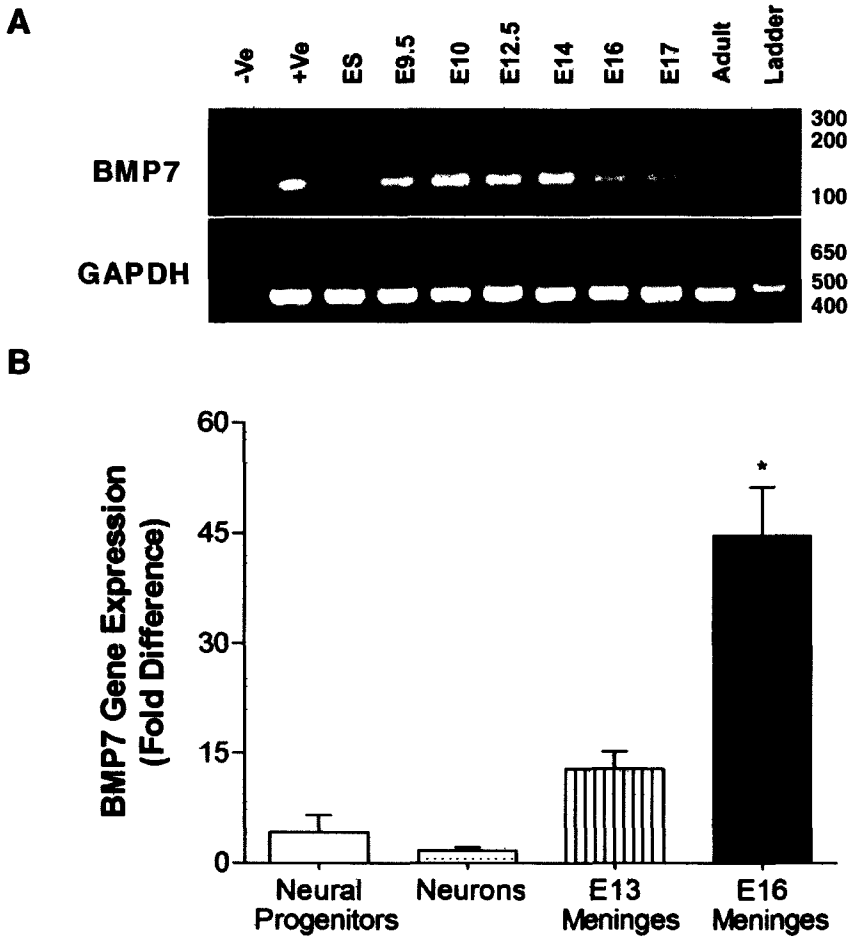

Figure 3.1: BMP7 is expressed in the brain throughout development. (A) RT-PCR analysis of BMP7 expression in mouse ES cells, E9.5 and E10 brain, and E12.5, E14, E16, E17 and adult cortices. (B) RT-qPCR was performed on neural progenitors (E10 brain), neurons (E16 CX), E13 meninges and E16 meninges to examine relative BMP7 gene expression. Fold differences were represented in arbitrary units relative to BMP7 gene expression in neurons and were normalized to ActB (β-actin) and GAPDH (1way ANOVA and *post hoc* Bonferroni's test; N=3, *P<0.001).

Next, the expression of various BMP receptors (BMPRIB, BMPRII, ActRI, ActRIIA and ActRIIB) was evaluated in the cortex by RT-PCR. The results demonstrated that all of the BMP receptors examined are expressed as early as E9.5 and continue until the adult stage (Figure 3.2), further emphasizing the importance of BMP signaling in cortical development.

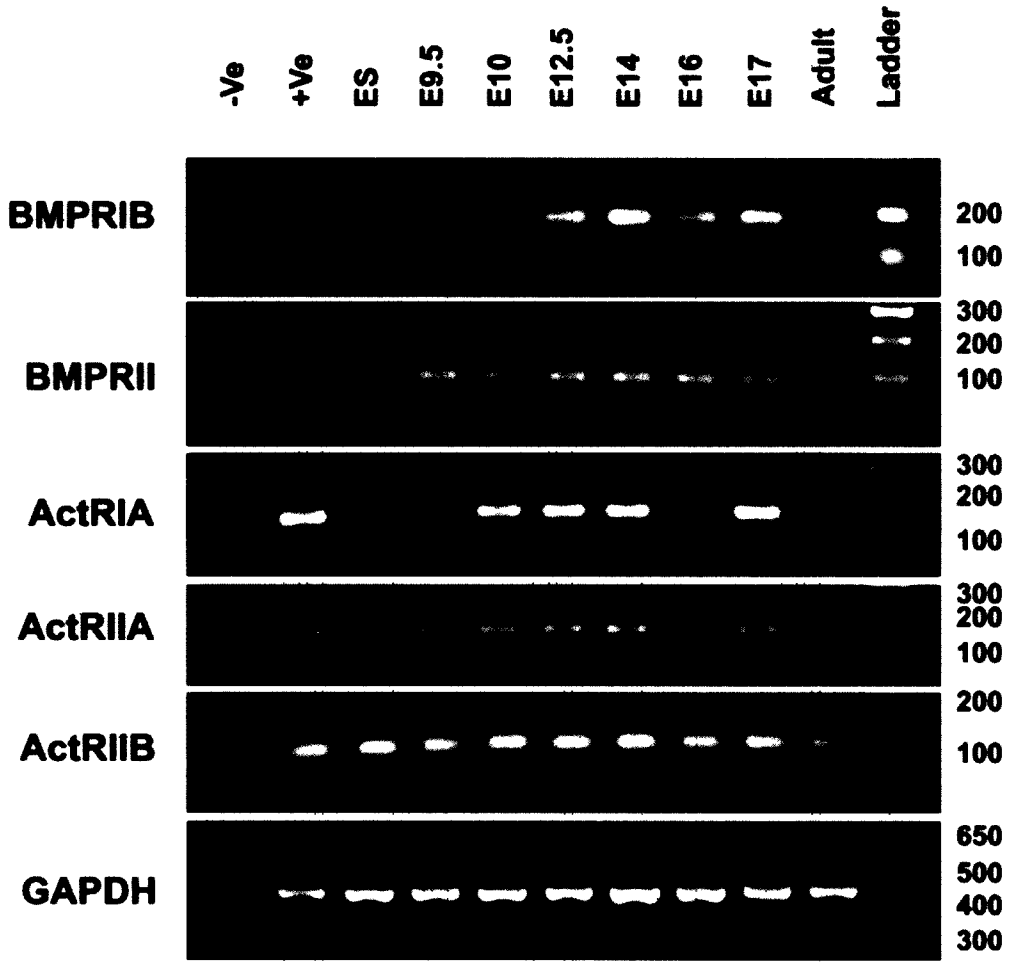

Figure 3.2: The BMP receptors are expressed in the developing cortex. RT-PCR was performed to detect transcript of BMPRIB, BMPRII, ActRI, ActRIIA and ActRIIB throughout cortical development. Samples included mouse ES cells, E9.5 and E10 brain, and E12.5, E14, E16, E17 and adult cortices.

3.1.2. The effects of BMP7 on neuronal and glial differentiation

To study the effects of BMP7 in neurogenesis, cortical progenitors were exposed to recombinant BMP7 every 24 hours for up to 5 days *in vitro* (DIV) and evaluated with several neural and neuronal markers. Parallel cultures were treated with 1 ng/ml, 10 ng/ml or 30 ng/ml of BMP7 to represent its physiological level in the human CSF (< 1 ng/mL) and the therapeutic doses used *in vivo* (10-1000 ng/mL).

To ensure that such a low level of BMP7 (1 ng/mL) triggers signaling in our model, Western Blot analysis was used to verify the presence of phosphorylated SMAD1/5/8 (pSMAD1/5/8) in the nuclear extracts obtained from neural progenitors (Figure 3.3). In contrast to the control, a prominent band at 65 kDa (representing pSMAD1/5/8) was detected as early as 30 min of BMP7 treatment in our cultures. This effect was maintained for at least 2 hours and followed by a lighter band after 24 hrs, determining a functional time course within which SMAD1/5/8 remains activated upon induction by a single treatment at 1 ng/mL.

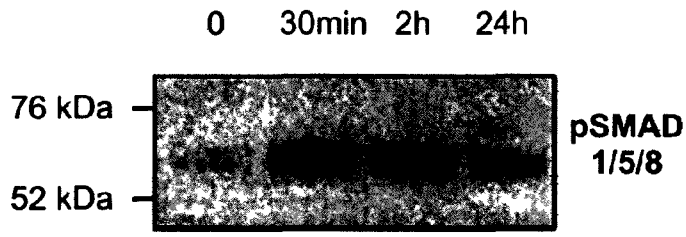

Figure 3.3: SMAD signaling in cortical cultures is activated shortly following treatment with BMP7. Western Blot analysis indicated that elevated pSMAD1/5/8 levels are present in the nuclear extracts from cortical progenitors as early as 30 min following treatment with 1 ng/mL. Western Blot performed by Roger Tremblay.

To measure the effect of BMP7 (1 ng/mL) on neuronal differentiation, five independent experiments were performed evaluating the percent of MAP2+ cells present following 5DIV (Figure 3.4A-B). Over 10,000 cells were counted for each condition or treatment. On average, control E13 cortical cultures contained 31.6 ± 3.4% MAP2+ cells, whereas BMP7 treated cultures consisted of 46.5 ± 3.4% MAP2+ cells (Figure 3.4C). Therefore, the physiological BMP7 level significantly enhanced neurogenesis by nearly 1.5-fold. Following 5DIV, cultures contained a small percent of GFAP+ cells, which was not significantly different between the control and BMP7 (1 ng/mL) treated cultures (1.9 ± 0.6% vs. 2.2 ± 1.1% GFAP+ cells in control and BMP7-treated cultures, respectively). Furthermore, since higher concentrations have been reported to enhance the generation of GFAP+ cells, we also assessed gliogenesis in the presence of 30 ng/mL BMP7 (Figure 3.4D). In cultures treated with 30 ng/mL BMP7 for 5DIV, a significant increase in the percent of GFAP+ cells was observed (1.3 ± 0.2% vs. 15.7 ± 1.1% in control and BMP7-treated cultures, respectively).

Several reports have claimed that Noggin, another member of the BMP signaling pathway, induces neural induction in the CNS by inhibiting BMP4 and other ligands [Lim *et al.,* 2000]. To determine whether BMP7 maintains its effect in neuronal differentiation in the presence of antagonists, cortical progenitors were treated with 30 ng/mL of Noggin (representing its physiological level in the human CSF [Chmielnicki *et al.,* 2004]) and further induced by BMP7. Our data showed that BMP7 maintains its ability to enhance neuronal differentiation, as evidenced by quantitative immunocytochemistry (Figure 3.5).

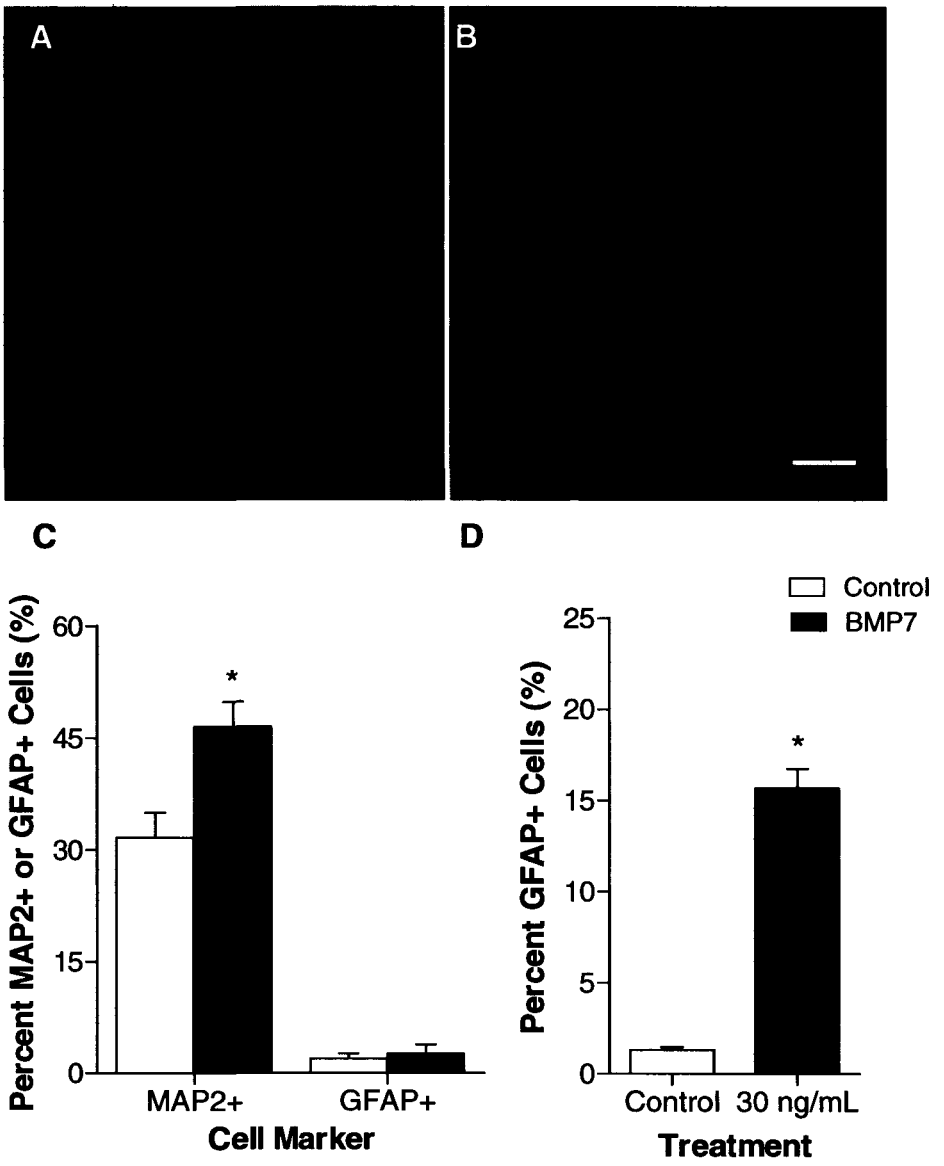

Figure 3.4: BMP7 treatment enhances neuronal differentiation. Immunocytochemistry indicated that E13 cortical progenitors have the capacity to differentiate to MAP2+ neurons (green) in the absence (A) or presence of BMP7 (B). The number of MAP2+ neurons was nearly 1.5-fold higher in cultures treated with 1 ng/mL BMP7 (C) (Unpaired Student's t-test and *post hoc* Bonferroni's test; N=5, *P<0.05). The percent of GFAP+ cells was not altered with 1 ng/mL BMP7 treatment (C), whereas the generation of GFAP+ cells was significantly increased in cultures treated with 30 ng/mL BMP7 (D). The percent of MAP2+ and GFAP+ cells were counted relative to Hoechst+ cells (blue) (Unpaired Student's t-test and *post hoc* Bonferroni's test; N=1, *P<0.001). Scale bar: 30 μm.

