# CHAPTER 4

# TO DETERMINE THE EFFECT OF MeCP2 DEFICIENT ASTROCYTES ON OLIGODENDROCYTES AND NEURONAL SURVIVAL, PROLIFERATION AND DIFFERENTIATION

## 4.1 Introduction

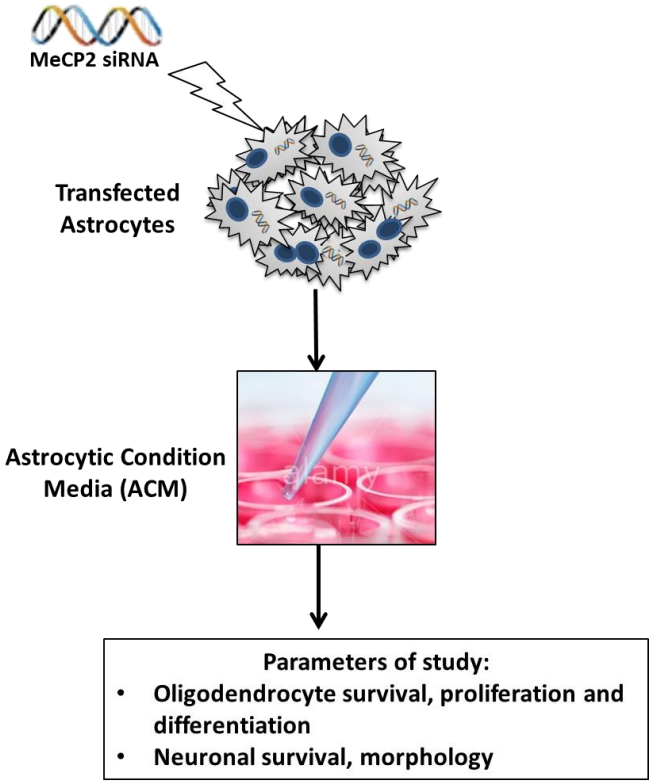
Astrocytes have for long been considered as merely supportive glial cell components in the nervous tissue which are markers of diseased tissue when reactive. Studies in recent times have explained their wide diverse functions in the healthy CNS. Astrocytes are well known for their house-keeping functions like providing nutrients and structural support around synapses, maintaining the integrity of the blood brain barrier (BBB) and buffering excess ions and neurotransmitters (Sofroniew and Vinters 2010).

Astrocytes play diverse roles in supporting other cells in the brain- oligodendrocytes and neurons. Astrocytes influence OPC survival, proliferation and maturation (Noble and Murray, 1984; Noble et al., 1988). They function in close proximity and are involved in oligodendrocytes development and biogenesis. Astrocyte secreted cytokines (Biancotti et al., 2008; Nair et al., 2008; Ishibashi et al., 2009) and neurotrophins (Miyamoto et al., 2015) govern oligodendrogenesis timing, development and function. The ratio of astrocytes to neurons varies among species and increases comparably with the increased complex neural network (Banaclocha, 2007). Astrocytes also promote neuronal maturation, synapse formation and neuronal survival during development (Drukarch et al., 1998; Sofroniew and Vinters 2010). Moreover, dynamic communication at the tripartite synapse coordinates neuronal activity (Halassa et al., 2009). Neurons when incubated with astrocytic condition media showed enhanced neuronal survival by eliminating excitotoxic molecules from the surrounding environment (Arai and Lo, 2010; Krum et al., 2002).

