Role of thioredoxin system in regulation of neural stem cell proliferation and differentiation

By

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Physiology and Pathophysiology

College of Medicine, Faculty of Health Sciences

University of Manitoba

Winnipeg

Acknowledgement

It is a great pleasure to express my gratitude to everyone who supported in making this thesis possible. First and foremost, I thank God, the almighty for everything provided.

I would like to thank my supervisor Dr. Eftekhar Eftekharpour for believing in my potential and giving me an opportunity in his research team. This lab has been a stage for learning new techniques and Dr. Eftekharpour has been appreciative about that. I am very thankful for his continuous guidance and patience. I always look up to him for his great qualities and he has been more than a mentor. Dr. Eftekharpour played a crucial role in morphing my life in Winnipeg.

I express my sincere thanks to my Master's thesis committee members. Dr. Soheila Karimi, Dr. Christine Doucette and Dr. Jun-Feng Wang for offering their kind and constructive critique; I could not imagine any better committee members for this thesis. I appreciate them for giving their kind help, precious time, valuable input and intriguing questions.

I extend my thanks to former and current lab members Santhosh, Nagakannan, Alysa, Khuong, Albert, Affan and Stefany for their help in the project, good company in day and night, stimulating conversations, fun activities and being good friends.

I like to thank Department of Physiology, Spinal Cord research Centre and the Regenerative Medicine Program for collaborations, sharing knowledge and resources. Open lab space helped the project move further with their collaboration. Journal clubs,

RMP seminars and fun competitions were thought stimulating. I extend my sincere thanks to Research Manitoba for their generous support for my studentship.

I like to thank city of Winnipeg for giving me amazing set of friends and new beginning in my life. Three years is long but all my friends made my stay the happiest. Thanks Ohana!

I thank my family the most of all for their sacrifice, unconditional love and continued support. I missed them all here in Winnipeg. My accomplishments are meaningless without them.

Thank you all!

Abstract

Neural precursor cells (NPCs) have been a hot research topic because of their regenerative potentials. These cells maintain a remarkable capacity for continuous neurogenesis throughout the life and therefore have inspired the quest for a cure towards central nervous system (CNS) repair, however their clinical application is severely restricted due to their limited proliferation. The role of Thioredoxin-1 as a neuroprotective antioxidant protein has been shown previously. This multifaceted protein has also been linked to increase in NPCs proliferation. For my MSc thesis, I hypothesized that enhancing Trx1 levels using a novel intracellular protein delivery approach may enhance cell proliferation and alter differentiation of NPCs. Using immunocytochemistry and Western blotting, I showed that Trx promotes cell proliferation and potentiates oligodendrogenesis in these cells. I have also examined downstream signaling mechanisms involved in these processes. My studies indicate that Trx therapy may represent a viable approach for induction of NPCs proliferation.

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List of Abbreviations

ASK1	Apoptosis signal-regulating kinase 1
ATM	Ataxia-Telangiectasia mutated
bFGF2	Basic fibroblast growth factor
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
BSO	Buthionine sulfoximine
CNS	Central nervous system
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DTT	Dithiothreitol
DMEM	Dulbecco's Modified Eagle Medium
DNTB	Dinitrothiocyanobenzene
Dcx	Doublecortin
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
EGF	Epidermal growth factor
ERK1/2	Extracellular signal–regulated kinase 1/2

FBS	Fetal Bovine serum
FoxO	Forkhead transcription family O
GFAP	Glial fibrillary acidic protein
GTP	Guanosine-5'-triphosphate
GUHCl	Guanidine hydrochloride
HEK	Human Embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV-1	Human Immunodeficiency Virus
HIF1α	Hypoxia inducible factor-1 alpha
LTR	Long Terminal Repeats
mTOR	Mammalian target of rapamycin
NAC	N-acetyl cysteine
Nrf2	Nuclear factor erythroid 2–related factor 2
PFA	Paraformaldehyde
PTEN	Phosphatase and tensin homolog
PBS	Phosphate buffered saline
PDGFR	Platelet-derived growth factor receptors

PINK1	PTEN induced putative kinase 1
TrK	Receptor Tyrosine kinases
RFP	Red fluorescent protein
TBHQ	Tert-Butylhydroquinone
Trx	Thioredoxin
Txnip	Thioredoxin interacting protein
TrxR	Thioredoxin reductase
TAT	Trans-Activator of Transcription
VEGF	Vascular endothelial growth factor
VSV	Vesicular Stomatitis Virus

Chapter 1: Introduction

1.1 Neural Precursor Cells

The central nervous system (CNS) consists of the brain and the spinal cord, which are comprised mainly of neurons, astrocytes, oligodendrocytes and microglial cells. Although the fact that all non-neuronal cells retain their proliferation capacity and act as reservoir for new cells in the time of need has been well accepted, the concept of neuronal proliferation and turnover was overwhelmingly opposed in the field of neuroscience for a long period. The evidence that proliferating cells contributing to post-natal neurogenesis was first discovered in mid 1960s (Altman and Das, 1965; Berry and Rogers, 1965); however, it took three more decades to discover proliferating precursor cells in the brain and spinal cord in the 1990s, which generated much curiosity amongst scientists to study them (Reynolds et al., 1992; Reynolds and Weiss, 1992). This subset of cells was termed neural stem and progenitor cells, collectively known as neural precursor cells (NPCs). A detailed study (Morshead et al., 1994) showed the specific localization of these cells in the brain and spinal cord tissue. NPCs can expand and maintain multi-lineage differentiation *in vitro* and can be isolated from the embryonic and adult brain (Reynolds et al., 1992; Reynolds and Weiss, 1992). The involvement of NPCs in development and normal tissue turnover, as well as memory formation and in neurotrauma provided ever-growing information about NPCs. Their discovery ignited an intense pursuit of an ultimate cure for treatment of any diseases associated with neural cell death; including neurodegenerative diseases, stroke and spinal cord injury. Understanding the NPCs biology can enhance our ability to promote functional repair after CNS injury.

The NPCs are a renewable source of undifferentiated cells that are located in sub ventricular zone (SVZ) and sub granular zone of hippocampus (SGZ) in the brain and throughout the ventricular neuroaxis lining central canal of the spinal cord (Figure 1) (Alvarez-Buylla and Temple, 1998; Johansson et al., 1999; Gage, 2000). Two cardinal properties of NPCs are discussed here; 1) Self renewal: the ability to maintain their own population and 2) multipotency: the capacity of differentiation into neurons, astrocytes and oligodendrocytes (Craig et al., 1996). Complex network of spatio-temporal factors regulates NPCs self-propagation and differentiation during embryogenesis and postnatal life by interaction of environmental cues and growth factors that lead to manipulation of several different transcription factors. Of all the cells in CNS, neurons are generated first during the embryonic development and ends before birth in most parts of the CNS with the exception of cerebellum (Espinosa and Luo, 2008). Differentiation of NPCs to the glial cells, astrocyte and oligodendrocyte, starts at the termination of neurogenesis during embryogenesis and continues during the postnatal life. These waves of differentiation provide the structural basis of brain architecture (Greig et al., 2013) and are driven by cellular genetic cues and guided by the local microenvironment or "niche". Additionally, secreted factors either promote or inhibit NPCs growth and differentiation. For instance, Neurogenin promotes neuronal differentiation while inhibiting astrocyte differentiation (Sun et al., 2001) and Bone morphogenetic protein (BMP) signaling inhibits neurogenesis in hippocampus while promoting gliogenesis (Yousef et al., 2015). Postnatal neurogenesis is very limited in the CNS and based on our current knowledge, it is restricted to specific areas of the brain. As such, adult brain-derived NPCs from SVZ and SGZ can differentiate into neurons, while spinal cord NPCs in

central canal are mostly gliogenic (astrocytes and oligodendrocytes) (Figure 1). This has been attributed to the microenvironment of these cells (Shihabuddin et al., 2000; Lie et al., 2004; Barnabe-Heider et al., 2010). Understanding the regulating factors in proliferation and cell fate determination of NPCs can have important implications for regenerative purposes.

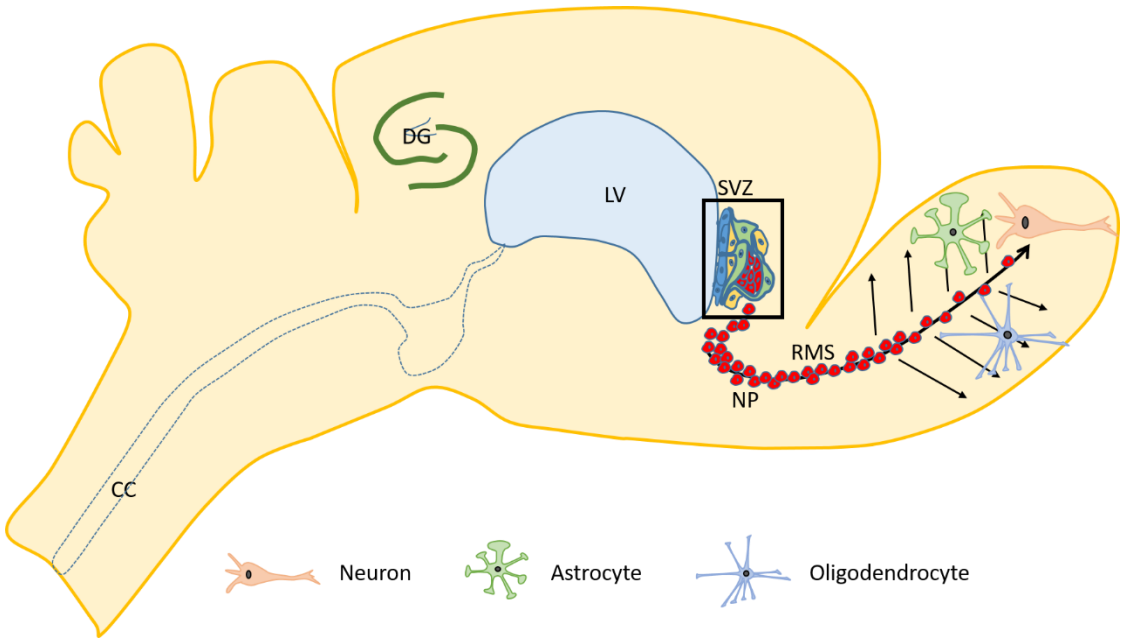


Figure 1: SVZ Neural stem cell pocket: The neural stem cells lining lateral ventricles have the ability to differentiate into neurons, astrocytes and oligodendrocytes. Under normal conditions in the rodent brain (Cassidy and Frisen, 2001), SVZ neural precursor cells differentiate to neural progenitors (NP) and migrate in the rostral migratory stream and then terminally differentiated into neurons, astrocytes and oligodendrocytes. During traumatic brain injury, the endogenous stem cells proliferate and migrate towards the injury site to replace the lost tissue. The other regions of stem cells reservoirs are Dentate Gyrus (DG), which is neurogenic niche and Central Canal (CC), which is a gliogenic niche during

During the last two decades reports on NPCs response to injury/trauma has raised the hope amongst the patients for the stem cell miraculous therapeutic effects. Trauma /stroke provides a mixture of promoting and inhibitory factors that affects the response of endogenous NPCs (Horky et al., 2006; Alizadeh et al., 2015); although an increase in NPCs proliferation in is observed, the extent of their contribution towards efficient cell replacement and tissue repair is very minimal. Identification of factors that increase the capacity of NPCs for proliferation may lead to identification of novel therapies that enhance their contribution to regeneration and repair. Several attempts have been made to induce NPCs proliferation and some factors have been identified to stimulate proliferation. This involves manipulation of gene expression and growth factors signaling (Craig et al., 1996). Physical activity has been also identified to induce NPC proliferation (van Praag et al., 1999).

1.2 NPCs and ROS: A basic understanding

Similar to every cells in mammalian body, NPCs also produce ROS which play vital roles in cell signaling. The original discovery of NPCs was attributed by their responsiveness to growth factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF2) (Tropepe et al., 1999). The effect of these growth factors is mediated through secondary messenger action of reactive oxygen species (ROS) and controlled oxidation of downstream signaling molecules. The level of ROS in the cell is tightly regulated by a complex network of antioxidant proteins. Recent literatures indicate that manipulation of ROS may have a significant role in regulation of NPCs proliferation. Certain genes maintain the stem cell pool by altering ROS levels. One such candidate gene is Positive Regulatory Domain-Containing Protein 16

(*Prdm16)*. Prdm16 is preferentially expressed in primitive stem cell pools. Decrease in Prdm16 level increases ROS levels and results in depletion of hematopoietic and neural stem cells. Prdm16 has promoter control over hepatocyte growth factor (Hgf) gene which combats oxidative stress. Thus ROS elevation was correlated with decreased Prdm16 and its downstream target Hgf (Chuikov et al., 2010). Understanding the basic knowledge of redox balance in NPCs will provide insight into the factors affecting their proliferation and differentiation which can be used for regenerative therapies. Evolution has conserved ROS production in aerobic organisms as a signaling system rather than a negative regulator (Schippers et al., 2012). The imbalance in production to utilization of ROS is pivotal. Excessive accumulation of ROS causes oxidative stress, resulting in cellular dysfunction/death and disease; yet physiological or normal non-lethal or ROS elevation results in activation of signaling pathways that promote higher rates of proliferation (Le Belle et al., 2011). Redox sensitive proteins provide another level of regulation abiding ROS levels with antagonizing effect in cell proliferation. Elevation in ROS levels due to stressful conditions, oxidize redox sensitive proteins, which cause decreased cell proliferation (Wang et al., 2013). Evidence suggests that, ROS has dynamic control over cell proliferation and differentiation. Understanding ROS production and regulation in NPCs provide widow of opportunity to optimize cell proliferation and differentiation.

1.3 ROS, cellular signaling and oxidative stress:

ROS are produced as a byproduct of aerobic cellular metabolism but play an important role in cellular physiology. The ratio between ROS production and neutralization is known as redox status of the cell. Every cell type maintains its own

redox balance, which defines the function of the particular cell type; e.g., immune cells involve in phagocytosis of foreign pathogens generate high levels of ROS (Brune et al., 2013), while hepatocytes are equipped with reducing antioxidants to neutralize the many xenobiotics entering our body through the gastrointestinal system (Bailey and Cunningham, 1998). The imbalance in production to utilization of ROS is pivotal in disease progression as excessive accumulation of ROS can cause oxidative damage, resulting in cellular dysfunction/death and disease; however, controlled non-lethal elevations of ROS plays an essential role in normal cellular process including promotion of cell proliferation in NPCs (Le Belle et al., 2011).

There are three major intracellular sources of ROS in the cells; mitochondria, cell membrane and endoplasmic reticulum (ER). One of the most common produced ROS, the highly reactive superoxide anion $[O_2\bullet]^-$ is formed in mitochondrial complex III (Cytochrome-C oxidase) and complex I, located in the inner membrane of mitochondria as one of the components of mitochondrial electron transport chain. Additionally, cytosolic superoxide is produced by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) located in the cellular plasma membrane and the endoplasmic reticulum (ER). Superoxide anions can react with nitric oxide (NO) to generate the highly damaging peroxynitrite ($ONOO^-$). Superoxide isenzymatically dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase located in the cytosol (SOD1) and the mitochondria (SOD2). Despite lower reactivity of H_2O_2, this ROS has a longer half-life and can diffuse across biological membrane. These properties make H_2O_2 an important player in cellular signaling. The damaging effects of H_2O_2 is resulted from its conversion to hydroxyl radical (OH^-) in a reaction

known as Fenton reaction catalyzed by Ferrous ion (Fe_2^+). The hydroxyl radical (OH^-) is the most reactive and damaging free radical in biological systems due to its capacity to extract electrons from other molecules, including fatty acids, proteins and nucleic acids resulting in formation of organic free radicals and irreversible damages to cellular macromolecules. Organic hydroperoxides (ROOH), once formed, can also promote the generation of hydroxyl radicals, further exacerbating oxidative damage. Therefore, scavenging of peroxides and ROS is a critically important task in maintaining cellular health. Amongst the different antioxidant systems thiols are specialized in clearance of peroxides.

The antioxidant properties of thiols are highly dependent on cysteine residues in their active sites. Cysteine residues are specifically susceptible to oxidation owing to their low redox potential allowing for easy proton extraction by ROS. Although controlled oxidation of protein is required for their proper structural folding in endoplasmic reticulum, excessive oxidation of thiol groups leads to irreversible damage to the proteins.

1.4 ROS as mediator in growth factor response and cell proliferation

Several extracellular signals such as growth factors trigger in cell proliferation. Recent advances in redox biology indicate the involvement of ROS as secondary messengers in this process (Chiu and Dawes, 2012). The growth factor-mediated signal transduction task is conducted by receptor tyrosine kinases (TrK). Such tyrosine phosphatases are vulnerable to oxidation which leads to their structural alteration and thus inhibition. This inhibition is reversible with reducing agents or proteins. Phosphatase at these sites is required to keep the receptors sensitivity for growth factors.

Elevated ROS levels in growing cells inhibit phosphatase activity thus TrK function is prolonged in low density seeding. Cell-cell contact is also another regulating factor in cell proliferation. Such 'Contact inhibition' is marked by decreased intracellular ROS levels which activate phosphatase activity to ameliorate growth factor signaling *in vitro* cell culture system (Pani et al., 2000; Limoli et al., 2004). Growth promoting external stimuli can elevate intracellular ROS levels, mostly originate from NOX in the plasma membrane. Mitochondria sense such extracellular stimuli as cytosolic stress/alteration to induce downstream signaling (Bae et al., 2011). Redox signals are generated through oxidation of cysteine residues in the active site of key signaling proteins. Reduced and oxidized state of these proteins function differently due to changes in their structures. For example oxidation of protein tyrosine phosphatases (PTP) has a key Cysteine residue in its active site which is prone to oxidation results in negative regulation of the receptor (Miki and Funato, 2012). NPCs respond to many external stimuli through their exclusive set of receptors. Growth factor responsiveness and possessing growth factor receptors for EGF and bFGF2 is associated with 'stemness' of the NPCs (Tropepe et al., 1999). One of the well-studied signaling pathways is mediated by EGF receptor, and can mark NPCs in the developing mouse brain (Tropepe et al., 1999). Activation of EGF receptors and enhancement of cell proliferation is known to be mediated by an increase in H_2O_2 levels via small GTPase Rac1 stimulated NOX1 (Sundaresan et al., 1996). H_2O_2 elevation is crucial to relay the changes in phosphorylation status of tyrosine receptor proteins (EGF receptor and phospholipase C-γ1) into activation of intracellular signaling pathways. Notably, inhibition of H_2O_2 production by increased catalase activity reduced proliferation of human epidermoid carcinoma cells (Bae et al., 1997).

Platelet derived growth factor (PDGF) receptor (PDGFR) also plays a role in development of CNS and neuroprotection (Pietz et al., 1996). PDGFR is known to regulate oligodendrocyte progenitor formation and neuronal specification (Raff et al., 1988; Smits et al., 1991). Naive NPCs do not express PDGFR but this receptor is rapidly increased upon stimulation of differentiation of NPCs that are fated towards immature neurons and oligodendrocytes. PDGF also stimulates neuronal fate adaptation in dividing neural stem cells by promoting expansion of immature neurons in differentiating NPCs (Erlandsson et al., 2001). The mitogen effect of PDGFR is similar to EGF receptor and is known to activate intracellular H_2O_2 levels through Nox1. The activation of NOX1 is also associated with induced proliferation in rat smooth muscle cells and human hepatic stellate cells (Sundaresan et al., 1995; Adachi et al., 2005). A direct relevance of nontoxic H_2O_2 addition (2-4μM) and high proliferation of neural stem cells has been reported where inhibition of Nox causes decreased NPC proliferation and neurogenesis (Le Belle et al., 2011). Conversely a previous report claims, that H_2O_2 treatment decreases NPC proliferation (Kim and Wong, 2009), however it is noteworthy that higher concentration of H_2O_2 was used in these studies. In the similar thought, O2A progenitor (Oligodendrocyte and type 2 Astrocyte bipotent progenitors) population expansion was increased by an antioxidant N-acetyl cysteine (NAC), which also reduces oligodendrocyte differentiation (Smith et al., 2000). Increase in mitochondrial generated superoxide flashes or knockout of SOD2 was associated with decreased NPCs in developing mice brain (Hou et al., 2012). NPC specific Ataxia-Telangiectasia mutated (ATM) knockout shows elevated intracellular ROS levels which negatively affected cell proliferation and neurogenesis (Kim and Wong, 2009). Neural

precursor cells can be quiescent and upon activation, they proliferate to give rise to more NPCs. ROS are generated by membrane bound NOX and mitochondria. Actively proliferating NPCs have high ROS. Similarly differentiating cells have more ROS than its ancestor progenitors due to metabolic shift from aerobic glycolysis to oxidative phosphorylation (Figure 2) (Prozorovski et al., 2008; Prozorovski et al., 2015). ROS levels in a concentration-dependent manner, modulate NPC proliferation and differentiation (Figure 2). Post mitotic exit of neuroblast to form immature neurons increases ROS levels and mitochondrial proteins for the metabolic shift (Tsatmali et al., 2005). ROS sophisticatedly governs cellular functions, yet the fine tuning of signaling is vastly unknown.

