CHAPTER FOUR

4. To determine the crosstalk of ERK1/2-ROCK signalling in OPCs

4.1 INTRODUCTION

Migration and process protrusion of oligodendrocyte progenitors involve continual remodeling of the cytoskeleton (Bacon et al., 2007; Bauer et al., 2009). Several intra-cellular signalling pathways are activated downstream of growth factors and chemo-kines in OPCs during migration and cytoskeletal rearrangements (Lee et al., 2000). PDGF-A, is one such growth factor which stimulate OPC migration through the activation of Extracellular signal-regulated kinase (ERK)(E. E. Frost et al., 2009). Apart from a ERK signalling, PDGF also initiate a signalling cascade by sequential activation of Fyn, Cdk5, and WAVE-2, which culminates in increased OPC migration (Miyamoto et al., 2008). The non-receptor tyrosine kinase Fyn phosphorylates Cdk5 which regulates PDGF-dependent OPC migration through the direct phosphorylation of WASP (Wiskott–Aldrich syndrome protein)-family verprolin homologous protein 2 (WAVE2) at Ser-137. WASP family proteins, downstream of Rac/Rho signalling pathway, are key regulators that links extracellular signals to actin reorganization. WASP and N-WASP specially bind to the Rho family small GTPase,Cdc42 and control the formation of filopodia (Symons et al., 1996),whereas WAVE proteins constitutively regulate the formation of lamellipodia and membrane ruffling through the Rho family small GTPase, Rac (Miki et al., 1998). Fyn activation results in inhibition of the small GTPase RhoA, an event associated with oligodendrocyte maturation (Liang et al., 2004; Wolf et al., 2001). The components which form myelin inactivates Fyn and activates RhoA in oligodendrocytes, thereby inhibiting their differentiation(Baer et al., 2009).

Furthermore, the downstream target of RhoA, a Ser/Thr p160 Rho associated protein kinase (ROCK) stimulates actin myosin contractility, focal adhesion and stress fiber formation involved in cell motility (Kippert et al., 2007). MLC phosphorylation, required for the formation and maintenance of stress fibers and focal adhesions, is regulated by Rho-kinase and MLC phosphatase downstream of Rho in cooperation with the Rho effector mDia (Amano et al., 1997; Amano et al., 2010).

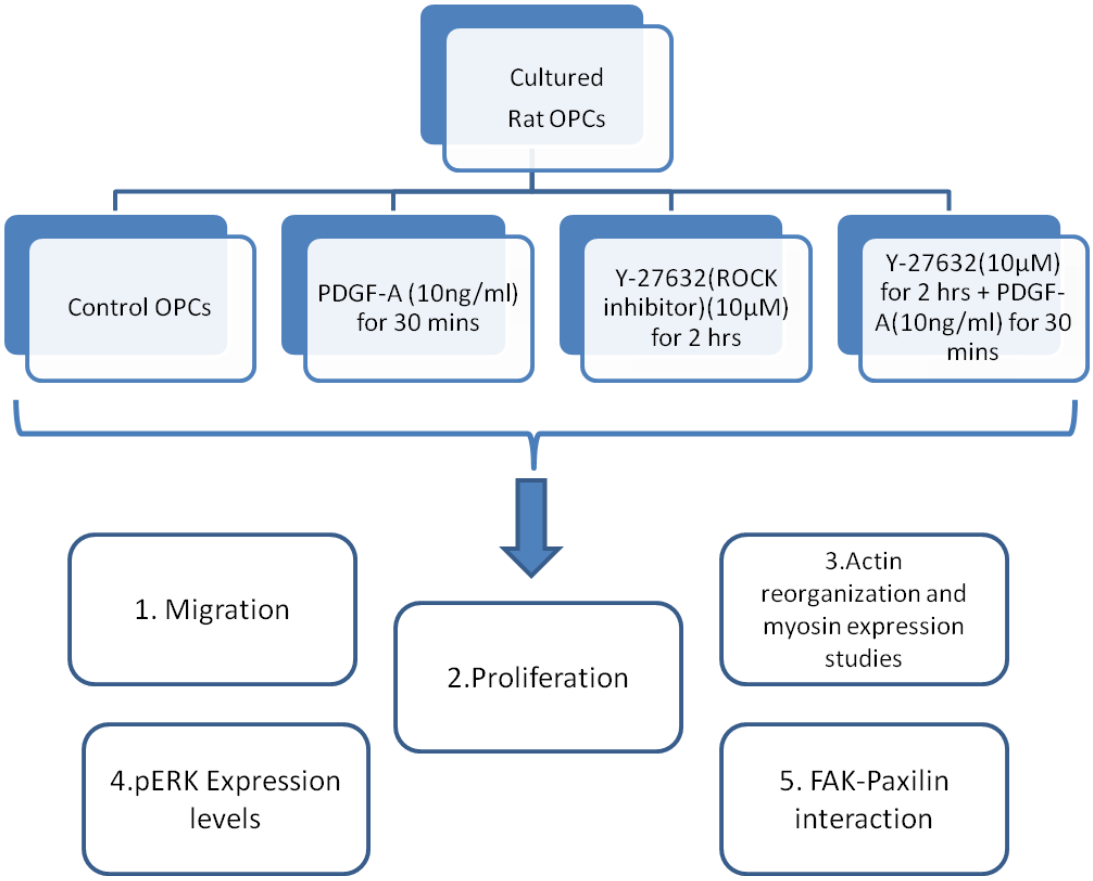
Netrin-1 dependent activation of RhoA and ROCK reduces process length during the course of migration. In contrast, once an OPC has stopped migrating and differentiated into a mature oligodendrocyte, netrin-1 induces a decrease in RhoA activity (Rajasekharan et al., 2010). ROCK inhibition has been demonstrated to

