Karaciğer yenilenmesinde demir-sülfür (Fe-S) küme proteinlerinin moleküler etkileşiminin anlaşılması

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by

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APPROVAL OF THE THESIS

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ETHICAL STATEMENT

I hereby declare that this thesis study titled ‘‘Understanding the molecular interactions of Fe-S cluster proteins in liver regeneration’’ has been prepared in accordance with the thesis writing rules of Eskisehir Osmangazi University Graduate School of Natural and Applied Sciences under academic consultancy of my supervisor (**Prof. Dr. Mediha CANBEK**) and second supervisor (**Dr. Ayşe Özmen Yaylacı**). I hereby declare that the work presented in this thesis is original. I also declare that, I have respected scientific ethical principles and rules in all stages of my thesis study, all information and data presented in this thesis have been obtained within the scope of scientific and academic ethical principles and rules, all materials used in this thesis which are not original to this work have been fully cited and referenced, and all knowledge, documents and results have been presented in accordance with scientific ethical principles and rules.

I further wish to declare that the frozen liver tissues and sera used in this study was provided by Prof. Dr. Mediha Canbek and Dr. Ayşe Özmen Yaylacı, from their previous project titled ‘‘The role of ubiquitin signalling pathway on liver regeneration in rats’’ with ethical clearance number (549/2016). And that permission to use the frozen tissues was granted by Eskisehir Osmangazi University (ESOGU) animal experiments local ethics committee (HADYEK) ethical approval certificate numbered 823/2021 dated 17.02.2021 (**appendix A**). 05/04/2023

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ÖZET

Karaciğer rejenerasyonunda moleküler düzeyde birçok karmaşık hücresel olay meydana gelmektedir (Chen ve ark. 2010). Karaciğer rejenerasyonu olaylarının zinciri yüksek derecede enerjiye bağımlıdır. Ana parankimal hücre olan hepatosit genellikle yüksek metabolik gereksinimlere sahiptir ve rejenerasyon hücrenin enerji durumundan etkilenir (Fausto ve ark. 2006). Mitokondri, hücrenin ana biyoenerjetik ve metabolik merkezi olarak apoptoz gibi hücre içi sinyalleşmesinde kilit oyuncu ve organın majör rezeksiyona tepkisinde temel rollere sahiptir. Son zamanlar da karaciğer ve doku rejenerasyonunda düzensiz biyoenerjetik olaylar, metabolik yeniden yapılanma ve onların ilişkili olduğu hücre içi sinyalleri ile dokunun yenilenmesi sırasında koordineli bir metabolizma-hücre bölünmesi düzenlemesi ile ilgili hücresel temellerinin araştırılmasına yönelik çalışmalar yeniden ateşlenmiştir. Rejenerasyon yaralanma sonrası doku iyileşmesinin önemli bir unsurudur (Locasale ve Cantley, 2011).

Anormal hücre bölünmesi davranışları sergileyen tümör-kaynaklı hücre hatları gibi patolojik anlamda çoğalan hücrelerin metabolik gereksinimlerinin anlaşılmasında büyük ilerlemeler kaydedilmiş olsa da (Vander Heiden et. al., 2009), doğuştan gelen doku rejenerasyonunun fizyolojisi hakkında çok az şey bilinmektedir. Memelilerde, özellikle karaciğer gibi sürekli olarak hücresel hasara maruz kalan dokularda doku homeostazı için rejenerasyon çok önemlidir (Fausto et. al., 2006).

Mitokondriyal biyogenez ve dinamiklerinin bu olaylarının merkezinde yer aldığı bilinmektedir. Mitokondriyal oksidatif fosforilasyon karaciğer rejeneratif sürecini besler ve karaciğerin enerji durumunun posthepatektomi karaciğer fonksiyonu ve klinik sonuç ile ilişkili olduğu bilinmektedir (Ozawa et. al., 1982; Satoh et. al., 1996; Mann et. al., 2002; Alexandrino et al., 2016). Çok sayıda çalışma, hepatektomiden sonraki ilk saatlerde kalan karaciğerde artan mitokondriyal DNA ve RNA'nın yanı sıra ETC enzimi sitokrom oksidaz (COX) gibi enerji metabolizmasında yer alan birkaç enzimin ekspresyonunun arttığını bildirmiştir (Koyama et. al., 1998; Sun et. al., 2007). Bu hepatektomiden sonraki ilk iki ile dört gün içinde karaciğerin enerji üretme kapasitesinde genel bir artışla sonuçlanır (Nagino et. al., 1989). Hücrelerdeki mitokondriyal kalite ve miktar, yeni organellerin oluşumu (biyogenez) ve yıkımının (mitofaji) dengesinden etkilenir (Stotland and Gotlieb, 2015).

Mitokondrinin biyogenezin en temel işlevselliği demir-sülfür (Fe-S) biyogenezdir. Demir-sülfür (Fe-S) küme biyogenezi; mitokondriyal biyogenezi ve mitokondriyel solunum zincirinin I, II ve III kompleksleri (mitokondriyel oksidatif fosforilasyon (OXPHOS) sistemi ve elektron taşıma zinciri) ile bağlantılı sistemlerdir. Ayrıca mitokondriyel biyogenez, kalite kontrol ve protein taşınmasının kontrolünde islevi görülmektedirler. Apoproteinlere da bir ko-faktörüdür. Krebs siklüsünde yer alan akonitaz ve süksinat dehidrojenaz enzimleri DNA polimeraz, DNA primaz ve birçok tamir proteinleriyle ilişkilidir. Bu temel mitokondrinin işlevi, DNA stabilitesi ile doğrudan bağlantılıdır. Aynı şekilde bu enzimler hücre siklüsü, hücre kontrol noktaları, siklinler ve siklin-bağımlı kinazlar (CDKs) ile ilişkilidir. Bu nedenle böyle kilit bir nokta da yer alan Fe-S proteinlerindeki mutasyonlarda nice biyolojik mekanizmalarda bozulmalara neden olabilmektedir. Fe'nin toksisite veya demire-bağlı DNA hasarı riski olmasına rağmen, hücrenin Fe-S küme'sini tercih etmesi bu konuyu daha da çok önemli hale getirmektedir (Fuss et. al., 2015). Ayrıca, NEET ailesinden hücre sinyalleşme, demir ve ROS metabolizmasında önemli rolü oynayan mitoNEET ve otofaji, apoptoz ve kalsiyum işlevlerinde rol alan MINER1, hücre proliferasyonunda temel rolü oynadığı bilmektedir. PHx hemen sonrasında, ALR-MitoNEET çapraz iletişimle IL-6, IL-6 tarafından STAT3 fosforilanması ve Kupffer hücreler tarafından da indüklenmiş nitrik oksit sentaz oluşturması sağlayarak JAK-STAT3 sinyal yolağı aktive ettiği bilmektedir (Gupta ve Venugopal, 2018; Ibrahim et. al., 2018). Yine ALR tarafından Ca^{2+} seviyeleri düşülerek endoplazmik retikulum stres azalttığı da geçmektedir (Nalesnik et. al., 2017). Ca^{2+} homeostazı ile yakın ilgilenen MINER1, tahminen ALR ile yakın bir temas olabileceği düşünülebilir. Sonuçta, ALR-mitoNEET-MINER1 metabolik genler olarak bir yana, demir, ROS ve O_2 metabolızması önemli oyunculardır.

Tüm hücresel temel süreçlerde Fe-S proteinlerinin olması ne kadar çok yönlü olduğunun bir kanıtıdır. Bozulmuş Fe-S biyosentezinden kaynaklayan bir mitokondriyel yetmezlik karaciğer, merkezi sinir sistemi, iskelet kası ve kalp kası gibi yüksek enerjiye bağımlı organlarda ciddi sonuçlara sebep olabilir. Ayrıca oksidatif fosforilasyonla ilgili kusurların klinik fenotiplerini de gösterebilir. Fe-S biyosentezi ve hücresel demir homeostazı arasındaki karmaşık ilişkiye bakıldığında, bozulmuş Fe-S, demir birikimine yol açabilir (Vanlander ve Van Coster, 2018).

Bu düşünce ile, PHx sonrasındaki, bazi temel hücre işlevi bilinen 6 Fe-S'li geni (MitoNEET, ACO1/IRP1, ACO2, SDH, MINER1, GLRX5), mitokondri biyogenezin ile

ilgili olan PGC-1α geni, IRP1 ile ilişkili olan HIF-2α ve mitoferrin genleri, ve hücre metabolizması ve proliferasyonu ile bilinen C-MYC geni ile karaciğer rejenerasyonunun çeşitli aşamalarında, özellikle erken hipoksik fazında yer alan genler arasındaki moleküler etkileşimleri araştırılmıştır. Amacımız, çeşitli zaman noktalarında (0, 6, 12, 24, 48, 72) karaciğerin rejeneratif sürecinde parsiyel hepatektomi sonrası mitokondriyal biyogenez ve biyoenerjinin bir gereği olarak Fe-S biyogenezinde yer alan genlerin moleküler etkileşimini ortaya koymaktır. Gen ekspresyonun ölçülmesi için RT-QPCR, proteinlerin miktarı ise western blot kullanılarak genlerinin ve proteinlerinin ifadesi bakılmıştır. Ayrıca, suksinat reseptörü olan (SUCNR1/GPR91) ölçülerek süksinat enzimi da bakıldı. PCNA immunohistokimyası yapılarak hepatocyte proliferasyonu da bakıldı

Çalışmamızda gen ifadesi yüksek olan ve istatistik açıdan anlamlı ($p \leq 0.05$) Fe-S genleri sırasıyla mitoNEET****; ACO1***; mitoferrin***; Miner1*; SDHA* ve bu genlerle ilişkili olan HIF-2α**** ve C-MYC**** olarak anlamlı bulunmuştur. PH gruplarında ise gen ifadelerinde istatistiksel olarak mitoferrin hariç daha anlamlı olduğu tespit edilmiştir. Protein ifadesi Western Blotting çalışması için gen ifadesi yüksek olan ve istatistiksel açıdan anlamlı ($p \leq 0.05$) Fe-S genleri olan DMT-1, mitoNEET, IRP 1 (ACO1) ve HIF-2α incelenmiştir. Karaciğer rejenerasyonun erken ve ara fazlarında etkili olabileceklerini, rejenerasyonun başlatmasında da gerektiren metabolik değişikleriyle beraber gelen sinyalleşmesinde ALR ile tetikleyen mitoNEET hem ROS hem de demir regüle ederek IRP1 ve HIF-2α ile ince bir moleküler etkileşimde olduklarında düşünmekteyiz. Bu moleküler etkileşim ile da ALR-mitoNEET tarafından JAK-STAT3, ALR-mitoNEET-IRP1-HIF-2α ile MAPK-ERK sinyalleşme tetikleyerek karaciğer yenilenmesinde moleküler rolü oynadıkları inanmaktayız. Ayrıca, CMYC ve SDHA'nin zıt çalışması rejenerasyonun başlatılmasında ve MINER1 ise Ca^{2+} sinyalleşme ile proliferayonunda önemli rolü oynadığını söyleyebiliriz. Bu çalışma ile Fe-S proteinin karaciğer rejenerasyonundaki rolü önemli ve karışık bir ilişki içerisinde temel fonksiyon olarak görev aldığı ortaya konmuştur.

Anahtar Kelimeler: karaciğer rejenerasyon, mitokondriyal dinamikler, demir-sülfür (Fe-S) küme, RT-PCR, western blotting, moleküler etkileşimler

SUMMARY

Liver-related diseases sometimes requiring transplant or not, continue to present a major burden to global health. Mitochondrial dynamics is central to the cellular bioenergetics of liver regeneration, a major aspect of liver transplantation. Iron-Sulphur (Fe-S) cluster biogenesis, as an essentiality of mitochondrial (biogenesis) dynamics, may be involved in regulation of several diverse cellular processes, key to hepatocellular proliferation. Fe-S cluster-containing genes such as (mitoNEET, MINER1, IRP1, ACO2, SDH, GLRX5) are known to play critical cellular functions. However, molecular basis of the cellular functions of Fe-S proteins and their interactions with partner genes (PGC-1α, MFRN, HIF-2α and c-MYC) remain to be explored in the context of liver regeneration.

After a 70% partial hepatectomy, some selected genes of interest were examined for their gene and protein activity using RT-PCR and Western Blotting assays. Succinate receptor activity and hepatocyte proliferation rates were determined with ELISA and PCNA immunohistochemistry tests respectively.

In our study, gene activities of mitoNEET, IRP1, MINER1 and SDHA as Fe-S genes and partner genes: mitoferrin, HIF-2α and c-MYC, were found significantly expressed ($p \leq 0.05$) in the PH groups, except for mitoferrin. Significant mRNA expressions ($p \leq 0.05$) of DMT-1, mitoNEET, IRP1 and HIF-2α, was also seen in the PH groups. PGC-1α and MINER1 were also significantly expressed intra-PH ($p \leq 0.05$). Expressional patterns of Fe-S gene relative to their molecular interactions with partner genes, seem to define their cellular functions. Such a gene interaction network of molecular interactions involving Fe-S genes with partner genes, may promote hepatocyte proliferation via metabolic rewiring of cellular bioenergetics via regulation of iron, oxygen and ROS metabolism coupled to the major molecular events of hepatocyte hypertrophy-hyperplasia dynamics in the early-intermediate phases of liver regeneration. Fe-S protein biogenesis and degradation is delicately balanced to guide the molecular basis of their cellular function.

Keywords: Liver Regeneration, Mitochondrial dynamics, Iron-Sulphur (Fe-S) cluster, RT-PCR, Western Blotting, molecular Interaction

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LIST OF SYMBOLS VE ABBREVIATIONS

Symbols	Descriptions
Kg	Kilogram
KD	Kilo Dalton
OD	Optical Density
µg	Micrograms
hr	Hour
µl	Microlitres
M	Molars
mM	Millimolars
ml	Millilitres
ng	Nanograms
X	Multiple
%	Percentage
°C	Degrees Celsius
Fe	Iron
Ca	Calcium
S	Sulphur
ΔCt	Change in Threshold Cycles

Abbreviations	Descriptions
ACO1/IRP1	Cytosolic Aconitase/Iron Regulatory Protein 1
AKT	Protein kinase B
ALR	Augmenter of Liver Regeneration
ALT	Alanine Transaminase
AMPK	AMP-activated Protein Kinase

LIST OF SYMBOLS VE ABBREVIATIONS (Continued)

Abbreviations	Descriptions
ATP	Adenosine Triphosphate
BECs	Biliary Epithelial cell
bHLH/LZ	Basic helix-loop-helix/leucine zipper
CAPN2	Calpain-2 Catalytic subunit
CISD	CDGSH Iron–Sulphur Domain
C-MYC	Cellular Myelocytomatosis oncogene
CoQ	Coenzyme Q
CREB	cAMP response element-binding protein
DNA	Deoxyribonucleic Acid
Drp1	Dynamin-related protein 1
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
EPO	Erythropoietin
ETC	Electron Transport Chain
ETF	Electron-transferring flavoprotein
ETF: QO	ETF: Ubiquinone Oxidoreductase
FNR	Fumarate Nitrate Reductase Regulator
G6Pase	Glucose-6-Phosphatase
GLRX5	Glutaredoxin 5
GLDH	Glutamate Dehydrogenase 1
GPR91	G-coupled Protein Receptor 91
GR	Glutathione Reductase

LIST OF SYMBOLS VE ABBREVIATIONS (Continued)

Abbreviations	Descriptions
H_2O_2	Hydrogen Peroxide
HCC	Hepatocellular Carcinoma
HGF	Hepatocyte Growth Factor
HIF-1a	Hypoxia-inducible factor-1
HIF-2α	Hypoxia Inducible factor 2α
HIFs	Hypoxia-inducible factors
IGFs	Insulin-like Growth Factors
IL-6	Interleukin-6
JNK-AP-1	Jun N-terminal kinase-activator protein-1
LIP	Labile iron pool
LPCs	Liver Progenitor cells
LPS	Lipopolysaccharide
MB	Mitochondrial biogenesis
MMP	Matrix Metalloproteinase
MINER1	MitoNEET-related protein 1
mtDNA	Mitochondrial DNA
NAF-1	Nutrient-deprivation autophagy factor-1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B
NOS	Nitric Oxide Synthase
Nrf-1α	Nuclear Respiratory Factor-1α
OXPHOS	Oxidative Phosphorylation complex
PCNA	Proliferating Cell Nuclear Antigen
PDGF	Platelet-Derived Growth Factor
PEPCK	Phospho-Enol-Pyruvate Carboxy Kinase

LIST OF SYMBOLS VE ABBREVIATIONS (Continued)

Abbreviations	Descriptions
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1α
PHx	Partial Hepatectomy
PPAR- γ	Peroxisome Proliferator–Activated Receptor-γ
PI3K	Phosphatidylinositol 3-kinase
PPARs	Peroxisome Proliferator-Activated Receptors
ROS	Reactive Oxygen Species
RT-QPCR	Real-Time Quantitative Polymerase Chain Reaction
SDHA	Succinate Dehydrogenase subunit A
Sirt-1	Sirtuin-1
STAT3	Signal converting and Transcription 3 Activator
STEAP3	Six transmembrane epithelial antigen of the prostate 3
SUCNR1	Succinate Receptor 1
TFAM	Mitochondrial Transcription Factor A
TfR1	Transferrin Receptor 1
TGF-α	Transforming Growth Factor-α
TGF-β	Transforming Growth Factor-β
TLR4	Toll-like receptor 4
TNF-α	Tumour Necrosis Factor-α
TOM	Translocase of the Outer Membrane
TZDs	Thiazolidinediones
uPA	Urokinase Plasminogen
UTRs	Untranslated Regions
VEGF	Vascular Endothelial Growth Factor

1. INTRODUCTION AND PURPOSE

Cell metabolism is quite central to cellular and biological processes, especially cell division. Unfortunately, fundamental molecular aspects of cellular metabolism during liver regeneration coupled to hepatic cell cycle and proliferation remain poorly understood. Given that liver regeneration can be both pathologic and physiologic, a thorough comprehension of molecular factors underlying such fundamentals is very promising (Solhi et. al., 2021).