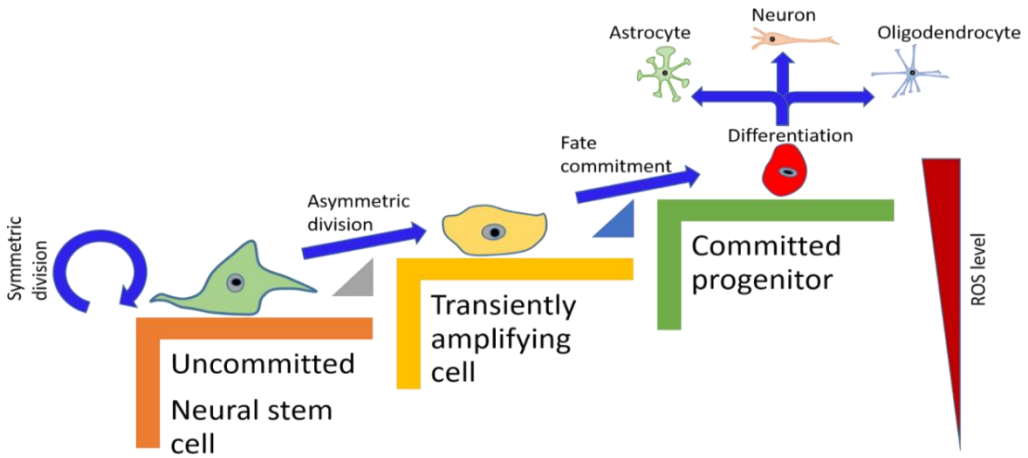


Figure 2: Fate commitment in NPCs and Redox regulation: The uncommitted neural stem cells lining the ventricular zone display low proliferative capacity. They self-renew by symmetrical division giving rise to two identical uncommitted NPCs; however asymmetrical division gives rise to an uncommitted NPC and a transient amplifying precursor cell. These transient amplifying cells turn into committed progenitors then form terminally differentiated neurons, astrocytes and oligodendrocytes. Uncommitted slowly proliferating NPCs exhibit low ROS levels and actively proliferating NPCs display significantly more ROS compared to primitive NPCs. Committed progenitors show progressively more ROS levels.

1.5 ROS regulated proteins and transcription factors

Redox signaling is a fast-paced and transient process in which protons are transferred between the oxidizing ROS and redox-sensitive proteins, such as tyrosine kinases and phosphatases (Randall et al., 2013). The interaction between ROS and these signaling molecules results in transduction of external signals to NPC proliferation and differentiation. A sophisticated antioxidant system is required to maintain the balance between ROS levels and available antioxidants. The concept of redox signaling is increasingly gaining importance in regulation of cellular proliferation and differentiation. Several transcription factors are known to be activated by the redox status of the cell. These include ATM, FoxOs, Nrf2, HIF1α and APE1 known to regulate redox driven signals with emphasis on NPC fate determination.

1.5.1 ATM regulates NPC self-renewal and differentiation

Ataxia-Telangiectasia mutated (ATM) is a serine/threonine protein kinase involved in redox balance, DNA repair and cell proliferation (Ditch and Paull, 2012). ATM was reported to be essential for adult neurogenesis as high levels of ATM expression is seen in neuronal progenitors, which decreases during differentiation and suggests a role for ATM in proliferating NPCs maintenance. Ataxia-Telangiectasia patients exhibit an aberrant neuronal differentiation, which is possibly due to excessive yet aberrant proliferation of neuronal progenitors (Allen et al., 2001). Under normal condition, elevated ROS levels activate ATM, which leads to downstream activation of p53, causing senescence in proliferating cells (Barlow et al., 1997). ATM regulates ROS levels in self-renewing NPCs, and its knockout increases ROS levels leading to oxidative stress, resulting in decreased proliferation of NPCs which can be rescued by

antioxidants or p38 MAPK inhibitor (Kim and Wong, 2009). This study suggests that excessive upregulation of ROS levels is detrimental for cell proliferation.

1.5.2 FoxOs

FoxO (Forkhead transcription family O), a group of transcription factors that have consensus binding site; is known to double life expectancy in *Caenorhabditis elegans.* Interestingly, there is a strong association with human longevity and the involvement of a genetic variant of FoxO3A. Animal studies suggest a correlation between FoxO3 and insulin and insulin-like growth factor signaling (Lin et al., 1997; Willcox et al., 2008). FoxO1 is essential in activation of redox sensitive Oct4, a key modulator of pluripotency in stem cells, and thus maintaining their embryonic pluripotency (Zhang et al., 2011). There is a cell-specific regulation of FoxO in NPCs where FoxO1 is exclusively expressed in slowly proliferating NPCs (type B) and is excluded in doublecortin positive (Dcx+) neuronal progenitors (type A); this indicates a cell stage-specific regulation (Kim et al., 2015). Thus FoxO1 is associated with repression of differentiation and the maintenance of stemness, and regulates the stem cell reservoir. FoxO3 is also known to maintain NPC pool homeostasis by regulating proliferation and differentiation (Renault et al., 2009). An evolutionarily conserved interaction of MST1-FOXO plays an important role in oxidative stress response in mammalian neurons. A similar interaction of orthologs CST1-DAF-16 is reported to increase life span in nematode worm (Lehtinen et al., 2006). The oxidative stress response is mediated through FoxOs in many stem cell types, including hemopoietic stem cells (Tothova et al., 2007). A direct deletion of FoxOs in neural precursor cells results in megalocephaly or enlarged brain, suggesting increased proliferation of NPCs

that can result in depletion of NPC pool in the adult brain (Paik et al., 2009). FoxO-null mice display an increased level of ROS in their NPC pool which result in reduced self-renewal of the pool (Paik et al., 2009). Interestingly FoxOs are induced in neurodegenerative diseases and spinal cord injury, which enhance recovery (Neri, 2012; Zhang et al., 2013). Cumulatively, the current literature indicates a regulatory role for FoxOs on NPCs proliferation and differentiation. This includes regulation of antioxidants systems which control ROS levels that are ultimately involved in maintaining the NPCs reservoir as well as their fate determination. Understanding such complexity can have potential application in aging and neurotrauma.

1.5.3 Hypoxia and HIF1α

Oxygen requirement is different amongst different cell types, and when a tissue receives less oxygen than its normal levels, pathologic hypoxia occurs. NPCs in the SVZ and SGZ are normally exposed to much lower oxygen pressure than perivascular regions of the brain, although this is considered a physiological hypoxia and has important implications during development (Simon and Keith, 2008). Hypoxia inducible factor-1 alpha (HIF1α) is an oxygen sensor that is degraded rapidly in normoxic conditions but its stability increases in reduced oxygen levels (Roitbak et al., 2008). The expression of HIF1α is regulated by the redox status of the cell (Yuan et al., 2011; Semenza, 2012). Under oxidative stress conditions ROS induces transactivation of NFκB which in turn activates HIF1α promoter. Upregulation of HIF1α e induces expression of its downstream genes including VEGF, p21, p53 and Bcl-2 (Bonello et al., 2007).

HIF1α is essential in maintaining neural stem cells in the adult and embryonic brain (Westfall et al., 2008; Li et al., 2014). This has been linked to upregulation of

VEGF as its receptor in NPCs which results in increased NPCs proliferation and neurogenesis (Schanzer et al., 2004). Overall, hypoxia plays a crucial role in defining stem cell properties and their increased proliferation, which can be utilized for novel therapeutic strategies.

1.5.4 PTEN-PINK1

Phosphatase and tensin homolog (PTEN) is a tumor suppressor gene, which is frequently mutated in many human cancers (Li et al., 1997; Steck et al., 1997), but also has been associated with social deficits and autism spectrum disorder in mice (Napoli et al., 2012). Loss of PTEN results in NPCs exiting cell cycle at the G_0 phase and increased self-renewal by AKT1 downregulation (Sinor and Lillien, 2004). PTEN deficiency causes hyperproliferation but does not cause stem cell depletion. Also, PTEN depletion causes cell proliferation independent of growth factor dependency (Groszer et al., 2006). PTEN is a redox sensitive protein which controls NPC proliferation. Growth factors such as EGF and PDGF stimuli can cause elevated ROS mediated by NOX which reversibly oxidize PTEN to produce cell proliferation (Kwon et al., 2004). This reversible oxidation-reduction modulates the proliferation of NPCs when required. As discussed before, Nox2 associated growth factor signaling for proliferation occurs via PTEN inhibition. A study reports that adult hippocampal progenitors are maintained by oxidizing PTEN upon FGF signaling (Dickinson et al., 2011). Interestingly PTEN knockout neurospheres does not show increased proliferation under elevated ROS conditions. Where neurospheres from wildtype and PTEN heterozygous mice respond to H_2O_2 induced hyper-proliferation (Le Belle et al., 2011). Thus, PTEN is involved in generation of cancer-initiating stem cells by adaptation of reduced growth factor

responsiveness. PTEN induced putative kinase 1(PINK1) is downstream of PTEN and is known to be involved in mitochondrial function. PINK1 knockout results in increased ROS generation and thus oxidative stress (Valente et al., 2004; Requejo-Aguilar et al., 2014). In a recent study, PINK1 is shown to increase during normal mice development and its deletion results in decreased gliogenesis, unaltering NPC proliferation, neuronal or oligodendrocyte differentiation. Given the fact that astrocyte differentiation pathway is altered but NPC proliferation is unaffected upon PINK1 knockout, it is an interesting finding about how ROS regulated protein controls gliogenesis. Mitochondrial ROS generation was not changed during NPC differentiation (Choi et al., 2016). This is not the case in mouse embryonic fibroblasts, where mitochondrial ROS levels and oxidative stress elevated upon PINK1 global knockout (Requejo-Aguilar et al., 2014). The difference in mechanisms for such phenotype is yet to be explored (Choi et al., 2016). Overall, understanding PINK1 will reveal the mechanisms behind reactive astrogliosis.

1.5.5 p53

p53 protein functions as a tumor suppressor and a negative controller of cell proliferation in NPCs by inducing p21, a key negative regulator of cell cycle and apoptosis in adult NPCs (Meletis et al., 2006). Although the role of p53 in differentiation of embryonic stem cells has been shown, its involvement in NPC differentiation remains to be identified (Lin et al., 2005; Tedeschi and Di Giovanni, 2009). Conversely a paralog of p53 known as p73 is crucial in NPC maintenance (Talos et al., 2010). A direct correlation of p53 in ROS generation in mouse embryonic NPCs has been reported (Forsberg et al., 2013). Knockout of p53 is associated with increased ROS, which in

turn activates the AKT-PI3Kinase pathway that promotes neuronal differentiation of NPCs (Forsberg et al., 2013).

1.5.6 Nrf2

Nuclear factor erythroid 2–related factor 2 (Nrf2) is a master transcription factor that induces a battery of genes that contain a conserved sequence known as the antioxidant response element (ARE) in their promoter (Ma, 2013). Nrf2 is dynamically regulated by ROS levels in neural stem cells (Madhavan, 2015). Under normal conditions, Nrf-2 is quickly ubiquitinated and destined for degradation, however when exposed to increased ROS levels during oxidative stress conditions, Nrf2 is translocated into the nucleus where it binds to ARE and increases a set of antioxidant proteins (Karkkainen et al., 2014), including members of glutathione and thioredoxin system. Quinone components such as tertiary butyl hydroquinone (tBHQ) can increase Nrf2 protein stability and thus increase cellular defense (Li et al., 2005). Upregulation of Nrf2 is known to enhance NPCs survival against any oxidative stress (Li et al., 2005). This has been shown in SVZ-derived NPCs in culture or after ischemia which correlates with induced neurogenesis (Karkkainen et al., 2014). The effect of Nrf2 is partially mediated by Notch1 signaling, which is conserved in neurogenesis but not in gliogenesis (Nye et al., 1994). The exact role of Nrf2 on NPCs in degenerative diseases remains to be investigated.

1.6 Effect of NPCs niche on proliferation

Detailed structural studies have revealed that the self-renewing population of NPCs is kept under physiologic hypoxic condition. Under these conditions, NPCs are maintained quiescent in the microenvironment but can be activated to proliferate in

waves during development and after or during injury. The interaction of ROS and redox sensitive proteins alongside with other factors (such as sonic hedgehog, Notch and Wnt signaling), are known to regulate these events (Donovan and Dyer, 2005; Ji et al., 2010). Different models have been proposed for maintaining the stem cell niche *in vivo* and several redox sensitive factors have been identified that influence stem cells response to changes in their niche (Riquelme et al., 2008; Wang et al., 2013). The underlying in vivo mechanisms remains to be identified.

The SVZ niche has been well studied in detail: The ependymal cells (Ep) line the ventricle and separate the cerebrospinal fluid from the SVZ. There are highly proliferating type A cells arranged in a cluster. These cells are migratory in nature and move in the rostral migratory stream to reach the olfactory bulb. As described in figure 3, Type B cells are large quiescent cells with long processes and line the sub ventricular zone arranged next to ependymal cells. They show glial phenotype and contain GFAP intermediate filaments. Type B cells possess innate stem cell characteristics and produce Type A cells and Type C cells. Type C cells are scattered in SVZ. They are transiently amplifying cells and divide once or twice to give rise to Type A neuroblast cells. (Garcia-Verdugo et al., 1998). Thus, the SVZ is a region with a heterogeneous cell population, each with a different proliferative capacity. The architecture of the SVZ favors such proliferation differences through several factors including ROS.

The ephrinB2-Jagged1 system has been identified as a regulator of NPCs quiescence in the interface of NPC-endothelial cells (Ottone et al., 2014). Stem cells differ in nature by their proliferation rate, differentiation efficiency and fate determination. Microenvironment plays vital role determining cell fate by physical

interaction with other cell types. The stem cell niche is often kept in oxygen poor environment; a gradient of O_2 level provided by vasculature also plays a fate decision for stem cells in adult mice SVZ (Panchision, 2009; Crouch et al., 2015). The cells which have physical interaction with endothelial cells of capillaries shows enhanced oxidative phosphorylation and thus enhanced ROS levels, which is also an indicator of growth factor signaling (Le Belle et al., 2011; Ottone et al., 2014).

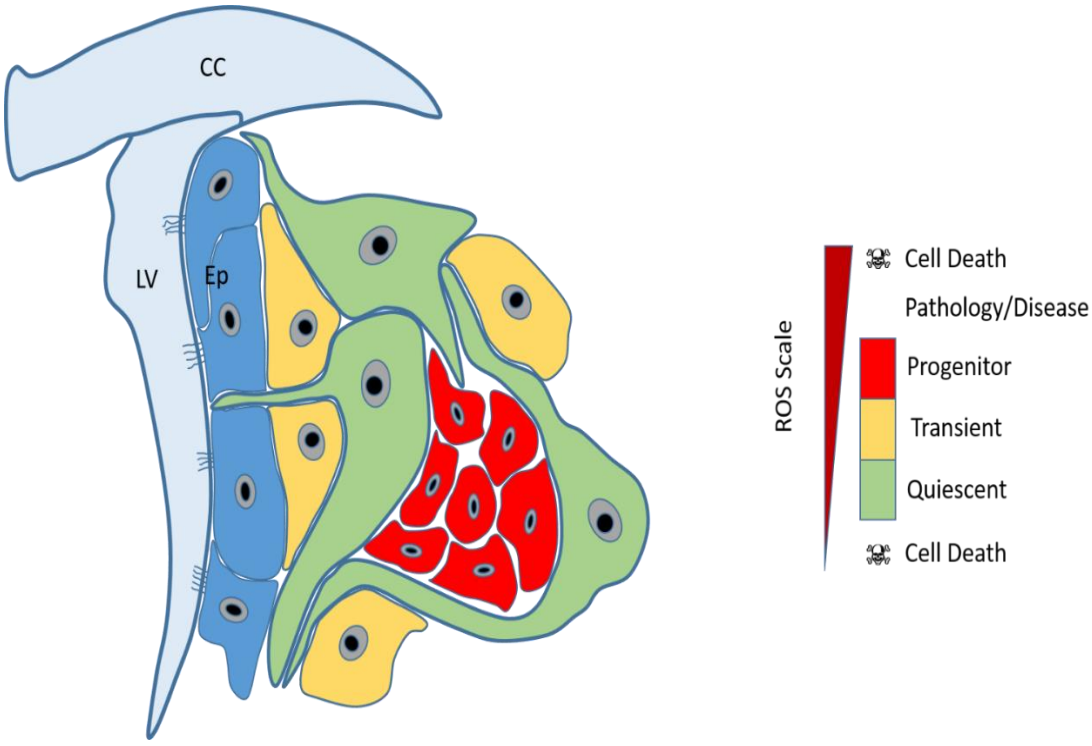


Figure 3: Niche maintenance in SVZ NPCs: Slow proliferating primitive NPCs align adjacent to the ependymal cell layer (Ep) and contact with lateral ventricle (LV). The transiently amplifying NPCs and progenitors are arranged in a distinguished manner (Tavazoie et al., 2008). The microenvironment of SVZ exhibit differential ROS levels based on their location. Cells adjacent to ventricles receive the least oxygen level and thus display low amounts of ROS. Progenitors receive blood circulation result in high oxygen availability and causing high ROS production partially because of the oxidative metabolism. This primitive NPC layer would be the source of the adult derived NPC pool resulting in life long generation of new neural cells. Modulating ROS levels in the microenvironment can result in disturbance of proliferation cycle of these cells. Increase in ROS levels result in activation of endogenous NPCs proliferation and deplete the NPC stem cell pool in certain CNS pathologies. (CC= Carpus callosum)

1.7 NPCs metabolism and Mitochondria

Cellular metabolism also affects NPCs proliferation and differentiation. The early embryonic NPCs, also known as primitive NPCs, are mostly dependent on glycolysis (Shyh-Chang et al., 2013). As development progresses and nutritional requirements change, there is a shift from glycolysis to oxidative phosphorylation (Homem et al., 2015). Quiescent stem cells residing in the SVZ are similar to a primitive cell type and depend on glycolysis. Oxidative phosphorylation is the main form of metabolism in proliferating NPCs. However, a forced disruption of mitochondrial metabolism in NPCs will switch their metabolism back to glycolysis, resulting in their quiescence via p53 inactivation (Bartesaghi et al., 2015). Increased oxidative damage in mitochondria that leads to mitochondrial DNA damage decreases self-renewal in NPCs, which can be rescued by the antioxidant N-acetyl cysteine (NAC) (Ahlqvist et al., 2012). A further study that was done by the same group in induced pluripotent stem cells resulted in similar observations including decreased proliferation with mitochondrial mutagenesis. This could be rescued with antioxidant therapy as showed by mild concentration of NAC and mitochondria targeted antioxidant mitoQ. Interestingly, higher levels of antioxidants resulted in decreased proliferation (Hamalainen et al., 2015). These studies suggest that high levels of ROS are detrimental for NPCs proliferation and differentiation. This may also imply the potential application of antioxidants for diseases that are associated with abnormal changes in NPCs proliferation (Hamalainen et al., 2015). Mitochondria are one of the major sources of cellular ROS and certain ROS can leak through the membrane affecting whole cell health. Mitochondrial structural dynamics are also known to affect ROS generation that

can influence the self-renewal and differentiation in NPCs(Khacho et al., 2016). Mitochondria can be found in elongated and fragmented structures which alters mitochondrial metabolism as well as ROS levels in NPCs. Sox2 positive NPCs contain elongated mitochondria and show lower ROS levels compared to Sox2 negative progenitors with fragmented mitochondria and higher ROS levels. Moreover, low ROS in Sox2+ NPCs result in reduced Nrf2 which enhances Notch signaling and thus NPCs self-renewal is maintained; whereas in Sox2 negative progenitors, ROS elevation activates Nrf2 signaling results in reduced self-renewal and induced differentiation. Antioxidant NAC reversed the condition and increased cell proliferation (Khacho et al., 2016).