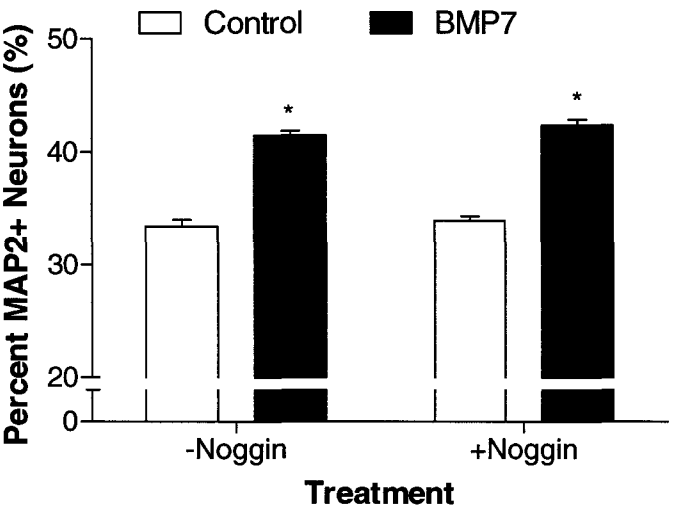

Figure 3.5: BMP7 treatment enhances neuronal differentiation in the presence of noggin. E13CX cultures treated with 1 ng/mL BMP7 in the presence physiological levels of the BMP antagonist noggin (30 ng/mL) contained similar percents of MAP2+ cells as the BMP7 only treated cultures. (2way ANOVA and *post hoc* Bonferroni's test; N=1, $P<0.05$).

3.1.3. Cell fate commitment, survival and proliferation

We hypothesized that the BMP7-induced differentiation occurs as a result of neural progenitors' commitment to a neuronal fate and not due to enhanced cell survival or proliferation. To test this hypothesis, parallel cell culture experiments were performed in the absence or presence of BMP7 in which the cells were stained with the markers for neural phenotype (nestin), survival (CFDA and PI) and proliferation (BrdU). The percent of cells positive for nestin, a marker for neural stem and progenitor cells, was reduced in cultures treated with 1 ng/mL BMP7 and to a much greater extent in cultures treated with 30 ng/mL BMP7 (both 2 and 5DIV) (Figure 3.6A-B). Further analysis of nestin mRNA expression levels was performed in control versus BMP7 treated cortical cultures (5DIV), using RT-qPCR (Figure 3.6C). These results suggest the BMP7-induced neuronal differentiation occurs as more progenitors commit to a neuronal fate.

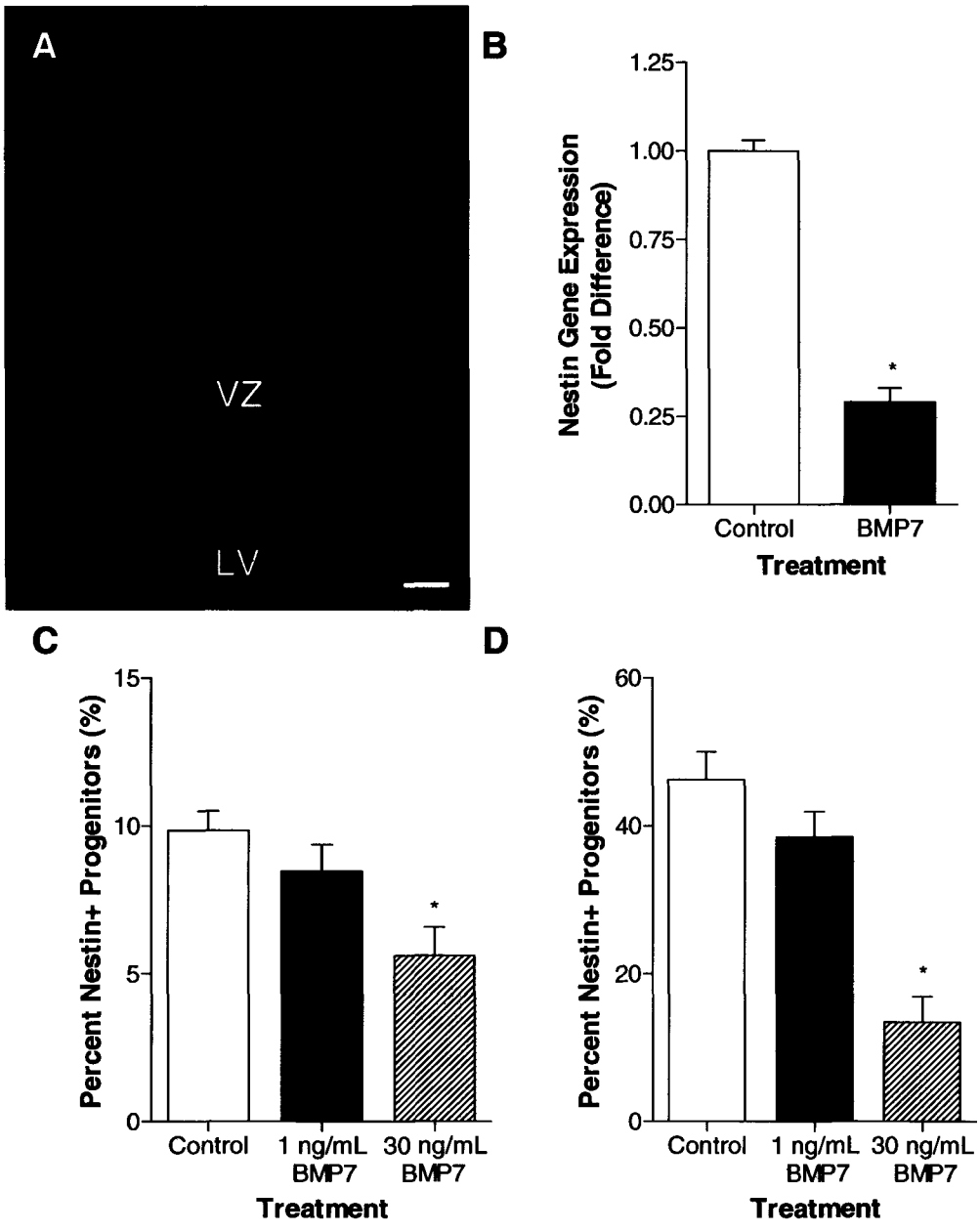

Figure 3.6: BMP7 targets nestin-positive cells. (A) Nestin+ progenitors (red) are present in the E13 ventricular zone (VZ) of the lateral ventricle (LV). (B) Nestin gene expression is significantly down-regulated in the E13 cortical cultures treated with 1 ng/mL BMP7 for 5DIV. Fold differences were represented in arbitrary units relative to the control and were normalized to ActB (β-actin) and GAPDH (Unpaired Student's t-test and *post hoc* Bonferroni's test; N=3, *P<0.001). E13 cortical cultures were stained for nestin and counterstained with Hoechst after (C) 2DIV and (D) 5DIV in the absence or presence of BMP7. The percent of nestin positive cells is significantly reduced in 30 ng/mL BMP7 treated cultures (1way ANOVA and *post hoc* Bonferroni's test; N=1, *P<0.001). Scale bar: 20 μm.

Although nestin down-regulation suggests a direct link between neuronal differentiation and cell-fate commitment in response to BMP7, cortical cultures were further examined to ensure that enhanced neuronal differentiation in the BMP7 treated cultures did not arise indirectly, as a result of an increase in neuronal survival and/or cell proliferation. The effect of BMP7 treatment on E13 cortical cell survival was measured using 5-CFDA, a fluorescent dye commonly used to quantify the percent of live cells in culture (Figure 3.7A-B). Our data showed that the level of cell survival is similar between the control (70.1 ± 2.2%) and BMP7-treated (70.7 ± 1.3%) cultures (Figure 3.7C) Complementary staining with PI was used to further distinguish CFDA positive live cells from PI positive dead cells in each culture (Figure 3.7A-B).

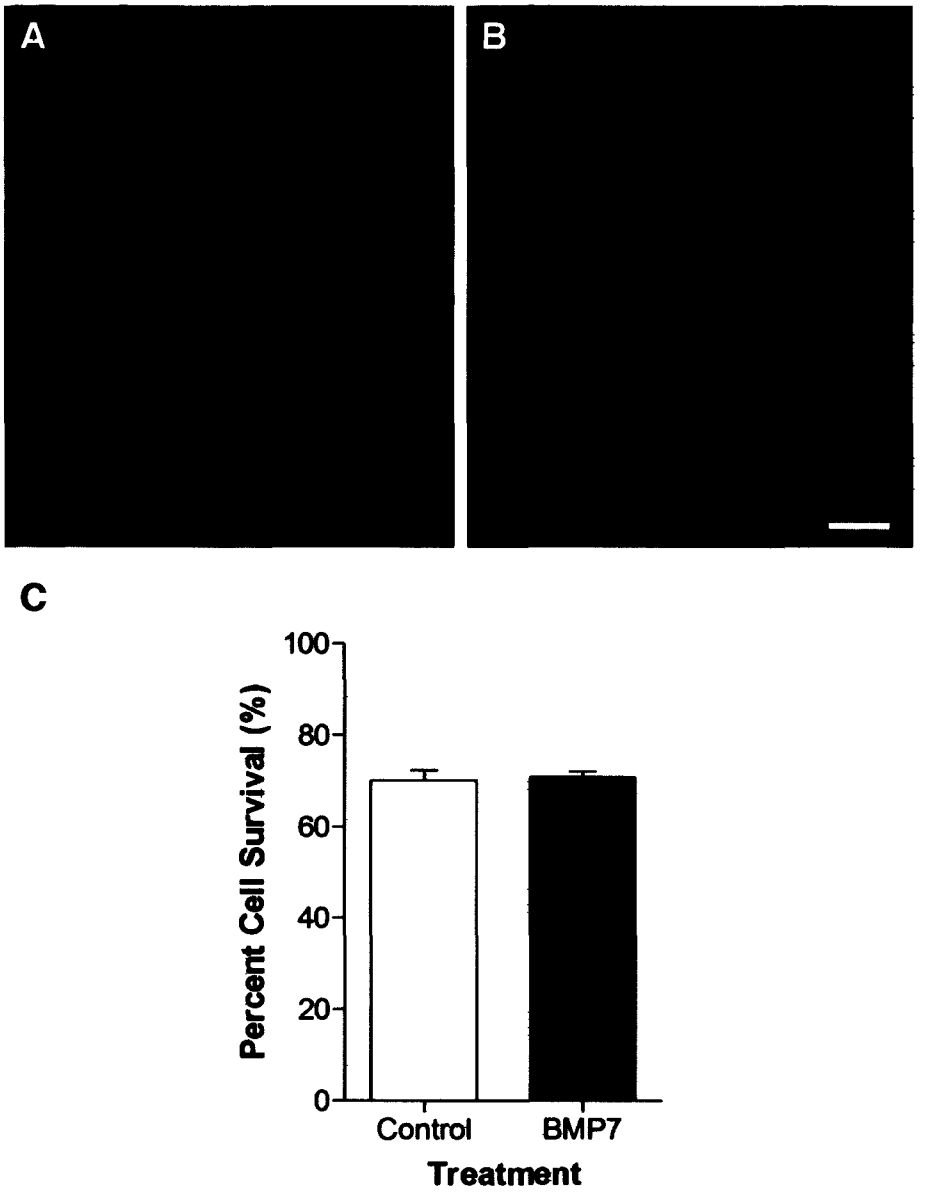

Figure 3.7: BMP7 treatment does not alter the survival of E13 cortical cells under normal culture conditions. (A) Control and (B) 1 ng/mL BMP7 treated E13CX cultures were examined with 5-CFDA (green) and PI (red) to determine cell survival and death, respectively, in the absence or presence of BMP7 for 1DIV (C) (Unpaired Student's t-test and *post hoc* Bonferroni's test; N=2, P>0.05). Scale bar: 30 μm.

To examine cell proliferation (DNA synthesis), E13 cortical progenitors were cultured in the absence or presence of BMP7, subjected to BrdU (for 20 hrs) and stained with BrdU antibody. Using these parameters, 33.1 ± 0.4% of control and 30.9 ± 0.6% of the BMP7-treated cells were BrdU-positive cells. Therefore, cell proliferation was not significantly altered by treatment with BMP7 at 1 ng/mL (Figure 3.8).

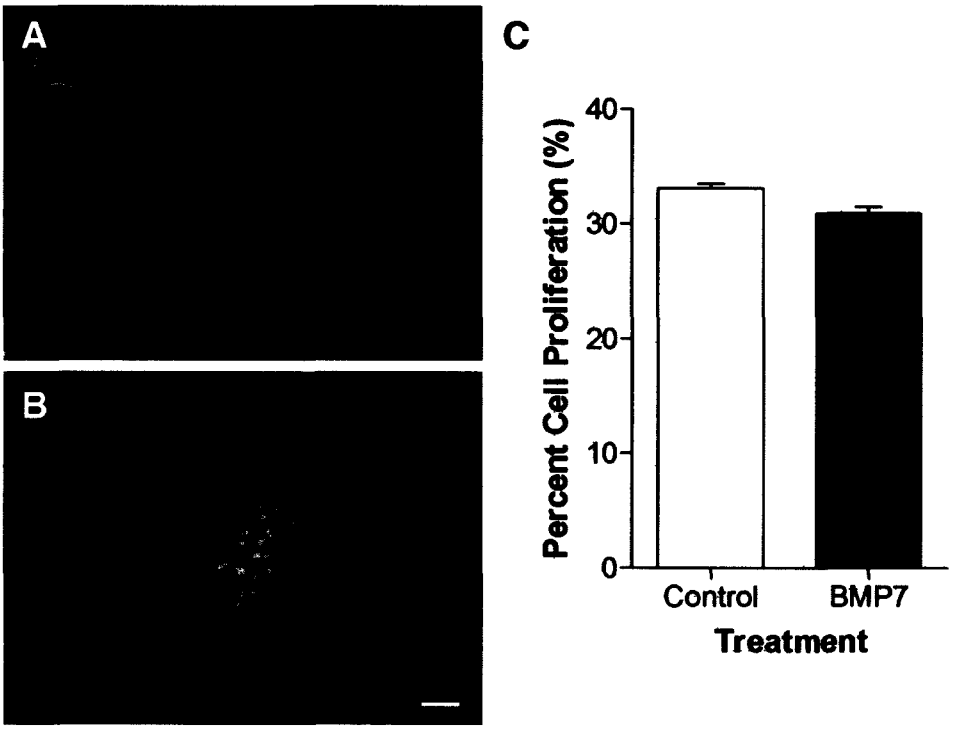

Figure 3.8: BMP7 treatment does not alter cell proliferation. (A) Control and (B) 1 ng/mL BMP7 treated E13 cortical cells were stained with BrdU (green) and Hoechst (blue) to determine the effect of BMP7 treatment on cell proliferation after 2DIV (C) (Unpaired Student's t-test and *post hoc* Bonferroni's test; N=2, P>0.05). Scale bar: 30 µm.