Recently, presence of MeCP2 was discovered in glial cells by several groups (Ballas et al., 2009, Maezawa and Jin 2010, Sharma et al., 2015, Tochiki et al., 2012, Vora et al., 2010). Several pathways associated with neuronal morphology, neuronal organization, development and transmission were reported to be disrupted in RTT rodent brains (Calfa et al., 2011; Armstrong et al., 2005, Pacheco et al., 2017). Furthermore, pathways related to glial functions such as glial morphology, migration and myelination of oligodendrocytes, astrocyte reactivation and apoptosis were also altered in MeCP2 deficient brains as relieved by extensive RNA sequencing and proteomics data (Pacheco et al., 2017). Loss of MeCP2 in astrocytes had a non-cell autonomous influence of neuronal morphology (Ballas et al., 2009; Maezawa et al.,

2009) and genetic rescue of MeCP2 in glial cells, particularly astrocytes, significantly improved the disease phenotypes thus confirming integral function of astrocytes in RTT syndrome pathogenesis (Cronk et al., 2015, Lioy et al., 2011, Okabe et al., 2012, Nguyen et al., 2013). These reports suggest the indirect and/or non-cell autonomous contribution of astrocytic MeCP2 in governing major neuronal and oligodendroglial functions which in turn plays a role in pathogenesis and progression of RTT syndrome as well as influencing myelination. Present study was undertaken to elucidate the role of astrocytic MeCP2 in regulation of oligodendrocytes survival, proliferation and differentiation as well as neuronal survival and morphology.

## 4.2 Strategy of work



## 4.3 Results

### 4.3.1 Astrocytic MeCP2 support OPCs survival and proliferation

Astrocytic MeCP2 expression was repressed using specific siRNA. Transfected cells showed 50-60% reduction in MeCP2 transcript and protein expression levels (results described in section 3.3.1). ACM was collected freshly from these transfected cultures, filtered and used for all further experiments. OPCs were treated with serum-free DMEM media as control, negative control ACM and MeCP2 knockdown ACM (MkACM) for 24 hours. Cell viability was measured by MTT assay with respect to appropriate controls. Results indicate increased cell viability by ACM compared to DMEM control indicating contribution of astrocytes in oligodendrocyte survival whereas cell viability was decreased in MkACM compared to negative control (Figure 4.1 A). OPC cell proliferation was also found to be inhibited in MkACM treatment compared to control as evident by BrdU incorporation in dividing cells (Figure 4.1 B, C). These observations may be attributed to differential secretion of cell soluble astrocytic factors in response to MeCP2.

### 4.3.2 Astrocytic MeCP2 support in Oligodendrocytes differentiation

OPCs isolated from mixed glial cultures were treated with negative control and MkACM and fixed for immunocytochemical analysis of PDGFRα. OPCs treated with MkACM showed enhanced immune-reactivity for PDGFRα compared to control (Figure 4.2 A). Further, OPCs were grown in DMEM:ACM (1:1) for 5 days after which MBP protein levels were analysed which were down-regulated in MkACM group compared to control (Figure 4.2 B,C). Data suggests that astrocytic MeCP2 favours oligodendrocytes to remain in their precursor stage as evident by increased PDGFRα levels and decreased MBP levels. These observations are in accordance with increased transcript levels of PDGF in MeCP2 knockdown astrocytes compared to control (section 3.3.2).

### 4.3.3 Astrocytic MeCP2 is involved in supporting normal neuronal growth and survival

Astrocyte-neuronal crosstalk has been well documented for various neuronal and astrocytic functions. To study the effect of astrocytic MeCP2 on neuronal survival, neurons were treated with DMEM, negative control ACM and MkACM for 24 hours. MTT assay was performed to measure the cell viability with respect to appropriate controls. Results indicate significant down-regulation of neuronal cell survival by MkACM compared to negative control ACM (Figure 4.3 A). Effect of ACM was also studied with respect to neuronal morphology. Qualitative analysis indicates shorter and aberrant dendritic process morphology observed in MkACM treatment compared to control (Figure 4.3 B). This atypical morphological observation is concurrent with decreased levels of neuronal markers- NF and Tuj1 (Figure 4.3 C, D, E).