induce the differentiation of OPCs into mature myelinating oligodendrocytes by accelerating the production of myelin proteins (Baer et al., 2009; Pedraza et al., 2014). In addition, inhibition of ROCK signalling promotes the remyelination in vivo demyelination model and suggests its role as a potential therapeutic target for the development of remyelination-promoting therapy(Baer et al., 2009; Ibanez et al., 2004; Kotter et al., 2006).

Rho GTPase activate ROCK which further stimulate myosin light chain (MLC) phosphorylation through inactivation of MLC phosphatase and also probably through direct phosphorylation of MLC. Moreover, ERK phosphorylates MLCK and causes increase in MLCK activity (Klemke et al., 1997; Webb et al., 2004). MLCK activity is necessary for myosin light chain (MLC) phosphorylation, which induces myosin contractility, driving integrin clustering and focal adhesion assembly (Burridge et al., 1997) However, as it has been observed that the phosphorylation of myosin light chain kinase (MLCK) occurs downstream the MAPK/ERK activity, several investigations have linked the Rho and ERK signalling pathways(Sahai et al., 2001; Zohrabian et al., 2009). The involvement of the ROCK pathway, along with MAPK, has been investigated in Glioblastoma multiforme (GBM) cell migration and proliferation. ROCK inhibition was found to repress the PDGF induced ERK activation, suggesting ERK act downstream of ROCK in GBM cells (Zohrabian et al., 2009).

The ROCK signalling inhibition plays an important role in promoting the oligodendrocyte maturation. However, role of ROCK signaling in early stage of oligodendrocytes development such as OPCs migration has not been studied. So this chapter is focused on role of ROCK and ERK signaling in PDGF-A induced OPCs migration. In the present study, the role of PDGF-A activated ROCK signaling during the OPC migration and cytoskeletal rearrangements was addressed. Further, the crosstalk of ERK- ROCK signaling was determined.

4.2 PLAN OF WORK



4.3 RESULTS

4.3.1 ROCK signalling regulates OPC migration

To examine the effect of PDGF-A regulated ROCK signalling in OPC migration, cells were treated with PDGF-A or Y-27632 (ROCK inhibitor) alone or in combination followed by analysis of cell migration by agarose drop assay. It was observed that OPCs treated with Y-27632 alone or along with PDGF-A shows reduced migration compared to control and PDGF-A treated OPCs (Figure.4.1 A & B).

4.3.2 ROCK inhibition regulates PDGF-A induced ERK activation

In order to investigate whether PDGF-A induce crosstalk of the ROCK-ERK signalling in the OPC migration, pERK levels were analyzed in cells treated with

PDGF-A or Y-27632 alone or in combination. The levels of pERK were found to be reduced in cell treated with Y-27632 alone or with PDGF-A compared to control and PDGF-A exposed OPCs (Figure. 4.2), suggesting that ERK act downstream of ROCK signaling in OPCs. Further it implicates that ROCK signaling regulate the PDGF-A induced ERK activation involved in OPCs migration.