3.2. Quantitative Analysis of BMP Receptors during Cortical Development

3.2.1. Quantitative Analysis of BMP Receptors *in Vivo*

Our RT-PCR data indicated that at least five of the six known BMP receptors are expressed in the mouse cortex (Figure 3.2). To further determine the level of each BMP receptor during cortical development, we dissected the cortices at E10 up to adult stages and applied them to RT-qPCR (Figure 3.9A). Notably, BMPRIA, which was not included in our RT-PCR data (Figure 3.2), was also present in the cortex at moderate levels relative to the other receptors (Figure 3.9A). The other BMP type I receptors, BMPRIB and ActRI were expressed in relatively low levels throughout cortical development with the exception of ActRI, which displayed a sudden upregulation in the E16 cortex. Receptor expression peaks were also observed in the E16 cortex for BMPRII and ActRIIA, which may be indicative of the onset of gliogenesis and the maturation of cortical neurons. Relative to the other BMP Type II receptors in E10 and E13 cortices, ActRIIB expression was approximately 3 to 15-fold higher. In contrast, there was a significant reduction in ActRIIB expression after E16 (Figure 3.9A), coinciding with the completion of neurogenesis and the onset of gliogenesis in the cortex. Furthermore, ActRIIB was consistently the least expressed receptor following birth (PN10 and Adult cortices). Together, the RT-qPCR data demonstrate that most receptors were more prominent in the embryonic cortex than after birth (Figure 3.9A). There was also a higher expression level for the type II receptors compared with the type I receptors early in development. Most importantly, ActRIIB was the most highly expressed BMP receptor, at nearly 80-fold higher than ActRI (the least expressed receptor) up until the onset of gliogenesis at E16.

These data were complemented with the detection of ActRIIB protein during cortical development, using Western Blot. Similarly, the downregulation of ActRIIB transcript was closely associated with decreasing ActRIIB protein levels as cortical development progressed. In particular, ActRIIB protein was present at E15 brain and cortex, whereas it was undetectable at PN7 and adult stages (Figure 3.9B-C). Together, these results suggest that ActRIIB may be directly involved in BMP7-induced cortical neurogenesis.

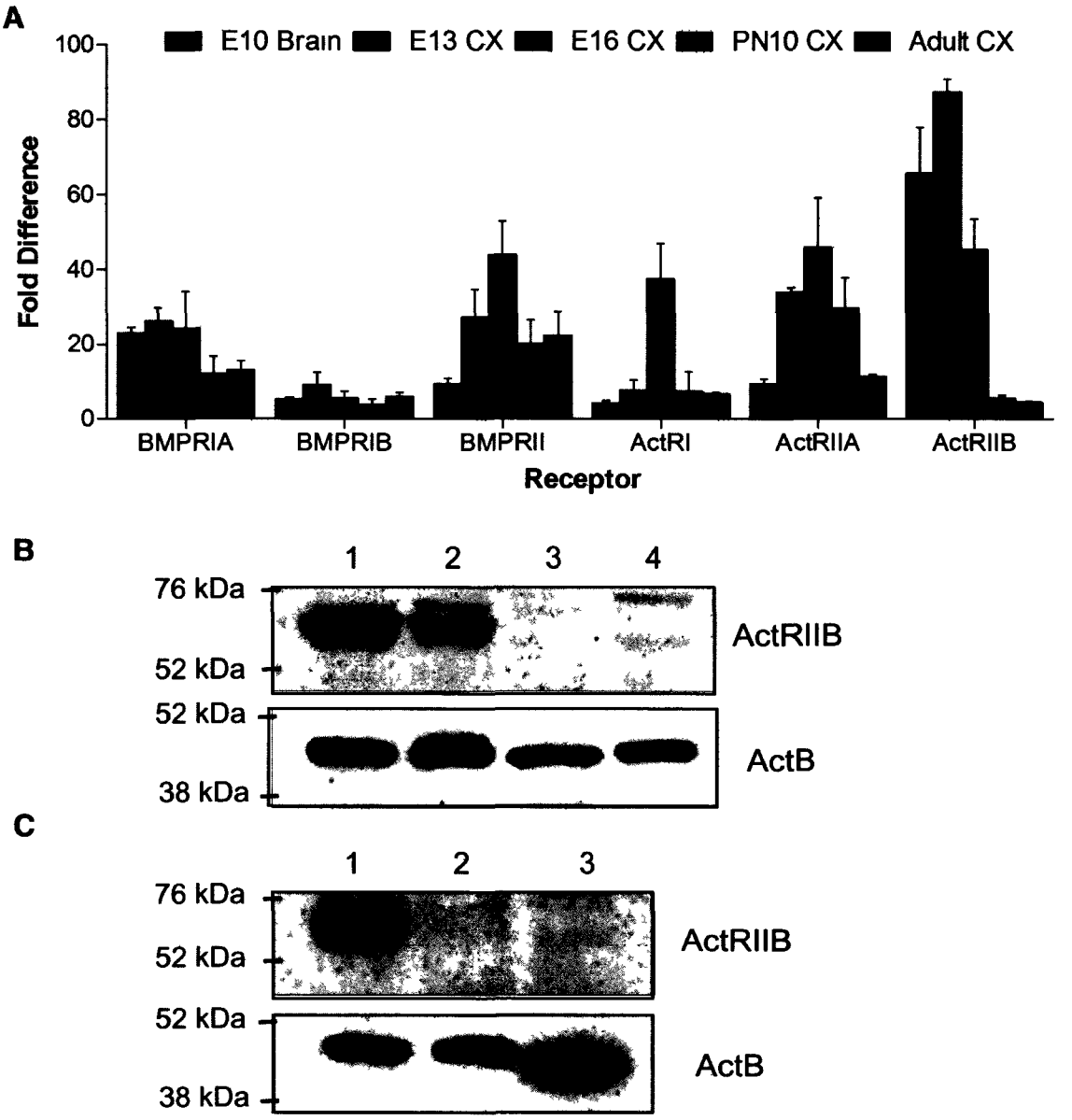

Figure 3.9: The BMP receptor ActRIIB is highly expressed during cortical development *in vivo*. (A) Comparative RT-qPCR analysis of E10 brain and E13, E16, PN10 and Adult cortices indicated that ActRIIB was the most abundantly expressed BMP receptor in the embryonic cortex, whereas its expression was significantly reduced in the postnatal and adult cortices. Fold differences were represented in arbitrary units relative to the least expressed BMP receptor (BMPRIB) and were normalized to ActB and GAPDH. (B) Whole brain lysates from E15 (lane 1), E18 (lane 2), PN7 (lane 3), Adult (lane 4) mice were analyzed for ActRIIB protein expression via Western Blot analysis. (C) Western Blot analysis of ActRIIB protein expression was also performed on E15CX (lane 1) and PN7 (lane 2) and adult (lane 3) neocortices. Consistent with RT-qPCR, ActRIIB decreased in the cortex as development progressed. ActB expression was used as a loading control.

3.2.2. Quantitative Analysis of BMP Receptors *in Vitro*

Parallel experiments were performed to quantify BMP receptor expression *in vitro*. Since ActRIIB was most abundant during the peak of neurogenesis (E13), cortical cultures were prepared at the same stage and followed for up to 7 weeks to fully assess ActRIIB expression *in vitro*. Similarly to the *in vivo* data, most receptors were highly expressed during neurogenesis *in vitro* (Figure 3.10). The type II receptor ActRIIB was the highest expressed receptor in cells cultured up to 5DIV and decreased with culture age, further suggesting a role for this receptor in neurogenesis. In addition, the type I receptor BMPRIA and the type II receptor BMPRII gradually decreased during the first 5 days in culture, however, in E13CX + 9DIV the expression of both receptors was significantly up-regulated. The observed shift in receptor expression may be related to the progression of gliogenesis or the maturation of neurons present in culture following 9DIV. Moreover, BMPRII up-regulation in the E13CX at 9DIV in culture may be related to its known role in dendritogenesis. Most importantly, ActRIIB remained the most abundantly expressed receptor in the E13 cortical cultures (up to 5DIV) and ActRIIB was significantly reduced as neural progenitors differentiated (Figure 3.10), further suggesting its role in the BMP7-induced neurogenesis. Together, the assessment of the receptors expression profiles *in vivo* (Figure 3.9) and *in vitro* (Figure 3.10) indicates that ActRIIB is the most abundant receptor in cortical progenitors, whereas its expression was significantly reduced within the time course required for neurons to differentiate. Although a suffieicnt period was used in our experiment to assess ActRIIB during neurogenesis, it was possible that as astrocytes form, its expression level may change due to the presence of both neurons and astrocytes in the culture. To assess this possibility, we further measured ActRIIB expression in pure cultures of neurons and astrocytes for up to 7 weeks. Our data clearly confirmed that ActRIIB remained down-

regulated in both neurons and astrocytes derived from cortical progenitors *in vitro* (Figure 3.11).

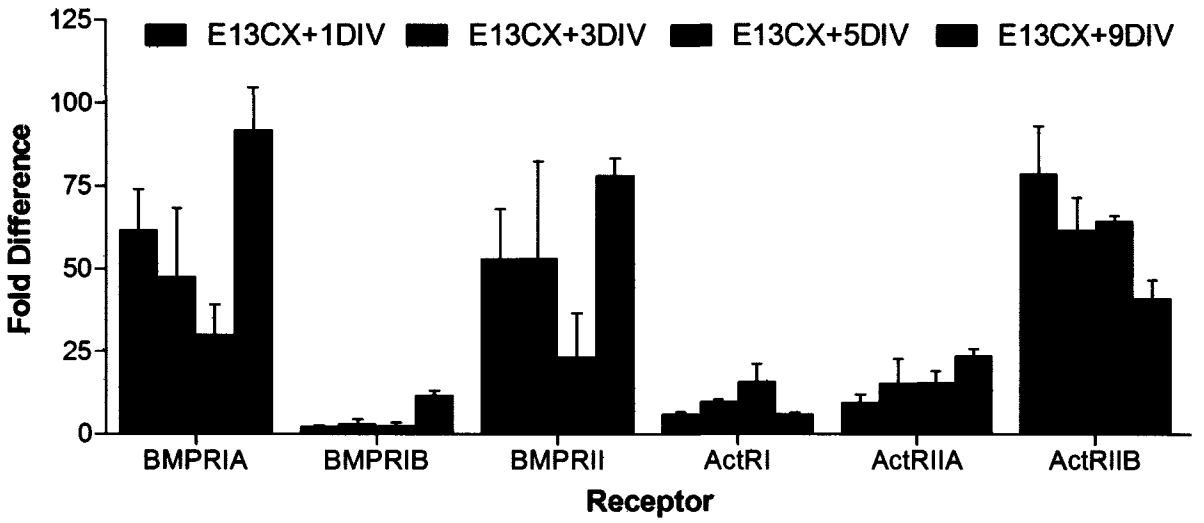

Figure 3.10: The BMP receptor ActRIIB is also highly expressed during cortical neurogenesis *in vitro*. Comparative RT-qPCR analysis of BMP receptor expression in E13CX cultures following 1, 3 and 5 days in vitro was performed. ActRIIB receptor expression remained high but significantly decreased with culture age and with the progression of gliogenesis. Fold differences were represented in arbitrary units relative to the least expressed BMP receptor (BMPRIB) and were normalized to ActB and GAPDH.

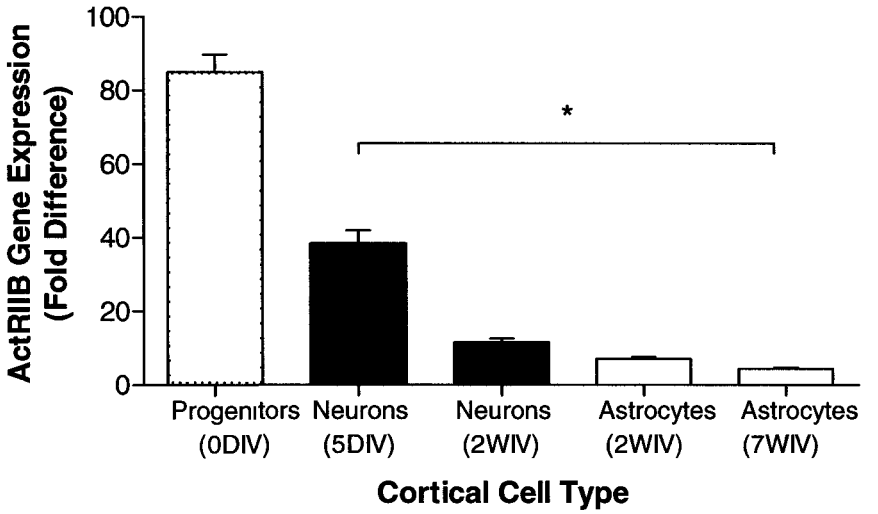

Figure 3.11: ActRIIB expression is significantly down-regulated as cortical progenitors differentiate. Comparative RT-QPCR analysis of ActRIIB receptor was performed in E13CX cultures, primarily consisting of neural progenitors (0DIV), neurons (5DIV and 2WIV) or astrocytes (2WIV and 7WIV). ActRIIB is highly expressed in neural progenitors and decreases significantly upon differentiation. Fold differences were represented in arbitrary units and normalized to ActB and GAPDH (1way ANOVA and *post hoc* Bonferroni's test; N=1, *P<0.01).

3.3. The Role of ActRIIB in BMP7-Induced Neurogenesis

Since ActRIIB is highly expressed during cortical neurogenesis, further studies were performed to examine its role in BMP7-induced neuronal differentiation.

3.3.1. Neutralization of ActRIIB and SMAD Activation

The role of ActRIIB in BMP7-induced neurogenesis was initially examined via receptor neutralization. Prior to examining the effect of an ActRIIB neutralizing antibody (ActRIIB nAb) on neurogenesis, its ability to block SMAD1/5/8 activation was first characterized using Western Blot analysis (Figure 3.12). Pre-treatment with 3 μg/mL ActRIIB nAb prior to the addition of 1 ng/mL BMP7 significantly reduced the phosphorylation of SMAD1/5/8 and its accumulation in the nucleus of E13CX cells at 1DIV.