## 4.4 Discussion

Earlier, reports have suggested of the interactions between astrocytes and oligodendrocytes. Cell-cell mediated contact of astrocytes aid in the maturation of oligodendrocytes (Sakurai et al., 1998) involving the interaction of α6β1 integrin on oligodendrocytes with astrocytic laminin (Corey et al., 2001); while myelin maintenance depends of astrocyte and oligodendrocyte connexions (Orthmann-Murphy et al., 2008; Lutz et al., 2009). A recent report suggests that myelin membrane formation by oligodendrocytes also depends on extracellular lipids delivered by astrocytes (Camargo et al., 2017). Apart from cell mediated contact, a number of astrocyte secreted factors have been implicated in regulating oligodendrocyte development.

MeCP2 is a widely studied global transcription regulator, mutations of which cause the neurodevelopmental disorder Rett syndrome (Amir et al., 1999). Majority of studies till date have focussed on the role of MeCP2 in neurons and linked to Rett syndrome; apparently due to its absence in glial cells (Shahbazian et al., 2002; Jung et al. 2003). However, contemporary findings have shown presence of MeCP2 in astrocytes, oligodendrocytes and microglia (Ballas et al., 2009; Maezawa and Jin

2010; Vora et al., 2010). While involvement of MeCP2 in individual glial cells is well documented, the effect of astrocytic MeCP2 on oligodendrocytes is largely unknown.

Present study focussed on the role of MeCP2 deficient ACM on oligodendrocyte survival, proliferation and differentiation. Results indicate that survival of OPCs increased in response to ACM compared to DMEM; while OPCs treated with MkACM showed significantly decreased survival levels compared to negative control ACM. Similar observations were recorded for OPC proliferation study by BrdU incorporation wherein exposure of OPCs to MkACM down-regulated proliferation of precursor cells compared to control. Astrocyte secreted factors like PDGF and FGF have been extensively studied in regulating OPC survival and proliferation. Astrocytes led to the expansion of OPCs in the rat optic nerve and this effect was mediated by growth factors (Noble and Murray, 1984) which were later identified as PDGF (Noble et al., 1988; Richardson et al., 1988) and bFGF (Bogler et al., 1990). Both of these are principle astrocyte derived factors which promote OPC migration, proliferation and survival (Grinspan 2002; Frost et al., 2009).

Influence of astrocytic MeCP2 was also observed in oligodendrocyte differentiation. In agreement to up-regulated PDGF transcript levels in MeCP2 knockdown astrocytes (section 3.3.2), immunocytochemical analysis demonstrated higher expression levels of PDGFRα in OPCs treated with MkACM compared to control. Moreover, MBP protein levels were down-regulated in mature oligodendrocytes treated with MkACM as compared to control. This suggests that MeCP2 in astrocytes maintains the progenitor state of oligodendrocytes by maintaining elevated levels of PDGF in OPCs and decreasing MBP in mature oligodendrocytes. These observations are also concurrent with decreased transcript levels of LIF and CNTF in MeCP2 knockdown astrocytes (section 3.3.2). CNTF (Stankoff et al., 2002, Nash et al., 2011) and LIF (Gard et al., 1995; Ishibashi et al., 2006) are astrocyte secreted factors which are known to promote oligodendrocyte differentiation and myelination. Furthermore, a number of signalling pathways linked to oligodendrocyte differentiation, including AKT and ERK (Pang et al., 2007; Guardiola-Diaz et al., 2012), were also activated in astrocyte conditioned media treated oligodendrocytes (Pang et al., 2013). Proximity of astrocytes enhances and remodels the myelination substrate of the neighbouring oligodendrocytes non-cell autonomously (Iacobas and Iacobas 2010).