1.8 Neural stem cells in Pathology

Acute neurotrauma, chronic neurodegenerative diseases and aging are shown to affect NPCs population (Hamilton et al., 2015) including increased proliferation in acute stroke and spinal cord injury (Barnabe-Heider et al., 2010; Marti-Fabregas et al., 2010). Conversely, depletion of NPCs or lack of their proliferation is a characteristic feature of chronic diseases such as Alzheimer's and Parkinson's disease (Hoglinger et al., 2004). All of these conditions are associated with aberrant ROS signaling. Mild oxidative challenge by buthionine sulfoximine (BSO) and other oxidants has been shown to decrease self-renewal, proliferation of NPCs and modulates multipotentiality by increasing astrocyte differentiation while decreasing neurogenesis. Increased Sirt1 is one of the factor for this effect at least in defected neurogenesis. Sirt1, a multifaceted NAD-dependent histone deacetylase, is involved in energy metabolism and transcriptional regulation (Leibiger and Berggren, 2006). Increased Sirt1 expression

decreases Mash1 expression in an epigenetic manner (Prozorovski et al., 2008). In another study with similar concept, H_2O_2 treatment promoted cell death and inhibited the protective autophagy in Sirt1 knockout cells in mouse embryonic stem cells (Ou et al., 2014). Such experimental conditions may be useful in mimicking the CNS pathological conditions with elevated oxidative stress. In reviewing this body of literature, one must appreciate the differential response of proliferating and quiescent NPCs under oxidative stress conditions.

Overall these studies indicate the involvement of ROS in the regulation of proliferation and differentiation of NPCs, and that the ROS-antioxidant balance is an important factor in this process. As NPCs have the potential for treatment of different disorders and neurotrauma in the nervous system, it is critical to use translationally related approaches to direct NPCs towards a specific phenotype. Therefore, for my thesis I am investigating the effect of thioredoxin on NPCs population, how proliferation and differentiation is altered. Although the effect of glutathione system on NPCs has been shown previously, my thesis examines the effect of thioredoxin protein transduction on NPCs. This may have therapeutic application for treatment of CNS pathology.

1.9 Thiol antioxidant systems

Protein oxidation-reduction is majorly governed by thiol-based protein systems such as thioredoxin and glutathione systems. Many potent antioxidants such as NAC mimic these thiols are used as therapeutic molecules (Moldeus et al., 1986). Thioredoxin (Trx) has direct interaction with several known proteins and an ever growing list of its substrates. These correlations provide a strong rationale for its importance in regulation

of major cellular activities. For instance, interaction of Trx with senescence associated proteins such as apoptosis signal-regulating kinase 1 (ASK1) and thioredoxin interacting protein (Txnip) prevents induction of apoptosis and cell cycle arrest respectively (Saitoh et al., 1998; Hwang et al., 2014). Therefore, major antioxidant systems must be investigated for their contribution to cell death prevention, maintaining cell proliferation and differentiation towards therapy.

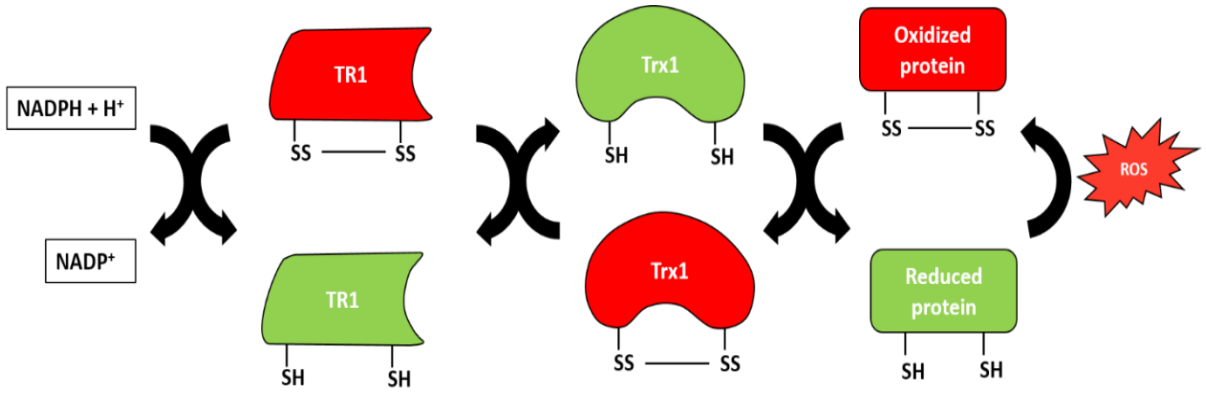


Figure 4: Thioredoxin system oxidation and reduction reaction: Cysteine residues are vulnerable to ROS and their thiol groups are oxidized resulting in conformational/functional modification. These oxidized proteins contain disulfide bonds (SS), that are encountered by reduced Trx1. Reduced Trx1 donates an electron to oxidized protein to reduce protein disulfide bond and becomes oxidized Trx1. Oxidized Trx1 is reduced by Thioredoxin reductase 1 (TrxR1). The reducing proteins are provided from NADPH, the major source for reducing protons.

1.10 Thioredoxin family of proteins:

Thioredoxin family of proteins consists of thioredoxin (Trx) 1 and 2, thioredoxin reductase (TrxR) 1 and 2. Trx1 and TrxR1 are found in cytosol but Trx2 and TrxR2 have a mitochondrial localization tag and therefore are specific to mitochondria (Holmgren and Lu, 2010). As figure 4 describes, there are two thiol groups in which Trx active site (Cys_{32}–Gly–Pro–Cys_{35}) that provide the electrons for reduction of oxidized proteins or ROS scavenging. Trx is oxidized in this process and is no longer capable of interacting with any targets until it is regenerated by the Trx reductase

enzyme using electrons from NADPH (nicotinamide adenine dinucleotide phosphate) (Holmgren, 1979). The intricate function of Trx must be delicately regulated by Trx inhibiting protein (Txnip), also known as Trx binding protein 2 (TBP2) and vitamin D3 upregulated protein1 (VDUP1). This protein directly binds to Trx to inhibit its function, subsequently resulting in exacerbation of oxidative stress (Hwang et al., 2014).

1.10.1 Thioredoxin1

Thioredoxin 1 is a 12kDa peptide with 105 amino acids. Trx1 is ubiquitously expressed in all living organisms (Holmgren, 1985). Homologous mutation of Trx1 results in defective embryonic implantation and is lethal (Matsui et al., 1996); whereas its overexpression increases life expectancy in mice (Mitsui et al., 2002). The active site of Trx1 is critical for all of its function and consists of a conserved sequence across all the Trx-like proteins (Lundstrom and Holmgren, 1993). The two cysteine residues in the active site work as a redox pair: the first Cys residue, (Cys32) donates its reducing proton to the target and a transient Trx-target protein bond is formed. The second Cys residue, Cys35 then becomes the reducing residue and donates its remaining proton to the target protein and itself binds to Cys32. In this process the target protein is released from Trx having received both protons, and oxidized Trxwill have an intramolecular disulfide bond between Cys32-Cys35 (Wu et al., 2014). This Trx is no longer capable of interacting with target molecules until it is regenerated by Trx reductase enzyme using electrons from NADPH (nicotinamide adenine dinucleotide phosphate). Few studies have taken advantage of Trx interaction with target proteins to identify its redox regulated targets. These groups have used a point mutation technique to replace the Cys35 with serine which is redox inactive. The Cys32-Ser35 Trx will initiate the

reduction of the target protein but since Ser35 is incapable of releasing the protein, a stable disulfide bond will be formed between the mutant Trx and its target (Hwang et al., 2014). Proteomic techniques have been used to identify tens of potential Trx targets (Motohashi et al., 2009). The biological importance of these interactions remains to be determined in biological systems.

Trx expression is regulated by two different systems: a constitutive promoter ensures its basal expression level, but a second inducible control also exists. Trx is also stimulated by antioxidant and oxidant response elements in its promotor; this makes Trx a first response protein under stressful conditions. This is well evidenced by Trx upregulation after stroke (Takagi et al., 1998) which indicates its potential protective effects. Protective roles of Trx can be attributed to its intracellular and extracellular localization;

1.10.1.1 Intracellular functions of Trx1

Higher levels of Trx1 in some cancers (Raffel et al., 2003) have been linked to enhanced cell proliferation and resistance to oxidative stress (Gasdaska et al., 1995; Mochizuki et al., 2009). This may be directly related to its involvement in reducing the active site of the ribonuclotide reductase enzyme which is required for and DNA synthesis (Figure 5) (Holmgren and Sengupta, 2010). Reducing capacity of Trx1 also regulates the DNA-binding capacity of some transcription factors such as Oct4 (Guo et al., 2004), that control cell proliferation. Trx1 overexpression in human adipocyte-derived mesenchymal stem cells induced proliferation via activating extracellular signal–regulated kinase 1/2 (ERK1/2) pathway (Song et al., 2011). A recent report indicated that Trx may promote NPCs proliferation after intraperitoneal administration

of recombinant human Trx (Tian et al., 2014). Protective effects of Trx1 can also be attributed to its anti-apoptotic properties mediated by its inhibitory function for Ask1 activation under normal conditions. Upon rise of oxidizing free radicals, Ask1 is released from Trx1 and induces the downstream programmed cell death mechanisms (Saitoh et al., 1998; Liu et al., 2000). Inhibition of Trx1, or TrxR leads to apoptosis and therefore this system is targeted in anti-cancer therapies (Cadenas et al., 2010).

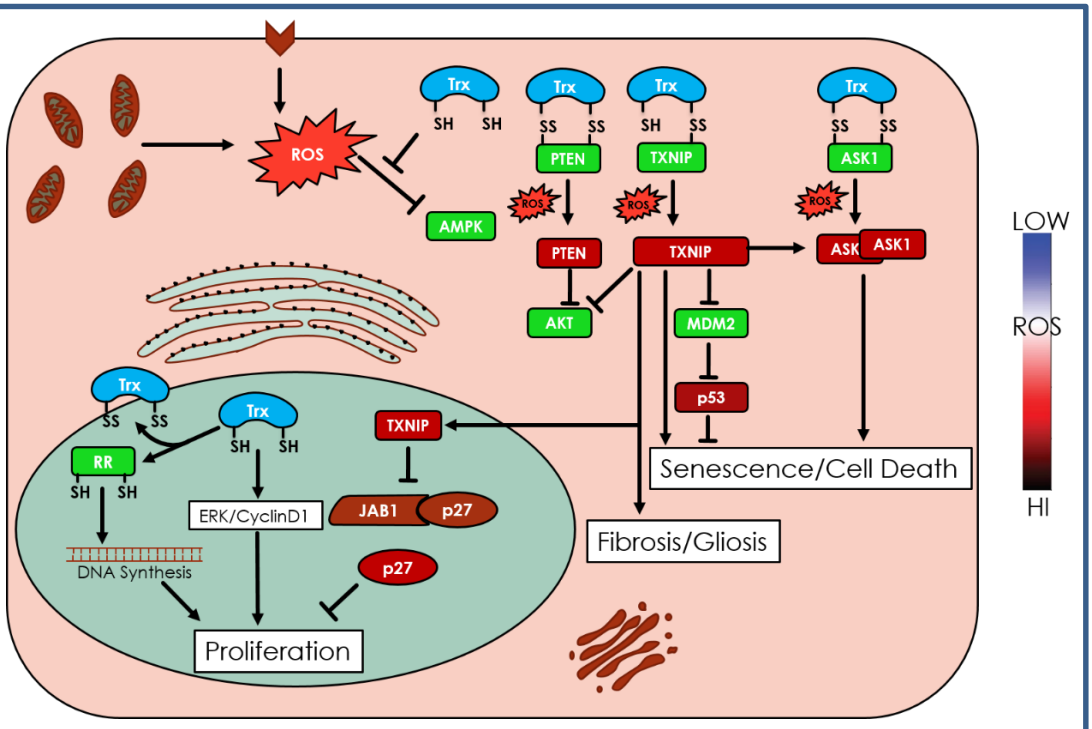


Figure 5: Thioredoxin in redox regulation and cell proliferation: Trx1 reduces ribonucleotide reductase (RR) which is an essential enzyme for DNA synthesis and therefore is directly correlated with cell proliferation. Trx1 facilitate pro-survival pathways such as AMPK and AKT. Trx1 physically interacts with senescence associated proteins such as PTEN, Txnip and ASK1 in a redox sensitive connection. ROS disrupts the physical interaction of Trx1 and other molecules and leads to senescence or apoptosis.

1.10.1.2 Extracellular function of Trx1

The majority of Trx1 functions have been studied in the context of intracellular compartments; although some immune cells secrete Trx into blood stream (Rubartelli et al., 1992). There is currently no receptor/transporter identified for Trx1, however minimal uptake of Trx has been reported (Gasdaska et al., 1995). Trx is secreted from normal and transformed cells through a "leader-less" pathway which does not follow the classical endoplasmic Reticulum-Golgi vesicle mediated secretion. Two secretory forms of Trx are reported; the full length protein with reducing capacity and the truncated protein (Trx80) with 80 amino acids, which lacks the redox activity. Both proteins have a mitogenic effect on peripheral blood mononuclear cells (Pekkari et al., 2003) and addition of Trx1 in the growth medium has increased cell proliferation in a series of solid tumors (Gasdaska et al., 1995). In the nervous system, Trx1 is secreted from astrocytes and that has been linked to neuroprotection under oxidative stress conditions (Hori et al., 1994). Mitotic role Trx1 after addition to the extracellular compartments has been also shown in vitro and in vivo in the brain (Tian et al., 2014). The effect may be due to its regulatory role on growth factor receptors, but this has not been well established. The dominant theory on Trx roles in extracellular environment is attributed to its downstream redox control (Truong and Carroll, 2012), as shown for tumor necrosis factor receptor (Schwertassek et al., 2007). The mechanism of this function remains unclear, as the extracellular environment has a higher (more oxidized) redox potential than the intracellular space and therefore the secreted Trx would be quickly oxidized. The oxidized Trx will require a reductase enzyme and a source of

proton donation such as NADPH; there is currently no extracellular reducing system identified for Trx1.

Protective effects of intravenous Trx1 administration have been reported in models of brain ischemia (Hattori et al., 2004) and inflammatory lung disease. These effects in CNS can be only explained by its anti-inflammatory effects, as Trx1 cannot cross the blood brain barrier, and very trace amounts of tissue deposition has been shown in these reports (Nakamura et al., 2009). Anti-inflammatory properties of Trx1 in models of lung disease are mediated through inhibition of neutrophil and monocyte chemotaxis that results in decreased cell death (Bertini et al., 1999). The mechanism of Trx1 action in NPCs or CNS pathologies remains to be identified.

1.10.2 Thioredoxin interacting protein

Txnip is a multifaceted protein that inhibits Trx1 activity by forming a protein-protein complex (Hwang et al., 2014). Txnip is known for its function in negative regulation of cell growth and metabolism; i.e. Txnip suppresses tumors and metastasis (Han et al., 2003). The negative effect of Txnip on cell growth in these cases may be partially due to its pro-oxidant role through inhibition of Trx antioxidative function (Park et al., 2013). A recent report indicates that down regulation of Txnip in astrocytes is associated with improved protection of neurons in an in vitro model of oxidative stress (Genis et al., 2014). As a natural inhibitor of Trx1, Txnip plays an effective role in downregulation of Trx1-mediated reactions. The interaction between Trx1 and Txnip is redox sensitive. Reduced Trx1 binds to Cys_{247} in Txnip by formation of a disulfide bond and oxidative stress can break this bond which leaves Txnip to execute the negative

regulation (Hwang et al., 2014). Thus enhancing Trx1 levels by inhibiting Txnip function may be beneficial for cell proliferation.

1.11 Upregulation of intracellular Trx1, a potential approach for translational purposes.

Evidence of therapeutic potential of Trx1 has been shown using protein transduction and gene delivery techniques. The advantage of using either one of these approaches is dependent on the specific condition; gene delivery is often used for long term effects and can be designed to specifically target a special cell type, while protein transduction is not cell-specific and can be used in acute conditions. Gene delivery is achieved using adeno- or retrovirus transfection with the gene of interest. This technique requires some time for the virus to successfully transfect the cells and for the transgene to be activated to produce a protein of interest in sufficient amount for therapeutic purposes. This may take up to a few months. Optimizing the dose and efficacy of the gene transfection are other variables that limit its application for clinical use. Recently, lentiviral therapy has been approved as a therapeutic approach for multiple diseases, however, it is still rigorously monitored (Persons, 2010) and cannot be used in acute conditions such as neurotrauma treatment, as it will require days-weeks to be expressed in vivo.

Protein transduction is based on the use of the purified protein of interest for fast delivery to the cell and tissue (Figure 6). The protein can enter the cell and will deliver the intended effect. This approach can be readily regulated for therapeutic purposes. Protein transduction is based on the use of short sequences peptide that can penetrate the cell membrane known as cell penetrating peptides. One of the most common peptide is TAT peptide. This is a derived from HIV-1 (Human Immunodeficiency Virus) TAT

(Trans-Activator of Transcription) protein that has been known to possess highly positive poly arginine repeats in its sequence. This cationic peptide facilitates cellular penetration through active endosomal intake (Erazo-Oliveras et al., 2014). The TAT peptide can be conjugated with high molecular weight cargo proteins for enhanced intracellular protein delivery (Nagahara et al., 1998). Protein transduction using TAT peptide also facilitates crossing blood-brain barrier to deliver bioactive proteins to CNS (Schwarze et al., 1999). TAT-mediated protein delivery has been successfully used for CNS injuries (Doeppner et al., 2012).

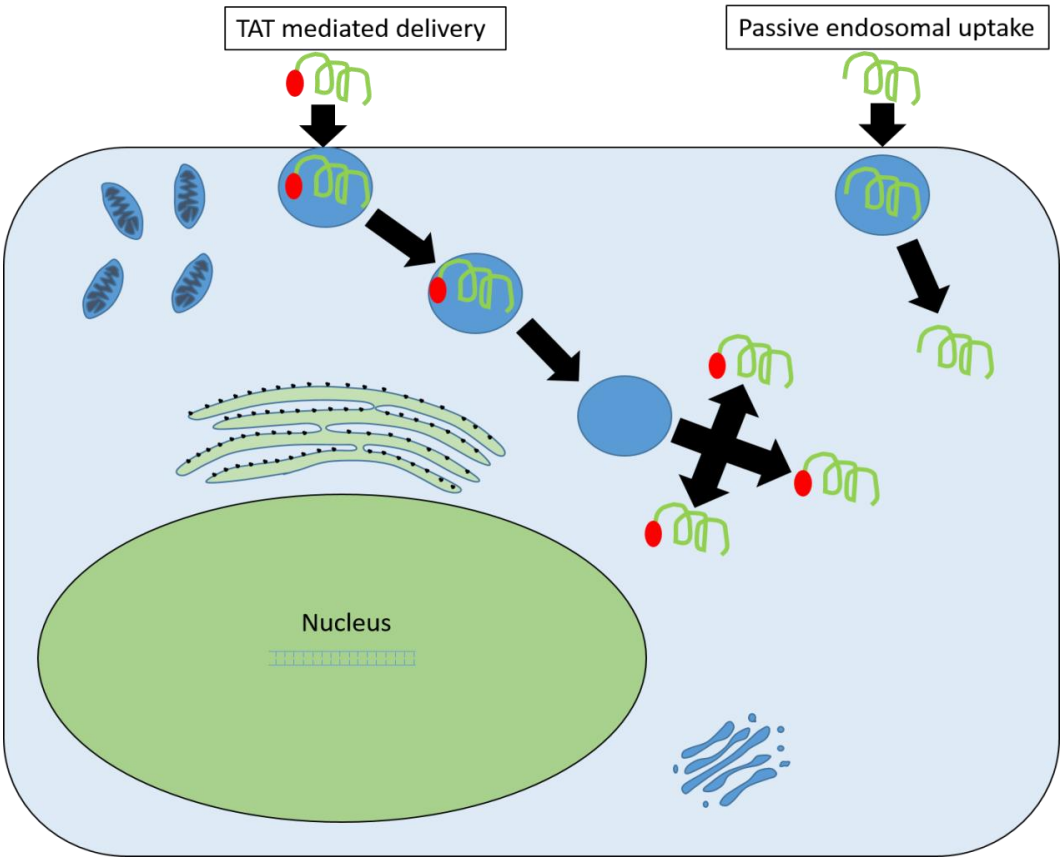


Figure 6: TAT mediated protein delivery: Conjugation of TAT sequence with the protein of interest enhances the intracellular protein delivery by increasing active endosomal intake due to the positive charge of the sequence. The cargo is then released into the cytosol. Non TAT peptide is also up taken by the cells but at a lower rate when compared to TAT mediated delivery (Erazo-Oliveras et al., 2014).