Interesting strides may have been made in understanding the metabolic needs of pathologic proliferation, for example in tumour-derived cell lines of pathologically-proliferating cells. This is a good example of how metabolism impacts cell division (Vander Heiden et. al., 2009). However, little is known about the physiology of innate tissue regeneration. Especially in mammalian tissues such as the liver tissue where constant toxic exposure causes cellular damage, regeneration is key to tissue homeostasis (Fausto et. al., 2006). Mitochondrial biogenesis and dynamics are at the centre of these events.

Liver regeneration is a complex re-growth process regulated by the restructuring of cellular bioenergetics and signalling pathways triggered by different stimuli such as transcription factors, cytokines secreted near the injury site or transferred to the liver via blood (Tao et. al., 2017). Liver mass recovery from these different stimuli has been demonstrated by studies through partial hepatectomy (PHx) in which hepatocyte proliferation after injury, is driven by 1-2 cycles of hepatocyte division.

Typical liver regeneration (LR) via PHx (liver resection) is accomplished via hypertrophy and/or hyperplasia of hepatocytes and biliary epithelial cells (BECs). In a 70% PHx, two-thirds liver resection results in immediate hepatocellular hypertrophy followed by hyperplasia. A one-thirds (1/3) PHx triggers only cell hypertrophy. Liver regeneration after 70% PHx in rats, is completed in about 5-7 days. Many complex molecular mechanisms are intertwined in the regenerative process (Taub, 2004). The 70% PHx model is mostly used to study the mechanisms controlling liver regeneration, because it enables mechanisms of cell cycle to be studied in vivo (Hu et. al., 2014).

Collectively, all the molecular signals serve as a physiological sensor of required liver mass and is termed hepatostat. Hepatostat can initiate and terminate liver regeneration (Manco et al., 2018). This phenomenon reflects the correlation between the needs of organisms and the mass of organs necessary for homeostasis (Michalopoulos, 2013).

The triggers of liver regeneration and cytokines as well as factors that modulate growth factors are closely related. In response to organ damage, beginning of each phase is initiated by a specific set of released molecules (Bhat et. al., 2019). This regenerative process; can technically and functionally be evaluated in 3 possible ways: the inter-relationships of the pathways linking the metabolic rewiring to cytokine stimulation required to initiate hepatocytes into the G1 phase, metabolic signals in the growth factor responsible for cell cycle progression and protein synthesis and DNA replication mechanism, none of which is still fully defined (Nelsen et al., 2003).

None of these alone is self-sufficient to drive regeneration. For regeneration is the cumulative effect of all of these put together, with domination of hepatocyte hypertrophy at the start of the process and cell division towards the end. At the beginning of liver regeneration, hypertrophy of hepatocytes dominates, and in later stages, regeneration mostly depends on cell division (Miyaoka et. al., 2012).

The chain of liver regeneration events is highly energy-dependent. Usually, hepatocytes have high metabolic requirements and thus regeneration is affected by the energy state of hepatocyte (Fausto et. al., 2006). Mitochondria, as the main bioenergetic and metabolic centre of the cell, are a key player in intracellular signalling such as apoptosis and have essential roles in the organ's response to major resection. Interests in studying the cellular bases of disordered bioenergetic events in liver and tissue regeneration, metabolic remodelling and their associated intracellular signals, and a coordinated regulation of metabolism-cell division during tissue regeneration have been recently aroused. Regeneration is an important element of tissue healing after injury (Locasale and Cantley, 2011).

During PHx-induced liver regeneration in rats, hepatocytes utilize mitochondrial oxidative phosphorylation efficiently for maximum ATP production to fuel cellular DNA synthesis and hepatocyte proliferation (Choi and Hall, 1974; Kamiyama et al., 1976).

Mitochondrial dysfunction caused a marked delay in liver regeneration (Rehman et al., 2011), indicating the essentiality of functional mitochondrial dynamics in liver regeneration (Weng et al., 2017).

These mitochondrial dynamics tie cellular proliferation to the ATP production-anabolic axis, to ensure increased production of essential metabolites (cell membrane phospholipids, amino acids, nucleotides and intermediate metabolites) and ATP. Catabolism of these essential metabolites must also be inhibited. Mitochondrial dynamics describe a well-coordinated cycles of mitochondrial fusion-fission (mitochondrial biogenesis) process (Tilokani et al., 2018) to regulate mitochondrial function. Observation of mitochondrial dynamics involvement in metabolic regulation of cardiac and skeletal muscle cell contractions (Abdelwahid, 2017), is a typical example linking mitochondrial dynamics to mitochondrial metabolism in mitochondria (Mishra and Chan, 2016; Wai and Langer, 2016).

Critical to the whole liver regenerative process is the mitochondrial biogenesis (Valdecantos et al., 2017). Mitochondrial biogenesis is a complex nuclear-mitochondrial genomic interplay (Hawley and Morton, 2014). Mitochondrial biogenesis is the process of either joining together two separate mitochondria to form a giant mitochondrion (fusion) or division of a mitochondrion into two separate mitochondria. Fusion-fission are the two typical changes in mitochondrial morphology. During high cellular metabolic demands, MB adapts the fusion-fission process to dynamic changes in mitochondrial morphology. Mitochondrial fusion is preferred over fission, for metabolic efficiency. Mitochondrial fission functions in favour of uncoupled respiration (Jacobi et. al., 2015).

These high cellular energy demands of liver regeneration and PHx-induced stress, stimulate the mitochondrial fusion process. Components are shared, non-faulty mtDNA sequences or mitochondrial protein complexes are made to compensate for the faulty ones, for maximum ATP generation. This could be a mechanism of mitochondrial quality and repair control system. With relaxed conditions in place, mitochondrial fission may set in as proof tool, to segregate functional (and non-faulty) mitochondria from dysfunctional (and faulty) mitochondria. This helps the cell to maintain a healthy mitochondrial network. Failure of the mitochondrial fission machinery in in high cellular stress conditions, activates the apoptotic machinery (Youle and Bliek, 2012; Aouacheria et. al., 2017; Trotta and Chipuk, 2017).

PGC-1α is a key transcriptional regulator of mitochondrial biogenesis and cellular energy metabolism (Cherry et al., 2014). Equally important to mitochondrial biogenetic process is the mitochondrial transcription factor (TFAM), which bind and wrap mtDNA, to initiate activation and regulation of mtDNA replication and transcription (Falkenberg et al., 2007; Scarpulla, 2008; Lodeiro et al., 2012). Cytochrome C oxidase II and other mitochondrial proteins, are encoded via mtDNA transcription (Freyssenet et al., 2004). Campbell and colleagues (2011) hypothesized that small amounts of TFAM are necessary to initiate mtDNA replication, whereas mtDNA expression is activated only at high concentrations (Campbell et al., 2012).

Iron-Sulphur (Fe-S) cluster (ISC) biogenesis is an essentiality of this mitochondrial biogenesis. being and coupled to DNA/RNA metabolism. ISCs are the basic components of mitochondrial respiratory complexes (I, II and III) used for electron transfer ubiquinone by NADH or FADH. and importantly required for functional DNA/RNA metabolism. Maintenance of genome stability is functionally bound to ISC biogenesis (Shi et. al., 2021). Defects in mitochondrial ISC biogenesis can result in nuclear genomic instability (Veatch et al., 2009).

Looking at the major role Fe-S clusters or proteins may play in mitochondrial oxidative phosphorylation and mtDNA biogenesis, not only ATP availability but also the molecular basis of the ATP production may be critical for cellular proliferation. An interesting link between mitochondrial and Fe-S cluster biogenesis, and post-PHx liver regeneration can be assumed. Fe-S biogenetic insufficiency to mitochondrial biogenesis and liver regeneration assumingly requires further investigation. This informed the basis of our study.

In our study, ten (10) genes of interest: PGC-1α, a mitochondrial biogenetic-related gene; six (6) Fe-S-containing genes/proteins (mitoNEET, ACO1/IRP1, MINER1, ACO2, GLRX5, SDHA); mitochondrial iron transporter (mitoferrin), HIF-2α and C-MYC were selected. Gene expression of β-actin as the reference gene, was also selected. Gene expression of all genes were analyzed using RT-PCR array after 70 % PH at different time periods of (0, 6, 12, 24, 48, 72) hours (hrs). MitoNEET, ACO1/IRP1, HIF-2α, mitoferrin, MINER1, SDHA and C-MYC were found to be statistically significant ($p \leq 0.05$) in the PH groups, except for mitoferrin. 4 genes (mitoNEET, IRP1/ACO1, HIF-2α, DMT-1) were selected for analysis of

their protein expression using Western Blotting method. An interesting but significant pattern of PGC-1α and MINER1 gene expressions, were observed intra-PH groups. Activity of succinate dehydrogenase enzyme was assessed by measuring the succinate receptor (SUCNR1-GPR91) expression. The rate of liver regeneration was determined based on the calculated liver weights and PCNA immunohistochemistry according to the various time points.

This study explored the molecular interaction of Fe-S genes and Fe-S biogenesis as a function of mitochondrial biogenesis after partial hepatectomy at various time points. Our work promises to shed light on the molecular involvement of Fe-S genes and proteins, and its interactions with other cellular players in hepatocyte renewal and repair.

2. LITERATURE REVIEW AND GENERAL INFORMATION

2.1. Literature Review

Extracellular and intracellular molecular signals guide the success of the liver regenerative system to correctly sustain initiating stimuli regenerative drive while instituting other terminative mechanisms to prevent tumourigenesis. These molecular mechanisms are defined by the actors recruited in a phase- and time-dependent fashion for liver regrow (Salameen et. al., 2022). Liver regeneration and tumourigenesis possess similar proliferative capacity. Unlike liver tumours, liver regeneration institutes biological, bio -chemical and -physical negative factors to terminate the regenerative process when threshold metabolic capacity have been gained in the liver.

Post-PHx injury, transient metabolic stress and related hepatic insufficiency may act as physiological sensors, to promote both the hepatocyte hypertrophy-hyperplasic processes of liver regeneration (Verma et. al., 2021). A series of transcriptionally and post transcriptionally-regulated molecular events are then activated to drive G_0 hepatocytes into the cell cycle, in such a manner that recruited cells are triggered in relation to particular phase at specific times (Loyer et. al., 2012). One such possible important molecular event in hepatocellular proliferation may be Fe-S biogenesis as an essentiality of mitochondrial dynamics and Fe-S proteins, owing to their versatile or irreplaceable cellular roles in iron metabolism, DNA synthesis, cellular bioenergetics, metabolism and several others (Rouault and Maio, 2017).

However, Fe-S protein and biogenesis, its molecular basis of cellular functions and effects of interaction network in physiological tissue regeneration, are still unknown. Understanding the interconnections or interdependence of these cellular functions of Fe-S proteins in liver regeneration, will not only aid in decoding the molecular basis of Fe-S proteins' functions but also guide to yet unproven molecular links between mitochondrial (Fe-S) protein biogenesis, cellular metabolism and hepatocyte hypertrophic-hyperplasic processes during liver regeneration.

In a study of PGC-1α expression after PHx observed over 0.5, 1, 2, 16, 24 and 40 hrs, PGC-1α was maximally induced at (2-4) hrs post-PHx evidenced by the highest mRNA and protein expression levels (Wang et. al., 2008). PGC-1α expression levels in G_0 hepatocytes and SH groups, were similarly observed. Therefore, PGC-1α may regulate mitochondrial oxidative and ROS metabolism via mitochondrial biogenesis-mediated cellular remodelling and organelle biogenesis (Austin and St-Pierre, 2012), to fuel liver regeneration.

MitoNEET is released in an ALR-mediated fashion to promote cellular proliferation (Nalesnik et. al., 2017). Besides its regulatory functions in mitochondrial iron, lipid and ROS metabolism, mitoNEET can transfer its cluster to IRP1 (Ferecatu et. al., 2014). Both mitoNEET and MINER1 as NEET proteins via cluster transfer, can activate CIAPIN-1 (cytokine induced apoptosis inhibitor-1) (Lipper et. al., 2015). GLRX5-mediated cluster transfer (Wingert et. al., 2005) has also been reported. However, mitoNEET, MINER1 and GLRX5 have all been shown to be involved in mitochondrial functions and metabolism via cellular iron homeostasis.

After 70% PHx, state of liver tissue may be both hypoxic and iron-deplete. IRP1- and IRE-IRP1 binding is activated to mobilize iron to meet these high iron-dependent metabolic needs (De Domenico et al., 2008; Hentze et al., 2010; Anderson et al., 2012). This may induce TfR1, DMT1-IRE, ACO2 (Kuhn, 2014) and HIF-2α (Patel and Simon, 2008). HIF-2α was reportedly expressed at (3-6) hrs after PHx (Weisener et. al., 2003). Mollbrink et. al., (2012) reported an initial increase in TfR1 and DMT-IRE at 4 hrs and the highest peak at 16 hrs for DMT1 and 24 hrs for TfR1 respectively post-PHx. Despite no net changes in mRNA levels in PH groups after PHx, tissue-specific DMT1 or TfR1 protein levels of up to 50-fold were observed. This indicated a strict posttranslational regulation of the iron carrier mRNAs (Rouault T. A. 2012).

Study of MINER1 gene in cancer models, showed its suppression to control breast cancer metabolism via increased TfR1-bound iron uptake and HIF-1α-associated cellular stress activation (Bian et. al., 2021). Disrupted MINER1 expression in mice was reported to resulted in upregulation of autophagy, cell death and premature aging (Chen et. al., 2021).

Exact molecular functions of NEET proteins (Bian et. al., 2021) and other Fe-S proteins are still unknown, and current topic of several investigations.

2.2. Liver Regeneration – A metabolic approach

The liver is the largest internal organ in the mammalian body, with adult liver weighing approximately 1.5 kg. The liver is composed of parenchymal cells (hepatocytes) and non-parenchymal cells. Parenchymal cells are composed of hepatocytes and constitute about 80 % of the tissue mass. While the non-parenchymal cells include endothelial cells, Kupffer cells, lymphocytes, stellate cells, etc. (Majno et. al., 2013; Rmilah et. al., 2019)

Epithelial tissues such as the intestines and skin having high cell turnover, usually require a pool of stem cells to restore cellular renewal and homeostasis. In the case of the liver with lower cell turnover, all mature hepatocytes are assumed to participate in cell division during normal liver homeostasis (Gilgenkrantz and Collin, 2011; Michalopoulos, 2017). Rodent model of seventy percent (70%) partial hepatectomy (PHx) however, exhibited otherwise, serving as a vital source of information on how the liver regenerates and thus hepatocyte proliferation.

In this model, where approximately 70 % of the liver is removed, mature quiescent adult hepatocytes of this remnant liver enter the cell cycle in a semi-synchronous manner (Gilgenkrantz and Collin, 2018; Rmilah et. al., 2019). Hepatocytes are stable cells, proliferatively quiescent in the G_0 phase (cell growth arrest) and rarely divide in their normal state. Upon stimulation (PHx), hepatocytes can rapidly make an entry into the growth phase (G_1) of the cell cycle, initiating cell division. Thus, hepatocytes hold the secret to the liver's unique ability to precisely regulate its tissue growth and mass. Oval cells (progenitor or stem-like cells) in adult liver have been reported to function as a supplementary reservoir of proliferative cells (Fausto, 2004).