With the gradual decrease in gliogenesis as the brain ages (Ge et al., 2012), neurons are found to be largely in contact with pre-existing mature astrocytes (Krzisch et al., 2015) which indicates the importance of astrocytes in neuronal maintenance. Astrocytes play a major role in neural progenitor proliferation (Song et al., 2002), synaptic formation in neuronal population (Allen 2013) as well as dendritic maturation and survival of new neurons in hippocampus (Sultan et al., 2015). Astrocytes are also important in non-neuronal contributions like learning and memory which require signal processing from astrocytes (Stehberg et al., 2012; Moraga-Amaro et al., 2014). Since early stages of myelination, astrocytes are found to be closely associated with axons (Ioannidou et al., 2014) and also induce oligodendrocytes to align their processes with axons (Meyer-Franke et al., 1999). ACM is known to contain great diversity protein components, extracellular matrix proteins and trophic factors (Christopherson et al., 2005, Mauch et al., 2001) which play diverse functions in regulating neuronal aspects. Soluble molecules contained in ACM induce synaptic formation in retinal ganglion cells (Pfrieger and Barres, 1997) while others like CNTF result in activation of neuroactive factors which influences neuronal excitability (Sun et al., 2016). A recent report also suggests that astrocyte-derived exosomes improves neuronal cell survival by prion protein (Guitart et al., 2018). A large number of studies have been reported on the involvement of MeCP2 in neurons. MeCP2 chiefly functions cell-autonomously and also non-cell autonomously in regulating number and length of dendrites, dendritic morphology as well as neuronal maturation (Armstrong, 1992; Belichenko et al., 1994; Armstrong et al., 1995; Kishi and Macklis, 2010). Deficiency of MeCP2 in neurons is also associated with reduced microtubule stability (Delépine et al., 2013). Current observations indicate decreased survival levels of neurons in response to MkACM. Similar to oligodendrocytes, these observations are also linked to non-cell autonomous effects of MeCP2 in astrocytes. Moreover, transcript and protein levels of neuronal cell specific markers were also found to be down-regulated in response to MeCP2 deficiency in ACM.

Very early reports have mentioned about the growth of axons on astrocyte cultures (Fawcett JW et al., 1989) and that immature astrocytes have a better capacity to support neurite outgrowth compared to mature astrocytes (Smith et al., 1990). Astrocytes are also known to influence the length and direction of axons in damaged

brain and spinal cord (Biran et al., 2003) as well as long term neurite orientation (Sørensen et al., 2007). Present study revealed qualitative observations of decreased dendritic arborisation and decreased dendritic outgrowth in neurons treated with MeCP2 deficient ACM compared to control. These observations are consistent with the hallmark pathologies observed in RTT human samples (Williams et al., 2014) and RTT mouse model (Belichenko et al., 2009). MeCP2-null astrocytes have also been reported to induce neuronal toxicity and aberrant neuronal morphology through non-cell autonomous mechanisms (Ballas et al., 2009; Maezawa et al., 2009). A recent report suggests that astrocytic MeCP2 could restore normal neuronal synaptic response even in absence of neuronal MeCP2 thus indicating towards a strong contribution by MeCP2 in astrocytes (Rakela et al., 2018) whereas preferential re-expression of MeCP2 in astrocytes improved RTT symptoms and rescued MeCP2-deficient mice (Kifayathullah et al., 2010; Zachariah et al., 2012).

## 4.4 Conclusion

Current study focussed on the effect of astrocytic MeCP2 in regulating oligodendroglial survival, proliferation and differentiation. OPC survival and proliferation were inhibited by astrocytic MeCP2 along with reduced differentiation of mature oligodendrocytes thus maintaining them in the progenitor stage. MeCP2 in astrocytes also influenced neuronal survival with decreased expression of neuronal marker protein and caused aberrant neuronal morphology. Since neurons and oligodendrocytes are the active participating cells in the CNS myelination, observations from this study would be crucial to understand further aspects and mechanism of myelination.