1.12 Rationale

Thioredoxin 1 is a key regulator of cellular redox status by controlling the reduction/oxidation of thiol groups in signaling proteins involved in many aspects of cell life including cell survival and proliferation. More than fifty years of extensive

research on Trx1 has identified several roles for cell survival, proliferation and immunomodulation. This makes Trx a good candidate to test for its therapeutic purposes. However, the role of Trx1 in NPCs proliferation and differentiation is poorly understood. These effects have been studied after Trx1 overexpression (gene delivery) or after its administration in *in vitro* and *in vivo* conditions (protein transduction). Interestingly, the intracellular Trx1 protein transduction method remains completely unexplored. Enhancing intracellular Trx1 levels may potentially alter the NPCs signaling system and promote their response under oxidative stress conditions. This can eventually be used as a therapeutic strategy for neurotrauma.

1.13 Hypothesis

Intracellular thioredoxin delivery will enhance cell proliferation in neural precursor cells and affect their differentiation capacity in *in vitro* conditions.

1.14 Objectives

1. To investigate the effect of intracellular delivery of Trx1 on cultures of neural precursor cells (NPCs) proliferation and survival in culture.
2. To assess the effect of *in vitro* administration of intracellular Trx1 on endogenous ROS levels in NPCs.
3. To compare the effect of extracellular (conventional) and intracellular Trx on cell signaling pathways in cultures of NPCs.
4. To assess the effect of lentiviral-mediated Trx1 overexpression in NPC proliferation and differentiation.

Chapter 2: Materials and methods

2.1 Plasmids, Recombinant Protein purification

Full-length human thioredoxin 1 cDNA (NM_003329.2) was used for these studies. Mouse and human thioredoxin 1 varies in two amino acids which is not in the active site. Both behaves equally in enzymatic reactions. Preference for human over mouse thioredoxin was done due to easy acceptance of human protein for clinically relevant models. The cDNA was constructed by Integrated DNA Technology (USA). This cDNA construct was cloned into the commercially available (Novagen) pET-28a(+) vector. To generate the construct for intracellular delivery of Trx, TAT-peptide was cloned at the N-terminal of Trx and was incorporated into the vector. Two control vectors were also generated to produce a TAT and a noTAT peptide in conjugation with redox inactive Trx protein in which the cysteine residues were replaced by serine ($C_{32}S$ and $C_{35}S$). In my thesis, the constructs without TAT sequence will be referred as extracellular Trx or E-Trx and those with TAT will be called as intracellular Trx or I-Trx. One-Shot TOP10 competent cells (Invitrogen, Canada) has been used for cloning and BL21(DE3) (New England Biolabs, Canada) for protein synthesis and purification. All the constructs contained a 6XHistidine at the N-terminal for purification using the Nickel (Ni-NTA) resin columns (Qiagen) and a red fluorescent protein (RFP) at the C-terminal end of the protein for easy visualization and tracking (Figure 7). Recombinant BL21 bacteria were grown in 600ml of Luria-Bertani (LB) media containing 50µg/ml kanamycin at 37˚C with 250RPM shaking. Protein production was induced using a final concentration of 0.5µM isoopropyl-B-D- thiogalactopyranoside (IPTG), when bacterial growth reached an optical density of 0.6-0.7 at 600nm absorbance using a

spectrophotometer for 6hrs at 37ºC. At the termination of the experiment, the bacteria were pelleted down at 6000RPM for 10 mins at 4˚C. A French Press was used at 1500 psi (pound-force per square inch) twice for complete mechanical lysis in the lysis/binding buffer (50 mM NaH_2PO_4, 350mM NaCl, 10mM Imidazole, pH 8.0). The lysate was treated with DNase-I 20units/ml and incubated for 15mins at 4˚C. Lysates were cleared of debris by centrifuging at 10000 RPM at 4°C for 15mins. The clear filtered supernatant was treated with 2mM DTT and was added to the pre-treated Qiagen Ni-NTA resin and incubated at 4º on a tube rotating mixer for binding. After sufficient binding, the resin-lysate mixture was added to the column. After settling of the resin, the columns were washed vigorously with binding buffer containing sequential increasing concentration of 10, 20 and 40mM imidazole to remove the unbound and unspecific recombinant proteins. The proteins were eluted in binding buffer containing 250mM imidazole. A P10 desalting column was used to remove DTT, imidazole and excessive salt from the protein. The protein was eluted in endotoxin free PBS and was additionally run through an endotoxin removal column to remove bound endotoxins. Finally, the proteins were dissolved in PBS, sterile filtered, aliquoted and after protein estimation (Pierce 660nm Protein Assay kit) were stored at -80˚C until used.

Figure 7: Thioredoxin 1 protein constructs: These fusion proteins contain a 6 Histidine residue tag at the amino terminal (H_3N-) that is cloned in line with the human Thioredoxin 1 followed by red fluorescent protein at the carboxy terminal (-COOH). To ensure intracellular delivery, a TAT sequence was added at the N-terminal. The fusion protein without TAT sequence is called as Extracellular Trx (E-Trx) and fusion protein with TAT sequence is called as Intracellular Trx (I-Trx). The mutant version of these constructs do not have cysteine residues and therefore lack Trx activity and are shown as mt E-Trx, and mt I-Trx.

2.2 Trx kinetic assay

Thioredoxin enzymatic activity is commonly measured using the insulin disulfide reduction/precipitation assay as described previously by (Holmgren, 1979). A kinetic enzymatic assay was performed using a commercially available kit (Imco corp, Sweden) according to the manufacturer's instruction. The assay buffer consists of 50mM MES (2-(N-morpholino)ethanesulfonic acid) buffer, 250mM NaCl and 2mM EDTA (pH 6.5). The substrate, bovine Insulin (Sigma), was diluted to prepare a 520µM stock and the fusion proteins were prepared in equal molarity (20µM) in the assay buffer. The reaction mixture was prepared in a clear 96-well plate by adding 25µl reaction buffer, 25µl of 520µM Insulin and 25 µl of 20µM fusion proteins to the well. A Synergy H1 microplate reader was used for this assay. The reaction was started by addition of 10µl of a 55mM DTT solution. The absorbance was recorded every minute for 30 minutes and the temperature was maintained at 25ºC. In this reaction, DTT is used to fully reduce Trx which then will reduce the insulin intramolecular disulfide bonds resulting in its precipitation that can be measured by changes in optical density at 650nm.

2.3 Trx endpoint assay

This assay takes advantage of Trx-mediated reduction of oxidized proteins. In this reaction, NADPH, and Trx Reductase is provided to ensure complete reduction of Trx in the sample (5µg total cell lysate in equal volume for all conditions). The reduced Trx then reduces the insulin disulfide bonds. In the next step DNTB [5,5'-Dithiobid-(2)-nitrobenzoic acid] is added that will quench the reduced thiol groups in insulin and a

yellow product (Thionitrobenzoate) is generated that can be measured spectrophotometrically at 412nm. The Trx endpoint assay kit (by Imco Corp., Sweden) was purchased from Cayman Chemical and was used according to the manufacturer instruction.

2.4 ROS Estimation in HT22 Cells

HT22 cells were grown as adherent culture in DMEM containing 10% Fetal Bovine serum (FBS), 2mM Glutamine and 1% penicillin streptomycin. Once the culture reached 80% confluency, the cells were passaged using TrypLE dissociation solution and seeded in 8 well multi-chamber slides at 20000 cells/well and were allowed to settle down overnight. The cells were treated with fusion Trx proteins for 12 hrs and then incubated with CellROX Deep Red (Molecular Probes, USA) at a 2.5µM (from stock solution 2.5mM) final concentration for 30 mins. The slides were then fixed with 3% Paraformaldehyde (PFA) and 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) stained. Imaging was done using Zeiss Axiovision at 644/665nm excitation/emission. Images were taken at random regions of multi-chamber slides. Quantification done by ImageJ software by defining individual cells with the ROS signal. Background noise was removed by confining area without any cells and subtracting with the signals. Signals were then plotted for comparison between conditions.

2.5 Generation of mouse SVZ neurospheres

Neural stem cells were isolated from the brain subventricular zone of embryonic day 18 or 4weeks old mice C58Bl/6 (University of Manitoba animal facility) according to our previously described methods (Eftekharpour et al., 2007). Briefly the sub ventricular zone was dissected in sterile conditions and transferred to artificial

cerebrospinal fluid (aCSF). The tissue is mechanically chopped using sterile forceps and triturated using 1 ml pipette tip. The suspension was spun down and the enzyme mix was added for tissue digestion by incubating at 37˚C for 5mins. DNAseI added to digest the sticky DNA. The enzyme was neutralized with DMEM containing 10% FBS and centrifuged at 1200RPM for 5mins. The Cell pellet was triturated using a fire polished pasture pipette, spun down and re-suspended with DMEM:F12 media. The single cell suspension was seeded in a 6 well plate at a density of $5x10^5$ cells/well in proliferation media DMEM:F12 with 20ng/ml EGF (Epidermal Growth Factor) and bFGF2 (Fibroblast Growth Factor-basic). The neural precursor cells are responsive to growth factors and form neurospheres, where other cells decay. Grown neurospheres were spun down and further passaged to maintain the precursor pool. Most of the experiments were performed with these clonally derived NPCs.

2.6 Lentivirus overexpression of thioredoxin 1:

2.6.1 Plasmids and bacterial cloning:

Packing vectors pGAG, pRev, pTAT2, pVSV-G (A kind gift from Dr. Rastegar, Regenerative Medicine Program, University of Manitoba) and Lentivirus Compatible overexpression vectors were procured and transformed into One shot TOP10 chemically competent E. coli. Using antibiotic selection, recombinant bacterial clones were formed. Single colony was picked from the antibiotic resistant LB AGAR plate and then grown in a small culture overnight with respective antibiotic (Ampicillin or Kanamycin). 2ml culture was used for plasmid isolation. Later the plasmids were validated using restriction digestion (Xho1-Xba1) for confirmation of plasmid specificity. Once confirmed, bacteria were grown in large culture to yield sufficient plasmid quantity.

Destination vector and mouse thioredoxin 1 wild type transient overexpression TrueORF plasmids (pCMV6-Txn1) were obtained from OriGene Technologies. pLenti Txn1 wildtype was made by restriction digestion of pCMV6-Txn1 with MluI-AsisI restriction enzymes, taking the insert from the digestion and ligate with the destination vector digested by the same enzymes. The ligated plasmid was inserted in One shot TOP10 competent *E. coli* strain and screened with specific antibiotic. The mutant Txn1(SS) gene block was procured from IDT and then inserted in the destination vector. Later both cloned plasmids were grown in large culture and with plasmid Midi preparation; plasmids were isolated and validated for the presence of insert with restriction digestion and/or using sequencing services.

2.6.2 Lentivirus production using HEK 293T cells:

2.6.2.1 Transfection:

HEK 293T cells culture: HEK 293T cells were grown in DMEM with 10% FBS and 2mM Glutamine in a T75 flask at initial density of 7.5 million cells. Packing vectors and overexpression vectors (total 50µg pDNA) were pooled in Opti-MEM. Lipofectamine 2000 (75µl) was added to a ratio of 1µg:1.5µl (plasmids to lipofectamine) Opti-MEM and incubated at room temperature for 5 mins. Then Plasmid mix was added to Lipofectamine and allowed to mix for 20 mins at room temperature. The transfection mix was then added to HEK cells in T75 flask and incubated in designated Lentivirus CO2 incubator for 16 hours. At the termination of this step the transfection media was replaced with DMEM:F12+GFs+Hormone mix and allowed to generate lentivirus for 48 hours.

2.6.2.2 Transduction:

The virus containing media was safely collected and spun down to remove any debris. Then the media was filtered using a 0.45µm syringe filter to completely remove any cells. The media containing virus was diluted with fresh media to have a 75% virus containing media. Then polybrene was added at 6µg/ml final concentration and this transfection media was incubated with the brain derived NPCs for 12 hours. Once virus has bound to NPCs, the non-bound virus containing media was removed and fresh media added. The cells were then allowed to express transduced thioredoxin 1 for three days.

2.7 Proliferation assays for NPCs

Neurospheres were dissociated and seeded at 20000 cells per well in a Matrigel coated 8 well chamber slide for cell quantification) or at 200000 cells per well density in 6 well plates, for real time QPCR or Western blotting. The cells were grown overnight and treated with E-Trx and I-Trx at the specified concentration. Occasionally commercially available rhTrx was used for comparative purposes. The control cells did not receive any protein treatment for 2 days. For 5-bromo-2'-deoxyuridine (BrdU) pulse-chase experiment, 8 well chamber slides were used. Cells grown for two days under experimental conditions received a single dose of 5µM BrdU for an hour. At this time, medium was removed and cells were immediately washed with room temperature PBS (2x) and were fixed with 3% PFA (15min) and washed with PBS for 10mins x 3 times and further processed for immunocytochemistry. To process the cells for molecular analysis, the 6-well plates were used. The medium was removed and rinsed twice with PBS. For complete cell lysis the plate was snap frozen at -80°C followed by addition of cell lysis buffer containing 1% NP40 and Halt protease and phosphatase inhibitor (as per manufacturer instruction). Cell lysis was completed using short burst of sonication

(10 seconds, repeated 3x, with one minute intervals) while the samples were always kept on ice. The lysate was cleared by centrifuging at 12000 RPM for 30 mins at 4°C. Clear supernatant lysates were separated and quantified for protein content.

2.8 Differentiation of NPCs

NPCs were grown at 20000 cells/ well in a multichamber slide for 24 hours in growth media (proliferation media) in Matrigel coated 8well chamber slides. Then the cells were introduced to cell differentiation media: containing 2%FBS in DMEM:F12 with or without E-Trx and I-Trx. The cells were allowed to grow for 6 days in these differentiating conditions. On the third day of differentiation, the cells were replenished with 50% fresh media. At the termination of this experiment (differentiation day 5), the chamber slides were fixed with 3% PFA and processed for immunocytochemistry.

2.9 Immunocytochemistry

Undifferentiated and differentiated cells in chamber slides were fixed in 3% paraformaldehyde (PFA). For immunostaining of BrdU pulse-chase slides, the cells were treated with 2N HCl+0.5% TritonX at 37˚C for 30mins to unmask BrdU antigen. Then the cells were neutralized with 0.1 M Sodium Borate (pH 8.5) for 10 min. After a brief wash with PBS, the cells were blocked in 5% milk+1%BSA+0.3%TritonX for one hour. Fluorescence immunocytochemistry was performed for the following antibodies: 1) cell proliferation markers: anti-BrdU (1:500; BDBiosciences), Ki67 (1:100; BDBiosciences), 2) Differentiation markers; for Oligodendrocytes- Olig2 (1:400, Chemicon) and astrocytes-GFAP (1:1000, Sigma) were used. All antibodies were incubated overnight at 4˚C in a humidified chamber. After brief washing cells were probed for respective secondary antibodies conjugated with Alexa flour 488, 568 or 647

(1:600, Life technologies). The slides were gently washed in the dark. The final wash was done with 1:15000 concentrations of 4,6-diamidino-2-phenylindole (DAPI) in PBS then mounted using Vectashield mounting media. Images were taken by Zeiss Axiovision and quantification was done using ImageJ software, according to the instructions.

2.10 Western Blotting:

Cells were scrapped in modified RIPA lysis buffer that contained 1% NP40 supplied with protease and phosphatase inhibitor. Cells were sonicated on ice and then centrifuged at 12000 rpm for 30 minutes at 4°C, supernatant was quantified for protein estimation using Pierce 660 nm Protein assay (Thermo Fisher). 20µg protein was loaded for each sample to detect Thioredoxin1 (1:2000 Santacruz), phospho/total ERK1/2, CyclinD1 (1:1000 Cell signaling) and normalized to β actin (1:5000 Santacruz). The blots were probed with respective secondary antibodies (1:3000 anti-rabbit or anti-mouse antibodies, Cell signaling) and chemiluminescent imaging is done using Microchemi 2.0 (Froggabio). Relative quantification was done using ImageJ software.

Chapter 3: Results

3.1 Thioredoxin1 protein validation

Generation of fusion Trx proteins from recombinant bacteria was validated by molecular and biochemical analysis. The purified E-Trx and I-Trx were detected as a single band in Coomassie blue stained gel and in immunoblots. Different concentrations of these proteins were used depicting a molecular weight of 40.8 kDa for E-Trx and 42.8 kDa for I-Trx that contain the TAT peptide (Fig. 8A&B). It was later checked for the

enzymatic activity of the recombinant proteins using the endpoint and kinetic assay as described before (Materials and methods section 2.2 and 2.3). It was observed that, similar activity levels between the two proteins that were comparable to the commercially available rhTrx. Mutant protein constructs were used as controls which did not show any activity in the kinetic assay (Fig. 9). To check the efficacy of TAT peptide for intracellular delivery, E-Trx and I-Trx transduction in cultures of NPCs were compared. Cultures were treated for 3hrs (short treatment) with I-Trx and E-Trx and the protein transduction was tested using multiple methods. Images were taken from fluorescent microscope for representation. Cells grown in microplates were scanned for the whole area under fluorescent microplate reader for red fluorescent protein (RFP) (Fig10B) or Trx1 protein quantification was by western blotting (Fig10C&D). Some of the E-Trx treated cells showed a very faint RFP signals whereas a strong signal was detected in all the cells in I-Trx treated NPCs (Fig. 10A). The RFP signals were quantified using microplate reader. My statistical analysis showed a significant difference in fluorescence intensity between E-Trx and I-Trx cells (Fig. 10B). To examine the long term protein retention, I used western blotting against Trx at three days after the short time (3hrs) protein treated samples. My experiments showed a significant protein retention (internalization of I-Trx) in comparison with E-Trx (Fig. 10C and 10D).

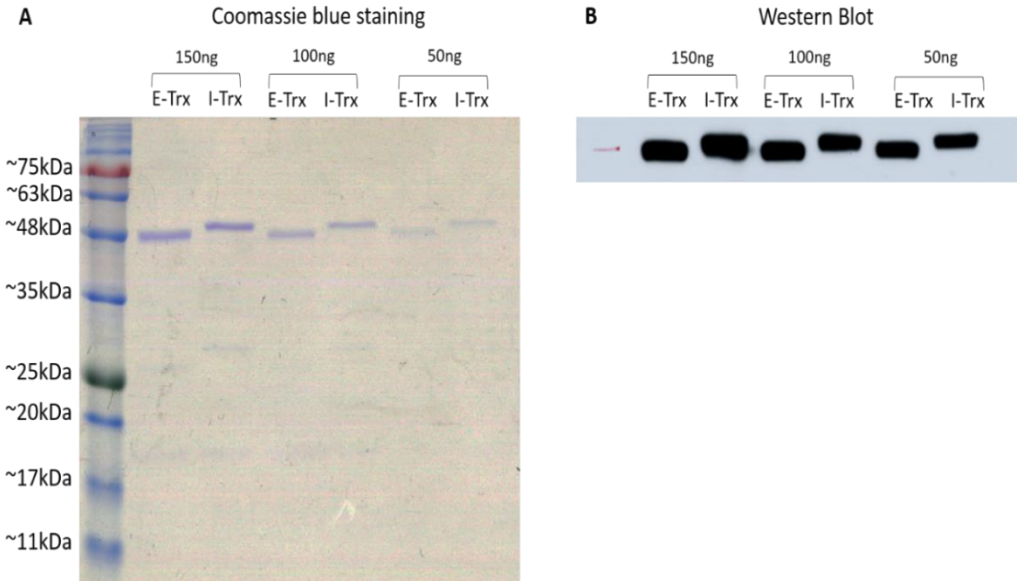


Figure 8: Thioredoxin 1 fusion protein production: E-Trx and I-Trx were produced and purified from recombinant bacteria. Purified proteins were validated for the specificity by running in SDS-PAGE. Fig.8A shows the purity of the recombinant protein loaded at different concentration of proteins (150ng, 100ng and 50ng). A similar gel was transferred to PVDF membrane to detect Trx1 (Fig. 8B). The molecular difference between E-Trx and I-Trx were clearly seen in the gel and by Western blotting.