Although differentiated hepatocytes have self-renewal ability, different hepatocytes subpopulations may have distinct proliferative abilities (Gilgenkrantz and Collin, 2018). Metabolically, the functional unit of the liver is the lobule. As depicted in Figure 2.1; periportal zone 1 hepatocytes specialize in gluconeogenesis and β-oxidation, whereas those located around the central vein (zone 3) are more important for glycolysis, lipogenesis, and detoxification, making hepatocytes functionally heterogeneous. This functional heterogeneity causes different gene expression subject to location in the hepatic lobule along the porto-central axis. This metabolic zonation permits the liver to respond to different

nutritional requirements or hepatotoxins. Recently, lineage-tracing experiments in an uninjured mouse, showed a population of proliferating and self-renewing hepatocytes in the centrilobular zone 3, adjacent to the central vein of the lobule (Font-Burgada et. al., 2015; Wang et. al., 2015).

A detailed study of the mouse liver homeostasis indicated that, situated around the central vein are the highly proliferative axin2-positive hepatocytes (pale green), more proliferative than the hepatocytes of mediolobular (green and blue) and periportal (pale blue) area. Degree of the injury is directly proportional to intensity of the response. After a mild acute damage, nondamaged hepatocytes would enter the cell cycle in mild acute damage while hybrid hepatocytes (pale blue) repopulate the liver in mild chronic damage. Severe chronic liver damage employs ductal cells and liver progenitor cells (LPCs), in a process known as ductular reaction. Ductular reaction is yet to be seen in liver regeneration or human pathologic conditions (Planas-Paz et. al., 2016; Font-Burgada et. al., 2015; Wang et. al., 2015).

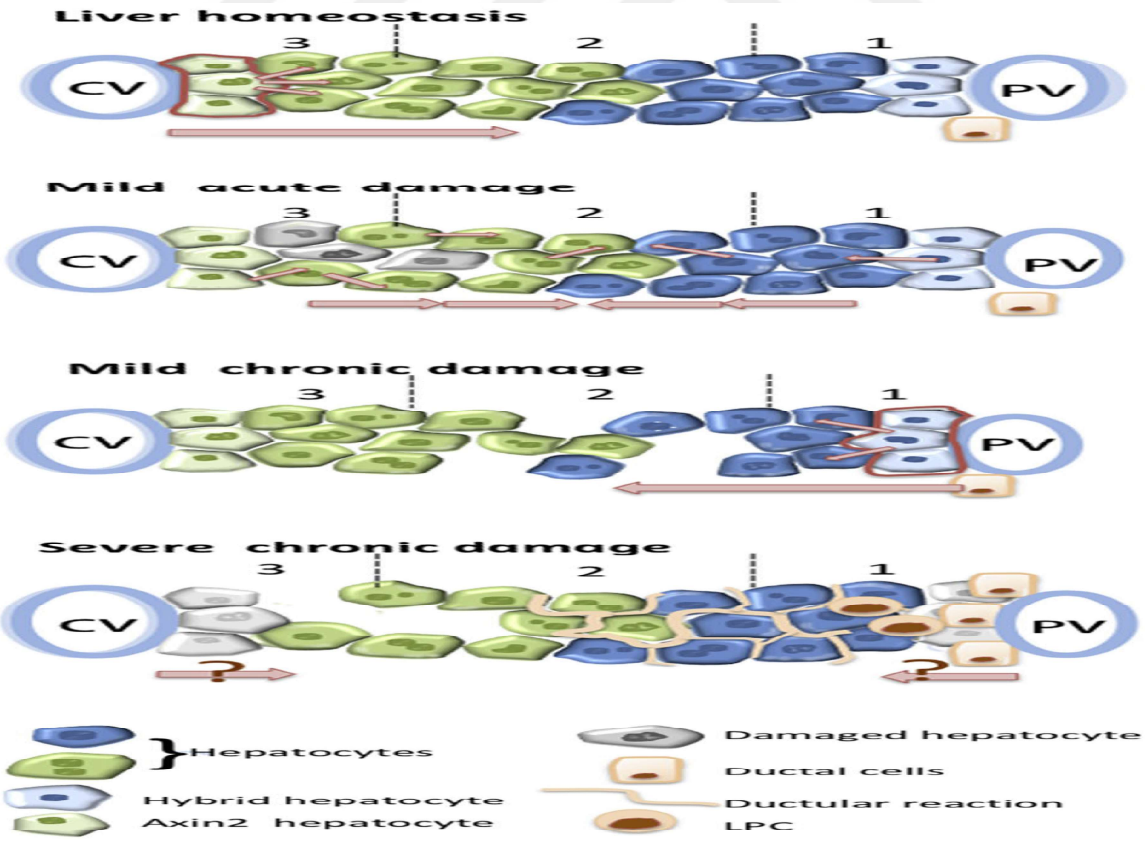

Figure 2.1 Cellular players in regeneration. Zones: 1-(periportal), 2-(intermediate) and 3-(centrilobular). CV– (central vein); PV- (portal vein) (Gilgenkrantz and Collin, 2018).

The type of injury may also determine the lobular structure as an anatomical unit with a diversified hepatocyte populations more susceptible to restoring the liver mass. Location, type and extent of injury determine the heterogeneous hepatic cells recruited. For example, proximity to endothelial cells was shown to be important for hepatocyte proliferation. The closer a hepatocyte is to an endothelium, the higher the proliferation (Ding et. al., 2010). Along the endothelial axis, all aligning hepatocytes may proliferate to regain the initial liver mass (Pu et. al., 2016).

For the purpose of tissue renewal or regeneration, a recovering tissue needs to regulate metabolism and cell division simultaneously (Locasale and Cantley, 2011). It can therefore be inferred that hepatocytes coordinate the regulation of metabolism and cell division simultaneously after a partial hepatectomy process to ensure hepatocyte survival and renewal. Studies to understand metabolic requirements of proliferating cells have mostly focused on tumour-derived cell lines displaying abnormal cell divisions (Vander Heiden et al., 2009). Little information exists on metabolic alterations during innate tissue regeneration. The mammalian liver is always exposed to unimaginable toxins and cellular damage as a result of its functions and requires regeneration for tissue homeostasis (Fausto et al., 2006). The hepatocytes in adult liver, though innately quiescent execute a broad range of demanding metabolic functions (Huang and Rudnick, 2014). However, during damage, they undergo one or more rounds of cellular division to induce compensatory hyperplasia or simply liver regeneration (Fausto et al., 2006).

These regenerating hepatocytes must both replicate and carry out its metabolic functions at the same time. It has become imperative to understand metabolic regulation during liver regeneration, metabolic needs of proliferating hepatocytes and effects of a failed hepatocyte proliferation on metabolic homeostasis. Transcriptional programs characterizing respective liver regeneration phases (priming, proliferation and termination) may be fairly known. Fausto et. al., (2006) implied the suppression of metabolic genes during liver regeneration (Fausto et al., 2006). This seems incomprehensible. Because a regenerating liver can never forfeit its function of maintaining metabolic homeostasis while regenerating. Thus, tissue renewal and repair must necessarily represent a metabolism-proliferation communication axis.

Decreased hepatic division during regeneration was seen to correlate with impaired metabolic homeostasis. Hepatocytes unable to undergo cell division during liver regeneration, were shown to have alanine transaminase (ALT) hyperactivation, a key indication of liver damage and metabolic regulation required to aid tissue recovery (Caldez et. al., 2018).

A good of understanding of the metabolic adaptations in proliferating hepatocytes during liver regeneration would help decode the metabolism-cellular proliferation crosstalk and offer clinical solutions to the issue of impaired hepatocyte division, usually seen in numerous liver diseases (Forbes and Newsome, 2016) and metabolic disorders (DeAngelis et al., 2005; Vetelainen et al., 2007).

2.3. Mechanism and phases of liver regeneration

Liver regeneration after PHx, is a highly-regulated and well-orchestrated process (Fausto et al., 2006), resulting in the liver gaining its full size and weight. This biological feat is of great clinical importance in the cases of liver injury, resection and organ transplantation. It is characterized by a rapid and well-synchronized passage of the quiescent hepatocytes at G_0 phase into G_1 phase of the cell cycle, in response to a regenerative stimulus received in the liver (Taub, 1996). Such a rapid cellular proliferation of the hepatocytes is the fundamental basis and driver of the liver regrowth.

In a clear demonstration of the liver's regenerative ability, PHx was initially described by Higgins and Anderson (Higgins and Anderson, 1931). In the PHx model, removal of two-third of the tissue mass initiates a unique response in which the remaining diploid hepatocytes enter the cell cycle (G_1-S-G_2-M) to compensate for the loss of liver tissue. This process of compensatory hyperplasia (rapid cell proliferation) lasts for approximately one week. Entry of cells into the G_1 phase corresponds to about 6 hours after PH and the G_1-G_2 transition is around the 12th hour. 24th hour post-PHx is the stage of highest DNA replication and finally mitosis occurring between 36-48 hours. Regeneration is completed 72-168 hours after PHx and the liver is reshaped (Stolz et. al., 1999).

The molecular mechanism of liver regeneration is highly-complex and intertwined. The regenerative response includes initiation, expansion, and termination in a manner of various well-co-ordinated biological events occurring within the same time range or in

sequence at the molecular, cellular, and tissue levels (Zou et al., 2012). Further studies involving this phenomenon led to identification of various key factors including but not limited to mitogens (growth factors, cytokines), co-mitogens and inhibitors (Gandhi, 2012).

Different responses are elicited with regards to the extent of liver resection (**Figure 2.2**). Hepatocytes increase their size or volume (hypertrophy) in response to one-third PHx, while hypertrophy and hyperplasia occur in two-thirds PHx. 80% to 90% liver resection recruits BECs (Biliary epithelial cells) to be dedifferentiated into LPC (liver progenitor cells) progenitor cells for liver repopulation. (Miyaoka et. al., 2012).

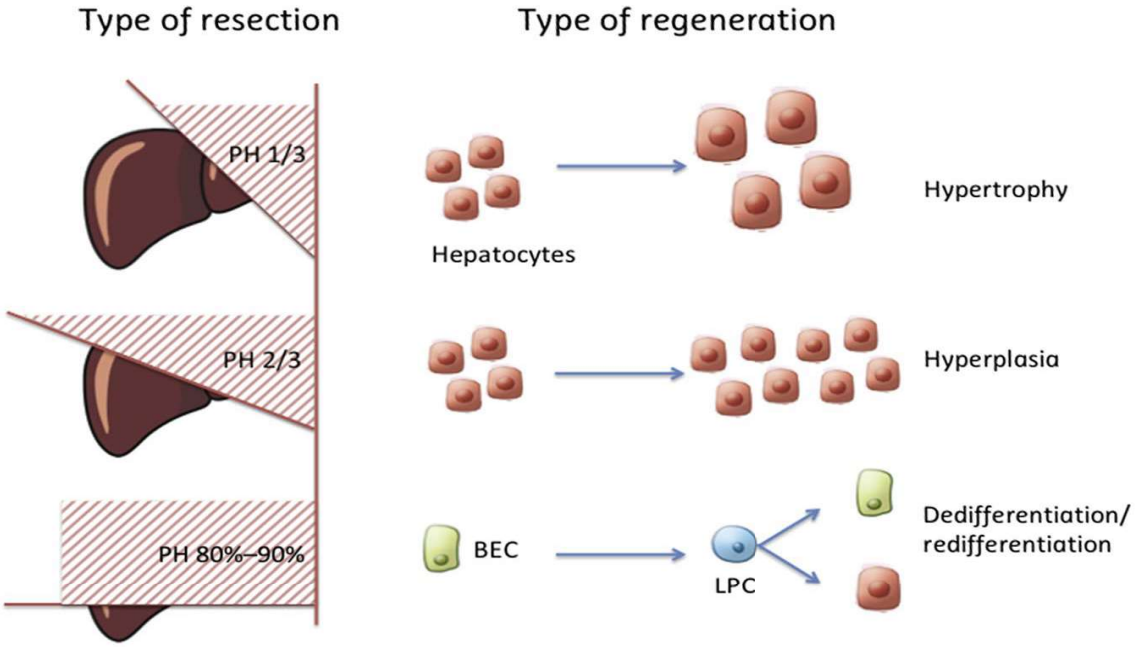

Figure 2.2 Types of liver resection (PHx) and mechanism of regeneration. BECs (Biliary epithelial cells) and LPC (liver progenitor cell). (Gilgenkrantz and Collin, 2018).

Mechanisms of hepatocyte hyperplasia still remain to be elucidated. Overturf et al., (1997) reported that, serially injected hepatocytes in mice were capable of more than seventy (70) rounds of cell division or replication (Overturf et al., 1997). It seemed impossible because, a single hepatocyte would theoretically be capable of completely regrowing a mouse liver. Miyaoka and colleagues, (2012), rather pegged the division rate of a regenerating hepatocyte at 1.6 times after 70% PHx in the most basic model, barring any probable change in cell size (Miyaoka et al., 2012). Numerous studies have observed otherwise (Minamishima et al., 2002; Haga et al., 2005; Haga et al., 2009).

Growth factors including but not limited to hepatocyte growth factor (HGF), transforming growth factor-α (TGF-α), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF), and cytokines such as tumour necrosis factor α (TNF-α), interleukin 6 (IL - 6) are usually expressed either at the site of injury or carried via the circulatory system to the liver. They elicit cell signalling pathways that have multiple biological effects. For instance, these factors activate the downstream cell cycle regulators such as STAT3, JNK-AP-1, PI3K/AKT, ERK and NF-κB, subsequently initiating the hepatocyte replication and regeneration (Iimuro et al., 2010).

A successful PHx liver regeneration model must therefore drive molecular players (such as IL-6 and TNF-α) to elicit hepatocytes entry into G_1 cycle and cause TNF-α secretion by Kupffer cells. This will in turn activate NF-κB in neighbouring hepatocytes and thus increasing IL-6 secretion. IL-6 would contrastingly activate the JAK/STAT3 (signal converting and transcription 3 activator) pathway to promote hepatocyte proliferation through receptor-binding. Accordingly, super-agonistic IL-6/soluble IL-6R fusion protein enhances liver regeneration (Hoffman et. al., 2020).

In insult-induced damage regeneration, oval cells acting as a reservoir of liver stem cells, instead of all damaged hepatocytes, differentiate into new hepatocytes and bile cells (Sun and Irvine, 2014).

The regenerative process includes three critical steps (Pahlavan et al., 2006). The early or initial phase is usually spanned with the first 30 minutes to 4 hours (0.5-4 hrs) after PHx. This marks the priming phase in which the quiescent hepatocytes (in G_0 state) are prepared for entry into the G_1 stage of the cell cycle, upon multiple stimuli. To initiate tissue repair and regeneration, inflammation must be activation. Activation of inflammatory response recruits a range of immune and inflammatory cells (Kupffer cells, liver macrophages, complement system cells, etc) and initiate a range of cytokine signalling necessary for tissue repair and renewal. This whole process would then cause the release of growth factors to aid in hepatocyte stimulation. IL-6 and TNF-α have been extensively studied in this regard. Kupffer cells are also known to release a group of cytokines and immunomodulatory factors with both stimulatory and inhibitory effects in hepatocyte renewal. The complement (C3a and C5a) can trigger either the LPS/TLR4 or the NF-κB

signalling pathway to elicit IL-6 and TNF-α secretion by liver macrophages (Cressman et. al., 1996; Yamada et. al., 1997; Tao et. al., 2017).

The next phase is characterized by the maintenance of the whole cell cycle process. The hepatocytes primed for regeneration, are moved beyond the G_1 point by mitogens and driven through the cell cycle (G_1-S-G_2-M) till the hepatocyte divide at mitosis. The proliferation phase spans 6^{th} hr to the 66 hours (6-66 hrs) after PHx. This stage of progression of cell proliferation is mainly supported by either the full mitogens or auxiliary mitogens. Whole mitogens such as HGF (hepatocyte growth factor), TGF-α (Transforming growth factor-α), EGF (epidermal growth factor), HB-EGF (heparin-binding EGF) and their co-receptor can strongly cause hepatocyte proliferation via activation of necessary cell signalling pathway (such as Ras-MAPK, PI3K/AKT, etc) to stimulate DNA synthesis. Auxiliary mitogens only function to support the whole mitogens. Some of these auxiliary mitogens may include bile acids, VEGF, noradrenalin, IGFs, oestrogen, serotonin, etc., (Tao et. al., 2017).

As signals indicating the achievement of the initial liver mass are received, hepatocyte proliferation is immediately stopped and regeneration terminated. This marks the termination phase of liver regeneration. It is assumed to occur within the (72-168 hrs) after regeneration. Hepatocytes return the G_0 (quiescent) stage. Negative factors such as (TGF-β) and activin take control (Liu and Chen, 2017; Mari and Morales, 2017).