4.3.3 ROCK inhibition has no effect in OPC proliferation and survival

Next, in order to understand the role of Rock inhibition on cell proliferation, BrdU assay was performed with following treatment groups: PDGF-A transiently (30 mins) or continuously (24 hrs), Y-27632 (2 hrs) with or without PDGF-A (30 mins). There were no significant differences observed in number of BrdU positive cells in group treated with alone Y-27632 or PDGF-A (30 min) or in combination compared to control (Figure 4.3). The significant increase in proliferation of OPCs was observed only in group treated with PDGF-A for 24 hrs, suggest that transient PDGF-A or Y-27632 treatment does not affect the OPCs proliferation. Further, to determine whether inhibition of ROCK signaling affects the viability of the OPCs. Cells were treated with range of Y-27632 concentration (1, 2, 5, 10 and 20µM) and viability was measured by MTT assay. It was observed that ROCK inhibition does not affect the viability of OPCs (Figure 4.4).

4.3.4 ROCK inhibition modulates actin-myosin expression

Stress fibers and focal adhesions confer contractility on cell that is mediated by Rho/ROCK signalling pathway. To address the mechanisms by which the inhibition of ROCK signalling exerts distinct effects on cell migration and cell polarity, OPCs were treated with Y-27632 and PDGF-A alone and in combination, followed by immunostaining of pMLC and F-Actin. The spatial distribution of MLC phosphorylation was seen uniformly in the cell centre as well as at the peripheral regions in the PDGF-A and control group. However, ROCK inhibited cells showed pMLC distribution in the peripheral regions and cellular processes. Stress fiber formations and actin polymerization was observed in the leading edges of OPCs treated with PDGF-A as compared to control. The stress fibers formation was found to be reduced at the centre of the cells treated with Y-27632 compared to control (Figure 4.5). Moreover, OPCs treated with Y-26732 showed number of cellular processes and branching, as it is known to induce OPC differentiation.

4.3.5 ROCK signaling does not modulate FAK-Paxillin interaction

Next, to investigate the effect of ROCK signalling on FAK-Paxillin interaction in OPCs, cells were immunostained with focal adhesion proteins FAK and Paxillin. FAK and Paxillin are cytoskeletal proteins which are members of the focal adhesion proteins involved during the cellular contraction and retraction of cell migration. PDGF-A was found to induce the interaction FAK-Paxillin as compared to the control and Y-27632 treated OPCs. Here, FAK-Paxillin was found to be colocalized at processes and cell bodies of OPCs. However, no FAK-Paxillin interaction was observed in cells exposed to Y-27632 (Figure 4.6).

4.4 DISCUSSION

Rho/ROCK signalling pathway has been an effective target for the initiation of remyelination in demyelinating pathologies. Direct and indirect contribution of Rho/ROCK signalling in oligodendrocytes maturation and myelination has been reported (Paintlia et al., 2005; Pedraza et al., 2014).The current study demonstrates the effect of ROCK signaling inhibition on PDGF induced OPCs migration and cytoskeleton reorganization.

The ROCK inhibited OPCs showed reduced migration as compared to the control and PDGF-A treated OPCs. Till now, no studies demonstrate the direct effect of rock inhibition on OPC migration which, therefore, needs to be explored. In contrast, ROCK inhibition has been shown to induce the oligodendroglial process extension and maturation. Migration and process protrusion of oligodendrocyte progenitors has been reported to involve continual remodeling of the cytoskeleton that is controlled by Rho-family GTPase, including Cdc42,Rac and RhoA (Bacon et al., 2007; Bauer et al., 2009).

PDGF-A signalling has been reported to activate Ras-ERK and Rho/ROCK pathway. Several investigations have linked the Rho and ERK signalling pathways to function in a cooperative manner to influence cell motility and growth (K.-W. Liu et al., 2011; Zohrabian et al., 2009).The present study demonstrate that expression of pERK is diminished in Y-27632 treated OPCs, suggest the ERK might act in downstream of ROCK signalling. However the effect of Y-27632 treatment on pERK level is modest in OPCs, implicate that role of ROCK signalling in OPC migration to be separate and no crosstalk with Ras-ERK pathway.