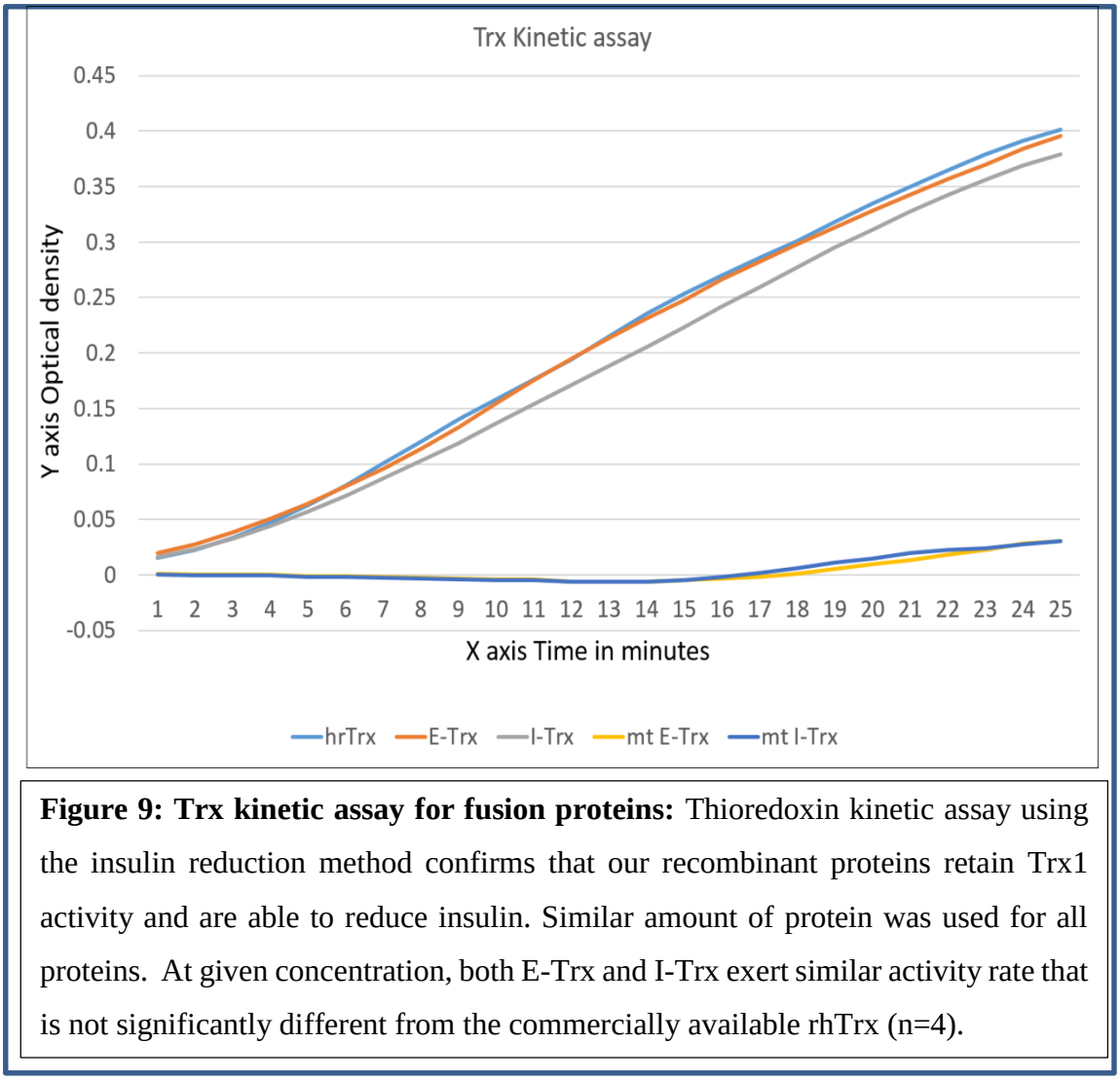


Figure 9: Trx kinetic assay for fusion proteins: Thioredoxin kinetic assay using the insulin reduction method confirms that our recombinant proteins retain Trx1 activity and are able to reduce insulin. Similar amount of protein was used for all proteins. At given concentration, both E-Trx and I-Trx exert similar activity rate that is not significantly different from the commercially available rhTrx (n=4).

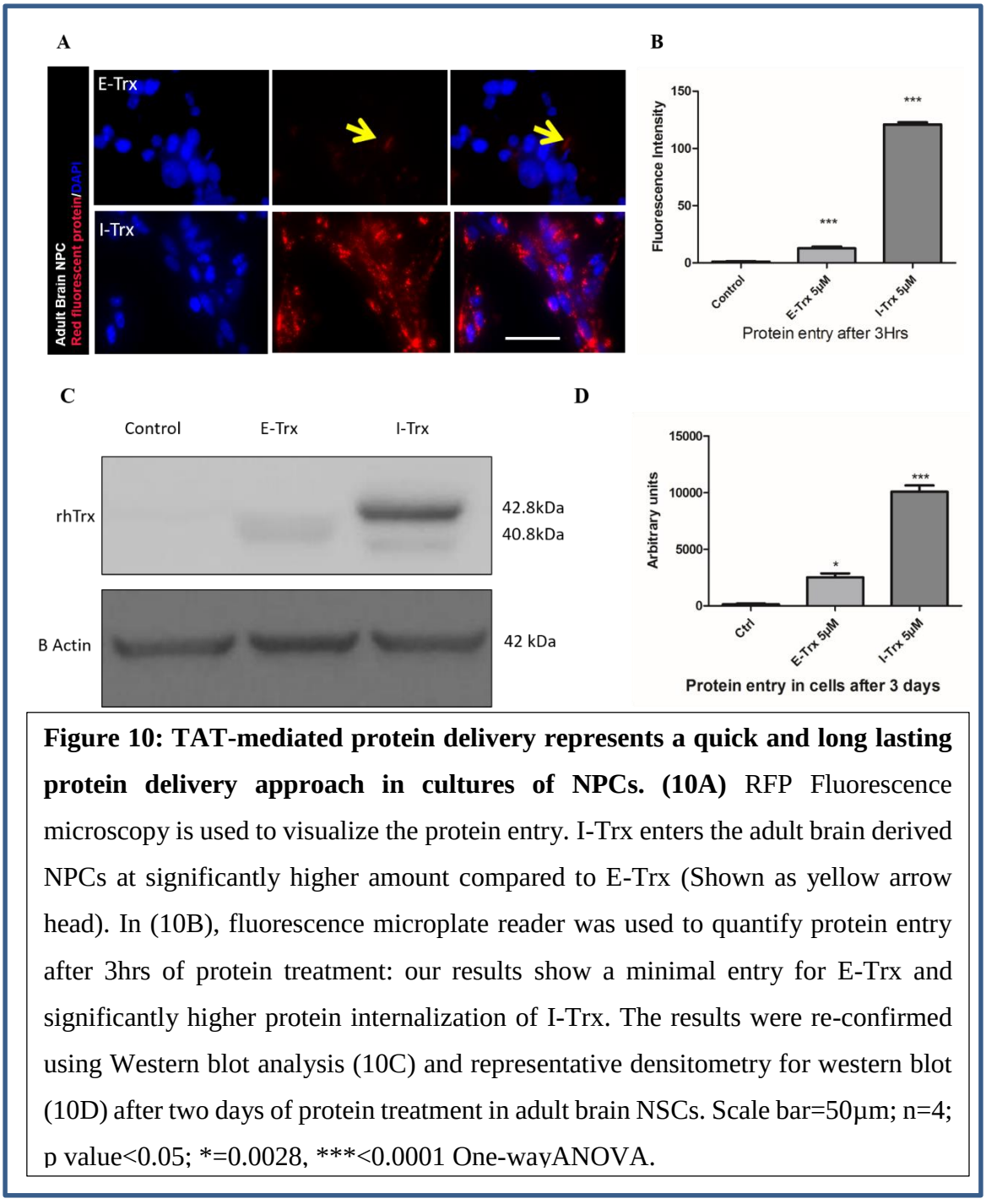


Figure 10: TAT-mediated protein delivery represents a quick and long lasting protein delivery approach in cultures of NPCs. (10A) RFP Fluorescence microscopy is used to visualize the protein entry. I-Trx enters the adult brain derived NPCs at significantly higher amount compared to E-Trx (Shown as yellow arrow head). In (10B), fluorescence microplate reader was used to quantify protein entry after 3hrs of protein treatment: our results show a minimal entry for E-Trx and significantly higher protein internalization of I-Trx. The results were re-confirmed using Western blot analysis (10C) and representative densitometry for western blot (10D) after two days of protein treatment in adult brain NSCs. Scale bar=50μm; n=4; p value<0.05; *=0.0028, ***<0.0001 One-wayANOVA.

3.2 ROS scavenging capacity of Trx1

Increased levels of Trx1 have been linked to decreased ROS levels, therefore it was asked whether recombinant Trx1constructs can maintain their ROS scavenging capacity. To test this, Matrigel coated mutichamber slides were used for NPCs. I-Trx and E-Trx were used in these experiments. A high background staining was observed due to a binding between the highly positive I-Trx construct and the Matrigel that interfered with microscopy. Therefore, HT22, an immortalized mouse hippocampal cell line was used that show high proliferation rate and can be cultured without any coating. The cells were treated with E-Trx and I-Trx for 12 hours after the initial seeding and ROS levels were assessed with CellRox Red treatment for 30minutes. Cells were washed and visualized using a fluorescent microscope using a 647nm (Far Red) filter. Signal intensity was measured for each cells using Image J. Result shows that Trx treated cultures had significantly lower ROS in comparison with the controls (Figure 11A and 11B). I-Trx treated cells showed lower levels than E-Trx-treated cells. As expected, the mutant proteins did not have any effects on ROS levels (Fig.11A&B). These results indicate that endogenous ROS levels might be regulated by intra and extracellular factors. Additionally, results suggest that any potential difference between I-Trx and E-Trx on cellular ROS levels may reflect their differential cellular entry.

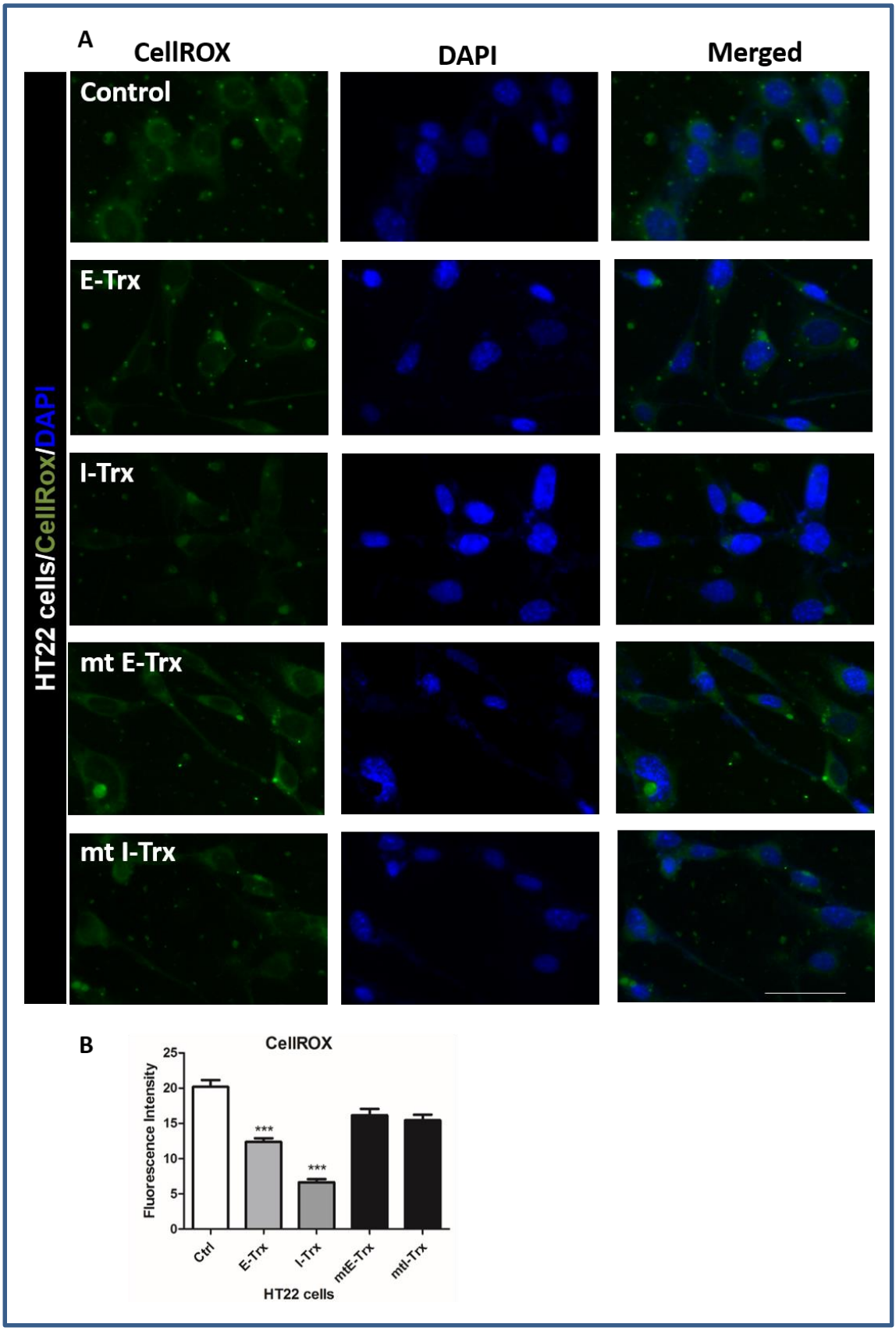

A
CellROX
DAPI
Merged
Control
E-Trx
I-Trx
mt E-Trx
mt I-Trx
HT22 cells/CellRox/DAPI
B
CellROX
Fluorescence Intensity
25
20
15
10
5
0
***
***
Ctrl
E-Trx
I-Trx
mtE-Trx
mtI-Trx
HT22 cells

Figure 11: Trx1 transduction decreases the endogenous ROS. Cultures of HT22cells were used for these experiments. Cells were treated with our Trx1 constructs for 12hrs. CellROX Deep Red was added during the last 30 minutes of the experiment. Fluorescent microscopy was performed and random images were recorded using optimized exposure time and intensity that was applied to all experimental groups. Representative fluorescent micrograph is shown in Fig.11A. The fluorescent intensity was quantified on single cells of by drawing boundary using Image J which showed a significant decrease in ROS levels in cells treated with wildtype I-Trx and E-Trx but not in cells treated with mutant Trx1 (Fig.11A). (Fig.11B). n=3; p value <0.05; One-way ANOVA.

3.3 Cell proliferation altered by Trx1 treatment

Once the proteins were validated, it was aimed to investigate the effect of these proteins constructs on NPCs proliferation. NPCs from adult, embryonic brain and spinal cord for cell proliferation were taken for this experiment. Proliferating cells were identified using two approaches: immune detection of Ki67-positive cells and pulse labelling of proliferating cells using BrdU at 2days in the presence of Trx constructs added to the proliferating medium. At the termination of experiment, cells were fixed and used for imaging. To quantify the proliferating cells, a minimum of 10 images randomly was taken and were used for cell quantification using Image J. Total number of Ki67/BrdU positive cells and DAPI-positive nuclei were counted and then the ratio was quantified Ki67 or BrdU positive cells. In these experiments, it was hypothesized that E-Trx and I-Trx have different capacities for regulation of cell proliferation. The effect of commercially available rhTrx on NPCs proliferation was used as positive control on embryonic day 18 brain and spinal cord derived NPCs. As compared to non-treated control, all Trx treatment showed a significant increase in cell proliferation

(Figure 12). E-Trx and commercial rhTrx showed a similar trend of increased proliferation, whereas I-Trx showed a significantly increased cell proliferation compared to commercial rhTrx and E-Trx (Fig. 12A-D).

Fig 12 A) E18 Brain NPC

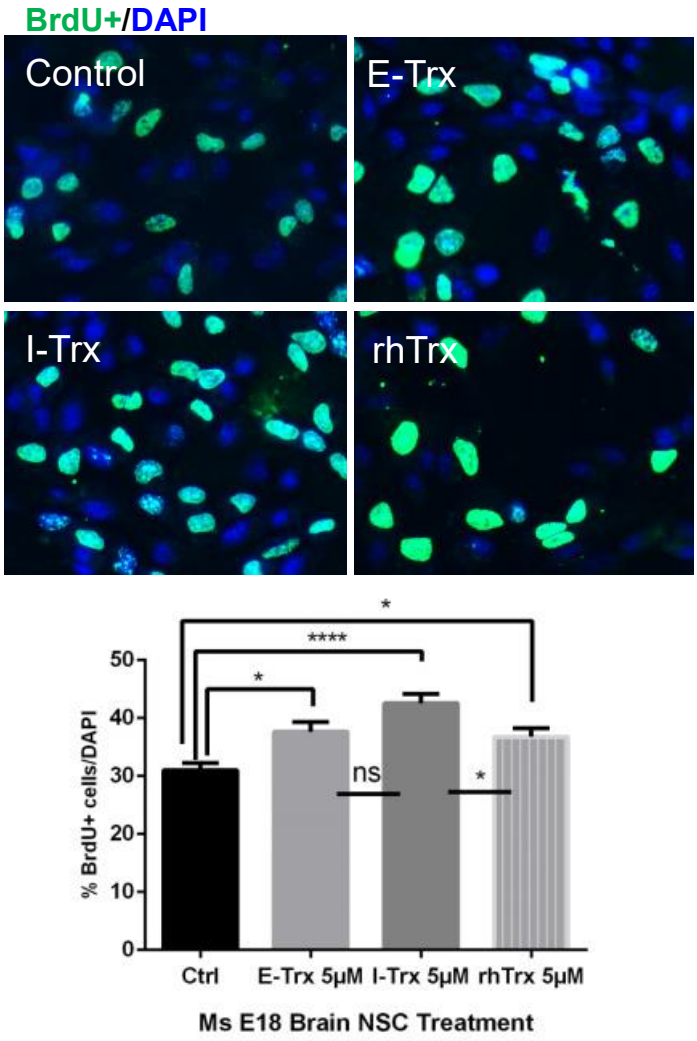
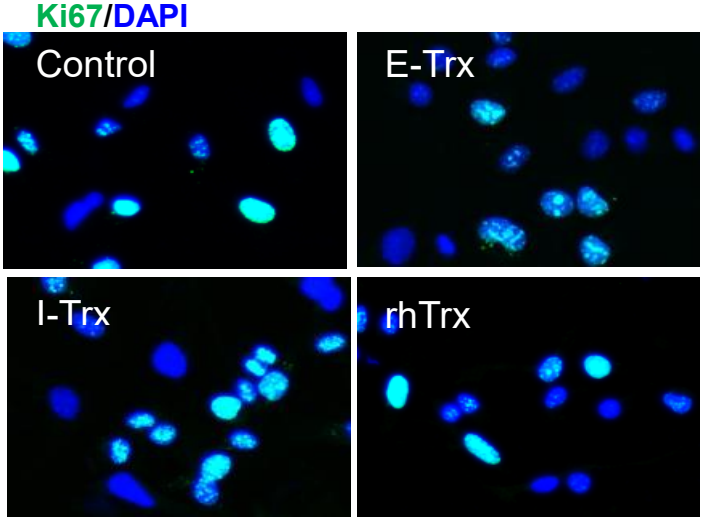