2.4. Mitochondrial dynamics and biogenesis

Mitochondria have primarily been considered as the ‘powerhouse’ of the cell, producing the energy required for cell metabolism by oxidative phosphorylation (OXPHOS) (McBride et. al., 2006; Kamer and Mootha, 2015). It was recently seen to be involved also in numerous other physiological processes such as programmed cell death, innate immunity, autophagy, redox signalling, calcium homeostasis and stem cells reprogramming (Nikoletopoulou et. al., 2013; Kamer and Mootha, 2015; Rambold and Pearce, 2018).

Mitochondria are enclosed by two membrane layers composed of different lipids and proteins, separated by an intermembrane space (IMS). Human mitochondria are believed to harbour over 1500 proteins, of which mtDNA encodes about only 13. This indicates the mitochondrial need for extra proteins and an efficient protein import mechanisms via the

membrane layers. Efficient membrane-regulated protein import may be very essential for mitochondrial biogenesis, function, and turnover (Koehler, 2000).

Internally, the mitochondria can be partitioned into 3 compartments: mitochondrial matrix (MM) enclosed within the inner mitochondrial membrane (IMM), the outer mitochondrial membranes (OMM) and the intermembrane space (IMS) between the MIM and the OMM. The MIM houses the mitochondrial electron transport chain (ETC) together with the ATP synthase. Embedment of the ATP synthase positions IMM as functional mitochondrial membrane potential barrier sustaining the proton gradient for ATP synthesis (Lapuente-Brun et. al., 2015).

And IMM also folds and protrudes at several points to form the cristae, increasing its surface area. On the other hand, MM serves as the predominant site of metabolic reactions and harbours the greater number of mitochondrial metabolic enzymes, together with the several copies of circular mtDNA. The mtDNA encodes the thirteen (13) OXPHOS protein subunits, as well as the tRNAs and rRNAs partaking in mitochondrial protein synthesis (Barshad et al., 2018).

The OXPHOS system is quite unique in animal cells, as the only system to have the structural components of its multiprotein complexes encoded by two genomes; maternal mtDNA and biparentally-transmitted nuclear DNA (nDNA). For the structural compostion of the OXPHOS, the mammalian mtDNA encodes 13 messenger RNAs (mRNAs), 22 transfer RNAs (tRNAs), and 2 ribosomal RNAs (rRNAs). Together with the almost 70 nDNA-encoded structural proteins, a functional OXPHOS complex is generated (Fernandez-Vizarra and Zeviani, 2021). Functionality of OXPHOS-encoded genes is therefore limited by a physical matching constraint, challenged by two very separate mechanisms imposing a close-fitting coevolution of both genomes. These result in variability in nDNA (sexual reproduction, mutation, and coexistence of two alleles) and in mtDNA (mutation, polyploidy, and segregation) (Lapuente-Brun et. al., 2013; Nissanka and Moraes, 2020).

The OXPHOS system is made up of four (4) electron transfer chain (ETC) multimeric enzymes, complexes I–IV, in addition to two mobile electron carriers, coenzyme Q (CoQ) and *cytochrome c*. In the biological names of the complexes; I (NADH: ubiquinone oxidoreductase), II (TCA cycle enzyme succinate dehydrogenase), III (ubiquinol:

cytochrome *c* oxidoreductase) and IV (ATP synthase) are the definitions of their cellular functions. NADH and $FADH_2$ are the main electron carriers. There is other two proteins that electron acceptors of the $FADH_2$. IMM-bound glycerol-3-phosphate, an intermediate glycolytic product in the cytosol and ETF or ETF: QO (electron-transferring flavoprotein or ETF: ubiquinone oxidoreductase) (Signes and Fernandez-Vizarra, 2018). ETF or ETF: QO accept $FADH_2$-generated electrons by 11 different mitochondrial dehydrogenases, usually involved in mitochondrial fatty acid and amino acid oxidation processes (Liesa et. al., 2013).

Electrons generated from NADH and $FADH_2$ are transferred to molecular oxygen (O_2), reducing it and forming water molecules in a complete process called cellular respiration. The electron transfer across the redox centres in the three complexes (I, III, IV) liberates energy, by which the protons are pumped across the IMM. The redox function of complex II is only associated with internal protonation activity within its own subunit A (Enriquez et. al., 2014). Protons are moved from the MM into the (IMS), causing an uneven protons distribution in the IMM. This creates a PMF (proton motive force) by which ATP synthase synthesize ATP generation by adding an inorganic phosphate to ADP (Tropeano et. al., 2020).

Newly synthesized ATPs are transported by the mitochondrial adenine-nucleotide transporter family, to the cytoplasm to be used as the chemical energy source for almost all the endergonic biochemical cellular processes. Mitochondria produce a daily ATP amount in humans based on the whole-body weight (around 72 kg on average) to meet the cellular energy needs. Mitochondrial dysfunctions lead therefore to dysfunctional metabolism, which results not only in ATP shortage, oxidative and metabolic stress, but also pronounced disease phenotypes (Nunnari, and Suomalainen, 2012; Suomalainen, and Battersby, 2018).

The OMM may relatively be more permeable to small peptides (<5 kDa) and small solutes bidirectionally, but carries a number of transporters, channel proteins and molecules involved in fusion and fission (MB). Notable among them is the channel protein, porin/VDAC and *t*ranslocase of the *o*uter *m*embrane (TOM) family, the principal OMM protein transporter (Koehler, 2000; Perry et. al., 2006).

Mitochondria were initially thought to be static and isolated structures but recent advances in live cell imaging showed otherwise. Mitochondria can modulate their

morphology to create a tubular network coordinated by fission and fusion events. The balance between these two opposite processes regulates mitochondrial number, size and positioning within the cytoplasm and is referred as ‘mitochondrial dynamics’ (Liesa et. al., 2009). A critical factor of mitochondrial dynamics is the mitochondrial biogenesis.

Mitochondrial biogenesis as a fundamental process of mitochondrial dynamics in cell biology, therefore seems critical for normal cellular regeneration and homeostasis to regulate mitochondrial function (Tilokani et al., 2018). A careful observation of how mitochondrial dynamics functions to metabolically regulate cardiac and skeletal muscle cell contractions (Abdelwahid, 2017), provided a typical example of the mitochondrial dynamic-metabolism link in mitochondria (Mishra and Chan, 2016; Wai and Langer, 2016). Increased ETC activity in yeast cell was observed to show elongated mitochondrial network (Egner et al., 2002) while energy substrates rather dictate mitochondrial elongation of human cancer cells (Rossignol et al., 2004).

Mitochondrial biogenesis is of major importance in the cells undergoing rapid cell division such as in hepatocytes dividing to restore a liver mass from injury (**Figure 2.3**) or in pathological states (such as rapid proliferative tumours). As part of their life cycle seen at the G_0 stage, mitochondria maintain a sustained and continuous rounds of fusion-fission, depolarization and autophagic degradation resulting from their local energy status and associated signals (Liesa and Shirihai, 2013). As external signals (global control signals) instruct the cell to enter the cell cycle, cycles of mitochondrial hyper-fusion in G_1–S and fragmentation at M phase cause significant mitochondrial population changes driven by the higher energy demands of the dividing cell. The need for the cellular components to homogenize and sequester during metaphase stage of the cell division cycle, increases the cellular energy demand. Understanding of the fundamentals of this mitochondrial phenomenon will surely help answer unresolved questions in cell biology and pave way for novel targeted therapies (Hyde et. al., 2016).

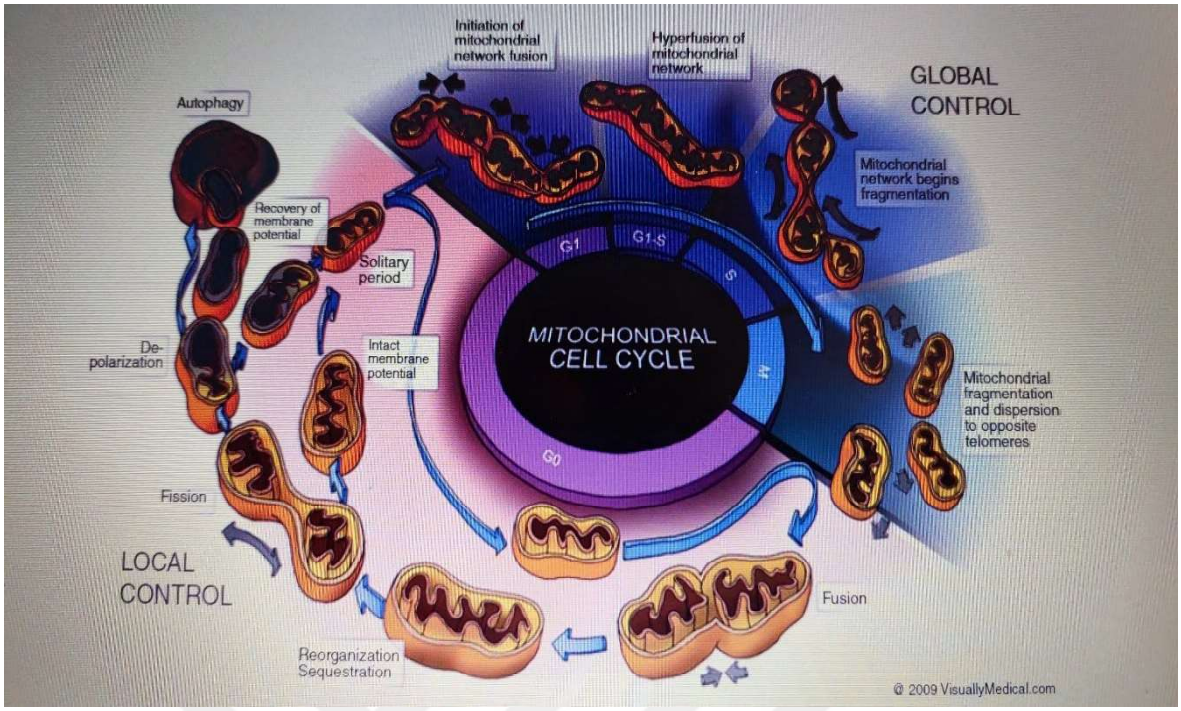

Figure 2.3 Mitochondrial life in the cell cycle (Hyde et. al., 2016).

MB is controlled by several molecular mediators. Prominent among them is PGC1α, a transcriptional gene coactivator and master regulator of MB (Cherry et al., 2014). PGC-1α is involved usually biological responses requiring a shift from glycolytic to oxidative metabolism, such as thermogenesis in brown adipose tissue (Puigserver et al., 1998), fiber-type switching in skeletal muscle (Lin et al., 2002), and fatty acid β-oxidation, together with gluconeogenesis in the liver (Puigserver and Spiegelman, 2003).

PGC1α encodes the regulatory proteins of mitochondrial biogenesis and function (Wu et al., 2009; Kelly and Scarpulla, 2004). Another key mediator of MB is TFAM, which binds and wraps mtDNA, activating and regulating mtDNA replication and transcription (Falkenberg et al., 2007; Scarpulla, 2008; Lodeiro et al., 2012). TFAM is essential for initiation of replication and mtDNA expression. This encodes portions of the mitochondrial proteins such as cytochrome c oxidase II (Freyssenet et al., 2004). Campbell et al., (2012) have stated that only small amounts of TFAM are needed for initiation of mtDNA replication, but only high concentration activate mtDNA expression (Campbell et al., 2012). Four (4) key proteins belonging to the dynamin-related family of proteins, have been particularly identified to possess unique functions in the fusion-fission process. In the fusion

process, mitofusin 1 and mitofusin 2 promote fusion of the OMM, while that of the IMM is executed by optic atrophy 1. DRP1 promotes fission of the OMM (Hoppins et. al., 2007).

MB encompasses also a well-coordinated complex nucleo-mitochondrial crosstalk, which couples new protein synthesis in both organelles to mitochondrial fission-fusion process (Hawley and Morton, 2014). Such a crosstalk may not only safeguard the integrity and quality of the newly synthesized mitochondria, but also explain control of nuclear respiratory factors (Nrf-1, Nrf-2) (Houten and Auwerx, 2004), TFAM (Jornayvaz and Shulman, 2010) and other downstream targets by PGC-1α. AMP-activated protein kinase (AMPK) as a key cell energy sensor, functions to regulate PGC-1α and thus mitochondrial biogenesis (Jornayvaz and Shulman, 2010).

The liver is extremely rich in mitochondria because of its critical cellular metabolic role, with each hepatocyte containing 1000–2000 mitochondria (Wiesner et. al., 1992). Thus, the major and central position of hepatocytes in this general systemic metabolism. Several studies have tried to observe mitochondrial dynamics in this context; perfused liver, isolated primary rat (Das et al., 2012) and mouse hepatocytes (Umbaugh et al., 2021). In a normal hepatocyte physiology, discrete globular or short tubular mitochondria were not only seen (Umbaugh et al., 2021), but fewer fusion events and little movement activity existed (Das et al., 2012). These events are indicative of less rapid alterations in mitochondrial dynamics of hepatocytes under physiological conditions.

The earliest events that stimulate liver regeneration and the distant signals that terminate this process are still not fully understood. Metabolic response to liver failure may be the earliest signal initiating regenerative hepatocellular proliferation. Mitochondrial dynamics is considered essential to the well-regulated bioenergetic and metabolic process of liver regeneration. After a liver injury (failure) or PHx, Weng et al., (2017) reported the importance of functional mitochondrial dynamics in liver regeneration (Weng et al., 2017). Mitochondrial biogenesis was shown to be critical in the liver regenerative process (Valdecantos et. al., 2017). Several studies have shown that pharmacologically inducing MB enhanced tissue regeneration and recovery in various rodent models of acute kidney injury (Rehman et al., 2013; Whitaker et al., 2013; Funk and Schnellmann, 2013; Jesinkey et al., 2014; Garrett et al., 2014; Khader et al., 2014) and other models of tissue injury (Finck and Kelly, 2007; St-Pierre et al., 2005; Funk et al., 2010).

Recovery of cell function after stress (such as PHx-induced stress) may facilitate regulation of mitochondrial regeneration, which is key to maintenance of respiration, metabolism and other critical cellular functions. Mitochondrial oxidative phosphorylation was observed to be enhanced in the remaining hepatocytes to fuel PH-induced liver regeneration in rats, linking ATP production to DNA synthesis and hepatocyte proliferation (Choi and Hall, 1974; Kamiyama et al., 1976). Failure to mobilize mitochondrial activities could lead to a marked delay in liver regeneration (Rehman et al., 2011).

ISC (Fe-S) biogenesis is an essential functionality of this mitochondrial dynamics (biogenesis) and critically dependent on the mitochondrial import pathways. Functional roles of ISC as key redox centres, make them major structural components of the OXPHOS complexes I, II, and III (Rouault, 2019). As its name implies, MIA (mitochondrial IMS assembly) pathway is employed in targeting most intermembrane proteins. It has two major components, one of which is IMS oxidoreductase Mia40. IMS oxidoreductase Mia40 has been shown to bind Fe-S clusters both in vivo and in vitro and work effectively in mitochondrial biogenesis (Kusminski et al., 2012; Landry et al., 2014; Vernis et al., 2017).

A critical consideration of the essential cellular and biological role of Fe-S clusters or proteins in mitochondrial oxidative phosphorylation and mitochondrial biogenesis; that of the molecular basis of mitochondrial oxidative phosphorylation and mitochondrial biogenesis in cellular energy (ATP) production; and the fundamental need of ATP production in cellular proliferation, it can be clearly inferred that ISC is indispensable to hepatocyte (cellular) proliferation. Fe-S biogenetic insufficiency can lead to dysfunctional mitochondrial biogenesis, which may critically affect liver regeneration. Association between impairment of mitochondrial biogenesis and liver regeneration after PH, particularly with respect to Fe-S cluster proteins, has rarely been investigated.

This study involving Fe-S cluster-containing proteins and related genes, sought therefore to reveal the possible molecular roles and interactions employed at various time points during hepatocyte (cellular) proliferation.

2.5. Iron-Sulphur (ISC) biology: biogenesis, cellular mechanisms and DNA metabolism

ISCs are redox-active prosthetic group or small cofactors composed of iron and sulphur atoms, oxygen-labile and usually bound post-translationally to proteins. They are ubiquitously expressed in all living cells as major players in most biochemical reactions, especially electron transfer (mitochondrial respiration) and occur mostly in three forms; (Fe_2S_2), (Fe_3S_4), and (Fe_4S_4). But, Fe_2S_2 and Fe_4S_4 (**Figure 2.4**) are essentially the two common forms in the mammalian cells synthesized via the cytosolic (CIA) and the mitochondrial (ISC), pathways respectively. Proteins containing the Fe-S clusters are termed as Fe-S proteins. Fe-S clusters demonstrate high capacity for accepting and donating electrons, thereby allowing efficient electron transport and subtle redox-tuning of protein properties. Their role as essential redox centres, warrants their incorporation into the OXPHOS complexes I, II, and III (Lapuente-Brun et. al., 2013). In most cases, Fe ions joined to sulphide ions, are coordinated by cysteine and histidine ligands (Rouault, 2019).