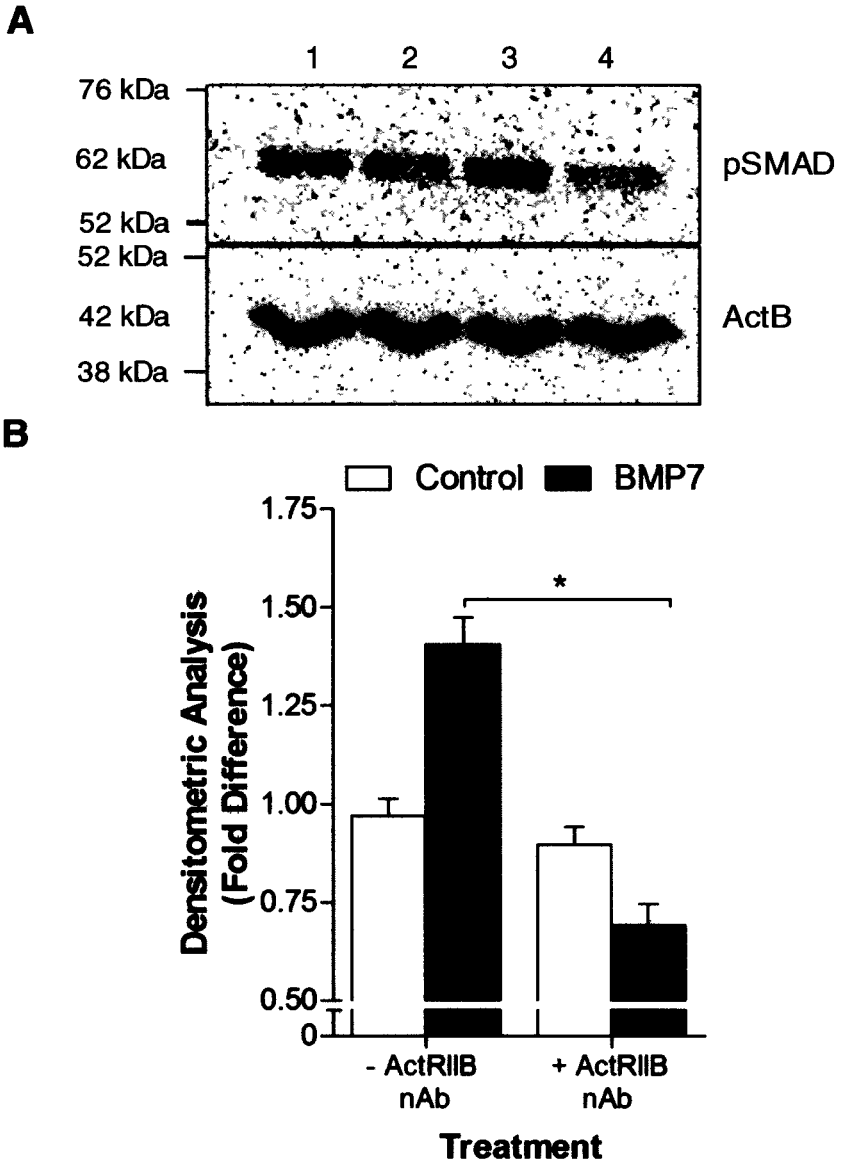

Figure 3.12: BMP7 signals through ActRIIB. E13 cortical control (lane 1), ActRIIB nAb (lane 2), BMP7 (lane 3) and BMP7 and ActRIIB nAb (lane 4) treated cultures (1DIV) were examined via Western Blot analysis following 1 hr treatment. Densitometric and Western Blot analyses indicated that co-treatment with BMP7 (1 ng/mL) and ActRIIB nAb (3 µg/mL) reduced activation of SMAD1/5/8 by BMP7. Fold differences were represented in arbitrary units (1way ANOVA and *post hoc* Bonferroni's test; N=3, *P<0.05).

3.3.2. Neutralization of ActRIIB and Neurogenesis

Next, the function of ActRIIB in BMP7-induced neurogenesis was examined via ActRIIB nAb treatment in culture. E13CX cultures were treated with 5 μg/mL ActRIIB nAb followed by adding 1 ng/mL BMP7 every 24-hrs up to 5DIV. Our results indicate that there was a significant reduction in the percent of BMP7-induced MAP2+ neurons in the presence of ActRIIB nAb (Figure 3.13). More specifically, the percent of MAP2+ cells was similar between the control and BMP7 treated cultures in the presence of ActRIIB nAb at 40.4 ± 1.1 % and 40.9 ± 1.5 %, respectively. In contrast, the percent of MAP2+ cells for the control and BMP7 treated cultures without ActRIIB neutralizing antibody was 39.1 ± 0.8 % and 46.6 ± 1.2 %, respectively. Therefore, these data suggest that ActRIIB and possibly SMAD signaling is important for BMP7-induced neurogenesis.

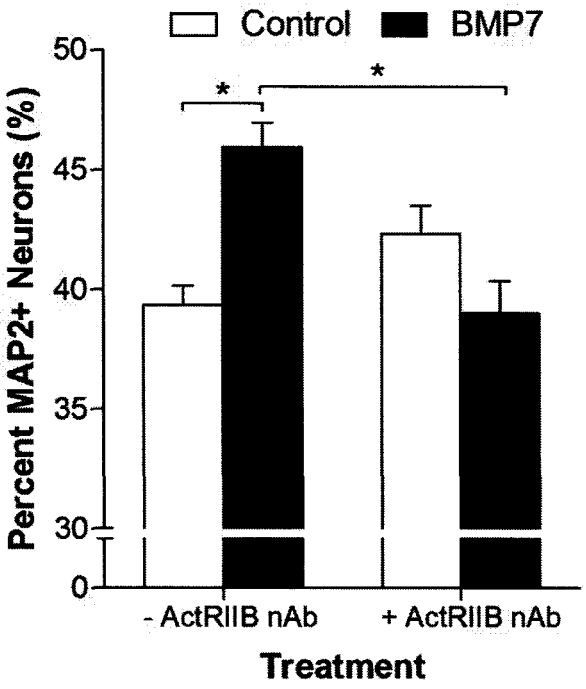

Figure 3.13: ActRIIB mediates BMP7-induced neurogenesis. Neutralization of ActRIIB in E13CX cultures treated with 1ng/mL BMP7 for 5DIV reduces the generation of MAP2+ neurons relative to cultures treated with BMP7 in the absence of ActRIIB nAb. The generation of MAP2+ neurons is similar between control, ActRIIB nAb alone and ActRIIB nAb + BMP7 treated cultures (1way ANOVA and *post hoc* Bonferroni's test; N=2, $P<0.01$).

3.4. Development of molecular tools for future studies

To set the base for future studies furthering our understanding of ActRIIB in BMP7-induced cortical neurogenesis, I developed and optimized a lentiviral system permitting shRNA-mediated knockdown of ActRIIB. In addition, to provide a useful tool for the study of BMP7 in neurogenesis and neuroprotection *in vivo*, a lentiviral-based BMP7 overexpression vector was developed.

3.4.1. Knockdown efficiency of ActRIIB shRNA lentivectors

A total of five different lentiviral-based shRNA vectors (pActRIIB-shRNA-A through E) targeting mouse ActRIIB were commercially obtained (OpenBiosystems) and tested to measure the knockdown efficiency of each vector in mouse striatal and cortical progenitors, which both highly express ActRIIB. Initially, Vectors A, B and C were tested in striatal progenitors, resulting in a 38%, 29% and 12% reduction in ActRIIB mRNA, respectively, after 5 days post-infection (DPI) (Figure 3.14A). Vectors D and E were further tested in E13CX cultures and had knockdown efficiencies of 67% and 23%, respectively (Figure 3.14B). Therefore, vector D (pActRIIB-shRNA-D) had the highest knockdown efficiency in progenitor cultures and was selected for further knockdown experiments. The empty vector, pLKO.1, was used as a control for ActRIIB-shRNA lentiviral infections.

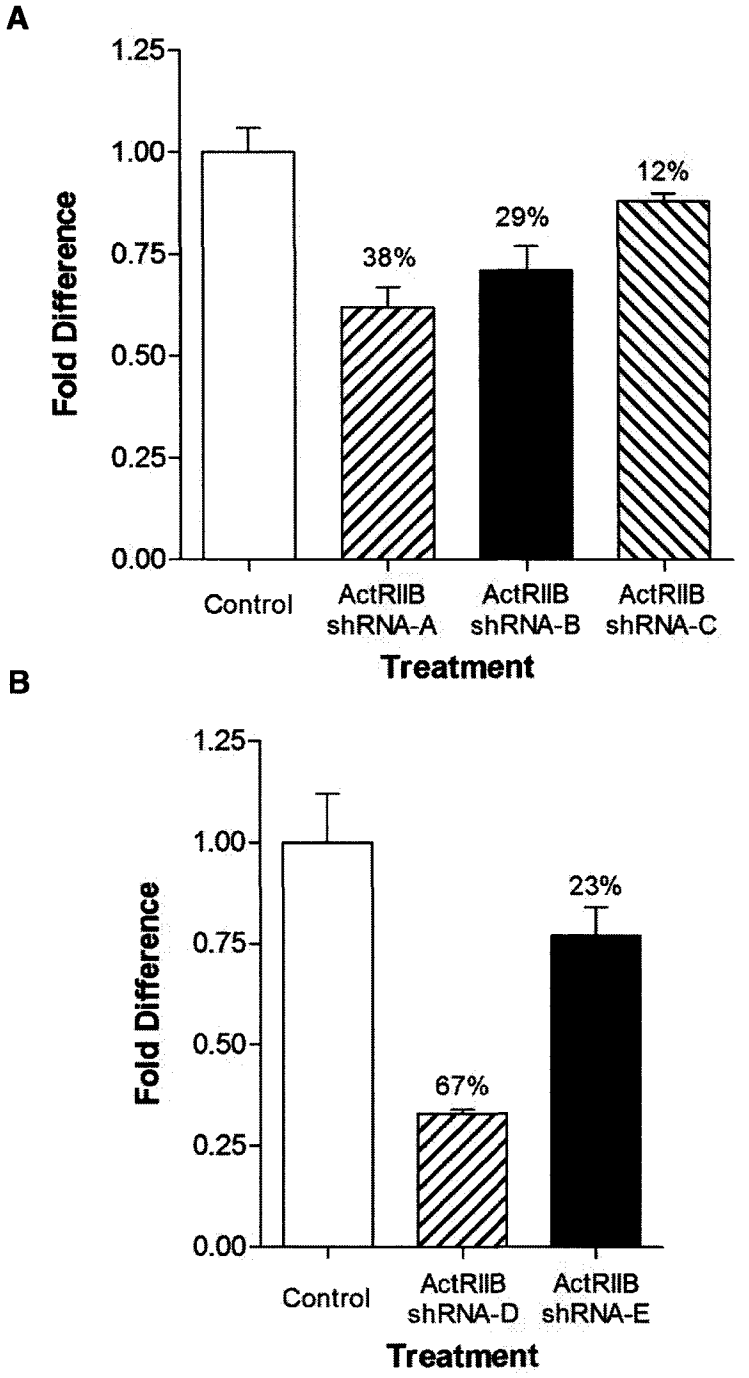

Figure 3.14: The shRNA lentivector pActRIIB-shRNA-D (Vector D) has the highest knockdown efficiency. QPCR was used to determine the knockdown efficiency of (A) pActRIIB-shRNA-A through C and (B) pActRIIB-shRNA-D and E in striatal and cortical progenitors, respectively. Vector D demonstrated a 67% knockdown of ActRIIB mRNA in E13 cortical cultures 5DPI. Fold differences were represented in arbitrary units.

3.4.2. Infection efficiencies of control and ActRIIB-shRNA lentivirus

Although the original shRNA vectors, pLKO.1 and pActRIIB-shRNA-D contained a puromycin resistance selection gene, treatment of E13 cortical cultures with puromycin was ineffective due the the short time-period of the experiments. Furthermore, a tracking system was desired to accurately assess infection efficiencies and neuronal differentiation in cells expressing ActRIIB-shRNA. Therefore, an EGFP gene was cloned in place of the puromycin resistance gene through a three step cloning process in both vectors (Figure 3.15A-D). The resulting vectors, pLKO.1-EGFP and pActRIIB-shRNA-EGFP were able to produce viable lentivirus (Control-Lv and ActRIIB-shRNA-Lv, respectively), which could be tracked with EGFP (under the control of the hPGK promoter) as early as 48-hrs post-infection. Following an extensive series of experiments, transduction protocols for both Control-Lv and ActRIIB-shRNA-Lv were optimized for E13 cortical cultures resulting in infection efficiencies greater than 70% (Figure 3.15B-C and E-F, respectively). Furthermore, the knockdown vectors in parallel with ActRIIB knockout studies will be used by other members of the lab in the next series of ActRIIB experiments.

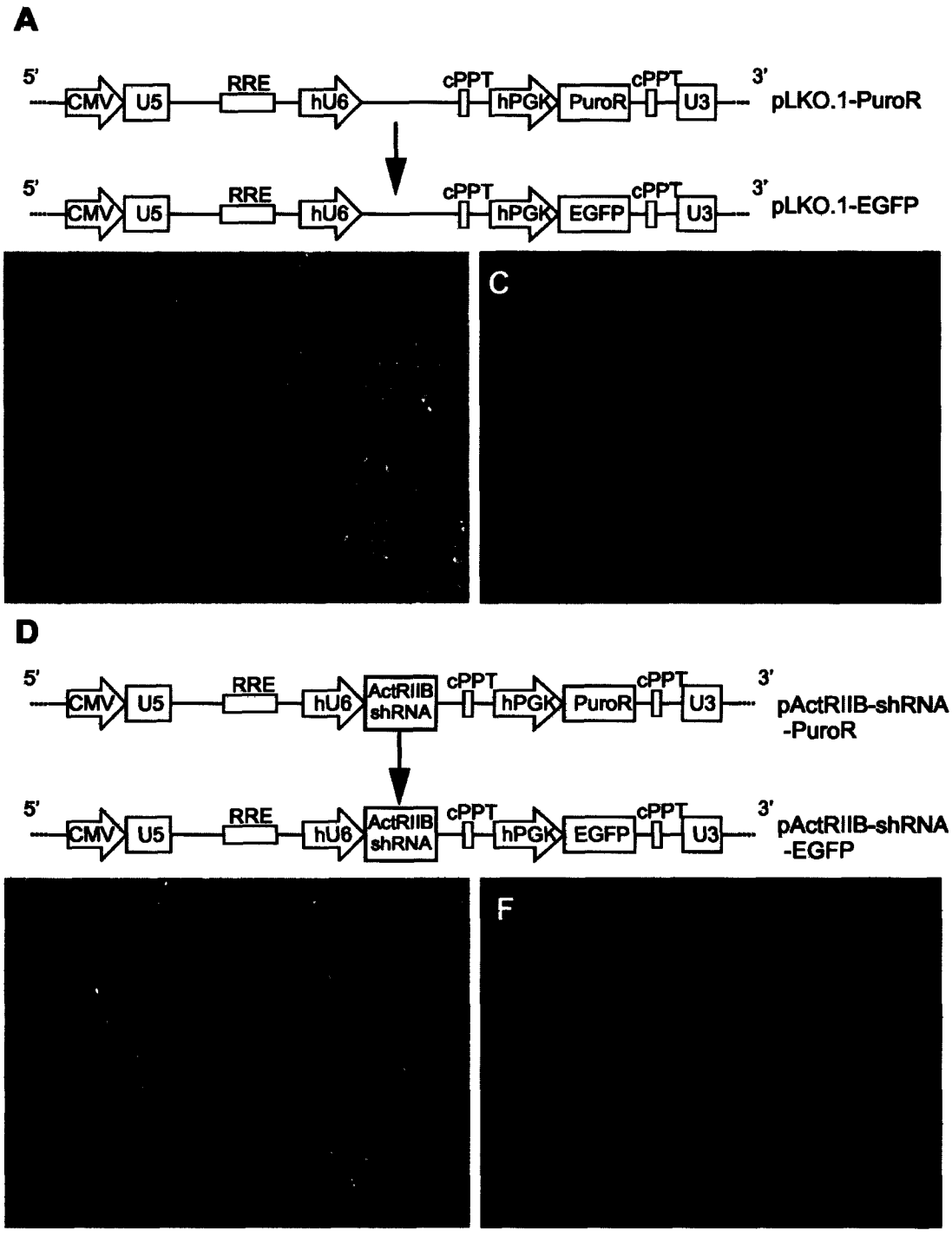

Figure 3.15: Enhanced Green Fluorescent Protein (EGFP) was used to assess infection efficiencies. The puromycin resistance gene in both (A) pLKO.1 and (D) pActRIIB-shRNA-D lentivectors was replaced with EGFP through a three-step cloning procedure. Infection efficiencies for both (B; Phase, C; FITC) pLKO.1-EGFP and (E; Phase, F; FITC) pActRIIB-shRNA-EGFP are greater than 70% in E13 cortical cultures following 5DIV.