Fig 12 B) E18 Brain NPC



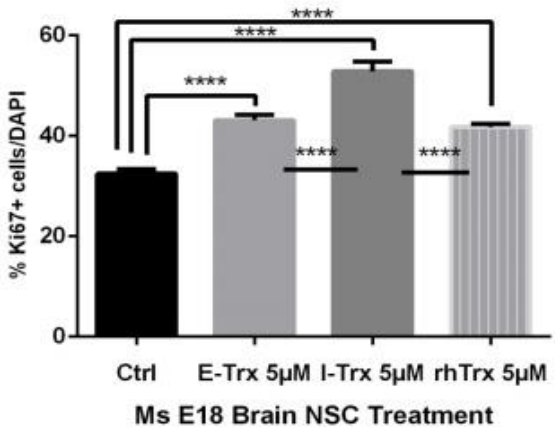
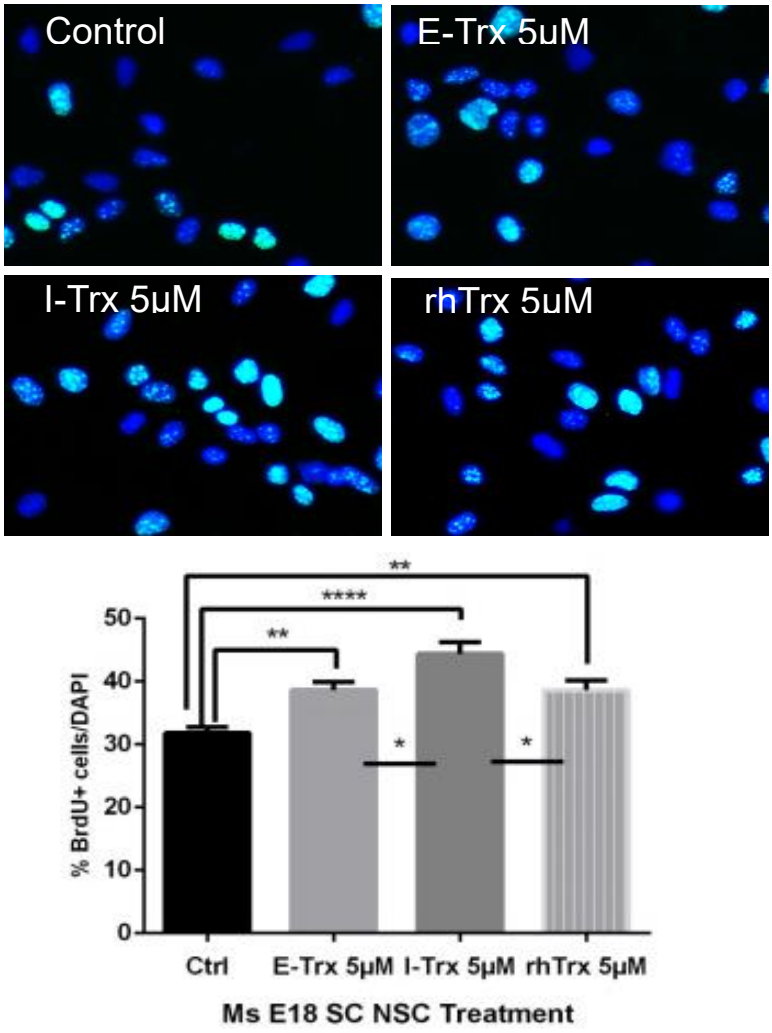


Fig 12C) E18 Spinal cord NPC
BrdU+/DAPI



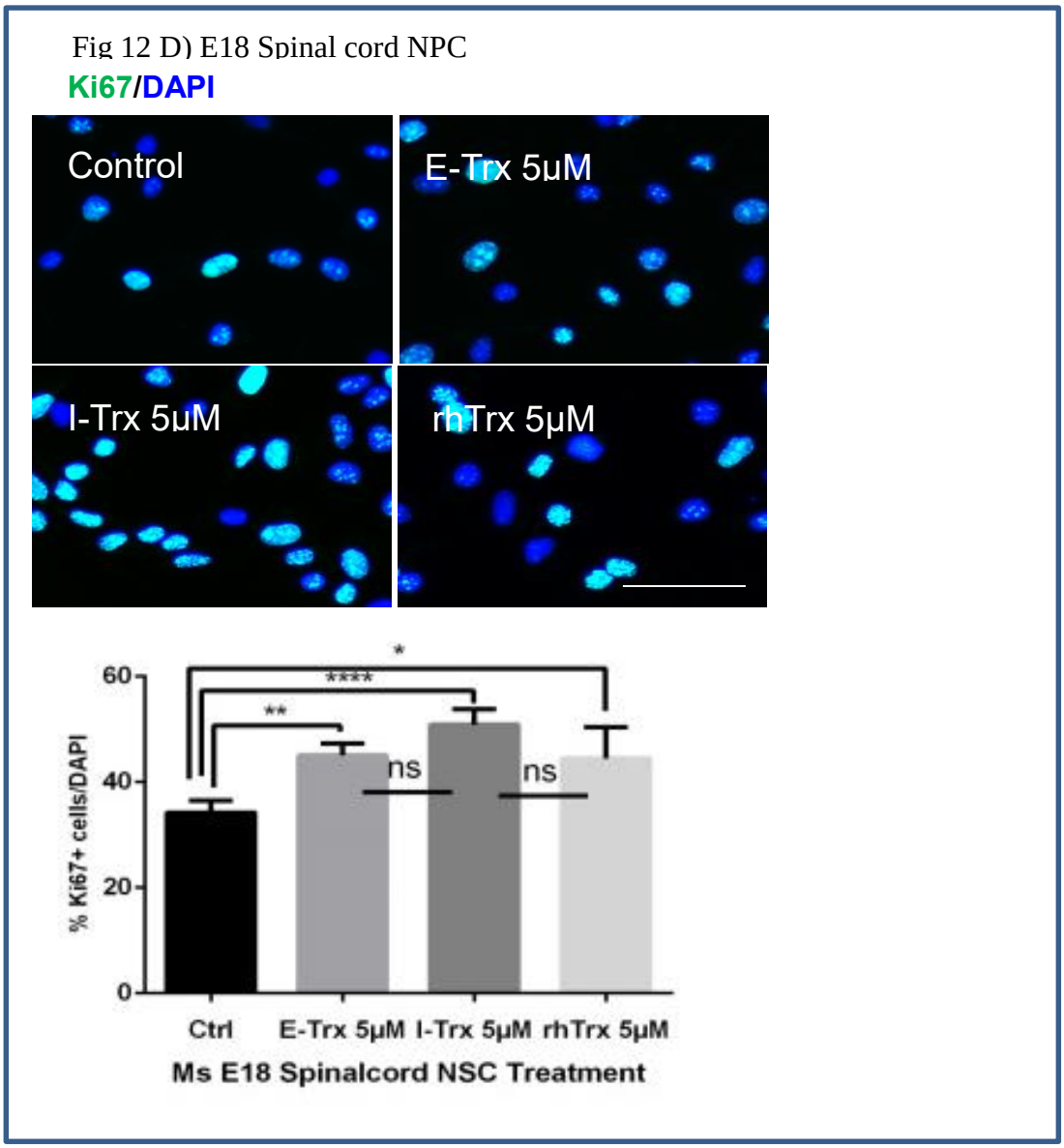
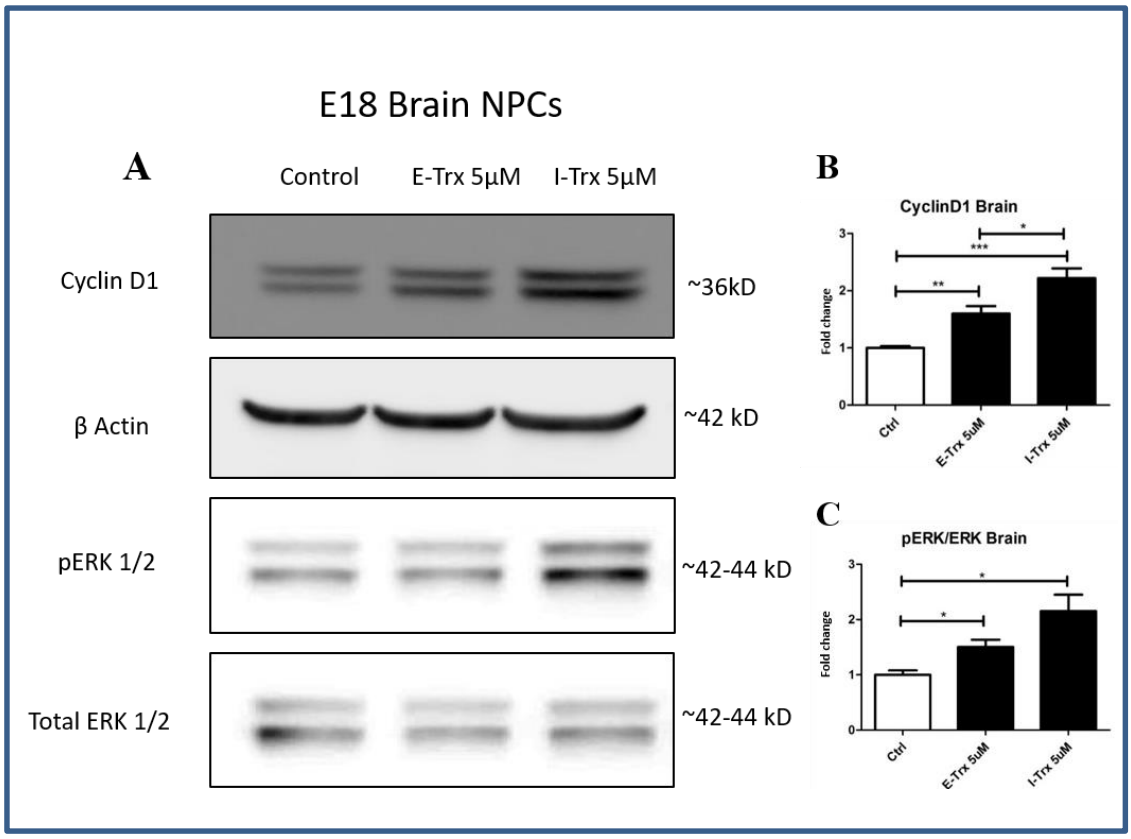


Figure 12: Trx-transduction enhances cell proliferation in E18 Brain NPCs. 12A) Immunofluorescence for BrdU shows an increased proliferation with all Trx protein treatment. E-Trx shows a similar pattern to the commercial rhTrx1. The results show that I-Trx was most efficient in increasing cell proliferation in embryonic NPCs. 12**B)** Ki67 immunostaining confirmed the results with BrdU that I-Trx mitogenic effects are significantly higher than other proteins. **12 C and D)** Similar trend of proliferation was shown for E18 Spinal Cord-derived NPCs (Scale bar=50µm) n=5; p value <0.05; One-way ANOVA.

3.4 Cell proliferation modulated ERK-CyclinD1 pathway

It has been previously shown that upregulation of Trx1 mediates activation of ERK pathway resulting in CyclinD1 activation and cell proliferation (Mochizuki et al., 2009). It was asked whether this signaling mechanism is involved in I-Trx and E-Trx mediated effects. Western blotting was used to assess changes in phosphorylation of this pathway. Phosphorylation of ERK and increased cyclinD1 marks the active cell proliferation. The data confirmed that compared to non-treated control cells, E-Trx and I-Trx treatment increase ERK phosphorylation which is required for increased transcription of CyclinD1 levels in E18 and adult brain-derived NPCs, Fig 13.



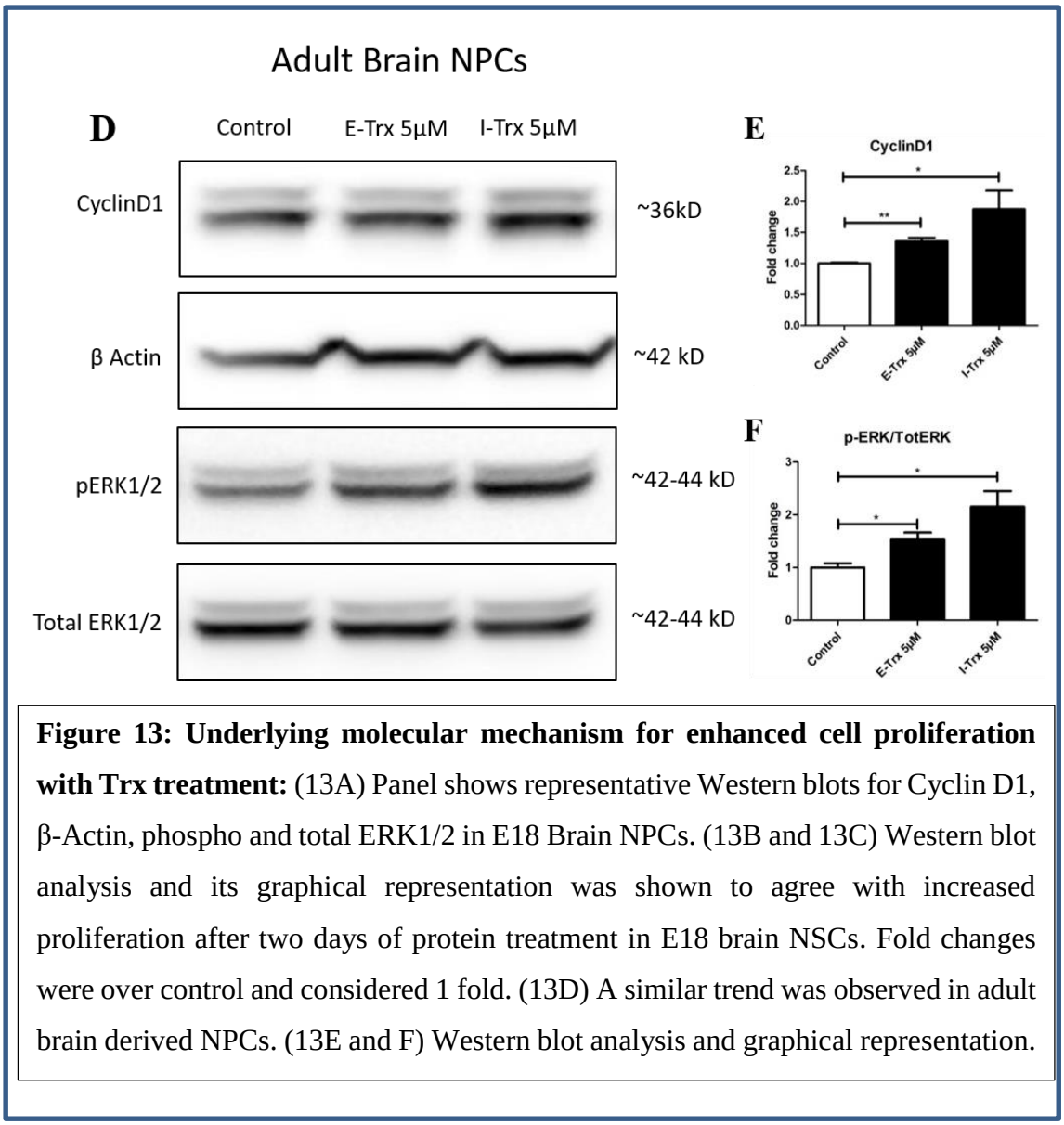


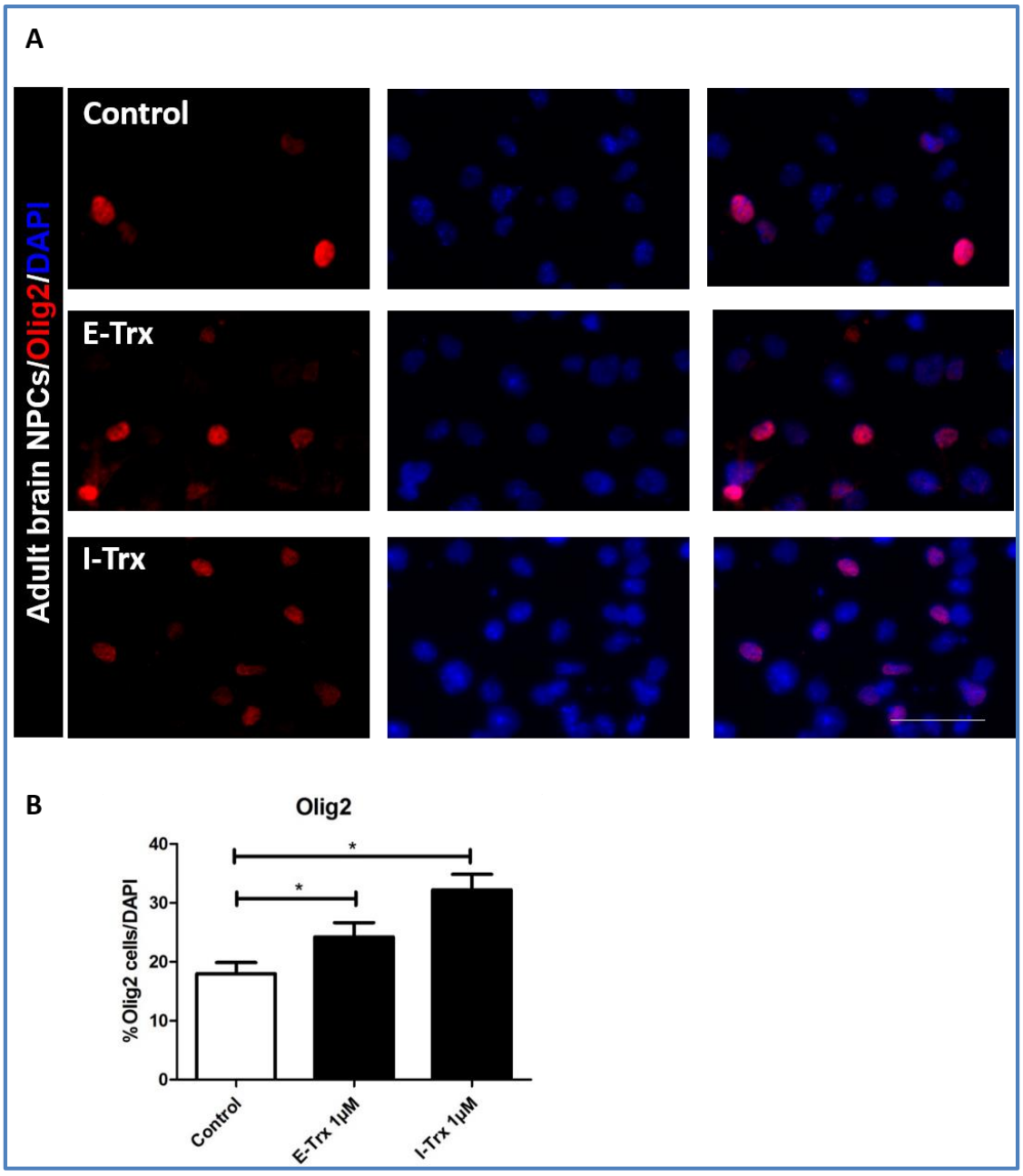
Figure 13: Underlying molecular mechanism for enhanced cell proliferation with Trx treatment: (13A) Panel shows representative Western blots for Cyclin D1, β-Actin, phospho and total ERK1/2 in E18 Brain NPCs. (13B and 13C) Western blot analysis and its graphical representation was shown to agree with increased proliferation after two days of protein treatment in E18 brain NSCs. Fold changes were over control and considered 1 fold. (13D) A similar trend was observed in adult brain derived NPCs. (13E and F) Western blot analysis and graphical representation.

3.5 Role of Trx on NPCs differentiation

Trx1 treatment has been previously shown to increase cell proliferation in NPCs in a model of stroke (Tian et al., 2014). This group showed that Trx1 treatment is associated with neuronal differentiation as shown with increased DCX-positive cells. The question asked was, whether glial differentiation is affected after Trx transduction.

Adult brain NPCs were grown in growth media for 24hrs and then transferred to the glial differentiation media (no growth factors, and 2% FBS). Trx1 constructs were added at this time. After 5-6 days of differentiation, the cells were stained for the astrocyte marker GFAP and oligodendrocyte lineage marker Olig2 (Figure 14A). The fluorescent images were taken using appropriate random sampling (10 random field and optimized exposure) and the ratio of GFAP or Olig2-positive cells was quantified; plotted against total number of cells (DAPI). The results suggest an increase in Olig2 cells in E-Trx which was further increased in I-Trx treated NPCs (Fig. 14A and B). Interestingly, GFAP-positive cells were decreased by treatment with E-Trx and I-Trx. I-Trx treatment cells showed the highest decrease in GFAP positive cells (Fig. 14C and D). Thus it is suggested that Trx treatment increased Oligodendrocytes and decreased astrocytes.

Reducing astrocytes and enhancing oligodendrocytes could be beneficial as reactive astrogliosis is one of a barrier in neurotrauma therapy. This result indicates a potential redox regulatory system for fate determination in NPCs which can be utilized for therapy.