Similarly, ROCK inhibition did not led to significant reduction in the proliferation of OPCs as compared to the control and PDGF-treated OPCs. In addition, ROCK inhibition had no effect on the survival capacity of OPC with or without PDGF-A. The results are consistent from previous data on Ras transformed cells which illustrated that ROCK inhibition did not affect the proliferation of Ras transformed fibroblasts (Sahai et al., 2001).

Further, activation of ROCK induces stress fiber formation and cell contraction in diverse cell types(Burridge et al., 1997). Potential mechanisms that led the differentiation of OPCs can be regulated by the activation of downstream signalling molecules such as phosphorylation of myosin light chain (MLC), which are responsible for the formation of stress fibers. In the present study, ROCK inhibition cause the reduction in the formation of stress fibers and were diminished in the centre of the cells with increased process extensions and branching as compared to the control. Further, pMLC levels were reduced by ROCK inhibition. The results supports previous data studies (Wang et al., 2012; Wang et al., 2008) that explains inhibition of ROCK, leading to the decreased levels of pMLC and altered generation of stress fibers; which are all responsible for increased process extension and differentiation contributing to myelination (Pedraza et al., 2014). These studies further support the hypothesis that ROCK inhibition regulates the acto-myosin levels which may affect the migration of OPCs.

Focal adhesions are dynamic actin sites of polymerization located at the cell periphery and more centrally in less motile regions and associated with the ends of the stress fibers. These focal adhesions comprises of high levels of Vinculin, Talin, paxillin , α-Actinin, VASP, FAL, phosphotyrosine proteins, and Integrins $\alpha_v\beta_3$ (Zaidel-Bar et al., 2004) and actopaxin. Focal adhesion assembly can be induced by MLC phosphorylation alone even when the Rho/ROCK activity is blocked in 3T3 fibroblasts(Totsukawa et al., 2000). In order to the study the association of FAK and paxillin, members of the focal adhesion assembly, when the ROCK activity is blocked, immunostaining of FAK and paxillin protein was carried out. However, FAK-paxillin interactions was not significant as compared to the control and PDGF-A induced OPCs, which suggests that ROCK inhibition may not be contributing to FAK- Paxillin interaction and focal adhesion formation in OPCs. These results further

demonstrate that other RhoA/ROCK mediated factors or other substrates phosphorylated by ROCK may be critical for the formation of focal adhesion assembly.

Moreover, Liu et al., 2012 explains that Slit2 protein, secreted by the floor plate, to be involved in regulating the dispersal of OPCs though the association of Robo1 and Fyn. The role of Slit2 in the RhoA activity has been widely reported. The chemorepellent effect of Slit2 on OPC migration was attenuated when the cells was treated with Y-27632.The active RhoA was found to be increased in OPCs treated with Slit2 that suggests the binding of Slit2 to Robos led to the activation of RhoA in the OPCs (X. Liu et al., 2012).

Although several studies indicate an active involvement of the Rho/ROCK signalling pathway in OPC differentiation and myelin dynamics, how ROCK regulates process extension and OPC migration is still incompletely understood. However, the present study explains the involvement of ROCK in OPC migration by phosphorylation of pMLC and stress fiber formation which further cause actin reorganization prior to migration.

4.5 CONCLUSION

ROCK signaling was found to regulate the OPC migration by formation of stress fibers which are responsible for the tail retraction and other cell shape changes due to increased contractility during the phase of migration. Further, ROCK inhibition was found to reduce the migration of OPCs and showed initiation of cell spreading and branching indicating OPC differentiation, which further suggest the involvement of ROCK signaling during OPC migration.