3.4.3. BMP7 Delivery

The short half-life of BMP7 (< 30 minutes) along with the transient nature of SMAD signaling, highlights the need for a longer-lasting and more consistent BMP7 delivery system to further examine its role both *in vitro* and *in vivo*. Moreover, BMP7 delivery *in vivo* via multiple intravenous or intraventricular injections, faces additional problems associated with the blood-brain barrier. Therefore, to help further our understanding of its role in the cortex, a reliable lentiviral-based BMP7 overexpression system was developed for use in future studies. In this study, a lentiviral-based BMP7 overexpression vector was constructed, utilized to produce viable lentivirus and assessed for BMP7 protein production and delivery *in vitro*.

3.4.4. Vector Construction and Lentiviral Titration

A lentiviral-based delivery system was engineered to produce recombinant BMP7 protein to be subsequently used to study the effects of BMP7 overexpression in the brain. The full length cDNA sequence encoding BMP7 was cut from pCMV-SPORT6-BMP7 and inserted into a third generation backbone (pTet07CSII-CMV-IRES-GFPq) lentivector, resulting in the construction of the 12,561 bp transfer vector, pLvBMP7-GFP (Figure 3.16A). The full length cDNA sequence for BMP7 encoded the 29 amino acid (aa) signal peptide, the 262 aa propeptide domain and the 139 aa mature BMP7 protein domain. Specifically, the BMP7 sequence was inserted downstream of a constitutively active CMV promoter and upstream of IRES-GFP to achieve high expression levels and to allow for viral titration and monitoring of the transfection and transduction, respectively. Using GFP as a reporter, the transfection efficiency in 293SF-PacLV packaging cells with pLvBMP7-GFP or pTet07CSII-CMV-GFPq transfer vectors was greater than 90% in all trials (Figure 3.16B).

Shortly following induction of transfected cultures, high lentiviral titers were achieved in the unconcentrated media of up to $3.7x10^7$ TU/mL, as measured by FACS analysis of serial infections in HEK293 cultures (Figure 3.17). In most cases viral titers were concentrated (at least 30-fold) to maximize transduction efficiency, while minimizing the infection volumes.

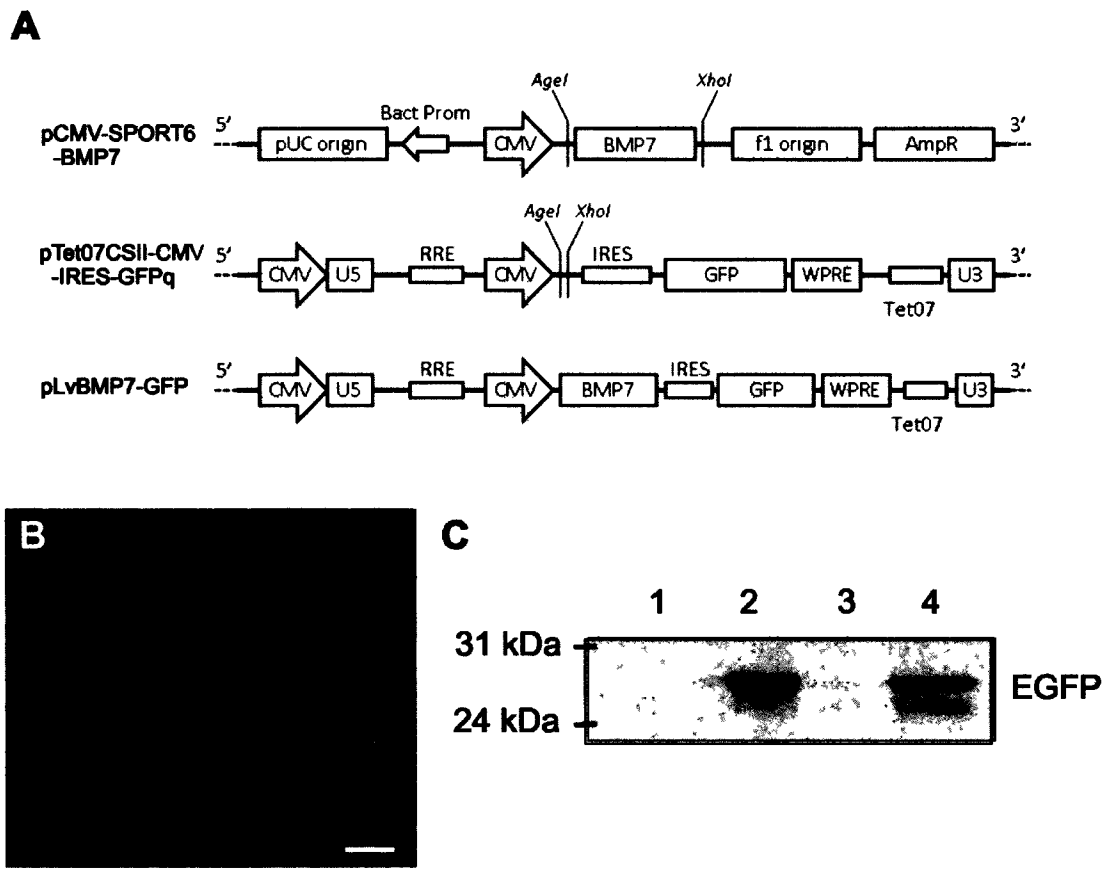

Figure 3.16: BMP7 lentivector design and transfection. A) To construct pLvBMP7-GFP, the sequence for mouse recombinant BMP7 was cut from the plasmid pCMV-SPORT6-BMP7 and inserted into the lentivector pTet07CSII-CMV-IRES-GFPq immediately upstream of the sequence encoding IRES-GFP. B) The BMP7-GFP expression was confirmed by fluorescence microscopy 18 hours after transfecting the packaging HEK 293SF-PacLv cells. C) Western Blot analysis verified EGFP protein in the lysates of transfected and infected HEK293 cultures (lanes 2 and 4, respectively). EGFP protein was absent in control (uninfected) lysates (lanes 1 and 3, respectively) . Scale bar: 50 μm.

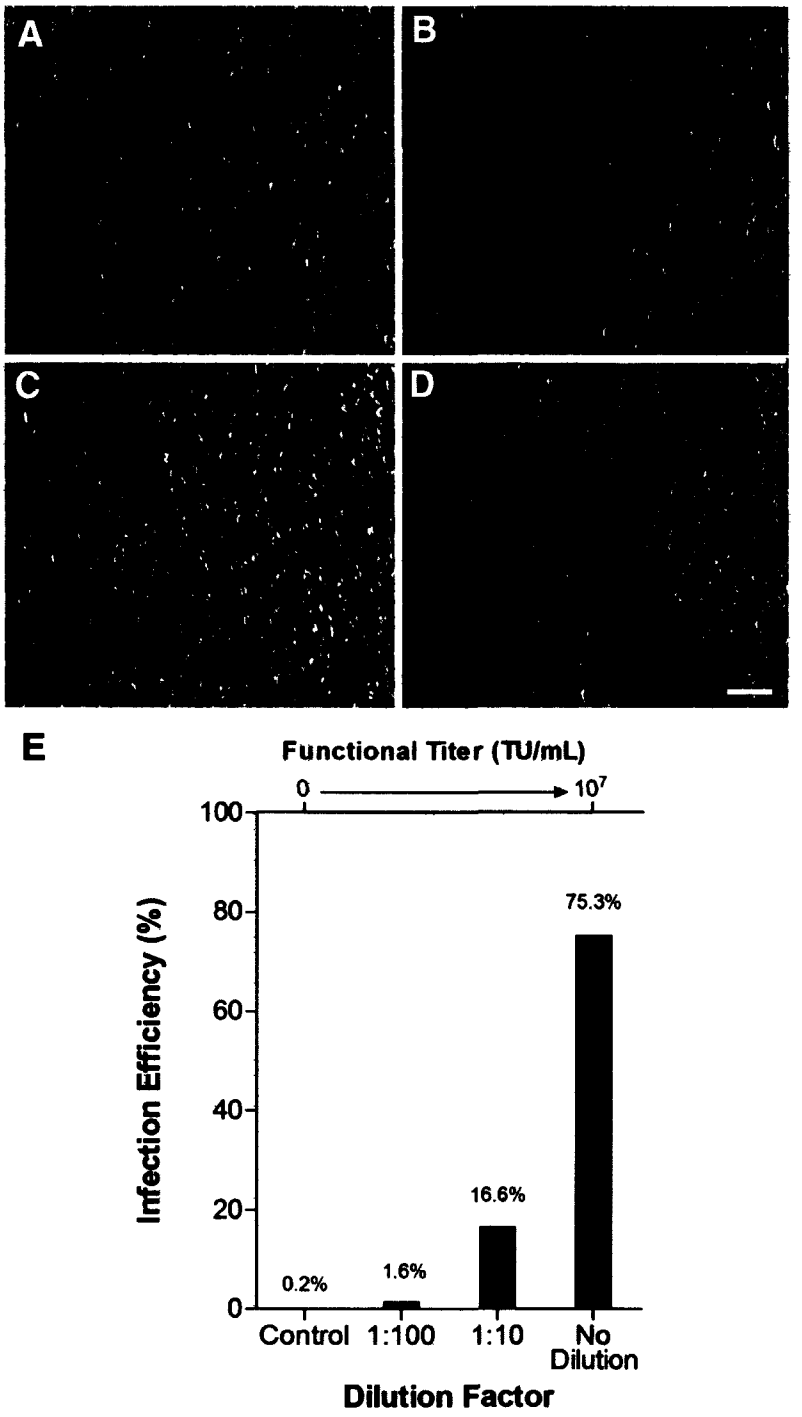

Figure 3.17: Titration of BMP7 lentivirus. The BMP7 titer was determined by FACS analysis of HEK293GPG cells infected with BMP7-GFP lentivirus (B-D), compared to control uninfected cultures (A). The infection efficiency was determined by obtaining the percent of BMP7-GFP positive cells in the cultures infected with different dilutions of supernatant in medium: 1:100 (B), 1:10 (C) or no dilution (D). Scale bar: 50 μm.

3.4.5. Infection of HEK293 Cultures

A minimum 75% transduction efficiency with BMP7 lentivirus was achieved in HEK293 GPG cultures based on a multiplicity of infection (MOI) of 100. Western Blot and ELISA verified the overexpression, correct processing and subsequent secretion of mature BMP7 protein into the supernatant (Figure 3.18A). The media collected from BMP7 lentiviral-transduced HEK293 cultures contained high levels of BMP7, whereas the protein was undetectable in the media collected from control cultures (transduced with GFP lentivirus). In particular, 223 ± 67 ng/mL of BMP7 was secreted over a 24 hour period by $1x10^6$ cells three days post infection (Figure 3.18B). In addition, the BMP7 lentiviral-transduced cultures consistently secreted LvBMP7 for up to 4 weeks following infection. Analysis of media collected from these cultures indicated that approximately 182 ± 24 ng/mL of mature LvBMP7 was released within 24 hours (Figure 3.18B). Therefore, the BMP7 lentiviral-transduced cells maintained the capacity to produce high levels of fully processed mature BMP7 protein for at least 4 weeks.

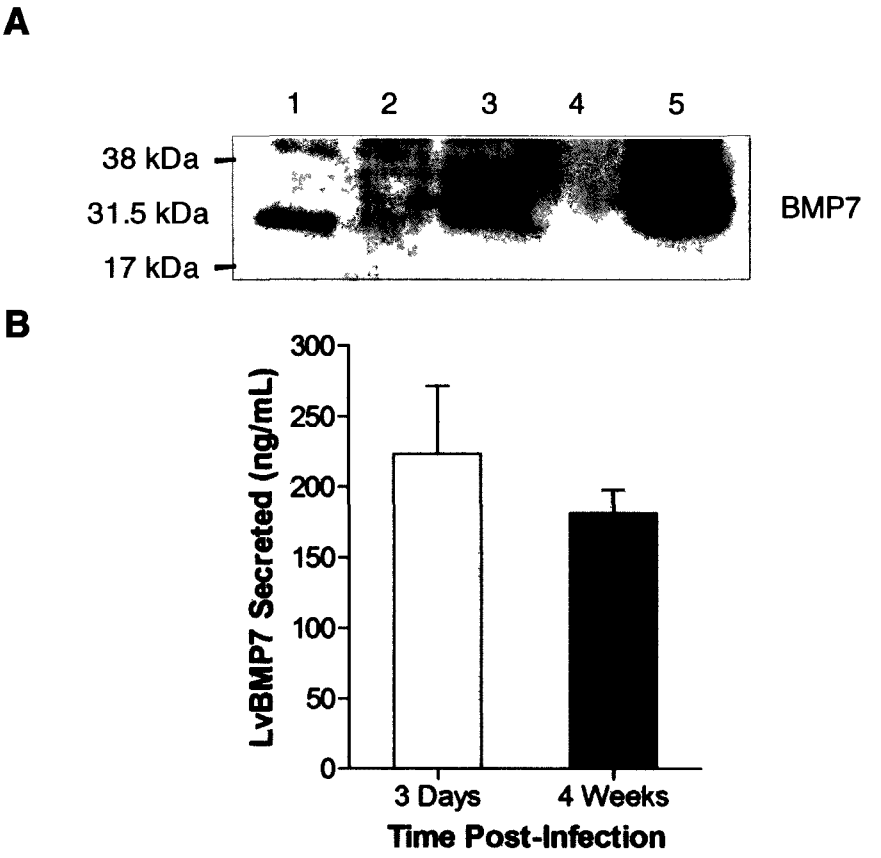

Figure 3.18: BMP7 protein production in HEK293 cells. The production of BMP7 protein was validated by Western Blot (A) and ELISA (B) of the infected HEK293GPG cultures. A) Western Blot analysis showed that BMP7 protein was present in the cultures as early as 48 hours following infection. Samples included: mouse cerebrospinal fluid (lane 1), cells infected with GFP-Lv (lane 2), medium from BMP7 lentivirus infected cultures (lane 3), medium (10x concentrated) from GFP-Lv infected cultures (lane 4), medium (10x concentrated) from BMP7 lentivirus infected cultures (lane 5). B) ELISA confirmed that a total of 223.6 ± 67.7 ng/ml of mature BMP7 protein was secreted over 24 hours, 3 days after infection. Cells continued to produce BMP7 protein (181.5 ± 23.7 ng/mL over 24 hours) at least 28 days post-infection.