A
Adult brain NPCs/Olig2/DAPI
Control
E-Trx
I-Trx
B
Olig2
% Olig2 cells/DAPI
40
30
20
10
0
*
*
Control
E-Trx 1μM
I-Trx 1μM

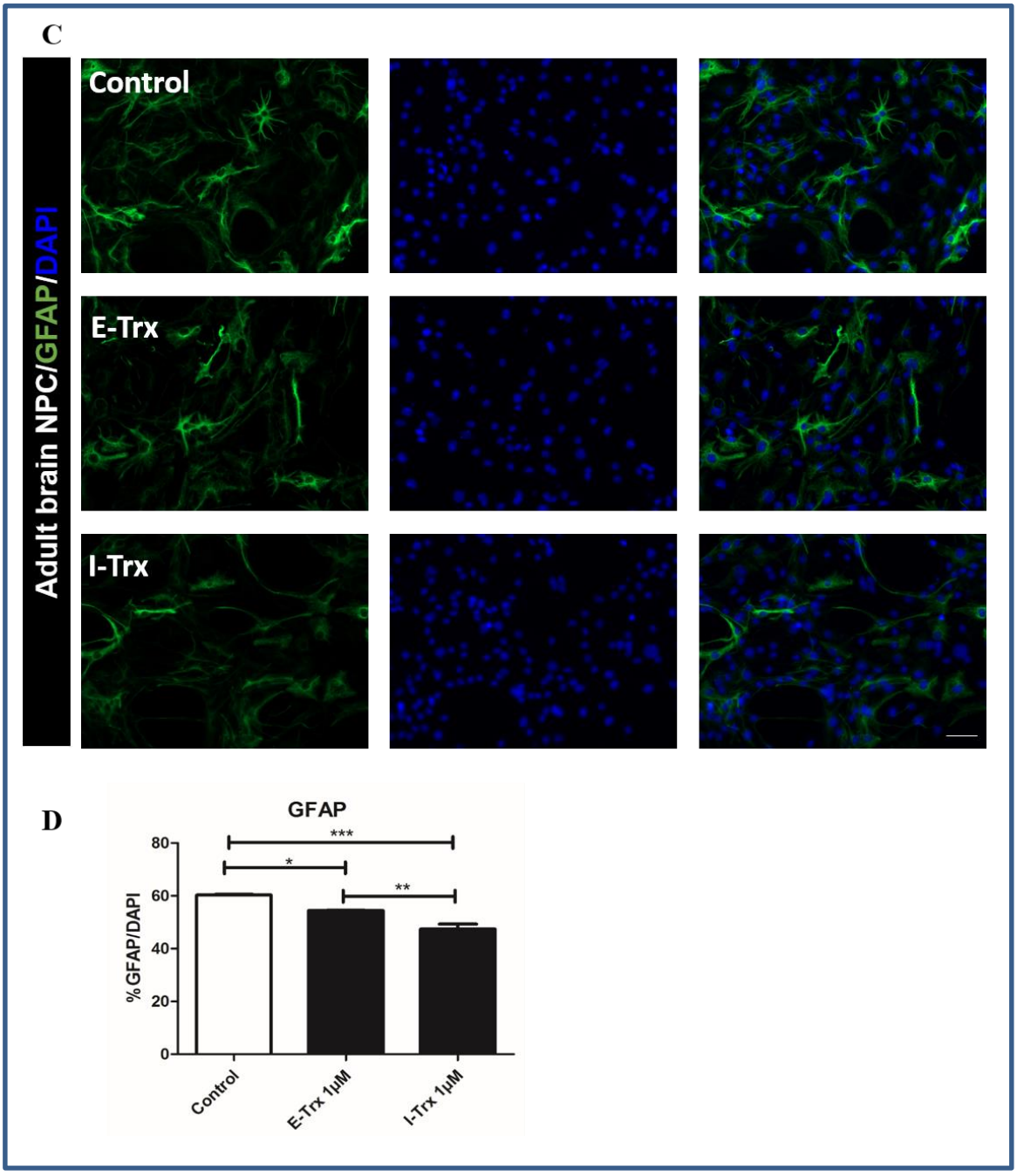

C
Adult brain NPC/GFAP/DAPI
Control
E-Trx
I-Trx
D
GFAP
***
*
**
80
60
40
20
0
% GFAP/DAPI
Control
E-Trx 1μM
I-Trx 1μM

Figure 14: Adult Brain NPCs differentiation after Trx treatment enhanced Oligodendrocyte differentiation and decreased astrocytes: (14A) Representative fluorescent micrographs depicting the effect of Trx transduction on adult NPCs fate-determination. Immunofluorescence of Olig2 (general marker of oligodendroglial lineage) increased with protein treatment. I-Trx shows significantly increased Oligodendrocytes compared to E-Trx. (14B) Graphical representation of Olig2 staining. (14C) GFAP staining showed that astrocyte population decreased with Trx treatment and I-Trx significantly reduced astrocytes compared to E-Trx. (14D) Graphical representation of staining in adult Brain NPCs (Scale bar=50μm). n=3; p value <0.05; Student's T-test.

3.6 Investigation of Trx effects on cell proliferation: the effect of gene delivery.

As an alternative approach to increase cytosolic Trx, we employed Lentivirus-mediated Trx overexpression. Previous work on adipose stem cells has shown that Trx1 and 2 gene delivery increases their proliferation. The question was, whether gene delivery may support the findings with I-trx intracellular delivery. Third generation lentiviral vectors were employed to deliver Trx1 gene into the NPCs. Viral packing vectors were validated using single/double restriction digestion according to the sequence information provided by Dr. Rastegar (Information not provided in this thesis). The digested product was run in a 1% agarose gel to check for the valid restriction digestion (Figure 15A). All the plasmids showed the fragment size match with the sequence information. Similarly, Trx1-wt (redox active wildtype) and the Trx1-mt (mutant construct containing serine) plasmids were validated using Asis1-Mlu1 double digestion Fig 15).

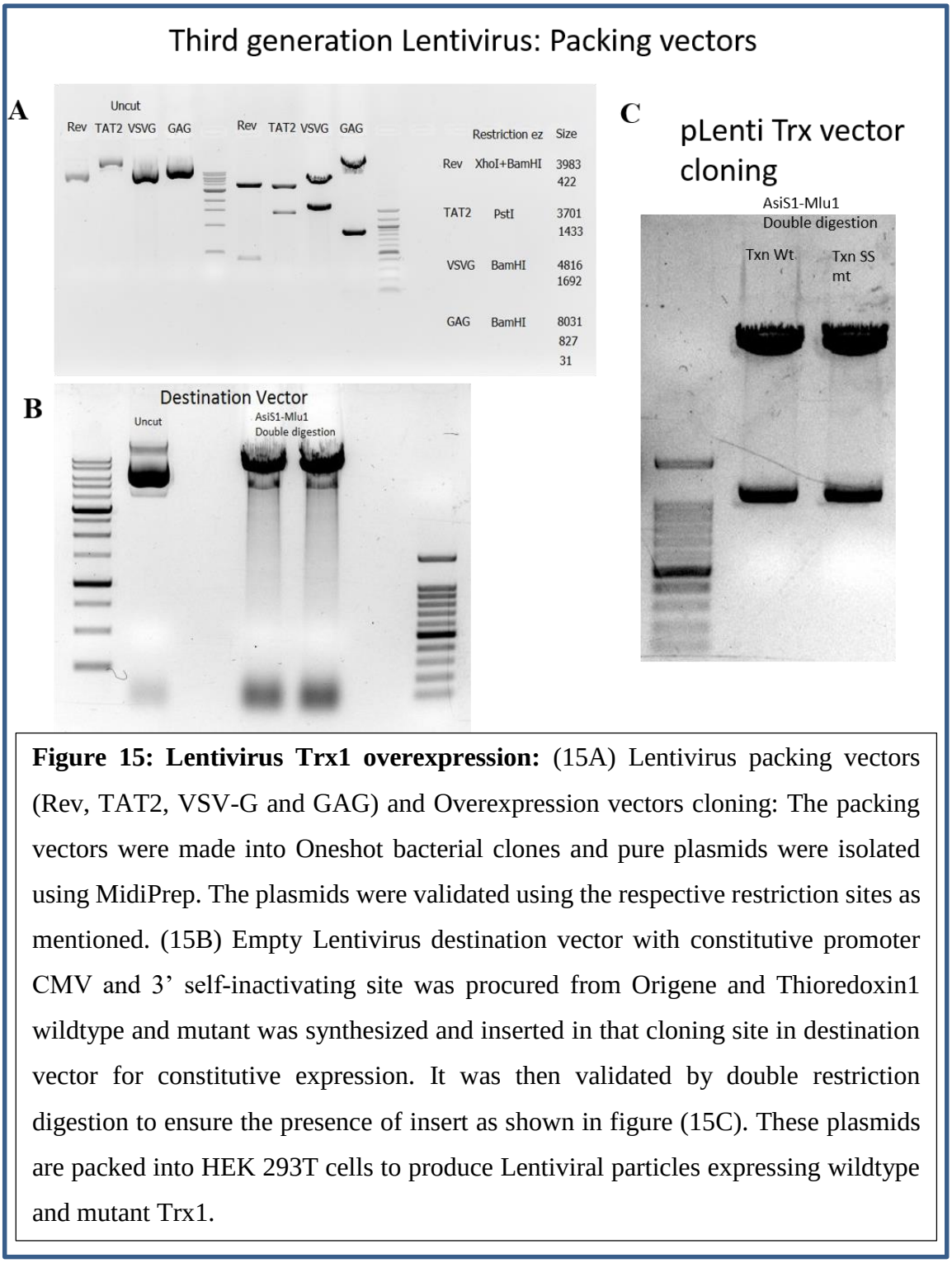


Figure 15: Lentivirus Trx1 overexpression: (15A) Lentivirus packing vectors (Rev, TAT2, VSV-G and GAG) and Overexpression vectors cloning: The packing vectors were made into Oneshot bacterial clones and pure plasmids were isolated using MidiPrep. The plasmids were validated using the respective restriction sites as mentioned. (15B) Empty Lentivirus destination vector with constitutive promoter CMV and 3' self-inactivating site was procured from Origene and Thioredoxin1 wildtype and mutant was synthesized and inserted in that cloning site in destination vector for constitutive expression. It was then validated by double restriction digestion to ensure the presence of insert as shown in figure (15C). These plasmids are packed into HEK 293T cells to produce Lentiviral particles expressing wildtype and mutant Trx1.

3.7 Trx overexpression validation

Two days after virus transduction, the NPCs were stained with anti-DDK antibody to check for the transduction efficiency. Counting DDK^+ cells reveal that >90% cells were detected positive for viral transduction (Fig. 16A). To validate overexpression, Western blot has been employed to detect Trx1 levels in NPCs. Western blot analysis of the cells, which were kept in proliferating condition and differentiated conditions, showed the overexpression of Trx1. Endogenous Trx1 at 12 kDa was unaltered in all three conditions, whereas overexpressed Trx1-DDK band appeared at 17 kDa only in Trx wt and Trx SS mut transduced cells (Fig.16B and C). Biochemical assay such as end-point enzymatic assay was used to detect wildtype and mutant Trx1 activity. The assay results suggested that, albeit overexpression, only Trx wt transduced cell lysate showed threefold increase in Trx activity where Trx SS mut lysate showed equal or lesser activity compared to destination vector transduced cells (Fig 16D).

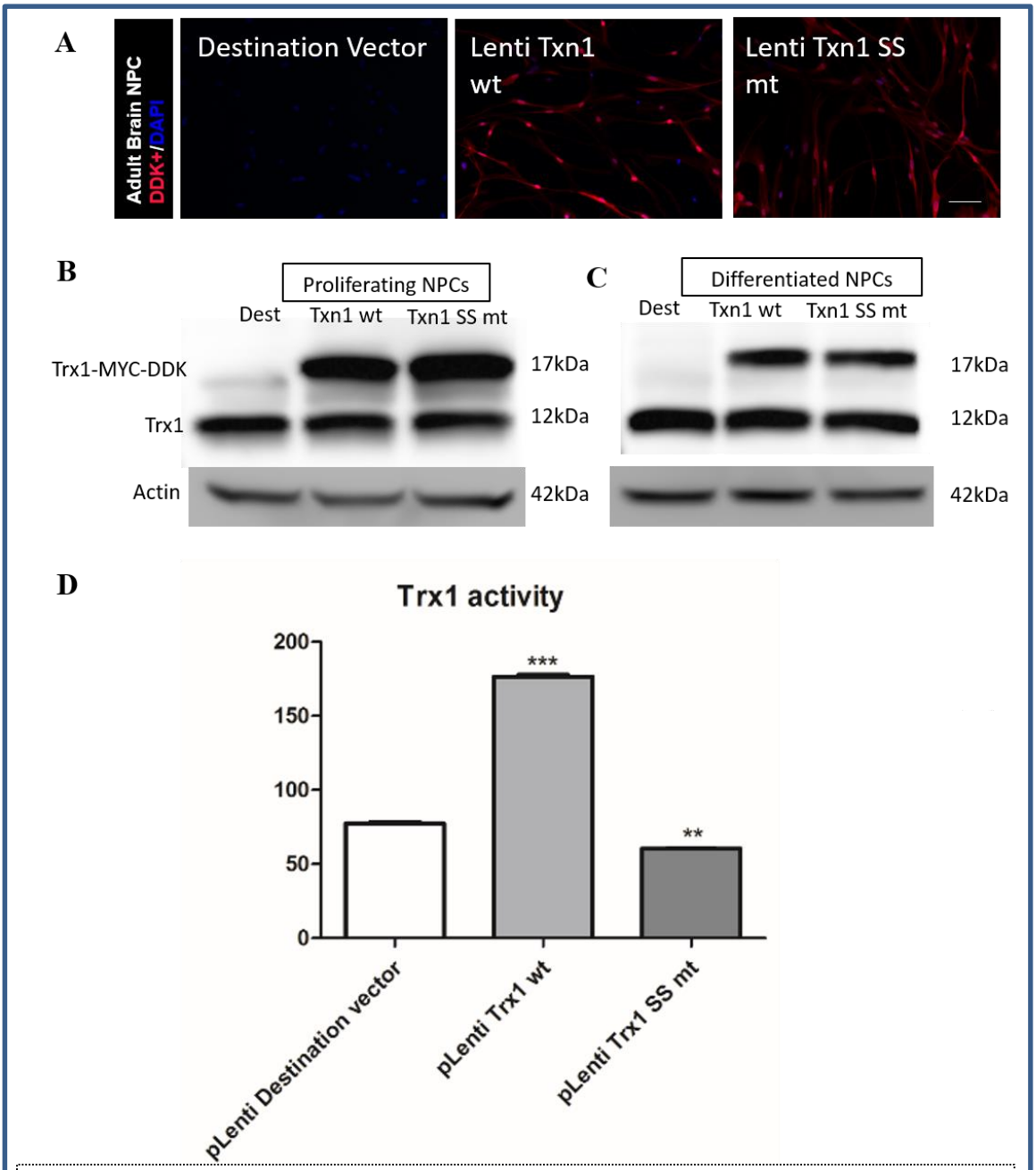


Figure 16: Trx1 Overexpression in adherent cultures of NPCs: 16A) Lentivirus particles containing Trx1 wildtype and SS mutant were produced in HEK cells. After three days transduction in NPCs, Immunofluorescence against DDK tag shows overexpression of Trx1 in both the cases (>90% Transduced) and not in controls (lentivirus with destination-vector infected NPCs). B and C) Similarly, Western blot analysis confirmed the successful expression of DDK-Trx construct which was associated with increased Trx enzymatic activity. n=4; $p<0.05$; One-way ANOVA.

3.8 Effect of Trx1-gene mediated delivery on NPCs proliferation

Once the transduction efficiency was validated, cell proliferation rate was tested after two days of Trx transduction. Ki67 staining was used and the ratio of Ki67-positive nuclei was quantified using a minimum of ten random microscopic fields. No significant differences were detected between the Trx1 (wt) overexpressing NPCs and the control cells (destination vector only); however, the NPCs overexpressing mutant Trx1 (SS) displayed significantly less proliferating cells (Fig.16 A and B). Western blot analysis showed a decrease in CyclinD1 levels although it did not reach statistical significance and Erk activation was equal in all the cases (Fig.18A, B and C).

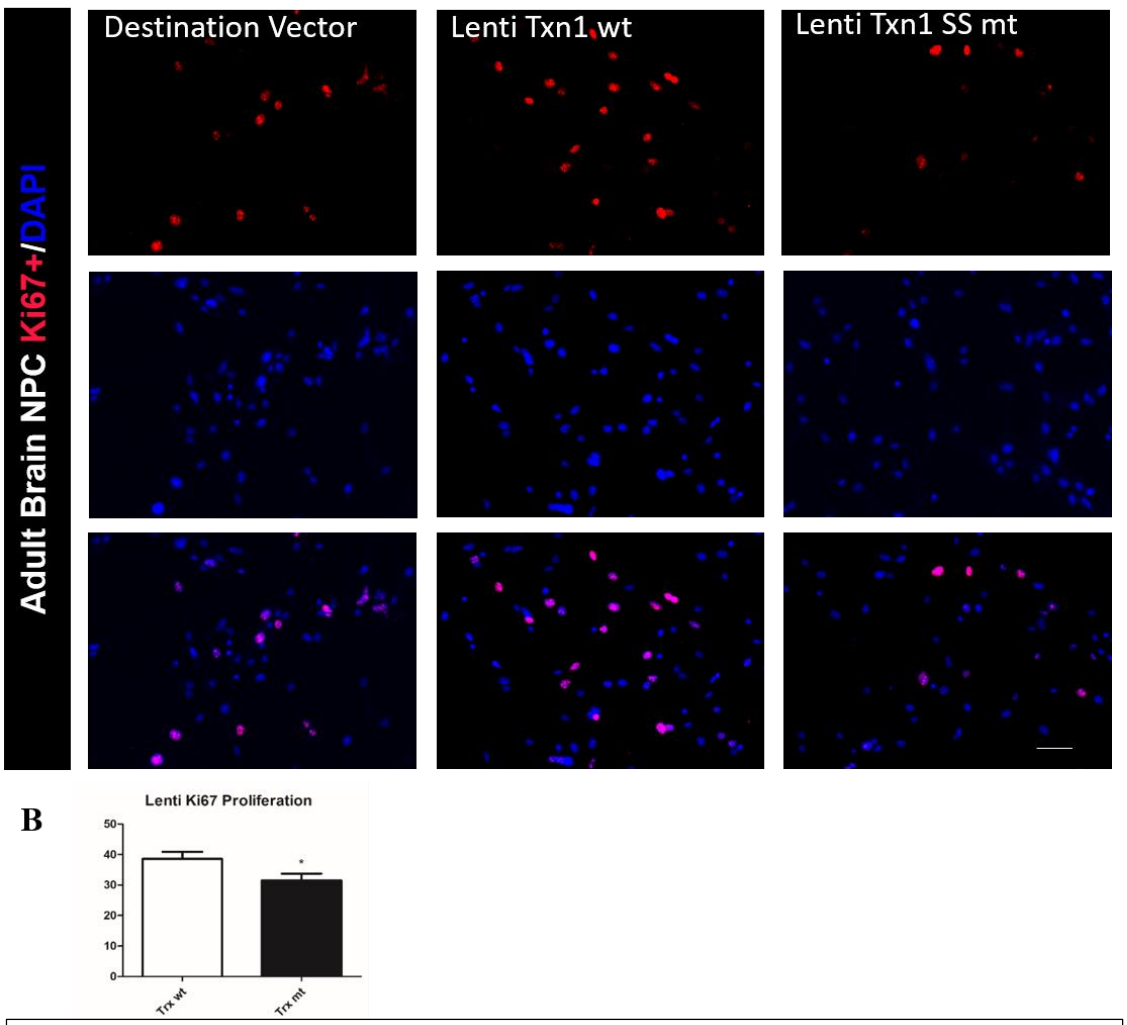


Figure 17: Overexpression of Trx1 did not increase Cell proliferation in adult brain derived NPCs. A) NPCs overexpressing wildtype and Trx1 as well as the control virus were grown in the proliferation medium for three days after virus transduction. Then the cells were fixed with 3% PFA and stained with antibody against Ki67. The results showed that cells overexpressing wildtype Trx1 had more proliferating cells when compare to the cells overexpressing mutant Trx, however no significant difference was observed between the control and Trx1 wildtype cells. The cells overexpressing the mutant protein had significantly lower proliferation rate. (n=4; $P<0.05$; Student's T Test)

3.9 NPC differentiation in Trx1 transduced cells

Trx1 overexpressing NPCs were exposed to six days of glial differentiation to test if Trx1 overexpression has any effect on glial differentiation similar to Trx1 protein treatment. Immunostaining was performed for two major glial lineages; Olig2 for Oligodendrocyte lineage and GFAP as an astrocyte marker. Oligodendrocyte lineage differentiation showed no detectable changes between the control cells (transfected with destination vector) and Trx1 wt overexpressing cells. Interestingly the number of Olig2-positive cells was reduced in Trx1 SS mt transduced cells. To investigate the effect of this manipulation, western blotting has been used for the above mentioned glial markers. However, the analysis did not yield any significant changes between Trx1 wt and Trx1 SS mt (p value 0.2546) Fig. 18 D and F). Although using cell counting, there was no detectable changes between the wildtype and mutant Trx overexpressing cells, quantification of GFAP signal intensity revealed a significant decrease in wt-Trx cells. However, western analysis did not reveal any changes (Fig 18 D and E). Trx1 overexpression was thought to alter glial differentiation pattern, yet there is no conclusive data supported by the hypothesis. This could be explained by not having a suitable control group and too small number of experiments.