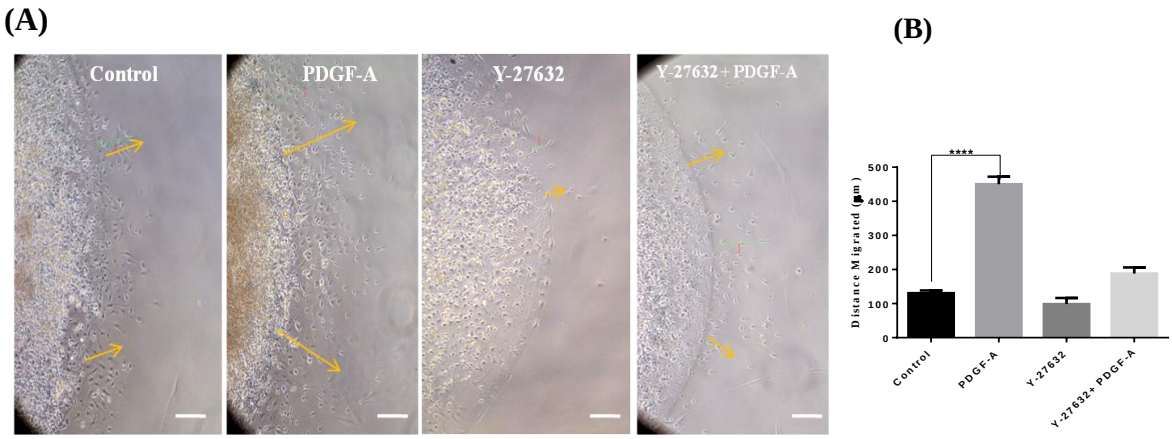
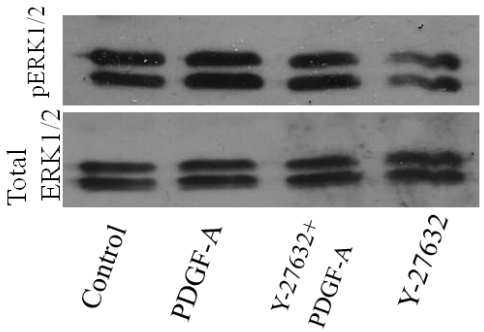


Figure 4.1 ROCK signaling regulates OPC migration: (i) Phase contrast photomicrograph showing OP migration from the edge of the agarose drop in Control (no treatment), PDGF-A (10 ng/ml) for 30 min and Y-27632(10µM) with and without PDGF-A (10ng/ml) (N=4) (Scale bar: 200 µm)**(ii)**The statistical data showing the distance migrated by the OPCs under given treatments conditions. Values represent mean ± SEM from 3–4 samples.*P<0.05;**P<0.01; ***P<0.001,****P<0.0001 compared to control ,one way ANOVA followed by dunnett test for multiple comparison



4.2 Effect of treatment with ROCK inhibitor in ERK activation in OPCs: Western blot analysis of pERK1/2 in OPCs in Control, PDGF-A for 30mins,Y-27632 for 2hrs andY-27632) (2hrs) with PDGF-A(30 mins) respectively from left to right lanes. The experiment was performed three times with similar results.