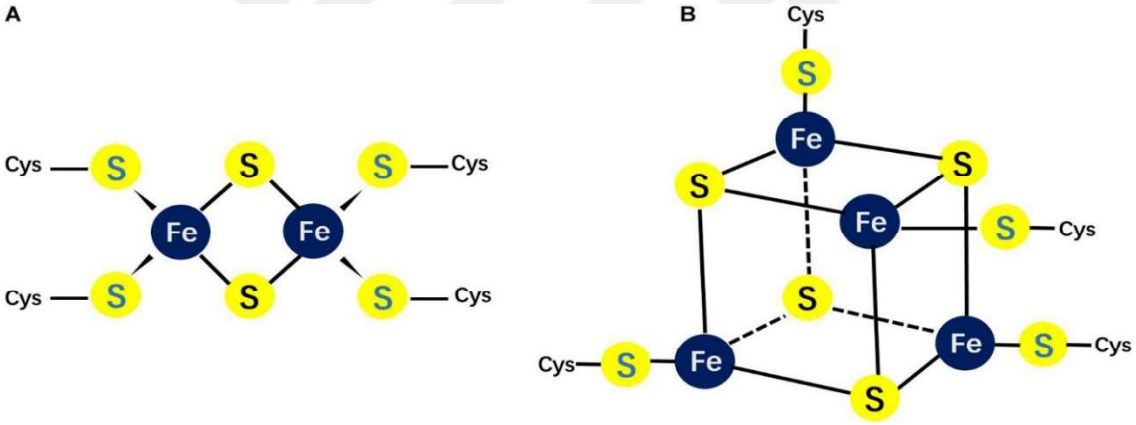

Figure 2.4 Structural shapes of Fe-S clusters (ISC) (A: Rhombic 2Fe–2S cluster; B: cubane 4Fe–4S cluster) (Shi et. al., 2021)

Data from the UniProt database (https://www.uniprot.org/) have indicated that over 200 known Fe-S proteins exist in human cell (). Johnson et. al., (2005) also reported some over 120 types of enzymes and proteins as ISC-containing proteins (Johnson et al., 2005). Bacteria are known to contain diverse Fe-S proteins (Andreini et al., 2017). Growing evidences have showed that increasing number of eukaryotic proteins actually contain Fe-S centres. ISC proteins occur mostly in the nucleus, cytosol, mitochondria and almost all eukaryotic organelles, where they perform fundamental biochemical processes. They are involved in processes as diverse as electron transfer (e.g., respiratory chain complexes), enzymatic reactions (e.g., aconitase), DNA/RNA metabolism (e.g., activities of DNA

metabolic enzymes; DNA polymerases α, δ, and ε, DNA primase, DNA2, glycosylases, Helicases, tRNA modification, etc). These broad involvements prove the versability of Fe-S proteins in all essential cellular processes. ISC also serve as cofactor of apoproteins (sulphur donors in lipoate and biotin cofactor biosynthesis). ISC proteins located in the mitochondria and nucleus, play various important role in regulation of gene expression in the cell (Volz, 2008; Netz, 2012; Arnold et al., 2016).

Any deficiencies or defects in mitochondrial ISC biogenesis can cause nuclear genomic instability (Veatch et al., 2009). Any chemical change to the Fe-S clusters was seen to have fatal consequences in living cells (Fuss vd., 2015). Impaired ISC biosynthesis can also result in mitochondrial failure, leading to a probable system failure in high energy-dependent organs such as the central nervous system, skeletal muscle, heart muscle, and liver. Clinical phenotypes of such impairment also are typical of defective oxidative phosphorylation (Vanlander ve Van Coster, 2018).

Eukaryotic Fe-S cluster biosynthesis machinery is basically classified into 3 systems; mitochondrial ISCU assembly, export machinery, and cytosolic Fe-S protein assembly (CIA) system. Currently, 9 CIA proteins and 20 mitochondrial ISC proteins are known to support major biogenesis steps, which are evolutionarily conserved from yeast to man (Lill vd., 2015). Biogenesis of Fe–S clusters initially occurred de novo in mitochondria (**Figure 2.5**) and is driven by the ISC complex machinery. Mitochondrial ISC biosynthesis pathway can be divided into at least twenty enzymatic steps (Vanlander ve Van Coster, 2018).

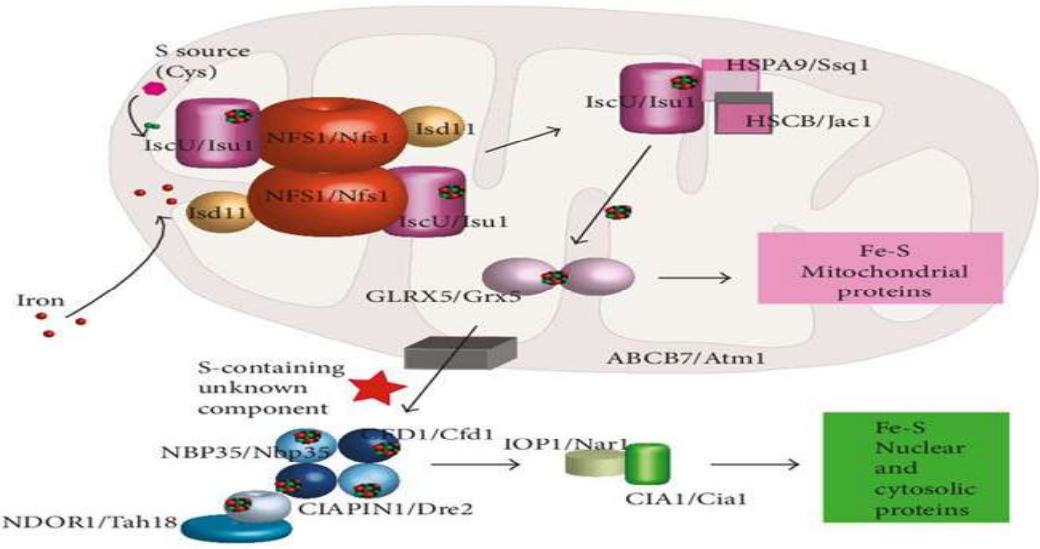

Figure 2.5. Mitochondrial Fe-S cluster biosynthesis (Vernis et. al., 2017)

Mitochondrial ISCU can be classified into three distinctive key steps; (Fe_2-S_2) cluster formation on a scaffold protein and its subsequent release by glutathione (GSH)-dependent chaperones. Further post-synthetic processing of synthesized product occurs intramitochondrially, then exported to the cytosol for further CIA processing and extramitochondrial Fe-S clusters assembly. The specific nature of the exported product is still unclear, but is believed to be glutathione-stabilized as ([Fe_2–S_2] ([Fe_2–S_2][GS]$_4^{2-}$) (Li and Cowan, 2015). Mitochondrial-synthesized and functional clusters are supplied via ABCB7 (an IMM exporter) protein (Lill et. al., 2015), to the cytosolic Fe-S cluster assembly as key precursor products. ABCB7 in conjunction with **ALR** (a FAD-dependent sulfhydryl oxidase), works to mediate this product export. An experimental study in yeast showed that absence of ALR did not impair the cytosolic Fe-S cluster assembly (Ozer et. al., 2015), bringing the functional role of ALR in the export system into dispute.

Mitochondrial machinery can synthesize (Fe_2–S_2) or (Fe_4–S_4) clusters and incorporate them into the appropriate apoproteins. Several proteins in the MM are purposely dedicated to the ISC synthesis. Fe-S protein biogenesis starts in the MM and involves the ISCU (scaffold protein) assembly machinery (Ye et. al., 2010). Mitoferrin-1 (SLC25A37) and Mitoferrin-2 (SLC25A28) transport ferrous iron (Fe) from the IMM into the mitochondrial matrix. SLC25A37 is abundantly found in erythroid cells while SLC25A28 is ubiquitously expressed in all cells (Paradkar et. al., 2009). How the iron is built into the Fe-S scaffold is yet to be found but in humans, frataxin (FXN) is known to bound the ferrous iron in the MM, playing partially an essential role. Iron-dependent ISC biosynthesis is basically dependent on FXN-ISCU molecular interaction (Stemmler et al., 2010).

NFS1 encodes the mitochondrial cysteine desulphurase enzyme in humans. In conjunction with subcomplex with ISD11, NFS1 provides the sulphur (S) component needed for the ISC biosynthesis (Boniecki vd., 2017; Cory vd., 2017). NFS1 converts cysteine into alanine, producing a persulphide used for the cluster formation on ISCU. NFS1-ISD11 interaction is necessary for desulphurase activity. FXN binds to the NFS1-ISD11-ISCU complex to donate the iron or activate transfer of sulphur to the Fe-S cluster assembly (Tsai and Barondeau 2010).

Ferredoxin reductase and ferredoxin as electron transfer chain, are also involved in cluster formation. ISCU initially forms Fe_2-S_2-containing (Fe_2-S_2) clusters, which are

released by HSP70-HSC20 chaperones and GLRX5 (monothiol glutaredoxin) and utilized for assembly of (Fe_2-S_2) proteins. For assembly of larger clusters such as (Fe_4-S_4, Fe_3-S_4), the molecular involvement of ISCA1, ISCA2, and IBA57 is necessary. Clusters formed, are donated to the recipient apo-enzymes (apo-proteins) such as the respiratory complexes, aconitase, and lipoate synthase via targeting factors such as IND1, NFU1, and BOLA3 (Bandyopadhyay et al., 2008; Lill, 2009).

Also, the CIA targeting complex involved in transfer and addition of Fe-S cluster to target apoproteins includes the Cia1 (human CIAO1) and Cia2 (also called human CIA2B, FAM96B, and MIP18) (Netz et al., 2014; Paul and Lill, 2014; Stehling et al., 2012).

Functional role of these proteins such as FAM96B (YHR122W, CIA2 in yeast) and MMS19 remain unclear, but are believed to show selective Fe-S cluster specificity for certain diverse Fe-S target apoproteins only. Through direct interactions, they selectively bind with many proteins involved in maintaining nuclear DNA integrity such as DNA polymerases (POLD1), primases, helicases of the DNA repair pathway (XPD or FANCJ) and of telomere maintenance (RTEL1) (Rouault, 2012; Lill et al., 2012).

MMS19 (and MIP18) as two major proteins, are physically and functionally associated with Fe-S cluster biogenesis, DNA replication and repair. As part of the mitotic spindle-associated MMXD complex, MMS19 in transferring Fe-S cluster assembly to ERCC2/XPD may be involved in chromosome segregation (Ito et al., 2010). In conjunction with Cia2, it promotes the progression of mitosis by enabling Fe-S clusters transfer to the motor protein KIF4A. This ensures KIF4A is correctly localized to the mitotic machinery components (Ben-Shimon et al., 2018). Cells with mutated MMS19 were observed to be hypersensitive to DNA damage and had elongated telomeres. Therefore, these target proteins seem relevant to various cancer-related diseases and aging.

Studies showed that MMS19 is required not only for the Fe-S cluster transfer to ISC apo-proteins, but for the stability of multiple Fe-S-containing DNA replication and repair proteins. MMS19 is a multiprotein complex and key component of the cytosolic Fe-S protein assembly (CIA) complex, mediating the insertion of the Fe-S cluster into DNA metabolic enzymes apoproteins (Ben-Shimon et al., 2018). MMS19 in linking the CIA components to Fe-S proteins target (ERCC2/XPD, FANCJ, and RTEL1), it enables DNA replication and

RNA polymerase II (POL II) transcription in nucleotide excision repair (NER) during homologous recombination-mediated double-stranded DNA breakage repair (Stehling et al., 2012; Gari et al., 2012; Seki et al., 2013; Weon et al., 2018).

It is clearly visible that Fe–S clusters are indispensable for DNA/RNA enzyme activity, but their role in processing nucleic acids is still elusive and remain to be fully deciphered. Despite the risk of iron toxicity to DNA damage, cellular preference for ISC complex right in the centre of DNA metabolism elicits a lot of interest.

Liver regeneration seems to be a convergence point for hepatocyte proliferation, cellular metabolism (essential metabolites/anabolites) and ATP production, linked to DNA metabolism. In cellular ATP production and metabolism coupled to DNA metabolism, is the possible molecular roles ISC proteins may play. Basically, this study sought to find some pressing answers to the molecular mechanisms underlying the expressions of different Fe-S genes and proteins at various time points (0, 6. 12. 24, 48 and 72 hrs) encompassing the whole regenerative period of liver regeneration. Molecular interactions of ISC proteins with other key genes involved in cellular signalling pathways during the different phases of liver regeneration is worthy of research.

2.6. Selection of genes of interest

Fe-S genes and proteins and associated genes with known cellular and biological functions, were selected and examined for their expressions at 0, 6, 12, 24, 48 and 72 hours of liver regeneration. These time points are representative of the 3 phases of liver regeneration.

In selection of genes of interest for examination of gene and protein expression in this study, their cellular functions were the possible molecular effects of the genes in liver regeneration known cellular functions The selected genes as depicted in (**Table 2.1**); have their full and cellular functions outlined.

Table 2.1. Selected Fe-S genes with associated genes

Genes of interest		
Abbrev.	Full name	Cellular and Biological functions
PGC-1α	PPAR)-γ coactivator-1α	Mitochondrial biogenesis,
MitoNEET (CISD1)		JAK/STAT-3 signalling, iron and ROS metabolism,
HIF-2α	Hypoxia inducible factor-2α	MAPK and HIF signalling, ROS and Iron metabolism
ACO1 /IRP1	Cytosolic aconitase/Iron Regulatory	Metabolic enzyme, regulation of iron metabolism
Mitoferrin		Iron transfer to mitochondria
MINER1 (CISD2)	Mito-NEET-related protein 1	Apoptosis, Autophagy, Ca^{2+} signalling
SDHA	Succinate Dehydrogenase enzyme subunit A	Succinate oxidation, succinate-fumarate conversion, Mitochondrial marker
C-MYC	Cellular MYC	Cellular proliferation and metabolism
ACO2	Mitochondrial aconitase	Mitochondrial TCA cycle enzyme
GLRX5	Glutaredoxin 5	ISC biosynthesis, Iron and ROS metabolism, antioxidant

2.6.1. Peroxisome proliferator-activated receptor (PPAR)-γ coactivator (PGC-1α)

Rapid cellular division demands high energy to power essentially cellular proliferation. Increased energy needs of the cells warrant increased mitochondrial mass (MB) in response to energy demands during hepatocyte proliferation. Turnover rates in mitochondria in the liver are remarkably higher than in other tissues. To meet these metabolic demands of the cells, MB must adapt the mitochondrial morphology in a fashion that utilizes mitochondrial fusion to increase metabolic efficiency while mitochondrial fission works in favour of uncoupled respiration (Jacobi et. al., 2015).

High cellular energy demand and stress stimulate mitochondrial fusion. Mitochondrial fusion enables effective sharing of components between functional

(‘‘healthy’’) and defective (‘‘sick’’) mitochondria, to correct defective mitochondrial DNA or protein complexes for efficient and maximum ATP production. Mitochondrial fission may occur both under stress-free or highly-stressed conditions. Mostly occurring under stress-free condition, mitochondrial fission separates newly-generated mitochondria from dysfunctional ones, to ensure a healthy mitochondrial network is maintained. However, in a highly-stressed cellular conditions, mitochondrial fission occurs to activate apoptosis (Youle and Bliek, 2012; Aouacheria et. al., 2017; Trotta and Chipuk, 2017). MB is therefore a mitochondrial adaptive mechanism, acting to repair mitochondrial insults in the mitochondrial strive to sustain energy homeostasis.

PGC-1α is a master regulator of mitochondrial biogenesis and metabolic processes (Sahin et. al., 2011; Luo et. al., 2016; Dumesic et. al., 2019). It is a transcriptional coactivator and inducer of nuclear receptors under different physiological and dietary conditions to ensure required cellular energy needs are met (Finck and Kelly, 2006). The PGC-1α-induced nuclear receptors are largely made up of PPARs (PPAR-α, PPAR-γ, and PPAR-δ) family. PPAR-α is important for adipogenesis and differentiation. PPAR-δ and PPAR-γ have essential roles in regulation of fatty acid oxidation. PPAR-γ is also a target of thiazolidinediones (TZDs). Liver regeneration after PHx in PPARα-knockout mice was found to be impaired, probably because of disordered hepatic lipid metabolism, cell cycle control and cytokine signalling (Hu et. al., 2019; Anderson et. al., 2002).