**Figure 4.1 (A)**

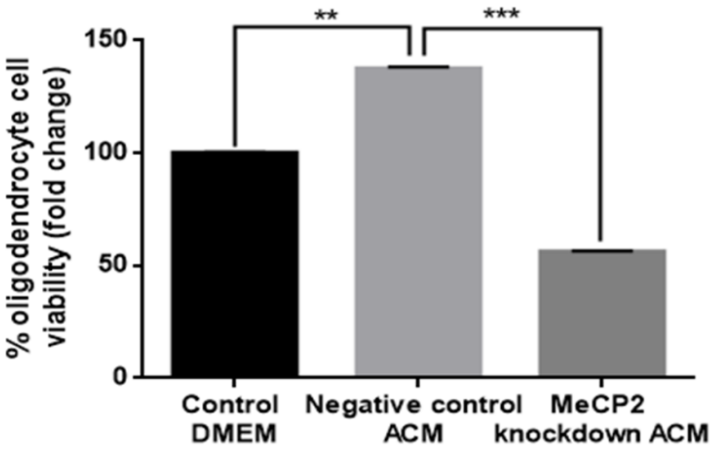
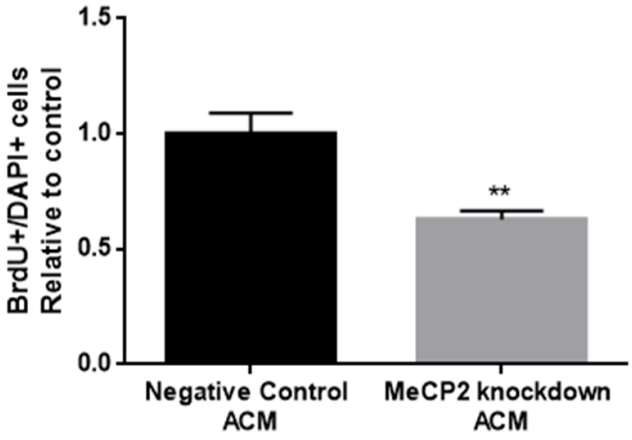



**Figure 4.1 (B)**



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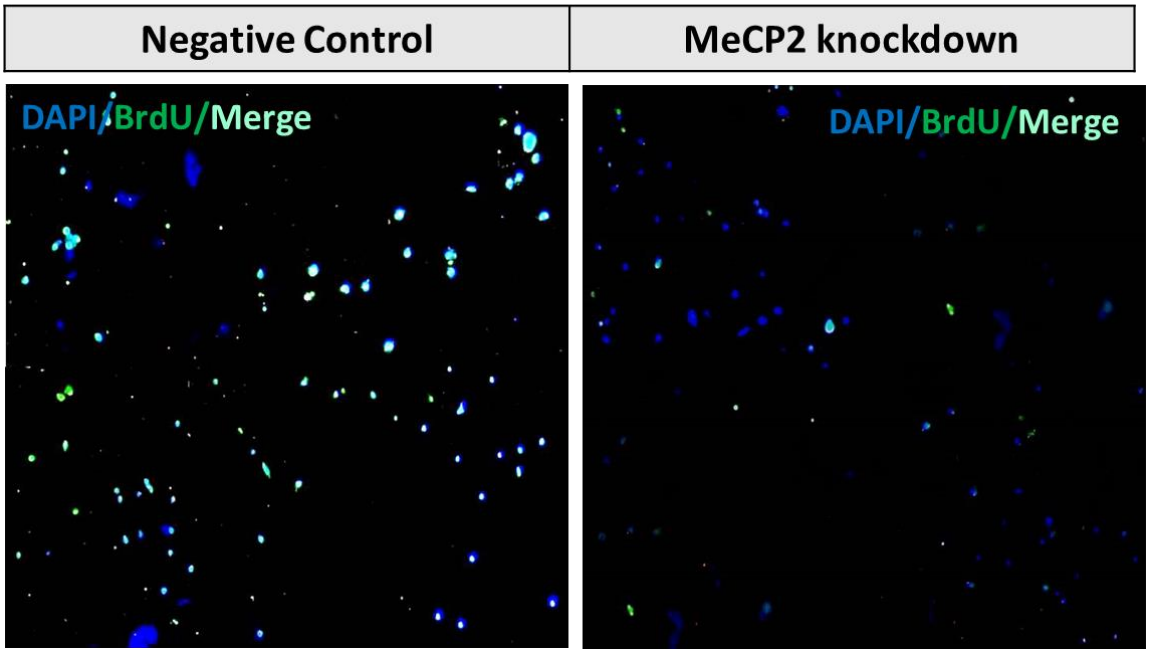
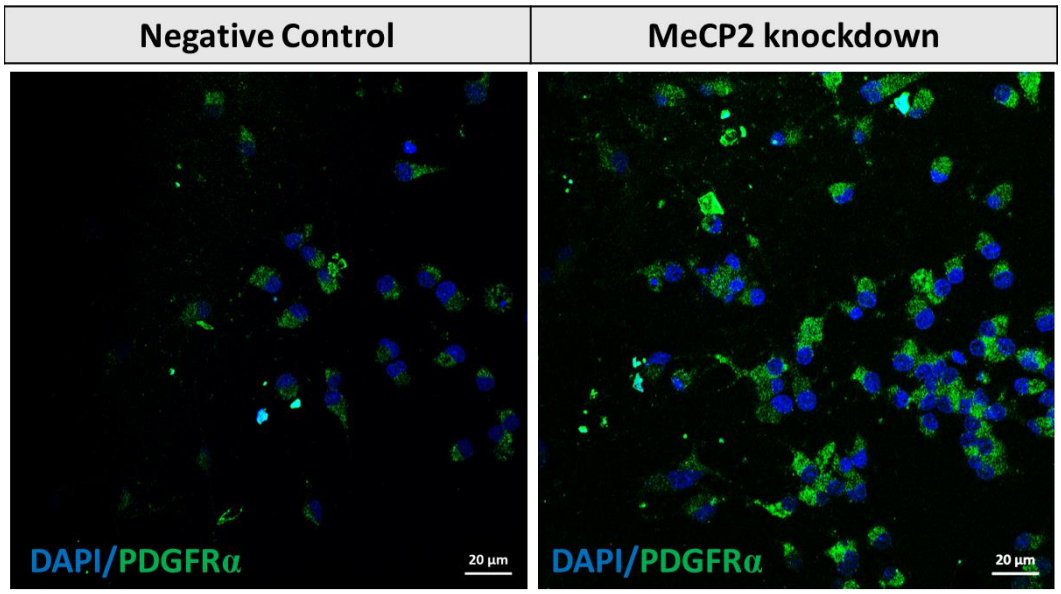