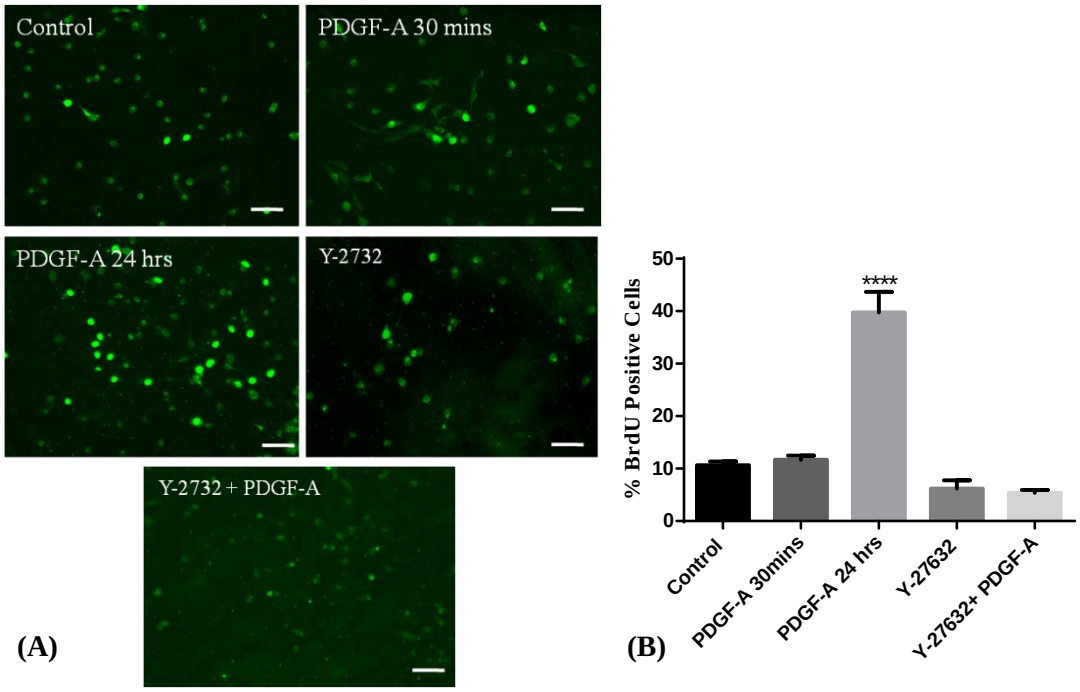


Figure 4.3 ROCK inhibition has no effect in OPC proliferation.(A) Representative fluorescence images of BrdU positive cells in OPCs treated with PDGF-A (10ng/ml) for 30 mins and 24hrs , Y-27632(10μM) for 2 hrs and Y-27632 followed by treatment of PDGF-A(10ng/ml) for 30 mins. Scale bar = 50 μm, **(B)** The statistical data showed percentage BrdU-positive cells calculated from panel A. *P<0.05;**P<0.01; ***P<0.001,****P<0.0001 compared to control ,one way ANOVA followed by dunnett test for multiple comparison

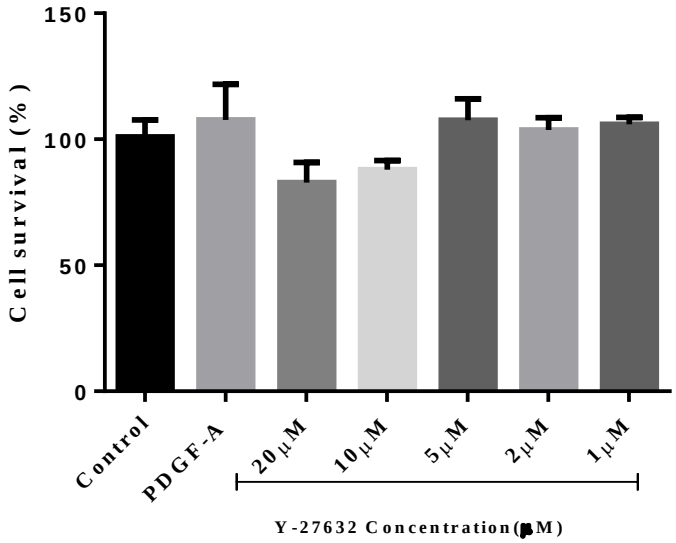
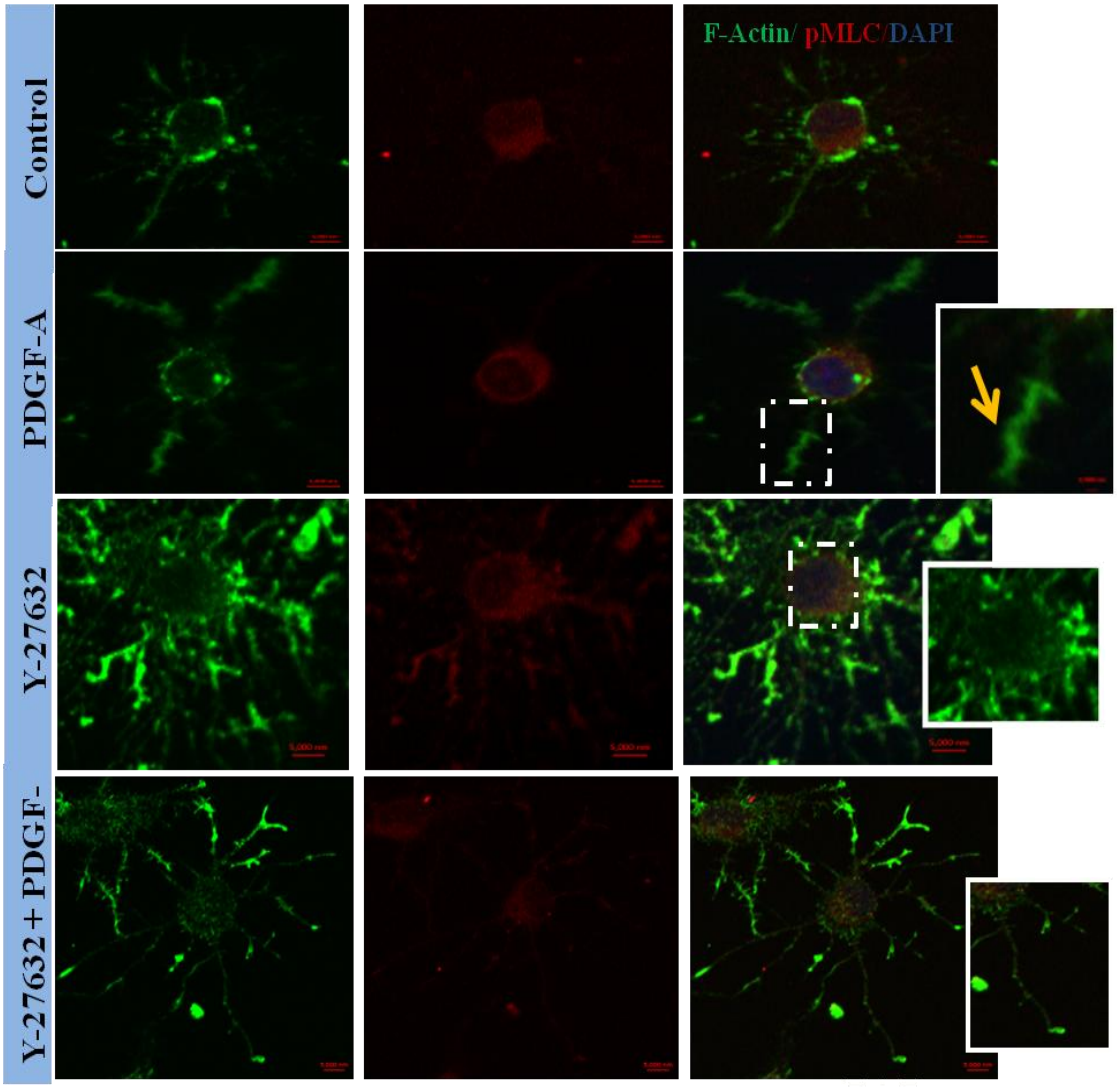