PGC-1α as a transcriptional coactivator, indicates indirect PGC-1α-nucleus binding. However, through interaction with some nuclear transcription factors, PGC-1α can coordinate major processes that lead to the induction of expressions of both mitochondrial and nuclear genes (NRFs) necessary for MB (Scarpulla, 2008). Particularly, PGC-1α was shown to induce nuclear respiratory factor 1 in the expression of ETC-encoding genes **(Figure 2.6)** (Baker et al., 2007; Scarpulla, 2008).

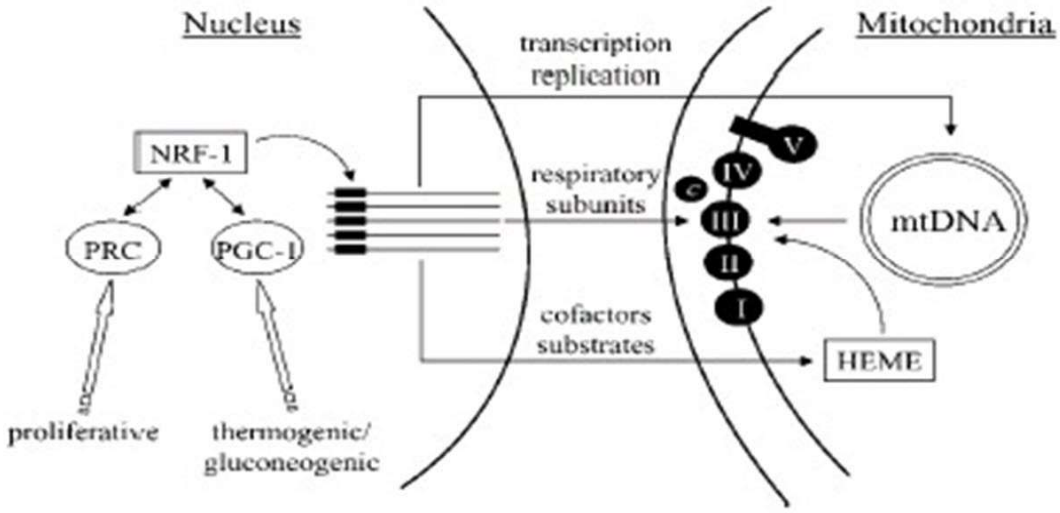

Figure 2.6 PGC-1α-Nrf-1 cross-communication in MB (Scarpulla, 2000-2013)

Importantly, PGC-1α itself can be activated by either the AMP-activated protein kinase (AMPK) or Sirt-1-mediated direct PGC-1α phosphorylation (Jager et. al., 2007). Sirtuin-1 can equally promote NAD^+-dependent PGC1α activation (Canto et. al., 2009). Thus, mitochondrial dynamics tightly regulates mitochondrial fission-fusion in such a manner that not only an orderly-organized mitochondrial network but the net mitochondrial morphology is carefully achieved (Chan, 2006). MB may be very fundamental to the cell, as to be considered a critical determinant of cell's fate, under certain cases (Jornayvaz et al., 2010; Scarpulla, 2008).

In fact, impairment of MB is thought to contribute to several forms of tissue injury. In the liver, there is some evidence that the stress caused by chronic ethanol feeding can induce MB (Han et al., 2012), though it is not yet clear what role this plays in alcohol-induced liver injury. In extrahepatic tissues, kidney cells treated with pro-oxidants showed increased MB after the initial stress, and overexpressing Pgc-1α enhanced recovery of mitochondrial function (Rasbach et al., 2007).

Pharmacologically inducing MB was observed to enhance tissue regeneration and recovery in various rodent models of acute kidney injury (Rehman et al., 2013; Whitaker et al., 2013; Funk and Schnellmann, 2013; Jesinkey et al., 2014; Garrett et al., 2014; Khader et al., 2014) as well as other models of tissue injury (Finck and Kelly, 2007; St-Pierre et al., 2005; Funk et al., 2010). It suffices to imply an upregulation of PGC-1α in the liver following

a PHx procedure, fatty acid and β-oxidation, and gluconeogenesis are increased. Thus, PGC-1α is a critical factor not only for mitochondrial biogenesis but also for a programmed activation of genes of energy production and metabolism in a regenerating liver.

2.6.2. MitoNEET (CISD1)

NEET proteins are a class of recently discovered iron–sulphur (Fe–S) cluster-containing proteins, ubiquitously found in all life. At least one highly-conserved 39-amino-acid motif called CDGSH Iron–Sulphur Domain (CISD) is characteristic of members of this protein family. CISD harbours a redox-active Fe_2–S_2 cluster, which is co-ordinated by an unusual 3-Cysteine–1-Histidine quartet, instead of the usual 4-Cysteine (Ferecatu vd., 2014; Tamir vd., 2015; Wiley vd., 2007).

MitoNEET (CISD1), MINER1 (CISD2) and MINER2 (CISD3) are the three known members of the NEET proteins in mammals (Karmi et. al., 2018; Sengupta et. al., 2018). MitoNEET and MINER2 are integral proteins of the MM with their CDGSH domain located in the cytosol, while MINER1 is an integral protein of the endoplasmic reticulum (Wiley et al., 2007). Factors underlying the basic molecular and cellular functions of NEET proteins are still unknown. However, NEET proteins are associated with fundamental cellular processes of RS homeostasis, Fe-S biogenesis, gene regulation, Ca^{2+} signalling (homeostasis), apoptosis, autophagy and aging, just to mention a few. Regulatory mechanisms behind these cellular roles are still unknown (Ferecatu vd., 2014; Kusminski vd., 2012; Landry vd., 2014; Salem vd., 2012; Sohn vd., 2013).

MitoNEET as a Fe_2–S_2 cluster-containing integral membrane protein of the outer mitochondrial membrane, it partakes in the cellular redox-sensitive signalling and Fe–S cluster transfer. It is a major regulator of mitochondrial oxidative capacity and iron homeostasis (Paddock et. al., 2007; Wiley et. al., 2007; Geldenhuys et. al., 2014). MitoNEET was shown to regulate cellular energy use, lipid metabolism, cancer cell proliferation and tumor formation (Kusminski et. al., 2012; Kusminski et. al., 2012; Sohn et. al., 2013). MitoNEET when oxidized, was seen to enable Fe_2–S_2 cluster transfer to apo-proteins, and consequently the released electrons from FMNH2 (reduced 1,5-dihydro form of flavin mononucleotide) passed on to oxygen or ubiquinone in mitochondria (Wang et. al., 2017; Li et. al., 2018; Tasnim et. al., 2020).

Ferecatu et. al., (2014) reported mitoNEET could repair oxidative insults to the ACO1/IRP1 complex. IRP1 mediates the iron metabolism-oxygen sensing cross communication. ALR is employed to stabilise mitoNEET and facilitate Fe-S cluster transfer (Ferecatu et. al., 2014). A similar study in drosophila was recently reported (Ganz et. al., 2005; Huynh et al., 2019) in which the CISD2 (Drosophila homologue of mitoNEET) together with AGBE (in human GBE1 [glycogen branching enzyme]) repairs the Fe-S cluster of the IRP1. CISD2-AGBE-IRP-1A was observed to be an important metabolic axis in Drosophila (Hernández-Gallardo and Missirlis, 2020).

MitoNEET is also known to interact with some other proteins relating to iron and redox metabolism, Fe-S biogenesis, metabolic enzyme and mitochondrial integrity proteins (Mittler et. al., 2019). Anamorsin (Lippper et. al., 2015) in iron regulation, glutathione reductase (Landry et. al., 2015) in redox regulation, glutamate dehydrogenase 1 (Roberts et. al., 2013) in metabolism and insulin regulation, and PARKIN (Kusminski et. al., 2016) in mitochondrial quality control. PARKIN is an activator protein in mitophagy (Kusminski et. al., 2012), and a mitoNEET-PARKIN interaction may function in regulation of mitochondrial stability.

ALR-mitoNEET crosstalk was shown to be a key molecular factor in the early phase of liver regeneration (Nalesnik et al., 2017). ALR was observed to promote in Kupffer cells the productions of IL-6, nitric oxide (NO) synthase (NOs) and TNF-α, in a concerted move to activate the STAT3 and MAPK signalling pathways (Nalesnik et al., 2017; Ibrahim and Weiss, 2019). What would a possible ALR-mitoNEET-IRP1 axis unravel in fundamental cellular processes? For mitoNEET is a known regulator of mitochondrial iron content, and its place in cellular iron metabolism, oxygen sensing and ROS, and may as well have been established.

Landry et. al., (2015) revealed that without disrupting the Fe_2-S_2 cluster, H_2O_2 could reversibly oxidized the reduced mitoNEET (Fe_2-S_2) clusters of a mouse heart cell extracts. This pre-suggesting that the mitoNEET (Fe_2-S_2) clusters response to oxidative signals via a redox transition undergo redox transition, as part of the mitochondrial regulation of energy metabolism (Landry et. al., 2015). In some previous studies, mitoNEET-overexpressing mice were observed to reduce mitochondrial ROS formation. However, deletion, absence or knock out of mitoNEET significantly upregulated oxidative phosphorylation and electron

transport (Victor et. al., 2009; Kusminski et. al., 2014). Such a role in mitochondrial ROS formation in conjunction with mitochondrial dysfunction (John et. al., 2011), presumably involves mitoNEET in human diseases such as diabetes, neurodegeneration, muscular atrophy and others (Taminelli et. al., 2008; John et. al., 2011; Chen et. al., 2019). In type-2 diabetes therapeutics, MitoNEET is identified as the binding domains in mitochondria for the thiazolidinediones (TZDs), insulin sensitizer drugs (Geldenhuys et. al., 2010; Colca et. al., 2004; Colca et. al., 2013).

Sepsis-mediated ROS excessive production is central to the disease process (Victor et. al., 2009). Thus, mitoNEET was seen to demonstrate anti-inflammatory and antioxidant behaviours in sepsis as well as other disease models (Faine et. al., 2011; Zhu et. al., 2011; Logan et. al., 2015). Inhibiting expression or activity of mitoNEET reduces inflammation and oxidative stress during inflammatory responses and sepsis.

2.6.3. Hypoxia inducible factor-2α (HIF-2α)

As a key member of the cellular (oxygen) O_2 signalling system. Hypoxia-inducible transcription factors (HIFs) belong to the Per-ARNT-Sim (PAS) family of basic-helix-loop-helix (bHLH) transcriptional factors, known to be very active in metabolism, angiogenesis, erythropoiesis, cell proliferation, and apoptosis in O_2-deprived states (Wenger, 2002; Schofield et. al., 2004). They regulate survival adaptations towards low O_2 availability.

Binding DNA as heterodimers, HIFs are structurally made up of two subunits: an O_2-sensitive α-subunit and the aryl-hydrocarbon receptor nuclear translocator (ARNT) as its β-subunit. The HIFs are usually identified with the α-subunit, as HIF-α subunits: HIF-1α, HIF -2α, and HIF-3α. They are known to be ubiquitinated by the pVHL (von Hippel-Lindau (VHL) tumour suppressor) in conditions of normoxia for proteasomal degradation. The loss of which stabilises the HIF-**α** subunits and constitutively activate the HIF signalling (Wang et. al., 1995; Ryan et. al., 1998).

HIFs are activated in an oxygen (O_2)-dependent manner or O_2-independent mechanisms via other cellular stresses and molecules (Majmundar et al., 2010). HIFs are oxidized by prolyl-hydroxylases (PHDs) under normoxia to cause their degradation, while low O_2 pressure would inhibit the hydroxylase activity, causing HIF stabilization and activation.

The liver expresses HIF-1**α**, HIF-2**α**, and HIF-3**α**, under various conditions of patho-physiology, showing HIFs as important mediators in normal liver function and disease. Physiologically, they are active players in liver regeneration and their active involvement in liver disease development such as fibrosis and hepatocellular carcinoma (HCC) are known (Nath, 2012). Angiogenesis, a term used for the reconstitution of the blood vessels, is an important process of tissue repair and regeneration. VEGF is an important angiogenic growth factor and HIFs their primary transcriptional inducers. During liver regeneration, the regenerating hepatocytes essentially provides its own VEGF (Shimizu et. al., 2001; Taniguchi et. al., 2001; Ding et al., 2010). The molecular mechanisms underlying such a timely hepatocyte's VEGF production during regeneration, and its probable molecular communication with HIFs in this cellular process, are still unknown (kron et. al., 2016).

Upon a sustained hypoxic stimulus, HIF-1a expression was seen to peak at 1 hr but drops to undetectable concentrations in 3 hrs. However, HIF-2a expression was observed much later, expressed continuously at the 3-6 hrs, exhibiting a coordinated cellular response to hypoxia by these two subunits (Weisener et. al., 2003). In mouse model, deletion of HIF-1α delayed the initial process of liver regeneration (Tajima et. al., 2009), while global PHD1 knockout accelerated hepatocyte cell cycle (Mollenhauer et. al., 2012), implying HIF-1α may be a promoter of hepatocellular proliferation. HIF-1α specifically activates glycolytic enzyme genes (Patel and Simon et. al., 2008). Some of the HIF-1α-activated glycolytic genes include phosphoglycerate kinase (Hu et. al., 2003), lactate dehydrogenase (Wang et. al. 2005), carbonic anydrase-9 (Raval et. al., 2005) and BNIP-3 (Grabmaier et. al., 2004).

But HIF-2α selectively activates VEGF, transforming growth factor-α (TGF-α), lysyl oxidase, Oct4 and Cyclin D1 (Baba et al., 2003; Covello et al., 2006; Erler et al., 2006; Gunaratnam et al., 2003; Hu et al., 2003; Wang et al., 2005).

In a different study, Titta et. al., (2019) also showed a similar increase in HIF-1α mRNA expression on day 1 of house gecko tail regeneration model, with leucocyte and erythrocyte cells domination in the tissue. HIF-1α mRNA expression was observed to reach its peak, with active cellular proliferation, migration and differentiation. In a simultaneous fashion, as HIF-1α expression decreases, HIF-2α mRNA expression and overall cellular activity increases. A more gradual but steady increase usually characterize HIF-2α expression (Titta et. al., 2019). While HIF-1α can be seen to be active initiators and

promoters in tissue regeneration process, HIF-2α primarily can be described as functional drivers of the proliferative process after the demise of HIF-1α to O_2 availability. Thus, HIF-1α and HIF-2α an play an overlapping role in overcoming tissue hypoxia.

HIF2 via various mechanisms, is known to upregulate the hepatic lipid biosynthetic pathways and lipid droplet surface protein ADFP, while suppressing the fatty acid β-oxidation (Rankin et. al., 2009). Qu et al., (2011) further provided proofs of dominant regulatory role of HIF-2α in hepatic lipid metabolism in vivo (Qu et al., 2011). HIF-2α inactivation suppressed hepatic steatosis development. These unique functions of HIF-2α in metabolic adaptation to hypoxia may be potential therapeutic target for fatty liver diseases (Rankin et. al., 2009). In fetal erythropoiesis and erythropoietin (EPO) production, hepatic HIF-2α is prerequisite, while constitutive HIF-2α activation in the adult liver causes polycythemia and vascular tumorigenesis (Rankin et. al., 2007; Rankin et. al., 2008).

HIF-2α has a mRNA with an IRE in the 5'-UTR. Similarly, to ferritin H IRE, an unusual sequence in HIF-2α IRE (**Figure 2.8**) shows a functional binding affinity functional to IRP1. HIF-2α preferential affinity to IRP1 (Zimmer et. al., 2008), may explain the observation that IRP1 translationally regulate HIF-2α in the kidney. Cellular iron levels also regulate the translation of HIF-2α mRNA, playing HIF-2α within the remarkable IRP-dependent Fe-O_2 metabolism (Sanchez et. al., 2007). HIF-2α IRE-IRP1 molecular interaction is affected by both oxygen and iron availability. Hypoxia not normoxia, may probably disturb the IRE–IRP1, even in iron availability (Zimmer et. al., 2008). Because under hypoxic conditions, Fe_4–S_4 clusters more readily form and inactivates IRP1 (Hanson and Leibold, 1998).

Similarly, hypoxic conditions also may cause HIF-2α stabilization, an active stimulus of EPO transcription. In conditions of deprived iron, HIF-2α mRNA translation may attenuate, disrupting the EPO synthetic machinery. Thus, IRP1 functionally regulate the Fe-O_2-sensing to HIF-2α expression, in control of erythropoiesis (Anderson et. al., 2013; Ghosh et. al., 2013; Wilkinson and Pantopoulos, 2013).

2.6.4. Iron Regulatory Protein 1 or Aconitase gene (IRP1/ACO1)

Cellular iron is an important player in various major fundamental biological mechanisms of energy metabolism, DNA synthesis, erythropoiesis, oxygen supply,

antioxidant system, inflammation, mitochondrial biochemistry and several others. Its functional versatility can only indicate a well-coordinated iron regulation in sync with other key metabolic regulatory pathways (Kuhn, 2014).