**Figure 4.1: Astrocytic MeCP2 support in OPCs survival and proliferation:** (A) OPCs were treated with DMEM control, Negative control ACM and MeCP2 knockdown ACM followed by measuring cell viability by MTT assay with respect to corresponding control. Statistical analysis was performed using one-way ANOVA followed by Bonferroni post hoc test. (B) A greater number of cells have incorporated BrdU in negative control ACM treated compared to MeCP2 knockdown ACM; evaluated using Student's t-test. (C) Proliferation of OPCs treated with MkACM was compared to control by double immunofluorescence labelling for BrdU and DAPI. Results are expressed as Mean ± SEM of 3-4 independent experiments (*$p<0.05$, **$p < 0.01$, ***$p<0.001$).

## Figure 4.2 (A)

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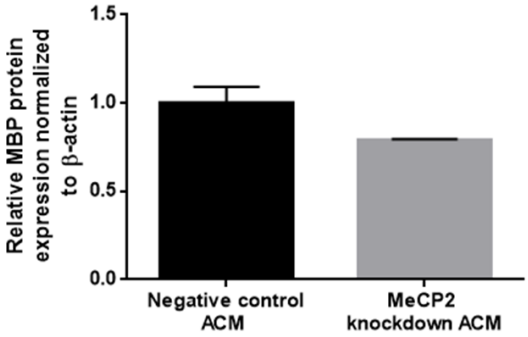