3.4.6. Infection of Primary Cultures

To further determine the suitability of a BMP7 transduction system, human amniotic fluid primary cultures were infected with the BMP7-GFP lentivirus. These cells were chosen based on the recent data from several laboratories, including ours, showing that they include subpopulations that share many of the characteristics reported for human embryonic stem cells. Thus, they have a great potential to be used for cell transplantation studies. As expected [Jeziereski *et al.,* 2010], human amniotic fluid cells required at least three days to demonstrate lentivirally-delivered gene expression. The level of BMP7-GFP expression was significantly enhanced within a week (Figure 3.19), and reached its maximum level after 2 weeks of infection. High levels of BMP7 expression were observed for at least 4 weeks (Figure 3.19). These data were further confirmed by ELISA, showing that human amniotic fluid cells were able to secrete mature BMP7 protein at the levels comparable to those of HEK293 cells. Both cell types have been previously used in the cell transplantation studies, emphasizing their potential applications in delivering beneficial factors such as BMP7 to the brain in a paracrine fashion.

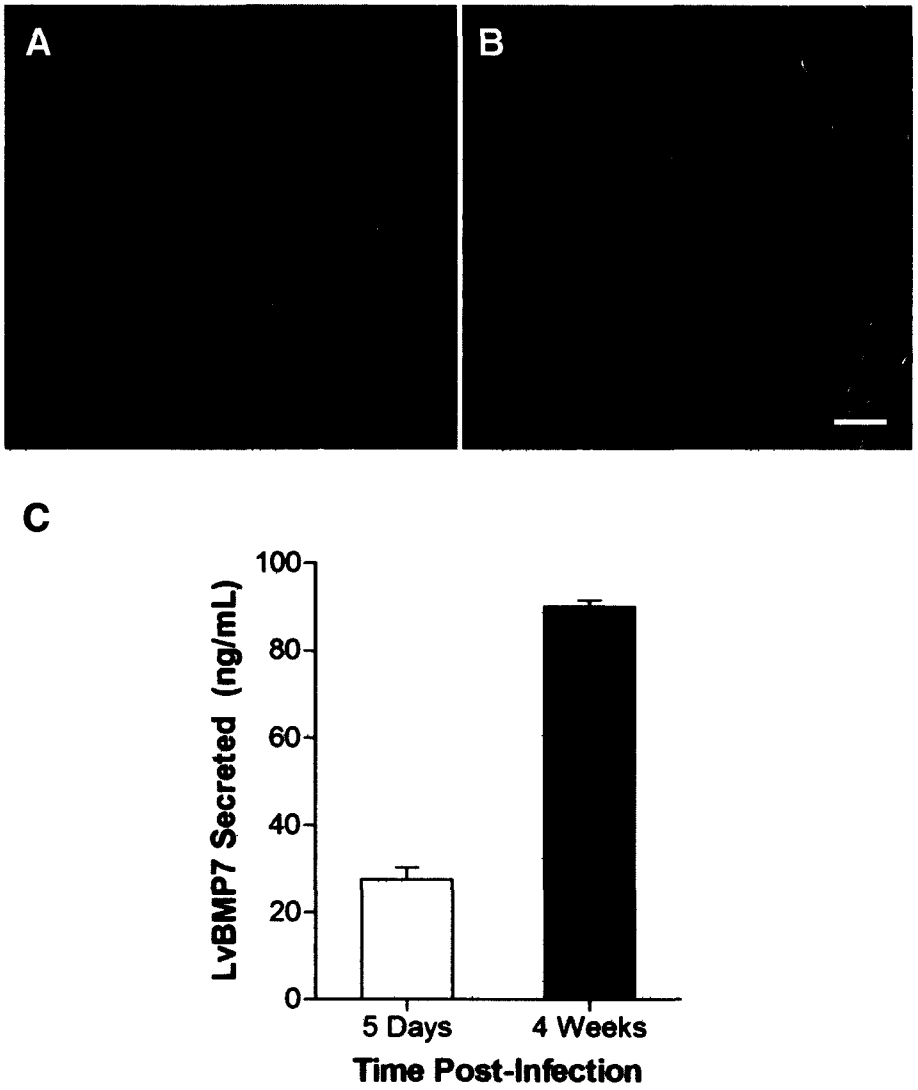

Figure 3.19: BMP7 production in human amniotic fluid primary culture. BMP7-GFP was expressed in human amniotic fluid cells 5 days after infection (A) and maintained for at least 4 weeks (B) throughout several passages, as evidenced by fluorescence microscopy. ELISA confirmed that significant levels of mature BMP7 protein can be obtained from the infected human amniotic fluid cells. The data represent the levels of mature BMP7 released over a 24 hour period at 5 days and 4 weeks post-infection. Scale bar: 50 μm.

3.4.7. Biological activity of LvBMP7

Next we examined the biological activity of LvBMP7 in neural progenitors via both SMAD phosphorylation and neuronal differentiation. Similar to previous experiments, cultures were treated with 1 and 30 ng/mL LvBMP7 to test the activity of lentiviral-derived BMP7 (LvBMP7). In comparison to the control cultures (infected with GFP lentivirus alone), significant (greater than 3-fold) SMAD1/5/8 activation was observed in the nuclear extracts of cortical cultures treated with 1 ng/mL LvBMP7 for 1 hour. Moreover, SMAD1/5/8 activation following treatment with 1 ng/mL LvBMP7 present in the culture media was comparable to that using 1ng/mL rhBMP7 (Figure 3.20A). Interestingly, SMAD1/5/8 activation was not significantly increased with the higher, up to 30 ng/mL, concentrations of LvBMP7.

In addition to SMAD activation, the functional activity of LvBMP7 was examined in terms of enhancing cortical neurogenesis in culture. Therefore, neural progenitors were treated with media containing LvBMP7, which was compared to rhBMP7. Relative to the control and Lv-GFP treated cultures, the number of MAP2 positive cells was significantly higher in cultures treated with either rhBMP7 or LvBMP7. Specifically, the number of MAP2 positive cells in cultures treated with LvBMP7 was enhanced by approximately 33 ± 8% and with rhBMP7 approximately 45 ± 6% relative to the control following treatment for five days *in vitro* (Figure 3.20B). Therefore, LvBMP7 was capable of enhancing the neuronal differentiation of mouse cortical progenitors, further indicating that LvBMP7 is biologically and functionally active.

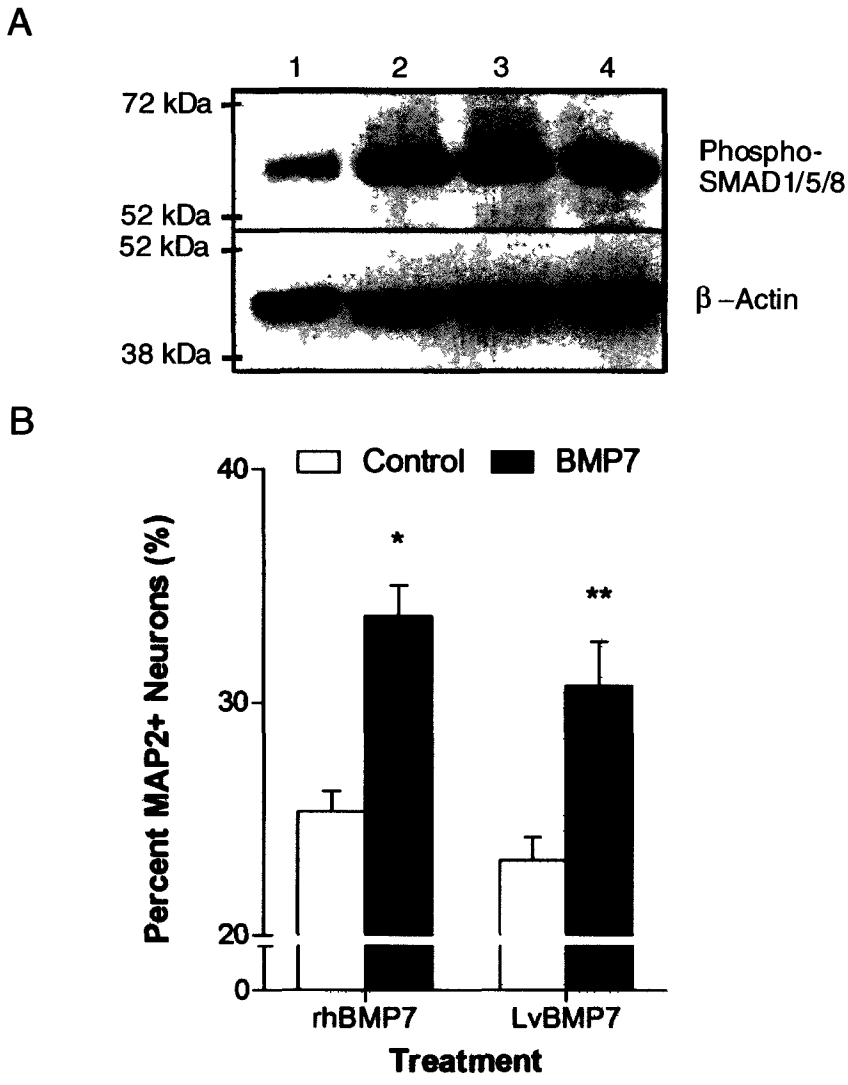

Figure 3.20: The biological activity of lentivirally-produced BMP7 protein. A) Western Blot showed that BMP7 initiated SMAD1/5/8 phosphorylation (activation) in neural progenitors. Cells were treated with the medium collected from control-GFP infected cultures (Lane 1), 1 ng/mL rhBMP7 (lane 2), 1 ng/mL LvBMP7 (lane 3) and 30 ng/mL LvBMP7 (lane 4). Lanes included un-infected control cells (lane 1), and the cultures treated with LvGFP (lane 2), 1 ng/ml rhBMP7 (lane 3) or 1ng/ml LvBMP7 (lane 4), using the same volume. B) There was a significant increase in the number of neurons in the BMP7 treated cultures, as determined by the ratio of cells positive for both MAP2 and Hoechst over the total number of Hoechst positive cells (2way ANOVA and *post hoc* Bonferroni's test; N=3, *$P < 0.01$).

DISCUSSION

4.1. Cortical Neurogenesis

The complex processes of neural cell proliferation, fate determination and subsequent differentiation into neurons and astrocytes during cortical development are regulated by a variety of intrinsic and environmental factors [Qian *et al.,* 1998, 2000; Bani-Yaghoub *et al.,* 2006, 2007; Dehay and Kennedy, 2007]. These factors allow cortical neurogenesis to initiate at around E10 and continue as the primary mode of differentiation up to E17 in mice. This process is complemented by gliogenesis contributing to astrocyte populations starting at E16 and increasing through post-natal development [Qian *et al.,* 1998, 2000]. Neural progenitors can also give rise to neurons and astrocytes in a similar chronological order *in vitro,* allowing the study of individual growth factors in neurogenesis and gliogenesis.

Bone Morphogenetic Proteins (BMPs) are among important growth factors that may regulate cortical development in a gradient-dependant manner. In particular, BMPs regulate apoptosis in early neuroepithelial cells residing in the neural tube, and cell differentiation in embryonic and adult neural progenitors [Panchision *et al.,* 2001]. For instance, BMP4 has been shown to promote caspase-mediated programmed cell death in the dorsal neural tube [Traister *et al.,* 2004]. The generation of Tuj1-positive and MAP2-positive neurons is enhanced in mid-gestation mouse cortical cultures following co-treatment with FGF2 and either BMP2, 4, 6, 7, 12 or 13 for up to 4DIV [Li *et al.,* 1998]. Additionally, BMP2/4 treatment enhanced the specification of late-born cortical GABAergic neurons at the expense of oligodendroglial lineage elaboration [Yung *et al.,* 2002], whereas transgenic overexpression of BMP4 is also reported to increase

astroglial differentiation at the expense of oligodendroglial lineage commitment [Gomes et al., 2003].

More recently, BMP7 has been shown to promote functional recovery following ischemia or mild stroke in the rat brain, suggesting a possible role in neuroprotection and cortical recovery [Chou *et al.,* 2006]. However, as BMP7 has been implicated in a variety of processes in the brain including neuroprotection, neurogenesis and gliogenesis, the mechanism by which functional recovery may occur, remains unclear. Since one prospective approach to enhancing functional recovery following cortical injury is through enhanced endogenous neurogenesis and/or induced neuronal differentiation of implanted progenitors, a thorough understanding of neurogenesis and the factors involved is necessary. Current studies suggest that BMP7 may regulate the differentiation of embryonic neural progenitors at low concentrations [Mehler *et al.,* 2000; Chang *et al.,* 2003]. However, due to the presence of additional growth factors and variable culture conditions, this notion requires further investigation in the context of cortical neurogenesis. Furthermore, since the various roles of BMP7 (and other BMPs) are regulated through the context and time-dependant expression of particular BMP receptors, antagonists and/or transcription factors, the mechanisms through which BMP7 functions during cortical development requires further examination.