Mammals have developed sophisticated mechanisms to maintain appropriate iron concentrations *in vivo.* Iron homeostasis in mammals is mainly regulated by a set of interlocking regulatory systems, including: hepcidin–ferroportin (FPN1)-mediated regulation of serum iron levels, iron regulatory proteins (IRPs)/iron-responsive element (IRE)-mediated regulation of intracellular iron homeostasis and hypoxia inducible factor-2α (HIF-2α)-mediated transcriptional regulation (Zhang et. al., 2014).

Proliferating and neoplastic cells were observed to display an unusually high affinity for iron and thus altered IRPs expression. Interleukin-6 (IL-6)-mediated inflammation equally alters iron metabolism (Sheikh et. al., 2007). IL-6 is a key player to initiate the liver cell proliferation after hepatectomy and needed for proper control of metabolic functions (Cornell et. al., 1990; Schmidt-Arras and Rose-John, 2016).

IRP1 acting as Fe-S protein sensor, regulates cytosolic iron metabolism in mammalian cells (Rouault, 2006; Muckenthaler et al., 2008). Based on whether it is composed of a (4Fe-4S) cluster or not, IRP1 exists in varying conformational forms. The first form of IRP1 (holo-IRP1 enzyme) has three iron atoms attached to its conserved cysteine residues, with the exposed fourth iron atom catalysing the citrate- isocitrate conversion. It thus functions as a cytosolic aconitase in this form (Philpott et. al., 1994). In the second form, the exposed ferric iron of the cluster enters into a reaction with superoxide or peroxynitrite, causing its removal as ferrous iron (Hausladen and Fridovich, 1994; Brazzolotto et. al., 1999). The (3Fe-4S) cluster form of IRP1, is thus an enzymatically inactive intermediate (Brown et. al., 2002) which necessitates cluster repair to either the first form or total breakdown. Processes to undertake either decision are tightly-controlled and never spontaneous. The (2Fe-2S) cluster-containing mitoNEET protein is essentially involved in the repair process (Ferecatu et al., 2014). Form 3 presents an open conformation of the protein (the apo-form) (Walden et al., 2006), binding the IRE stem-loop mRNA structures present in several iron transcripts. Such a IRP1-IRE bond enables more cellular iron uptake while reducing iron storage, to meet increased cellular iron demands during rapid proliferation.

After IRP1-IRE bonding, translation of multiple transcripts with IRE near the 5′-end region such as Ferritin H and L transcripts, HIF-2α, erythrocytic ALAS and ferroportin, are repressed by IRP1. Contrarily, IRP binding to IREs at the 3′-UTR protects the endonucleolytic degradation mRNA transcripts and transferrin receptor 1 is the most well-known such transcript. Apo-IRP1 undergoes a large conformational change that creates a complex IRE-binding pocket, in which the bulge C of the IRE binds to a pocket in domain 4, and three residues of the loop make finger-like binding projections into regions of domain 3 that become accessible after conformational change. The length of the upper base-paired stem of the IRE derived from NMR structural solution (Addess et al. 1997) optimizes the distance between the main IRP contact points, the unpaired C of the stem, and residues A15, G16 and U17 of the loop, resulting in high affinity binding (**Figure 2.7**).

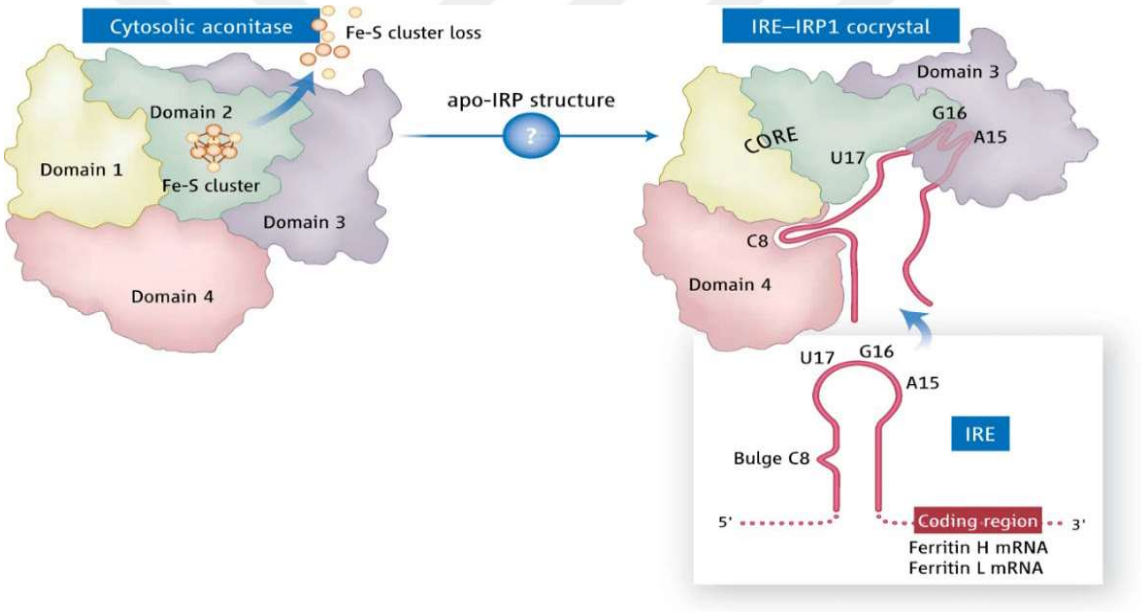

Figure 2.7 ACO1-IRP1 interchanging states (based on cellular physiological needs and iron bioavailability) (Rouault, 2006)

It has been observed in many animal cells that the need to absorb to enough iron through the transferrin receptor (TfR) is balanced against the hazardous reactivity of free iron atoms with the iron storage protein ferritin. Cellular iron homeostasis is maintained by post-transcriptionally regulating genes encoding TfR and ferritin through the IRP-IRE system. In cells lacking insufficient bioavailable iron, IRP1 usually loses its [4Fe-4S] cluster taking (apo-IRP1) form. This conformational change permits binding to IRE stem-loops in the 3′ untranslated region (UTR) of TfR mRNAs, stabilizing these transcripts to produce

more TfR. Apo-IRP1 also binds to the 5' UTR of various mRNAs, including transcripts encoding ferritin subunits and ferroportin (the primary cellular iron exporter), blocking their translation. The net effect of these interactions is that cells will soon capture more transferrin-bound iron, while they diminish their capacity to store this iron in ferritin or export it through ferroportin (Sheftel et. al., 2010; Kuhn, 2015; Muckenthaler et. al., 2017). This response is appropriate when iron is needed for other purposes in the cell, namely to furnish multiple iron cofactor-containing enzymes.

IRP1 fundamentally, can therefore be said to exist between the iron regulatory apo-state (IRP1-IRE) which binds iron transporters mRNAs (eg., ferritin); and the enzymatic cytosolic aconitase state of the Fe_4-S_4 cluster-containing holo-form.

Numerous studies relating to iron metabolism have identified various IRE-containing mRNAs (**Figure 2.8**), located either in 5' or 3'-UTR (Kuhn, 2014). Similar to ferritin H and L mRNA (Rouault, 2006), mRNAs of erythroid 5-aminolevulinate synthase (Cox et. al., 1991; Dandekar et al., 1991), ACO2 (Kim et. al., 1996; Schalinske et. al., 1998), ferroportin (Abboud and Haile, 2000; Mckie et. al., 2000), HIF-2α (Sanchez et. al., 2007; Zimmer et. al., 2008) and Drosophila's succinate dehydrogenase subunit B (SDHB) (Kohler et. al., 1995; Melefors, 1996) were shown to harbour their IREs in the 5'-UTR and iron-regulated. But in the duodenum and erythroid cells, non-IRE splice variant forms of ferroportin were observed to bypass this iron-control mechanism (Cianetti et. al., 2005; Zhang et. al., 2009).

Some spliced-DMT1 variants of have their mRNA with IREs in 3'-UTR (Gunshin et. al., 2001; Hubert and Hentze, 2002), enabling higher mRNA expression in iron-deprived conditions (Gunshin et. al., 2001), in similarity to TfR1. IRP-IRE molecular interaction may result in a stabilized mRNA. An intestine-specific IRP-deleted mouse showed decreased TfR1 mRNA expressions (Galy et. al., 2008). Like TfR1, tissue-specific availability of non-IRE-spliced DMT1 variants may determine its protein levels.

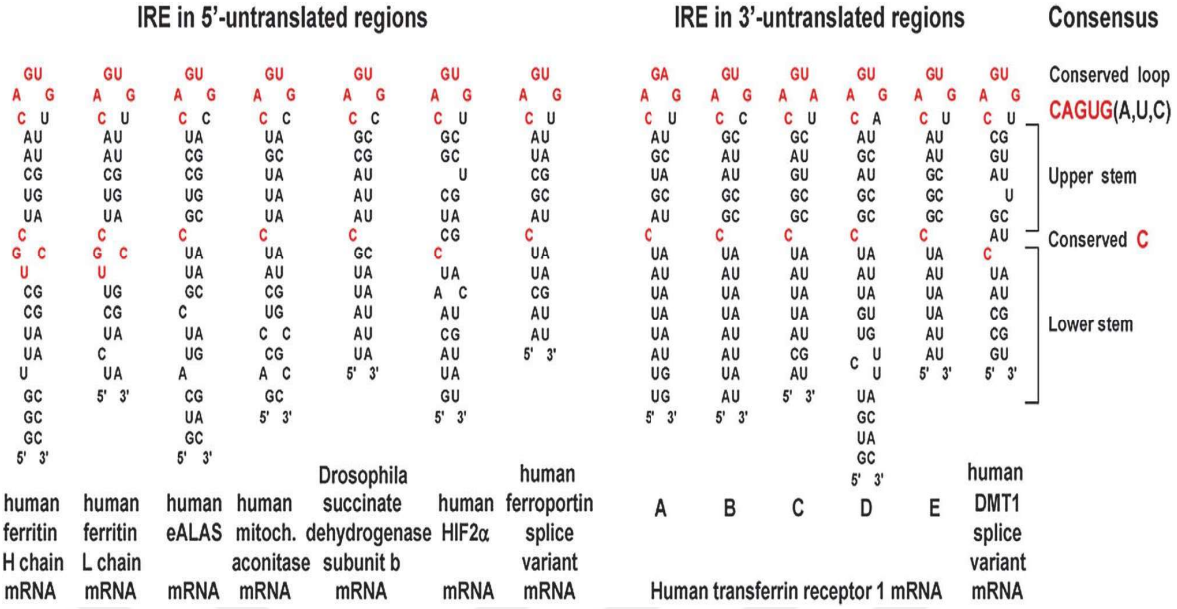

Figure 2.7b. IREs of some well-known iron metabolism-related mRNAs (Kuhn, 2014)

IRP1 was found to essentially link the mechanism oxygen-sensing which induces HIF-2α expression with an iron-sensing mechanism to control erythropoiesis. In an IRP1 This may probably be the main reason for the preferential IRP1 binding of HIF-2α- has been substantially shown to selectively bind IRP1 (Kuhn, 2014).

A recent study in Drosophila showed CISD2 (Drosophila homologue of mitoNEET) to be a functional player in the AGBE-IRP1 physical interactions. AGBE is a single orthologue of the glycogen branching enzyme (GBE) 1 in vertebrates (GBE1) (Huynh et. al., 2019). Similar human GBE1-IRP1 physical interactions was also revealed (Stark et. al., 2006; Wan et. al., 2015). However, the possible role of CISD2-GBE pathway (**Figure 2.9**) in the functional repair and shifting of IRP-1A between states, was recently reported in (Hernández-Gallardo and Missirlis, 2020). Huynh et. al., (2019) also showed that nuclear translocation of Fe_4-S_4-IRP-1A downregulate iron metabolism-related genes, thus a declined iron uptake. These outcomes may indicate a new role for iron metabolism in modulation of glucose metabolism.

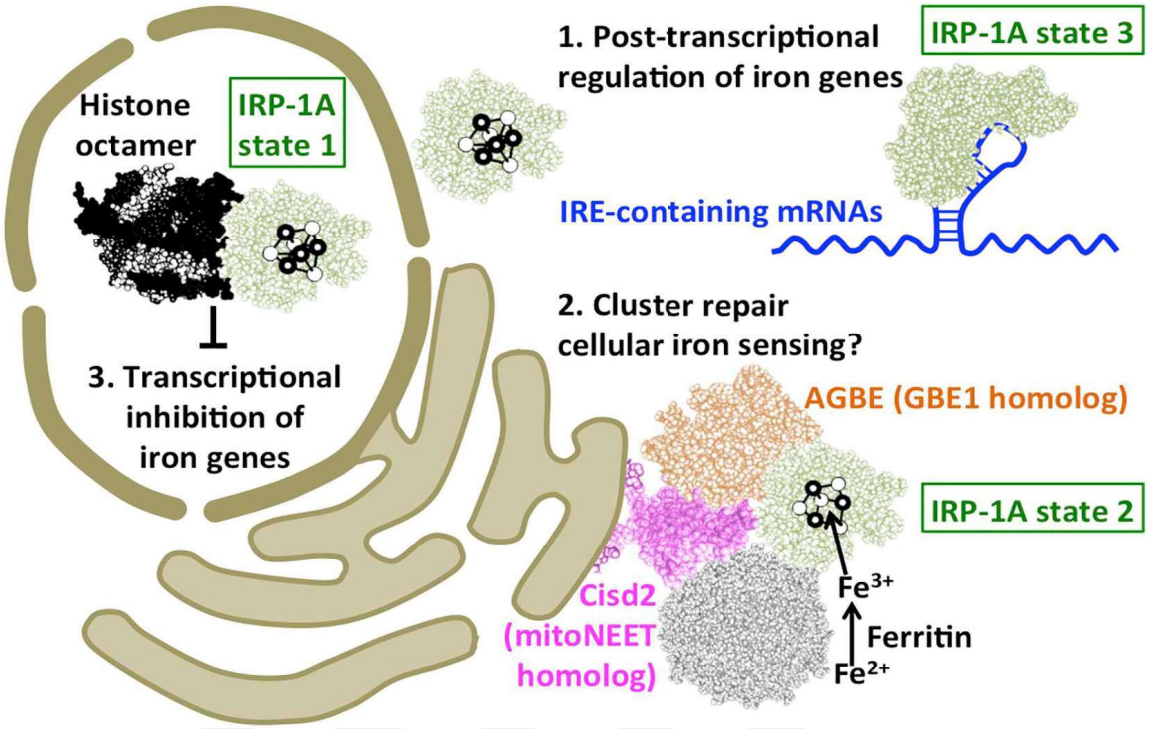

Figure 2.9 IRP-1A-mitoNEET-AGBE1 cross-communication (Waterhouse et. al., 2018; Hernández-Gallardo and Missirlis, 2020)

As depicted in (Figure 2.8.) above; Hernández-Gallardo and Missirlis, (2020) hypothesized that (i.) apo-IRP-1A (IRP-1A state 3) may bound IREs (IRE-IRP-1A) to induce expression of iron transporter genes with the net effect of preparing cells to receive iron (Lieu et. al., 2001; Nemeth and Ganz, 2006; Muckenthaler et. al., 2017). (ii.) Fe_3-4s-IRP-1A (state 2), as a result of oxidative damage may either disassemble or be repaired by CISD2-AGBE complex (Ganz et. al., 2005; Huynh et al., 2019). (iii.) IRP-1A (state 1) upon locating to the nucleus, binds histones to downregulate iron genes transcription reducing iron uptake (Ganz et. al., 2005). Ferritin was proposed to be partake in the CISD2-AGBE-mediated IRP-1A repair process as iron sensor (Hernández-Gallardo and Missirlis, 2020).

IRPs have proved to be indispensable to the overall systemic iron metabolism. IRPs-knockout was seen to embryonically lethal in mice while conditionally deleting either of the $IRP^{-1/-2}$ in the liver significantly decrease in Fe-S and heme biosynthesis, and consequently mitochondrial function due lack of iron mobilization (Galy et. al., 2010).

2.6.5. Mitoferrin gene

High iron demand during rapid cellular proliferation is essential to meet the various metabolic iron needs of Fe-S biogenesis, DNA synthesis, heme biosynthesis, redox metabolism, electron transport and many others (Ali et. al., 2022). Mitochondria plays an essential role in iron metabolism, just as iron is also fundamental to mitochondrial dynamics and functions (Upadhyay and Agarwal, 2019). Iron concentration must be tightly regulated to curb the Fenton and Haber-Weiss-mediated ROS formation (Lawen and Lane, 2013). An optimum intracellular iron concentration must therefore be tightly regulated. Thus, IRP/IRE system functions to maintain this critical post-transcriptional regulation (**Figure 2.10**), while regulation of RNA binding activity of IRPs is by intracellular iron concentrations itself (Pantopoulos, 2004; Wang and Pantopoulos, 2011). IRE is an evolutionarily conserved long hairpin-loop structure, made up of 30 nucleotides (Wilkinson and Pantopoulos, 2014; Hentze et al., 2004).