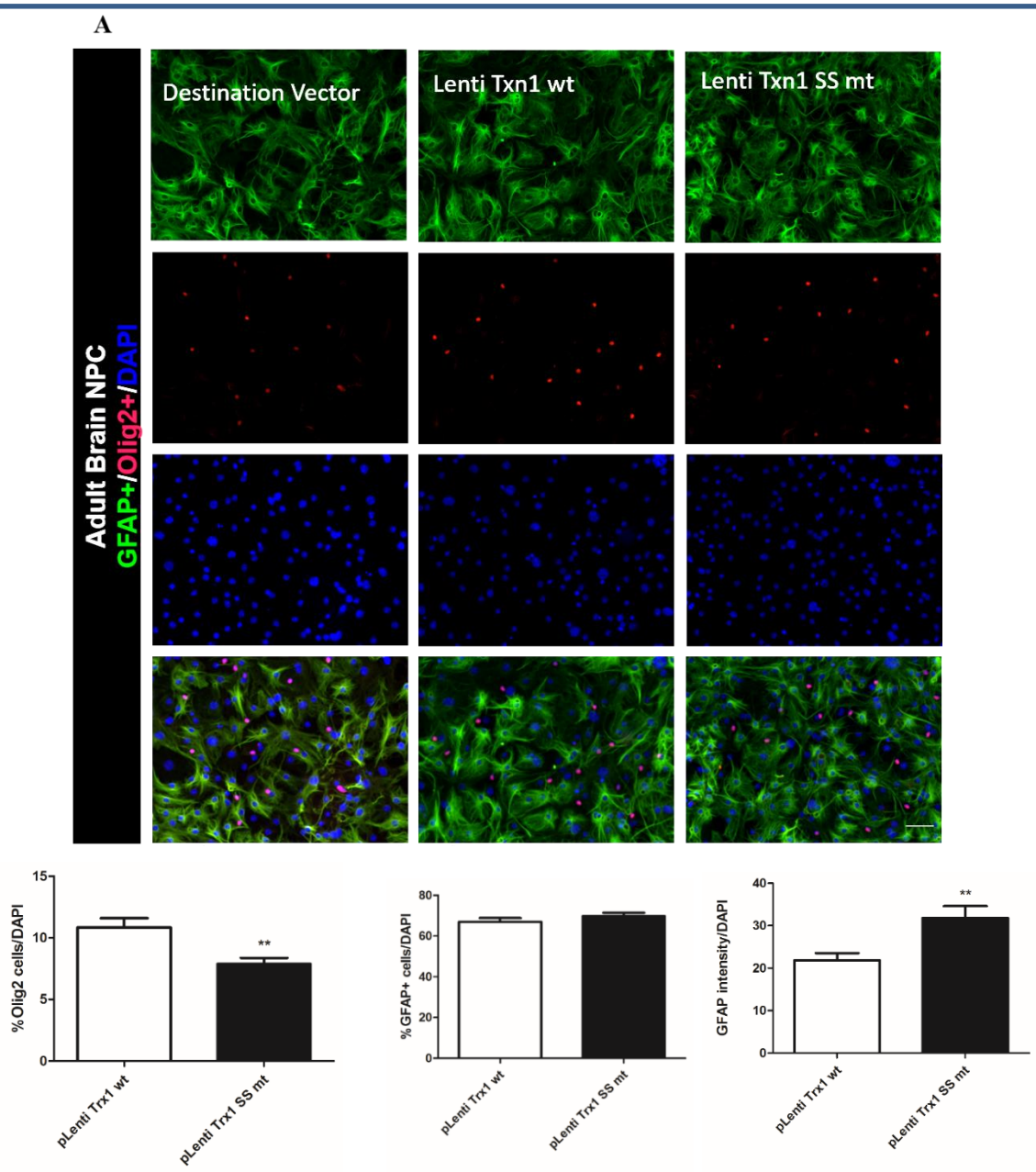
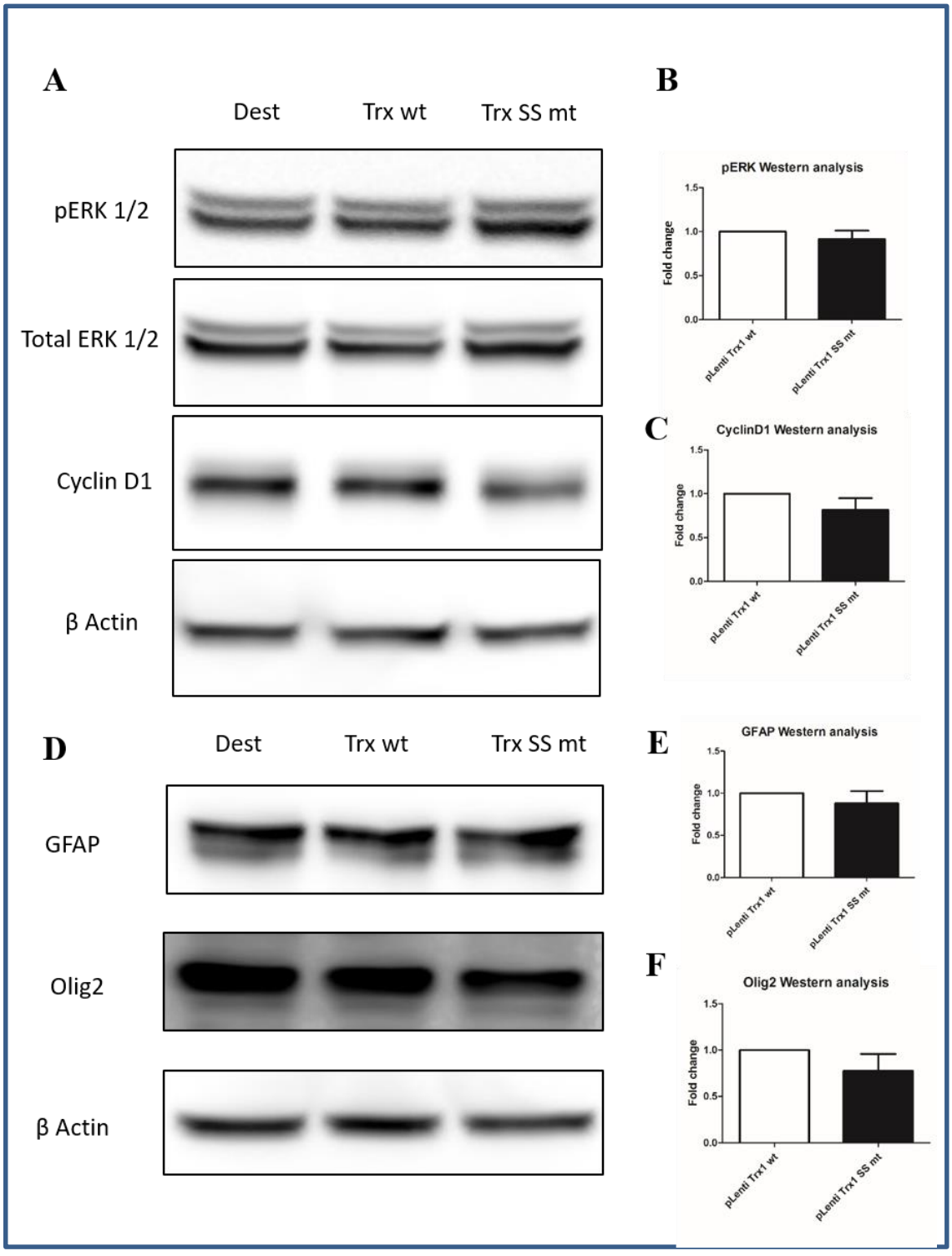


Figure 18: The effect of Trx overexpression on glial differentiation: The NPCs overexpressing wildtype, mutant and controls were maintained for 6 days in differentiation medium. The cells were fixed then stained to detect Olig2 and GFAP positive cells. A). Trx1 wildtype overexpression promotes Oligodendrocyte differentiation. B). No significant changes were detected in GFAP+ cells between Trx wildtype and mutant overexpressing cells, however there was a significant decrease in GFAP intensity in wildtype Trx overexpressing cells. n=4 $p<0.05$; Student's T Test.


A
Dest
Trx wt
Trx SS mt
pERK 1/2
Total ERK 1/2
Cyclin D1
β Actin
B
pERK Western analysis
Fold change
1.5
1.0
0.5
0.0
pLenti Trx1 wt
pLenti Trx1 SS mt
C
CyclinD1 Western analysis
Fold change
1.5
1.0
0.5
0.0
pLenti Trx1 wt
pLenti Trx1 SS mt
D
Dest
Trx wt
Trx SS mt
GFAP
Olig2
β Actin
E
GFAP Western analysis
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1.5
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pLenti Trx1 wt
pLenti Trx1 SS mt
F
Olig2 Western analysis
Fold change
1.5
1.0
0.5
0.0
pLenti Trx1 wt
pLenti Trx1 SS mt

Figure 19: Western blot analysis for Lentivirus overexpression on Cell proliferation and differentiation: A) The effect of Trx overexpression on NPCs signaling was assessed using ERK phophorylation and CyclinD1 levels as mentioned before. B and C) The results were inconclusive for phospho ERK levels. Similarly, there were no significant change in CyclinD1levels. D) Western blotting for markers of glial differentiation of NPC s did not show and significant changes for Olig2 and GFAP levels. (18 E and F) n=3.

Chapter 4: Discussion

Neurotrauma is a debilitating condition and the CNS is poorly understood for its plasticity after an insult. Endogenous stem cells are believed to support new generation of lost tissue; however, activating the quiescent NPCs and guiding them towards optimal differentiation and promotion of functional recovery is a main hurdle for therapeutic applications. With all the challenges, no therapy has been completely efficient to revert CNS damage. Here, I attempt to show that TAT-mediated Trx delivery is a promising approach to increase NPC proliferation, which promotes oligogenesis and decreases astrogliosis. These three main findings are currently the main topics for SCI treatments. The TAT-mediated protein delivery was rapid and enhanced the intracellular cargo content. Here I confirm a viable method for protein delivery to have a swift action when it is required, such as neurotrauma. In this TAT mediated protein delivery, we overcome the issue of thioredoxin1 passively being internalized through endosomes to have Trx action. Protein production using prokaryotic system is largescale and can be efficiently used. The activity of the fusion protein was not affected by the modifications. A potential problem associated with TAT-mediated protein delivery in the in vitro condition is the continuous protein entry into the cell (Figure 10). The huge protein influx could cause a stress to the cells *in vitro* yet optimizing the dosage will be necessary to keep it at the physiological concentrations. For the potential application in neurotrauma treatment such as stroke or spinal cord injury the use of mini-osmatic pump to deliver the protein at a constant rate of delivery would be required.

ROS levels have been debated to have an impact on cell proliferation with two schools of thought. ROS as endogenous source, act as second messenger in relaying cell

signaling but the injury-induced niche also produces lethal levels of ROS which cause cell death. In the first school of thought, ROS levels were reported to have an inverse correlation with cell proliferation. As a second messenger, ROS levels are key in cell proliferation (Le Belle et al., 2011). Increased ROS level after any neurotrauma can deplete the stem cell pool and migrating stem cells die in the injury environment due to hostile environment; which result in poor recovery. We need to understand a fact that, NPC is a general term to define a population of cells with different cell cycle progression. NPCs made of primitive quiescent cells with low cell proliferative capacity and progenitor population which divides very rapidly (Ottone et al., 2014; Homem et al., 2015). Trx1 scavenges ROS indirectly by providing the reducing equivalents for peroxiredoxins, the main H_2O_2 scavengers of the Trx system. Protein reducing capacity can influence the cytosolic ROS levels and reduce crucial proteins involve in cell cycle progression. As the results suggest (fig 11), ROS levels followed the trend as much Trx1 internalized, it could be argued that decreasing intracellular ROS levels could decrease NPC proliferation. But ROS scavenging by Trx1 is through peroxiredoxins and would neither affect free radical formation by NOX nor growth factor signaling. Thus Trx1 functions basically as protein reducing antioxidant and influencing ROS levels indirectly.

Cell proliferation is one amongst many other cell protective roles of Trx1 by acting as antioxidant or by triggering downstream action through redox sensitive protein interaction (Saitoh et al., 1998; Mochizuki et al., 2009; Hwang et al., 2014). Similar to the existing reports, my thesis project also supports the role Trx1 in cell proliferation of NPCs via activation of ERK1/2-CyclinD1 axis. This also can be extrapolated that the

E-Trx and I-Trx constructs in this study are able to interact with its natural substrate ASK1 in a redox manner. Similarly, Trx1 also been associated with proliferation by inhibition of Txnip, a negative regulator of proliferation (Yoshida et al., 2005). The differences between E-Trx and I-Trx in respect to proliferation in our results (Figure 12) come from the cellular internalization of Trx with ERK activation, probably combined with effect of redox balance and protein interaction (Figure 13). Neurotrauma activates endogenous NPCs proliferation, but under the inhibitory niche of the injury site, the survival decreases and thus impairs functional recovery. Trx treated NPCs provide enhanced proliferation and survival which can be utilized for regeneration purposes. This has to be tested by delivering Trx1 in injury site to look for recovery. The potential pitfall of these experiments did not distinguish the difference between extracellular versus intracellular Trx1 action. In T cells, Trx1 acts like an growth factor in yet it is not via EGF receptor (Gasdaska et al., 1995). So far, not much studies focused on extracellular function of Trx1. A good future experiment would involve identifying the receptor for Trx1 and blocking it to see the effect. So far there were no identified receptors found and according to one report, EGF receptors were not responsible for increased proliferation. Further experiments are needed to validate redox-sensitive protein interaction such as ASK1 and Txnip to back up our claim.

Neural precursor cells (NPCs) serve as a renewable source of producing new neurons, astrocytes and oligodendrocytes; thus providing plasticity, repair and altered functions in the central nervous system (CNS). NPC differentiation is a key function in routine maintenance of CNS especially in the case of any neurotrauma. Though NPCs are packed with regenerative capacity, their contribution towards repair after CNS

trauma remains limited due to injury site hostile microenvironment. ROS levels and inflammatory signals divert NPCs more towards reactive astrocytes than oligodendrocytes in CNS injury (Barnabe-Heider et al., 2010; Gauthier et al., 2013). Astrogliosis though confines the injury site initially, it causes a permanent physical barrier, which is less feasible for any intervention for regeneration. ROS levels could alter NPC fate towards astrocytes and a previous report suggests that increased Txnip is also associated with reactive astrogliosis (Perrone et al., 2010). The role of Trx1 in anti-inflammation and the negative regulation of Txnip could be reflected in decreased astrogliosis. In my thesis I show that, I-Trx significantly decreases GFAP positive astrocytes and increases Olig2 positive oligodendrocytes (Figure 14). This suggests that elevated Trx1 is sufficient to keep ROS at low levels and combats Txnip effect, resulting in less astrocyte differentiation. As Olig2 marks both committed oligodendrocyte precursor cells as well as uncommitted NPCs, my experiments did not differentiate any changes between mature and immature oligodendrocyte progeny. Therefore, future experiments must be focused on quantification of precursors and mature oligodendroglial cells. Currently, there is one report available on Trx1 and NPC differentiation that discusses about neurogenesis and astrocyte differentiation (Tian et al., 2014), I have yet to elucidate the mechanism behind increased Olig2+ cells. Using markers for oligodendrocyte terminal differentiation markers, such as CNpase, will give us further information about mature oligodendrocyte population after Trx1 treatment.

Lentivirus-mediated gene delivery provides a continuous expression of Trx1 under the CMV promoter. It has been shown from my data that I was able to overexpress wildtype or mutant Trx1 in NPCs. The lysates showed enhanced Trx activity with

wildtype but not with destination vector or Trx mutant transduced cells (Figure 16). Trx1 overexpression was observed when the cells were proliferating as well as after differentiating NPCs for six days. This shows the continuous source of overexpression. Although I anticipated a difference between destination vector transduced NPCs and Trx1 wt overexpressing NPCs abiding Trx activity, but the results were not supportive. This could be a problem associated with the vector design and lack of proper control. The destination vector which was used as control vector, has MYC-DDK under a constitutive promoter (CMV) yet there was no expression. Destination vector just marks the viral integration to target cells. Continuous overexpression of protein could be stressful for cells. It is possible that destination vector integrated NPCs were less stressed when compared to Trx1 overexpressing cells due to continuous expression. In the literature review, no studies compare between destination vector versus gene overexpression. A previous study using lentiviral transduction for Trx1 used eGFP as a control, where the control plasmid express eGFP (Chou and Sytwu, 2009). Instead of an another gene, I used a mutant Trx1 as a comparable control. Thus I made Trx1 without active site for this study where mutant protein does not show any redox activity. All the comparisons were done using Trx1 wildtype versus Trx1 SS mutant overexpressing NPCs. Comparing Trx wt transduced cells with Trx1 SS mut transduced cells, I observed a lower cell proliferation in mutTrx transfected cells (Figure 17). This shows the overexpression of wildtype Trx1 is responsible for the proliferation not the mutant Trx1. The results are not conclusive as western blot analysis did not show a significant data on proliferation changes but it showed a trend of mutant Trx1 overexpression decreased CyclinD1 levels (Figure 19). It is possible that, two methods

of enhancing Trx1 used in this study has its own differences. Still more experiments with proper control are needed for a conclusive result.

Lentivirus overexpression did not alter much of NPC differentiation (Figure 18). ICC staining results show no differences in astrocyte marker GFAP but decreased Olig2 positive Oligodendrocytes. Western blot for Olig2 and GFAP did not show any significant trend as shown in image analysis. The overexpression has been validated, and tested for differentiation. No significant change has been observed due to small sample size with the experiment. This emphasizes the need for more experiments to increase the number of samples for statistical analysis.

Chapter 5: Conclusion

In summary, this is a mechanistic study to show an accessible method of largescale recombinant thioredoxin 1 production using prokaryotic system. Trx1 with cell penetrating peptide has shown to rapidly and efficiently o enter the NPCs. I also show that ROS levels have been altered by Trx1 significantly where I-Trx exhibits a superior effect compared to E-Trx. Mutant proteins did not have any significant effect on ROS levels. I-Trx shows a better protective role in NPC cell proliferation and differentiation that is significantly higher than E-Trx and commercial recombinant-human Trx1. I also validated lentiviral mediated Trx1 overexpression method to replicate previous reports on Trx1 effect on cell proliferation that could be utilized for neurotrauma. Yet further studies are required to be conducted to reveal the molecular mechanisms of TAT-mediated Trx1 delivery, which could provide a viable therapeutic approach for stroke and spinal cord injury.

5.1 Limitations and Future directions

Thioredoxin 1 as shown from multiple groups, has proven to be cell protective in multiple aspects; yet it is poorly understood as a therapy for neurotrauma. This present study provides two viable methods of protein delivery into the cells. Here I attempt to examine NPC proliferation and differentiation after Trx treatment under normal condition. Protein treatment using TAT delivery method provides us an instantaneous protein delivery but it is limited by protein degradation. As literature suggests, TAT delivery enters endosomes and a portion escapes to cytosol (Erazo-Oliveras et al., 2014). I show that TAT delivery provides improved protein entry compared to passive internalization. Future experiments should also focus on protective capacity offered by I-Trx delivery by exposing the cells to oxidative stress conditions such as H_2O_2 treatment. Additionally, the downstream molecular events that may be responsible for Trx1 effects such as its interaction with Txnip, Ask-1 and other targets remain to be tested for I-Trx effects using immunoprecipitation or protein mass spectrometry. Although my thesis was specifically focused on cultures of NPCs to identify the downstream signaling system for Trx effect, future studies should also examine its potential effects in an in vivo model for normal and neurotrauma models. Continuous delivery of I-Trx1 may hold the key to fully understand the potential therapeutic effects. To compare the efficacy of protein transduction vs. gene delivery future experiments should focus on using appropriate lentiviral constructs to fully capture the capacity and therapeutic value of I-Trx delivery system.

Although TAT delivery system is widely used for protein delivery in preclinical systems, a major flaw for this system is the lack of target specificity; as the cargo will

be delivered into all the cells and organs. Therefore, a more selective cell penetrating peptide must be used for future studies. Similarly, for targeted gene therapy, one must design Trx1 overexpressing lentivirus particles under cell type specific promoter to target endogenous stem cells. Injecting virus in post injury spinal cord will lead to overexpression of Trx1 in one particular cell type. For example, injecting lentivirus particles in spinal cord containing Trx1 expression under olig2 promoter will exclusively overexpress Trx1 in oligodendroglial progeny and stimulate their proliferation as well as selectively increase their survival in the injured tissue. These cells may contribute more efficiently towards repair of damaged spinal cord axons.

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