4.2. BMPs and their Receptors

The transcripts for both BMPs (BMP2, 4 and 7) and the BMP receptors (BMPRIA, BMPRIB, BMPRII, ActRIA, ActRIIA and ActRIIB) have been previously studied in the rat brain [Söderström *et al* 1996; Funaba *et al.,* 1997; Mehler *et al.,* 1997; Zhang *et al.,* 1998]. To further advance our knowledge in this area, the expression of BMP7 and its receptors were initially examined during key stages of cortical

development via RT-PCR. Initially, we studied the stages such as neural tube patterning at 9.5 and E10, cortical neurogenesis at E12.5 and E14, and the initiation of gliogenesis at E16 and E17 [Published in Bani-Yaghoub *et al.,* 2008]. BMP7 and BMP receptor expression was further examined in adult neocortices, where neurogenesis is limited to the subventricular zone (SVZ) and gliogenesis is most prevalent. The presence of BMP7 transcript throughout cortical development suggests a possible autocrine role in cell maintenance and/or differentiation, which was followed by more recent studies, confirming the presence of BMP2, 4 and 7 transcripts in the neural tube at as early as E9 [Denesh *et al.,* 2009; Sun *et al.,* 2010]. However, BMP7 was found to be the most highly expressed bone morphogenetic protein in the early telencephalon (E9 to E10.5). Furthermore, the same study also indicated the presence of BMPRIA, BMPRIB and BMPRII transcripts during neural tube formation and patterning, suggesting their involvement in neural cell maintenance and differentiation.

Further experiments were performed to examine whether the presence of BMP7 transcript correlated with mature BMP7 protein in embryonic cortical tissues. Mature BMP7 protein was not detected in E13 cortical tissue, as measured by Western Blotting and ELISA. These results are in agreement with previous findings showing that BMP7 protein is undetectable in the human neural tube, but it is present in the embryonic meninges [Vukicevic *et al.,* 1994]. In addition to meninges, BMP7 protein has been detected in cultured astrocytes and to a much lesser degree in neurons derived from later stages of cortical development (E18.5 and PN1) [Chang *et al.,* 2003]. Consistent with the literature, we detected low levels of BMP7 in both meningeal tissue and cultured astrocytes (E15), suggesting a possible role in paracrine signaling. In contrast, BMP7 protein was not detected in either the conditioned media or lysates of cultured E13 progenitors and neurons. Interestingly, our results closely resemble the expression

patterns of BMP2 and 4, which are present in cultured astrocytes but absent in cultured neural progenitors, neurons and oligodendrocytes [Lu *et al.,* 2009].

It is known that paracrine signaling occurs in the cortex via the cerebrospinal fluid, which is secreted by the choroid plexus located in the ventricles of the brain. The choroid plexus is responsible for producing a wide array of growth factors important in development and neural cell maintenance such as the insulin-like growth factors (1 and 2), brain-derived neurotrophic factor and various BMPs. More specifically, it is reported that BMP7 mRNA is expressed in the choroid plexus for production and secretion into the CSF [Dattatreyamurty *et al.,* 2001]. Since human CSF contains low levels of BMP7 (~ 0.7 ng/mL), we examined mouse CSF via Western Blotting and ELISA [Dattatreyamurty *et al.*, 2001; Gautschi *et al.,* 2007]. Surprisingly, we only detected BMP7 in the CSF under reducing conditions using Western Blot analysis, whereas the fully-processed mature protein was undetectable using ELISA. Since ELISA is performed under non-reducing conditions, it is possible that other proteins in the CSF interact with BMP7 interfering with its detection in ELISA. For instance, the BMP7 prodomain has been reported to remain non-covalently associated with the mature peptide for targeting purposes [Gregory *et al.,* 2005], possibly reducing the detection limit of the BMP7 antibody. In addition, the half-life of BMP7 in both the CSF and serum of rats is very short (less than 30 minutes) following intracisternal or intravenous administration [Dreibelbis *et al.,* 2002; Vukicevic *et al.,* 1998; Grgurevic *et al.,* 2007]. Therefore, the short half-life of BMP7 in both the CSF and serum may also contribute to its limited detection via ELISA.

The biological activity of BMP7 can be measured by examining SMAD signaling *in vitro*. Emulating the physiological level of BMP7 in the CSF [Gautschi *et al.,* 2007], 1 ng/mL of BMP7 was added to cortical progenitors every 24 hours and tested for its role

in neuronal differentiation. As expected, the physiological level of BMP7 resulted in the presence of phosphorylated SMAD1/5/8 in the nucleus as early as 30 minutes up to 2 hours following treatment of E13CX cultures. Similar to the control, 24 hours following BMP7 treatment pSMAD1/5/8 levels were significantly reduced in the nucleus further indicating the transient nature of BMP signaling, it's short half-life in culture and the necessity to add BMP7 at least every 24 hours to the culture medium.

4.3. BMP7 in Cortical Neurogenesis

We examined E13 cortical progenitors in the presence of low to high concentrations of BMP7 (1 and 30 ng/mL) *in vitro*. All the experiments were performed in the absence of serum and other growth factors to limit variables that may either negatively or positively affect neurogenesis (with the exception of N2 supplement, which was added to cultures to prevent extensive cell death). Under these conditions, we clearly demonstrate that 1 ng/mL BMP7 is sufficient to enhance neurogenesis in cultured cortical progenitors. In particular, we show a nearly 1.5-fold increase in the number of MAP2-positive neurons in BMP7 treated vs. control cultures. These results are in agreement with the studies by Chang *et al.,* (2003) and Mehler *et al.,* (2000), suggesting a concentration-dependant role of BMP7 in cortical differentiation. More specifically, low concentrations of BMP7 enhance neurogenesis, whereas higher concentrations (greater than 10 ng/mL) favor gliogenesis in the embryonic cortex.

In contrast, in the adult SVZ BMPs inhibit neurogenesis, whereas noggin (expressed by adjacent ependymal cells) creates a niche promoting adult neurogenesis [Lim *et al.,* 2000]. Moreover, SVZ cells grafted into the striatum differentiate primarily to astrocytes [Lim *et al.,* 2000], suggesting that high levels of endogenous BMP7 present in the striatum and the dorsomedial cortex favors gliogenesis. This notion was further

supported by the addition of noggin to promote the differentiation of the grafted SVZ cells to Tuj1 positive neurons [Lim *et al.,* 2000]. It is possible that treatment with noggin potentially reduces the availability of BMP7 to its receptors, thereby mimicking low-concentration BMP7 conditions and promoting neurogenesis rather than gliogenesis. Analogous mechanisms have been described in olfactory neuroepithelial cultures where high-doses of noggin completely blocked BMP-induced neurogenesis, whereas low-doses of noggin allowed neurogenesis to occur [Shou *et al.,* 2000].

Similarly, Chang *et al.,* (2003) also demonstrated that conditioned media from neuronal cultures promoted the differentiation of neural stem cells into neurons, whereas astrocyte conditioned media promoted gliogenesis. The addition of noggin to cultures treated with astrocyte conditioned media resulted in increased neurogenesis, further supporting a concentration-dependant role for BMP7. Although the precise concentrations of BMP7 were not established, immature BMP7 was shown to be present in higher concentrations in the astrocyte conditioned media compared to that of the neuron conditioned media. Surprisingly, however, the addition of noggin to cultures treated with neuron conditioned media resulted in increased gliogenesis at the expense of neurogenesis, suggesting a very specific concentration of BMP7 is required for neurogenesis. Thus, the presence of other neurogenic or gliogenic factors in the conditioned media of neurons and astrocytes may also contribute to these processes. In support of this notion, other studies have shown that neural progenitors expressing neurogenin, a bHLH transcription factor, are directed toward a neuronal fate following BMP treatment, whereas activation of the Notch pathway promotes differentiation into astrocytes [Song *et al.,* 2001; Morrison *et al.,* 2000]. Taken together, BMP7-induced differentiation occurs both in a concentration- and context-dependant manner. However,

neurogenesis is not entirely dependent upon BMPs, as other secreted factors seem to play critical roles in cortical development.

As neural progenitors differentiate into neurons or astrocytes, the expression of nestin, an intermediate filament expressed in neural progenitors is down-regulated [Ortega *et al.,* 2009]. Therefore, we evaluated the consequence of BMP7 treatment on the expression of nestin to determine whether enhanced neuronal differentiation coincides with nestin downregulation. In cultures treated with BMP7, significantly fewer nestin–positive cells were present along with reduced nestin mRNA expression relative to the control. These data indicate that BMP7-induced neurogenesis may occur at the expense of nestin-positive neural progenitors. Consistent with our results, Ortega *et al.,* (2009) indicated that following intrauterine injection of BMP7 into the lateral ventricles of E14 mice, nestin immunoreactivity was significantly reduced in the ventricular zone, where neural progenitors are situated. Furthermore, radial glia are targeted by BMP7 and undergo premature differentiation into astrocytes following BMP7 treatment [Ortega *et al.,* 2009]. Notably, in this study an excessive amount of BMP7 (1 μg) was injected intraventricularly into E14 mouse embryos, which based on the results from our lab and those of others, high concentrations of BMP treatment would favor gliogenesis.

Another important aspect of BMPs, is that they have also been shown to support cell survival and regulate proliferation [Brenderlau *et al.,* 2004; Mathieu *et al.,* 2008; Panchision and Mckay, 2001]. For example, BMPs promote the survival and proliferation of chondrocytes during late stages of ossification [Gamer *et al.,* 2009]. More relevant to the brain, there is extensive evidence, including that from our lab, indicating a neuroprotective role for BMP7 in the brain enhancing neural cell survival during H_2O_2 and/or glutamate cytotoxicity [Tsai *et al.*, 2007]. Furthermore, it has been demonstrated via BrdU incorporation in the adult rat brain that BMP7 treatment promotes proliferation

following ischemia [Chang *et al.,* 2003], whereas proliferation is reduced in embryonic neural progenitors via BMP-induced phosphotidylinositol phosphatase-dependant cell cycle arrest [Mathieu *et al.,* 2008]. In an effort to rule out indirect effects on neurogenesis and gliogenesis, cell survival and proliferation studies were performed in the absence or presence of 1 ng/mL BMP7. Following 2-5DIV, neither cell survival nor cell proliferation was affected with low concentration BMP7 treatment. Hence, BMP7-induced neurogenesis did not occur as a result of enhanced cell proliferation or survival.

4.4. ActRIIB in Cortical Development

The expression of multiple BMPs and BMP receptors in the cortex suggests both redundant and distinct roles for each BMP ligand and receptor. BMP2 and 4 share approximately 60% of their sequence homology to BMP7, however they differ in receptor affinity. In particular, BMP2 and 4 have a higher affinity for Type I receptors, whereas similar to TGF-β and activin, BMP7 actually retains preference for Type II receptors, while maintaining its own distinct interface. Therefore, in addition to the temporal and spatial activity of BMPs and their antagonists, the expression patterns of the BMP receptors add an additional level of regulation [Greenwald *et al.,* 2003].

To explore BMP7 signaling at the level of the receptor, we established a comparative analysis of all six BMP receptors throughout cortical development both *in vivo* and *in vitro*. Samples were obtained from the mouse E10 neural tube, E13, E16, PN10 and adult cortices, representing critical stages of cortical development. Comparative RT-qPCR demonstrated that the Type II receptor, ActRIIB, is the most highly expressed receptor in neural progenitors at E10 and E13 (at least 3-fold higher than the other BMP Type II receptors). In contrast, its expression is significantly reduced in the E16 cortex and to a greater extent in the PN10 and adult cortices, when it

becomes the least expressed of the BMP receptors. Likewise, ActRIIB is highly expressed in the chick neural tube and is significantly reduced becoming restricted to rhombomeres 2 and 4 of the chick hindbrain [Stern *et al.,* 1995]. Taken together, ActRIIB expression is the most abundant during cortical neurogenesis, whereas it is significantly down-regulated during gliogenesis and postnatal development.

We complemented these experiments by *in vitro* assays to determine the expression level of ActRIIB up to 7 weeks. Consistent with our *in vivo* results, ActRIIB was the most highly expressed receptor in E13 cortical progenitors in culture. However, its expression was significantly down-regulated in cultures maintained up to 9DIV, resembling the receptor expression patterns observed in E16 cortices. Under the culture conditions employed, neurogenesis occurred within the first 3-5 days *in vitro* with minimum gliogenesis, whereas the rate of gliogenesis exceeded that of neurogenesis in older cultures. Moreover, by 9DIV (when ActRIIB expression was significantly reduced), there were relatively higher numbers of glial cell clusters present. Parallel studies demonstrated that ActRIIB expression is down-regulated following differentiation. Taken together, our *in vivo* and *in vitro* data suggest that BMP7-induced cortical neurogenesis is at least partially mediated through ActRIIB.

Further analysis of the other BMP receptors in E13 cortical cultures was performed *in vitro*. Although lower than ActRIIB expression, both BMPRIA and BMPRII were also abundant in E13 cortical cells cultured up to 9DIV. Indeed, these receptors showed a significant upregulation at day 9, a time point at which astrocytes are more abundant in culture. Constitutively-active BMPRIA (caBMPRIA) expression has been shown to enhance proliferation of neural progenitors [Panchision *et al.,* 2001], whereas BMPRIB, which was slightly upregulated in cultures at 9DIV, is reported to promote mitotic arrest leading to apoptosis in early-born neural progenitors and differentiation into

neurons or astrocytes in late-born mid-gestation neural progenitors [Panchision *et al.,* 2001]. However, BMPRIB knockout mice are viable and have no evident defects in the brain [Yi *et al.,* 2000], emphasizing functional redundancy among BMP receptors. Our results also showed that BMPRII is significantly upregulated in both cortical tissues and cultures, shortly following the onset of gliogenesis. Since BMPRII regulates dendritogenesis independent of SMAD signaling [Le Roux *et al.,* 1999; Withers *et al.,* 2000; Guo *et al.,* 2001; Lee-Hoeflich *et al.,* 2004], its upregulation in our model may contribute to other aspects of cortical neurogenesis such as neurite outgrowth.

4.5. BMP7 Signaling Through ActRIIB

To the best of our knowledge, ActRIIB has not been closely examined in the context of BMP7 in the cortex. However, studies in non-neural tissues have revealed functional significance of BMP7 signaling through ActRIIB. For instance, compared to BMP2 and 4, BMP7 preferentially associates with ActRIIB [Greenwald *et al.,* 2003], encouraging its importance in our model. In the presence of ActRIIA or ActRIIB, BMP7 effectively signals through BMPRIB and to a greater extend ActRIA (not BMPRIA), thereby contributing to BMP-mediated osteoblastic differentiation in primary human mesenchymal stem cells [Lavery *et al.,* 2008]. Furthermore, ActRIIB is primarily responsible for mediating BMP7-induced adipogenesis in mesenchymal progenitors. Finally, BMP7-ActRIIB interactions may also be involved in the regulation of apoptosis as observed in human cell lines [Kusumegi *et al.,* 2003].

As an important step to identify the role of ActRIIB in the E13 cortex, the receptor was neutralized with an ActRIIB-targeting antibody and evaluated for SMAD activity. The daily addition of high doses (5 μg/mL) of ActRIIB neutralizing antibody to cortical cultures partially blocked BMP7-induced SMAD1/5/8 activation and accumulation in the nucleus.