Figure 4.4 ROCK inhibition does not affect OPC survival: Effect of ROCK inhibition on OPC survival was determined by MTT assay in cells treated with PDGF-A for 30 mins and different concentration of Rock inhibitor(Y-27632) i.e, 20µM,10 µM,5 µM,2 µM,1 µM.The statistical data showed percentage of viable cells with respect to control. Values represent mean ± SEM from 3-4 samples. *p<0.05;**p<0.01; ***p<0.001;****p<0.0001 compared to control.

(A)



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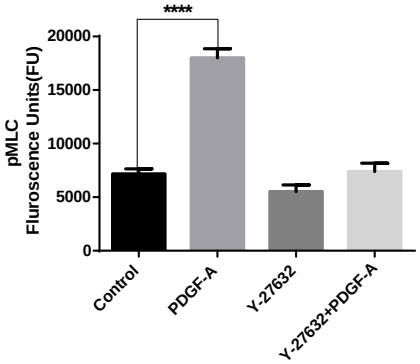

Figure 4.5 ROCK inhibition regulates actin reorganization and expression of myosin in OPC cultures: **(A)** Immunostaining of the F-Actin (Phalloidin) and pMLC (TRITC-Red)in Control, PDGF-A (10ng/ml) ,Y-27632 (10µM) with or without PDGF-A treated OPCs **. (B)** Statistical analysis of pMLC fluroscence recorded in given experimental groups. The experiment was performed three times with similar results. The insets images shows the zoomed out images of the cellular processes showing interactions.