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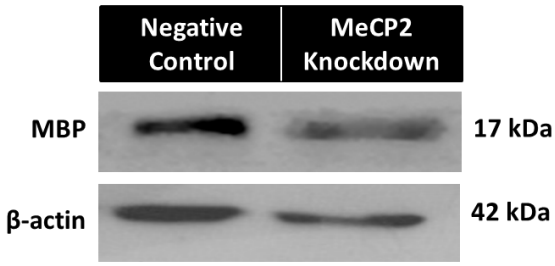
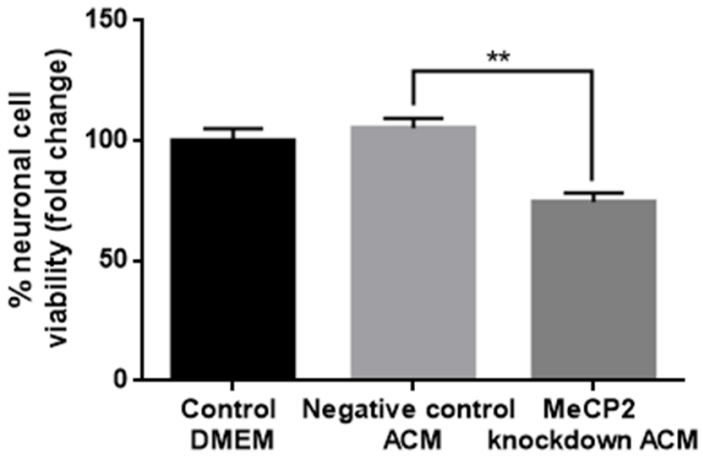
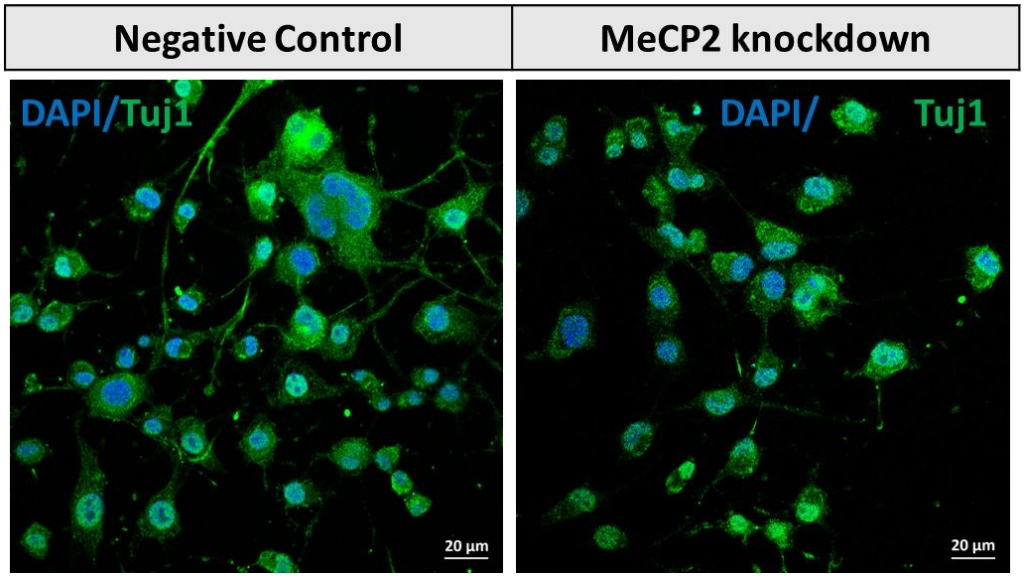