As a result BMP7-induced neuronal differentiation was relatively reduced. Although not significant, a higher percent of neurons was observed in the ActRIIB nAb control relative to the non-treated control. It is possible that ActRIIB may be involved in the regulation of the other BMP receptors, which may become more sensitive to other BMPs, following ActRIIB neutralization. For example, analogous mechanisms exist in pulmonary smooth muscle cells, such that expression of BMPRII inhibits BMP7-mediated activation of SMAD signaling, whereas siRNA-mediated knockdown of BMPRII enhances BMP7 signaling by 5-fold [Yu *et al.,* 2005].

To further examine the role of ActRIIB in BMP7-induced neurogenesis, lentiviral vectors (lentivectors) containing ActRIIB-targeting short-hairpin RNA (shRNA) sequences were obtained (OpenBiosystems). One of the five different ActRIIB-targeting shRNA lentivectors tested, pActRIIB-shRNA-D resulted in a knockdown efficiency of nearly 70% in cultured cortical progenitors, which was sufficient for our study. The lentivectors consisted of a dual-promoter backbone with the shRNA sequence under the control of the human U6 promoter and a puromycin resistance selection gene under the control of the human PGK promoter. As it was preferred to limit all factors that may alter cell survival, proliferation or differentiation, puromycin was omitted from the cortical cultures. Therefore, the puromycin resistance gene was removed from pActRIIB-shRNA-D and the sequence encoding for enhanced green fluorescent protein (EGFP) was inserted downstream of the hPGK promoter, to be able to evaluate the infection efficiency in each experiment. The resulting lentivectors, Control-EGFP-Lv and ActRIIB-shRNA-EGFP-Lv, successfully transduced cortical cultures at efficiencies greater than 70%, as observed via EGFP expression. The high transduction efficiencies in conjunction with the ability to significantly knockdown ActRIIB receptor expression levels

will provide an effective tool in future studies, further examining ActRIIB in BMP7-induced neurogenesis.

4.6. BMP7 Lentivector and Gene Delivery

In addition to its role in cortical neurogenesis during embryonic development, BMP7 may enhance functional recovery in the brain following injury or stroke [Chou *et al.*, 2006]. Although little is known about the mechanisms, BMP7 overexpression has been shown to enhance cell survival following the addition of cytotoxic levels of H_2O_2 in culture. Thus, BMP7 may also enhance neuron survival in the presence of toxic levels of H_2O_2 in the brain following injury or stroke, thereby assisting functional recovery [Tsai *et al.*, 2007]. Given its role in neurogenesis and functional recovery following injury or stroke, a reliable and consistent delivery method for BMP7 remains essential for future studies and applications in the brain.

Currently, BMP7 delivery to the brain requires direct administration through multiple injections over the duration of treatment. In addition, due to the short half-life of BMP7 (less than 30 minutes in CSF and the blood), multiple treatments would be necessary to achieve consistent BMP7 delivery [Vukicevic et al., 1998; Grgurevic et al., 2007]. In sensitive physiological systems such as the brain, multiple injections of a particular factor may lead to secondary tissue damage exacerbating the existing condition and altering end results. Therefore, rather than administering BMP7 through crude and potentially dangerous injections, it may be consistently expressed and delivered to target cells in the brain following a single lentiviral infection [Broussau et al., 2008].

Thus, a BMP7-lentivector was constructed and characterized for future work *in vivo*. Similar to the design of the lentivector ActRIIB-shRNA-EGFP, EGFP was desired to allow for the assessment of infection efficiency and cell tracking. However, there is evidence that even small modifications to either the N or C terminus of BMP7 may alter its biological activity [Swencki-Underwood et al., 2008]. Therefore, to prevent the possible problems associated with BMP7-EGFP fusion proteins, the sequences encoding for BMP7 and EGFP were separated by an Internal Ribosomal Entry Site (IRES).

A robust lentiviral production system, using HEK 293SF-PacLV cells was used to achieve high titers of lentivirus in our studies [Broussau et al., 2008]. The lentiviral titer was determined via FACS analysis to be as high as $3.7x10^7$ TU/mL in the unconcentrated supernatant, and a single lentiviral-based infection at an MOI of 100 resulted in 75% BMP7-GFP positive cells. These results suggest that the transduction efficiency of BMP7 lentivirus was significantly higher than that reported by Song et al., (2008) for adeno-associated viral (AAV) BMP7 delivery (< 10% for MOI: 100 and 68% for MOI: 10000). Furthermore, since Song et al., neither measured the level of BMP7 protein nor tested its biological activity, it is difficult to compare the efficiency of the AVV-based BMP7 delivery system with the one presented in this study. As early as 3 days post-infection, high levels of BMP7 (223.6 ± 67.7 ng/mL over 24 hours) were obtained and consistently produced for at least four weeks in both human HEK293 cell lines and amniotic fluid primary cultures. Thus, even without purification and concentration steps, abundant levels of BMP7 were produced, using the lentiviral system. More importantly, similar to our previous results, 1 ng/mL of lentivirally-produced BMP7 was sufficient to induce neuronal differentiation in cortical cultures. Therefore, in addition to limiting the

number of BMP7 proucing cells, the concentration of LvBMP7 can be diluted to physiological levels for use in future neurogenesis studies.

Several laboratories have developed adenoviral-based BMP7 delivery systems to regulate cell survival and differentiation in various tissues [Hidaka et al., 2003; Zhu et al., 2004; Zhao et al., 2005; Zhang et al., 2007; Tsai et al., 2007]. Adenoviral vectors have been known for their more work-intensive production methods, relative to the third generation lentiviral vectors. They also lack the machinery required for genomic integration; therefore, the transgene is not replicated or transferred during mitosis. Nevertheless, adenoviral-based BMP7 delivery methods are beginning to find applications in both neuronal [Tsai et al., 2007] and non-neuronal in vitro and in vivo models [Zhu et al., 2004; Zhao et al., 2005; Zhang et al., 2007]. For instance, Tsai et al., (2007) used a first generation BMP7 adenoviral vector to facilitate neuroprotection in the E15-E17 neuronal-glial mixed cultures exposed to H_2O_2 toxicity. This neuroprotective effect was associated with an induced differentiation of cultures into endothelial, oligodendroglial and calcitonin gene-related peptide (CGRP) positive neuronal cells via Smad1 upregulation. Since neuropeptide CGRP serves as a potent microvascular vasodilator in the central and peripheral nervous systems, it has been further suggested that virally-transduced BMP7 overexpression may have beneficial effects on CNS injury [Tsai et al., 2007]. Taken together, lentiviral BMP7 delivery systems may provide suitable tools to study regeneration in the nervous system and non-neuronal tissues.

5. CONCLUSIONS

5.1. Main Conclusions

The main focus of this study was to determine the role of BMP7 in cortical neurogenesis. I hypothesized that BMP7 enhances cortical neurogenesis. The data presented in this study clearly demonstrated that treatment with low concentrations of either recombinant or lentiviral-derived BMP7 (1 ng/mL) enhances neuronal differentiation, whereas higher concentrations (30 ng/mL) favor gliogenesis. Further analyses via immunocytochemistry and RT-qPCR showed that BMP7-induced cell differentiation occurs at the expense of nestin-positive neural progenitors. Comparative analysis of BMP receptor expression indicated that ActRIIB expression is several fold higher than any other BMP receptor during the peak period of cortical neurogenesis (E10-13). In contrast, ActRIIB expression was down-regulated during the onset of gliogenesis and was the least expressed BMP receptor in the post-natal brain. Consistent with the *in vivo* data, a similar trend was also observed in E13 cortical cultures where ActRIIB was highly expressed in neural progenitors and significantly down-regulated following differentiation into either neurons or astrocytes. Since ActRIIB was highly expressed during neurogenesis, it was hypothesized that ActRIIB mediates BMP7-induced cortical neurogenesis. Therefore, neutralization of ActRIIB was performed and resulted in both a reduction of SMAD1/5/8 activation and BMP7-induced neurogenesis to control levels. Moreover, the presence of neurons in both control cultures and cultures treated with ActRIIB neutralizing antibody, suggested that other pathways are involved in regulating cortical neurogenesis. To further examine the role of BMP7 and ActRIIB in neurogenesis, a lentiviral-based shRNA delivery system was developed and optimized in cortical cultures. Using a similar strategy, a lentiviral-based delivery system was developed and optimized to produce biologically-active BMP7 for

future *in vitro* and *in vivo* studies. The lentiviral-derived BMP7 mimicked the effect of commercially available BMP7 in the cortex, paving the road for its application for delivery into the brain. However, in future studies in the brain, it may be desirable to have an inducible BMP7 expression system, which could be easily accomplished with the cloning of a tetracycline-regulatable promoter in place of the current constituitively-active CMV promoter. Together, the data presented in this study clearly demonstrate that the physiological level of BMP7 enhances the differentiation of cortical progenitors into neurons. Furthermore, our data strongly suggest that BMP7 primarily utilizes ActRIIB to mediate its effect on neurogenesis.

5.2. Future Studies

To advance our understanding of ActRIIB in BMP7-induced neurogenesis, our lab has obtained ActRIIB heterozygous knockout mice ($ActRIIB^{+/-}$; a gift from Dr. V.R. Rosen, Harvard Medical School). To date, mating of the ActRIIB heterozygous knockout mice has resulted in two sets of progeny (embryonic and post-natal), which have been genotyped, sectioned and prepared for further analysis (Figure 5.1). To further assess the role of ActRIIB in the developing cortex, future studies will focus on examining cortical structure and neural cell differentiation in both homo- and heterozygous ActRIIB knockout brains during different stages of development.

In addition, since we suggest a receptor-specific function for BMP7 in cortical neurogenesis, an interesting question is how ActRIIB may transduce distinct signals inside the cell. As there is insufficient evidence supporting the notion that distinct actions of BMPs occur via differential activation of SMAD1/5/8 proteins [Panchision *et al.,* 2001], other signaling pathways may be involved in either a cooperative or competitive fashion. For example, it has been suggested that different pathways may be activated,

depending on the mode of BMP receptor oligomerization. In other words, preformed receptor complexes consisting of both two type I and type II receptors tends to activate SMAD signaling, whereas BMP binding to individual (non-complexed) type I or type II receptors tends to activate the p38 mitogen-activated protein kinase (p38-MAPK) pathway [Zhao *et al.,* 2005; Nohe *et al.,* 2002]. Similar to the SMAD1/5/8 pathway, the p38-MAPK pathways are involved in a number of cellular responses such as cell maintenance, proliferation and differentiation.

Therefore, in addition to identifying the precise role of ActRIIB in BMP7-induced cortical neurogenesis, multiple receptor genes and signaling molecules may need to be inactivated or knocked-down. Similarly, to demonstrate the functional significance of BMP7, cortical cell numbers and phenotypes need to be examined in the brain-specific conditional knockouts. Together, these experiments will provide more information about the role of BMP7 in the cortex.

Under the assumption that intrinsic mechanisms pre-program progenitor cell commitment to generate neuronal and glial cell types, BMP7 signaling in early progenitors may participate in neurogenesis by down-regulating the expression of Sox2, a gene known for its critical role in the cortex [Bani-Yaghoub *et al.,* 2006]. Interestingly in human embryonic stem (hES) cells, BMP signaling through SMAD1/5/8 has been demonstrated to moderately repress transcription of Sox2 mRNA [Greber *et al.,* 2008]. Current studies in our lab indicate four putative SMAD1/5/8 binding sites, two of which have been demonstrated via ChIP to interact with SMAD1/5/8 in cortical cells following BMP7 treatment. As there is strong evidence suggesting that BMP7-induced Sox2 down-regulation may be directly related to enhanced neurogenesis, further investigation of this target gene is underway.

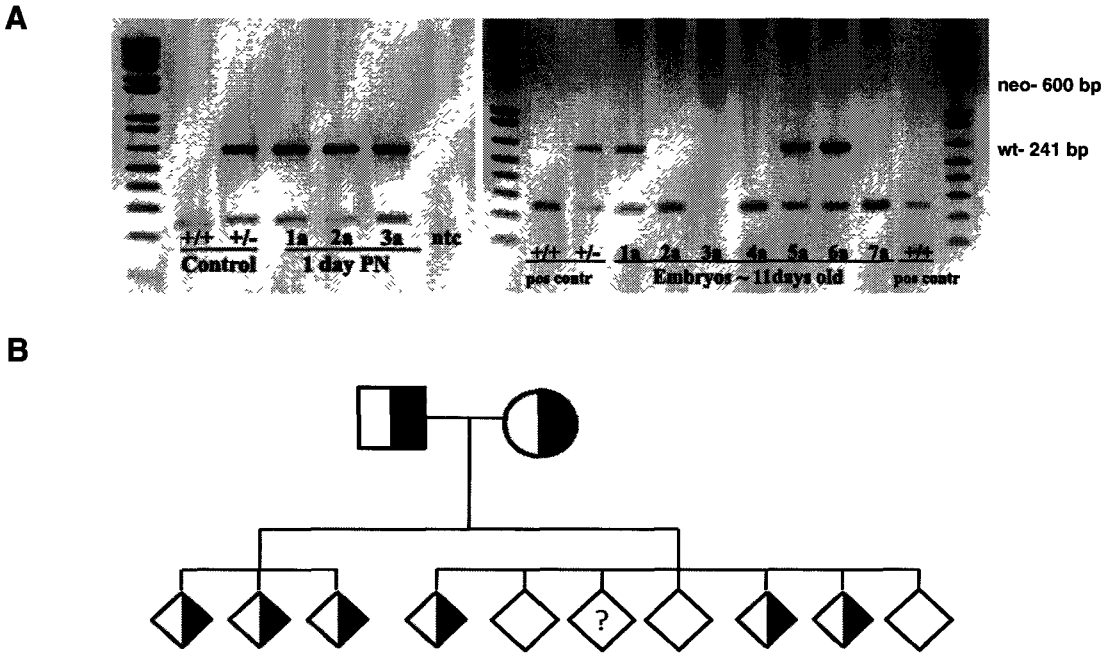

Figure 5.1: (A) Genotyping and (B) pedigree analysis of embryos from mated heterozygous (+/-) mice. Three post-natal pups and seven embryos (E11) were obtained and genotyped to screen for ActRIIB hetero- and homozygous knockouts to further examine ActRIIB in cortical development. All post-natals and embryos 1a, 5a and 6a were heterozygous knockouts, whereas 2a, 4a and 7a were wild-type. Tissues were taken from the tail, ear and/or foot. Legend: square: male; circle: female; diamond; sex undetermined; no shade: wild-type (+/+); half-shade: ActRIIB heterozygote (+/-); full-shade: ActRIIB homozygote (-/-) (Genotyping performed by Dr. Maria Ribecco-Lutkiewicz).

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