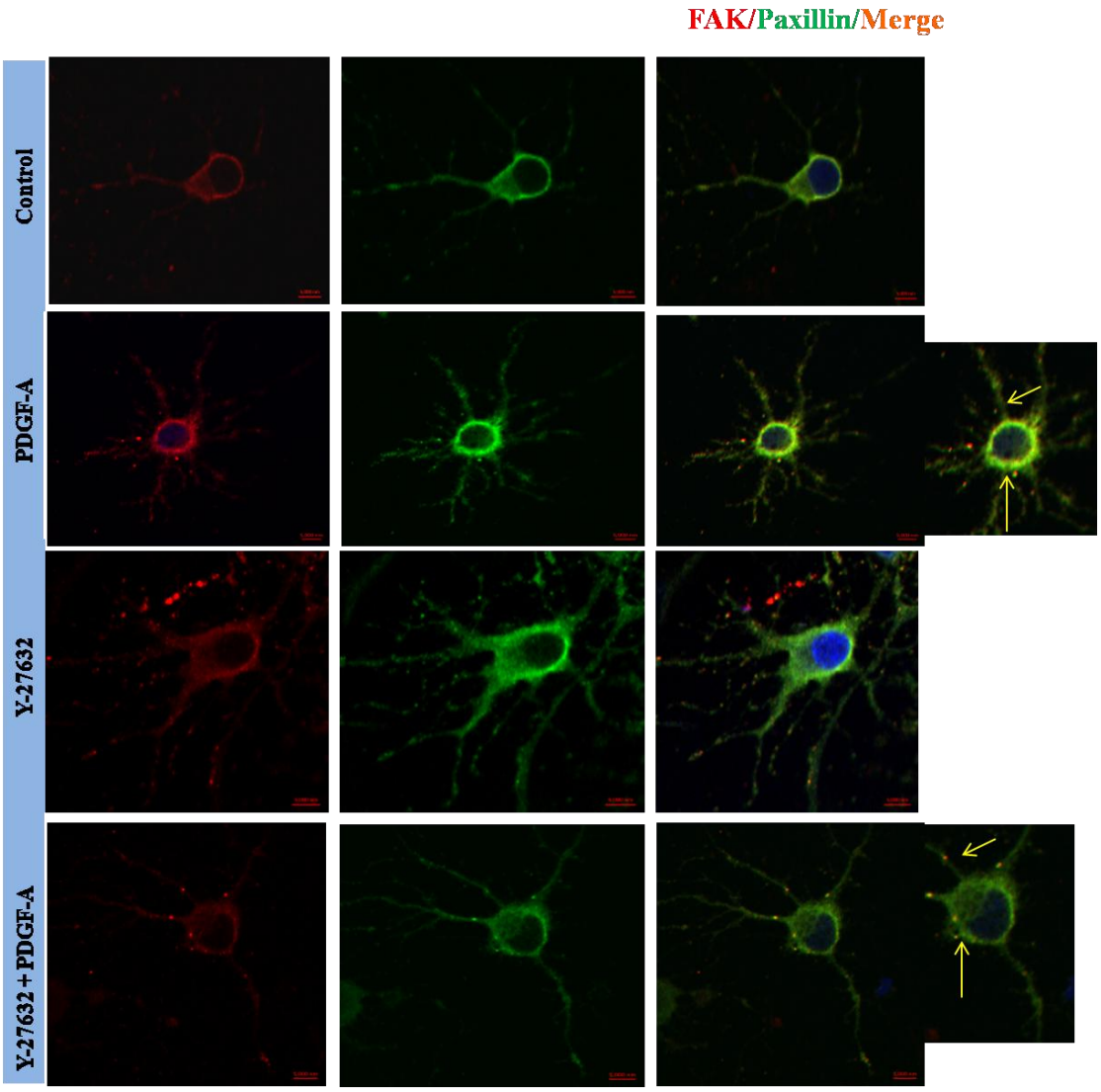

Figure 4.6 Effect of ROCK inhibition in FAK-Paxillin interactions: Association of FAK and Paxillin in OPCs was visualized by Immunostaining with anti-FAK (TRITC-Red) and anti-Paxillin (FITC-Green) antibodies in Control, PDGF-A (10ng/ml),Y-27632 (10µM) with and without PDGF-A treated OPCs . The experiment was performed three times with similar results. (Scale bar-. 1000 nm) The insets images shows the zoomed out images of the cellular processes showing interactions.