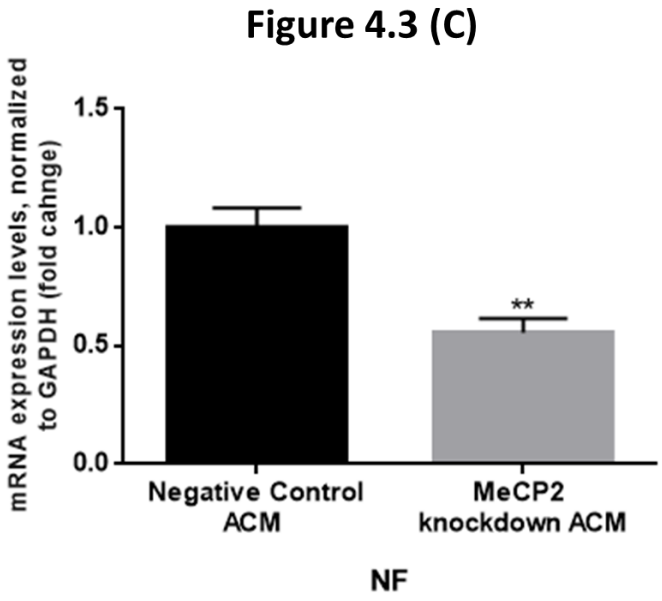

**Figure 4.2: Astrocytic MeCP2 support in Oligodendrocytes differentiation:** (A) OPCs were treated with Negative control ACM and MkACM followed by immunocytochemical analysis of PDGFRα with respect to control. (B) Graphical representation of relative change in MBP protein levels. Statistical analysis was performed using student's t-test. (C) Representative western blot of MBP. Results are expressed as Mean ± SEM of 2-3 independent experiments (*p<0.05, **p < 0.01, ***p<0.001).

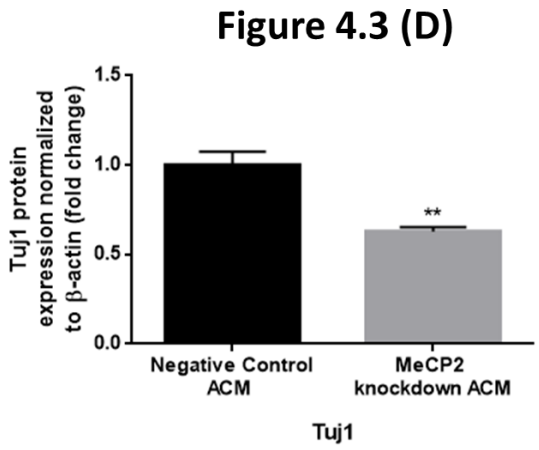
## Figure 4.3 (A)




## Figure 4.3 (B)





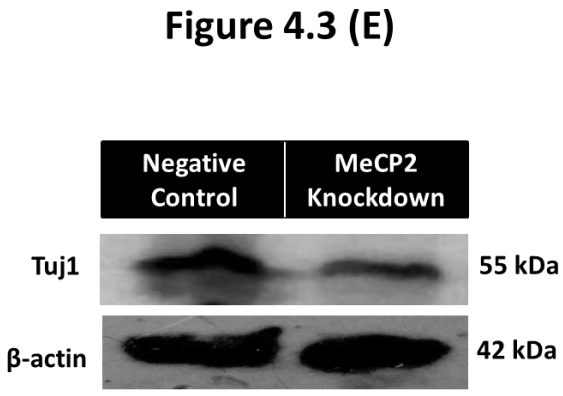




**Figure 4.3: Involvement of Astrocytic MeCP2 in supporting normal neuronal growth and survival:** (A) DRGN were treated with DMEM control, Negative control ACM and MeCP2 knockdown ACM followed by measuring cell viability by MTT assay with respect to corresponding control. (B) Immunostaining of DRGN with Tuj1 as dendritic marker (green) and DAPI (blue) demonstrates shorter and aberrant dendritic process morphology when cultured in MkACM compared to control. Scale bar: 20μm. (C) Decreased transcript levels of neuronal marker, NF in MkACM compared to control. Expression levels of NF mRNA were normalized to GAPDH and relative to control. (D) Quantitative analysis of change in Tuj1 protein levels in

negative control ACM and MkACM, normalized to β-actin and relative to control. (E) Representative western blot of Tuj1 protein levels. All results are expressed as Mean ± SEM of 3-4 independent experiments and analysed using student's t-test and one-way ANOVA followed by Bonferroni post hoc test wherever applicable (*$p<0.05$, **$p < 0.01$, ***$p<0.001$).