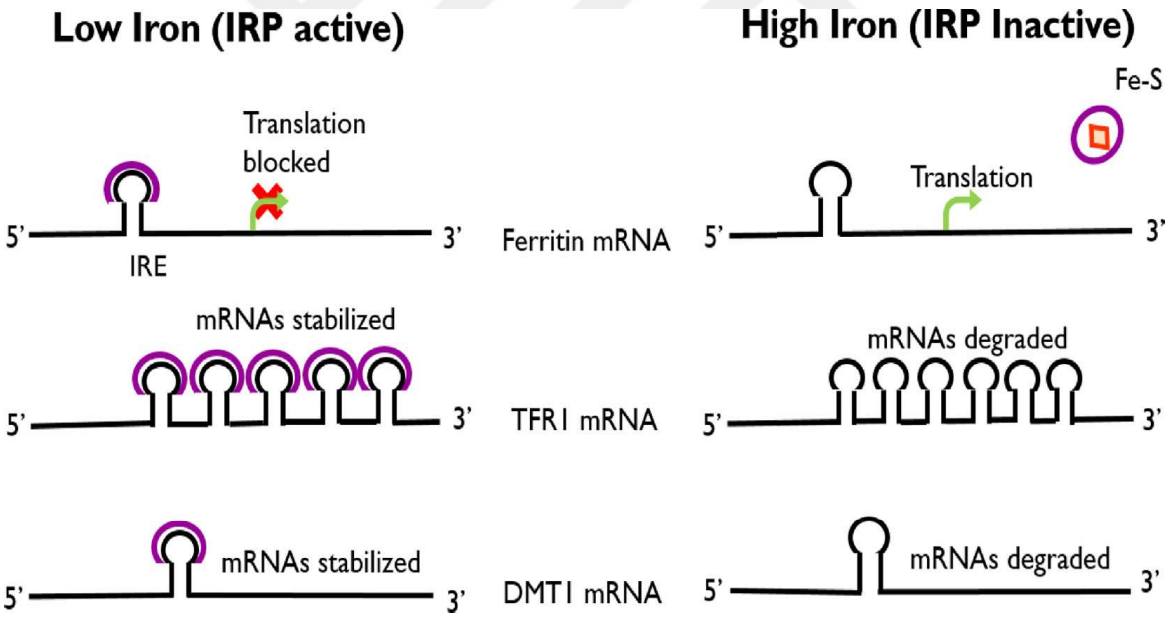

Figure 2.10 IRE/IRP system and DMT-1 regulation

Although mitochondrial iron is received from the cytosol and endosome (Richardson et al., 2010; Sherman et al., 2018), little is known about the mechanism of its delivery (Lane et. al., 2015). Previous studies have made proposals in this regard. The use of; (i) mitochondrial membrane potential (Lange et. al., 1999); (ii) voltage-dependent anion channel (VDAC) (Gincel et al.,2001; Szabo and Zoratti,2014); and (iii) DMT1 in outer mitochondrial membranous (Wolff et al., 2014; Wolff et al., 2018). Also, direct inter-

organellar iron transfer may transiently occur between the endosomes and mitochondria, in a fashion known as the ‘‘kiss and run’’ model (Sheftal et al., 2007; Das et al., 2016; Hamdi et al., 2016).

The inner mitochondrial membrane harbours the solute transfer carrier family 25, responsible for import of specific essential metabolites. Mitoferrin as a solute carrier protein, regulate the critical iron balance between the cytoplasm and mitochondria (**Figure 2.11**). In humans, the mitoferrin protein family is grouped into the mitoferrin 1 (SLC25A37) and mitoferrin 2 (SLC25A28), performing their functions in specific cell types. Many other identified eukaryotic homologues of the proteins exist (Shaw et. al., 2006; Christenson et. al., 2018). In non-erythroid cells, mitoferrins act as redundant mitochondrial iron importers required for Fe-S biogenesis and synthesis of heme-containing protein in non-erythroid cells. However, in high mitochondrial iron demand, mitoferrin 1 is essentially preferred for heme biosynthesis in the erythroid cells (Paradkar et. al., 2009).

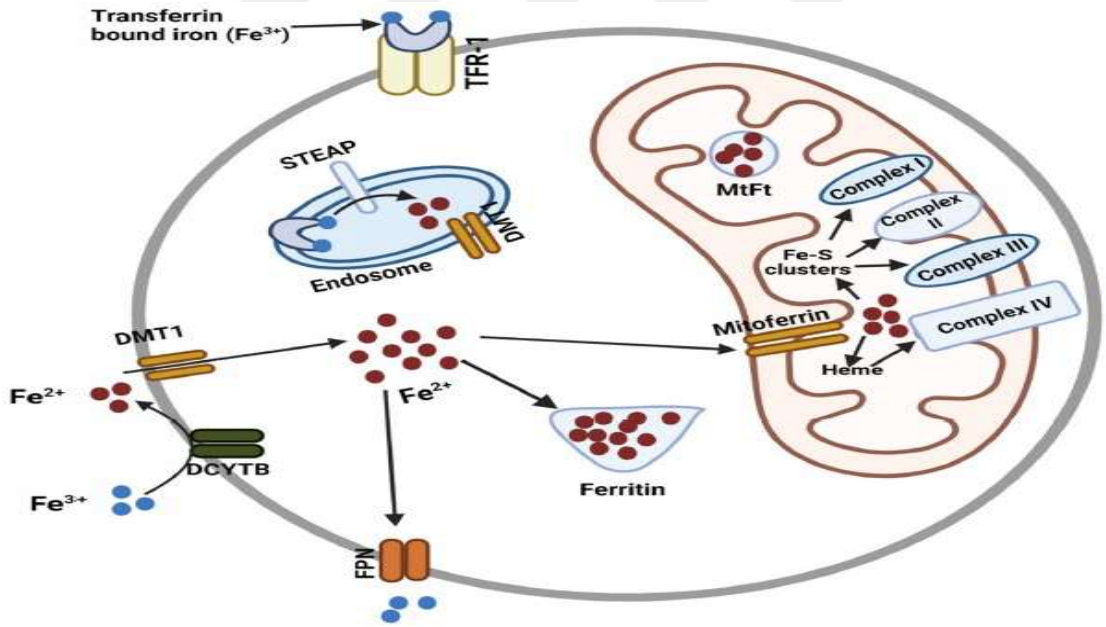

Figure 2.11 Cellular iron homeostasis, Fe-S cluster and mitoferrin regulation (Ali et. al., 2022)

High affinity for iron in active mammalian cell proliferation makes its highly dependent on iron import proteins such as the transferrin (TfR) and DMT-1. Mitoferrin plays a larger role in mitochondrial iron homeostasis. Mitoferrin loss in adult mouse hepatocytes was thus seen to seriously limit liver regeneration, owing in part to reduced iron import and availability (Seguin et. al., 2020).

2.6.6. MINER1 (NAF-1 or CISD2)

MINER1 is an integral protein of the endoplasmic reticulum (Wiley et. al., 2007). Diana Molino et. al., (2020) recently showed that MINER1 is localized at endoplasmic reticulum (ER), mitochondria, mitochondria-associated membranes (MAMs), ER-mitochondrial interface subdomains (Chen et al, 2009; Ferecatu et. al., 2014; Geldenhuys et. al., 2014; Diana Molino et. al., 2020). MINER1 is involved in cellular regulation of Ca^{2+} homeostasis, autophagy and apoptosis. While it partners Bcl-2 to regulate autophagy and apoptosis (Chang et. al., 2010; Tamir et. al. 2014), molecular binding of MINER1 to CAPN2 (calpain-2 catalytic subunit) activates a Ca^{2+}-mediated apoptosis (Lu et. al., 2014).

Both MINER1 and mitoNEET are both Fe_2-S_2 cluster donors, and mitoNEET can donate Fe_2-S_2 cluster to MINER1. Subsequently, mitoNEET-MINER1 complex may function as a relay system to transfer Fe_2-S_2-cluster between the mitochondria and cytosol (Karmi et. al., 2017).

Functionally, MINER1 (like mitoNEET) via redox mechanisms, can donate its clusters/electrons to molecular partners (BCL-2 and CAPN2) (Zuris et. al., 2011; Tamir et. al., 2015; Tan et. al., 2016). Cluster donation and redox status could cause changes in NAF-1 conformations (Bak and Elliott, 2013; Benson et. al., 2013). Thus, cluster availability and redox status were proposed to guide molecular binding of NEET proteins to interacting partners (Chang et. al., 2010; Tamir et. al., 2015).

Chang and colleagues (2010) in demonstrating how MINER1-Bcl-2 bonding functions to regulate autophagy, proposed that MINER1 holo-form may bind to Bcl-2 at the ER interphase, Bcl-2 binding to BECLIN 1 is activated and stabilized. This stops the autophagic process. On the other hand, nutrient stress may cause cluster loss in MINER1 (apo-MINER1). Apo-NAF-1 cannot bind to Bcl-2. Thus, a stabilized molecular interaction of Bcl-2-BECLIN 1 is established, which then initiate the autophagic process (Chang et. al., 2010).

Cellular mechanism of actions of NEET proteins remains largely unknown. But the cluster availability and protein's nature (holo-form/apo-form) in activation of autophagic/apoptotic pathways may underlie their cellular mechanism of actions (Tamir et. al., 2015).

Other studies however, showed that suppressing MINER1 expression and thus the resulting low MINER1 protein levels, also activated the autophagic or apoptotic process (Sohn et. al., 2013; Holt et. al., 2016). At low protein levels, MINER1-Bcl-2 bonding is impossible. Binding of MINER1-Bcl-2 complex to the ER domain is similar to that of BH3-containing proteins, and tends to be competitive (Tamir et. al., 2014).

MINER1 as a member of the NEET proteins, may therefore be functionally to tie Fe-S availability (or iron) to apoptotic (or ferroptotic) and/or autophagic cell death. MINER1 may be utilising the autophagic process, to regulate cellular survival or death pathways (Chang et. al., 2010; Sohn et. al., 2013; Holt et. al., 2016).

Maintenance of the intracellular Ca^{2+} homeostasis by MINER1 was shown to be an important contributory factor in regulating mitochondrial function in the tissues of adipose (Wang et. al., 2014), liver (Shen et. al., 2017) and heart (Yeh et. al., 2019, 2020; Shen et. al., 2021). By the same regulatory mechanisms via Ca^{2+} homeostasis and mitochondrial function, MINER1 could help heal corneal wounds and maintained epithelial integrity of the cornea, demonstrating that MINER1 is important to corneal regeneration (Sun et. al., 2021).

2.6.7. Succinate dehydrogenase gene (*SDH)*

Recently classified as a tumor suppressor (Aspuria et. al., 2014), SDH is a mitochondrial enzyme catalysing the oxidation of succinate to fumarate via flavin adenine dinucleotide (FAD) redox reaction in the tricarboxylic acid (TCA) metabolic cycle (Moreno et. al., 2020). SDH gains electrons by this process, and transfers them to the electron transport chain (ETC) for reduction of ubiquinone to ubiquinol (Rutter et. al., 2010). This ability of SDH to connect TCA to ETC makes it indispensable in cell metabolism. This functional unit allows for the maintenance of ROS.

SDH complex also known as the mitochondrial respiratory complex II, is located within the inner mitochondrial membrane (**Figure 2.12**). It is made up of basically four (4) subunits (SDHA, SDHB, SDHC, SDHD) as a component of the TCA. During succinate oxidization, SDH gains electrons and transfers them in a serial fashion through all the 4 subunits till the final electron acceptor in the ETC. These electrons from reduced FAD (FADH2) and ubiquinol are transferred to complex III to complete the process for adenosine triphosphate (ATP) production, cellular energy currency. Regulation of SDH enzymatic

activity through these subunits, aids in cellular respiration, hypoxic response, gene expression, etc. Altered SDH activity results in reduced electron flow, increased oxygen toxicity, and accumulated succinate, which can give rise to cancer and other diseases (Dalla Pozza et. al., 2020; Bandara et. al., 2021).

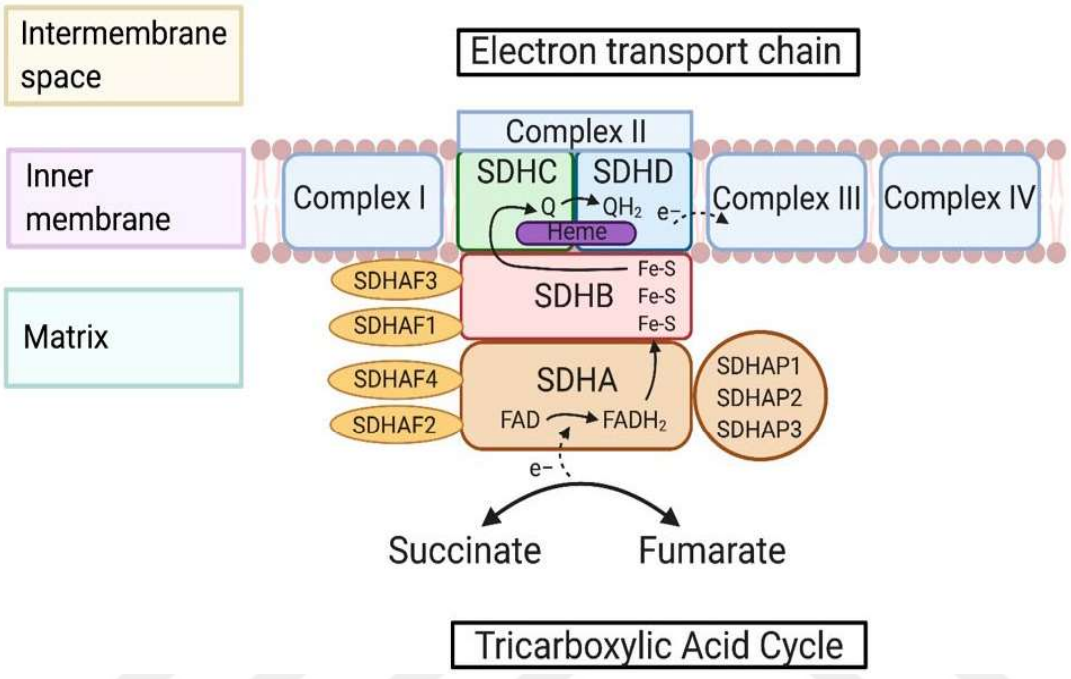

Figure 2.12 Structure, maturation, and assembly of SDH complex (Moreno et. al., 2020)

The four succinate dehydrogenase subunits are very common to many bacterial and mammalian mitochondria. Of these subunits; two, a flavoprotein (FAD) subunit (SDHA) and iron-sulfur protein subunit (SDHB) are hydrophilic and embedded within the mitochondrial matrix. While the remaining two subunits (SDHC and SDHD), are hydrophobic and membrane-bound heme b-containing subunit, serving as the ubiquinone binding site (Xue et. al., 2003). The reversible oxidative reaction of succinate to fumarate by SDH releases two (2) electrons. These 2 electrons are transferred to SDHA to protonate FAD to $FADH_2$, in turn releasing the 2 electrons to the Fe-S clusters of SDHB. Functional SDHA are FAD-dependent. SDHA-FAD co-binding promotes increased redox potential for sufficient catalytic activity and overall stability complex (Kim and Winge, 2013).

SDHAF1, SDHAF3 and SDHAF2 function as assembly factors to aid in the maturation of SDHA and SDHB. SDHB stores the Fe-S clusters provided by SDHAF1 and SDHAF3, and aid in electron transfer to ubiquinone from FAD. Frataxin is a key

mitochondrial protein providing the iron for Fe-S cluster biogenesis. Its deficiency causes mitochondrial iron accumulation leading to Friedreich's ataxia (Delatycki et. al., 2000) and decreased activities of Fe-S proteins (SDH malfunction) (Stehling et. al., 2004). SDHAF1 and SDHAF3 function to help SDHB maturation via Fe-S clusters transfer and shielding SDHB from respiratory distress and oxidants, to support respiratory growth (Na et. al., 2014). SDHAF2 in conjunction with dicarboxylate, is responsible for stabilization of the active site of SDHA. SDHAF4 acts as a chaperone of flavinylated SDHA and blocks excessive ROS production via direct interaction prior to SDHA-SDHB complex formation (Van Vranken et. al., 2014). Metabolic functions of SDHAP1-3 (known SDHA pseudogenes) are yet to be found.

Heme b-containing SDHC and SDHD is the site ubiquinone reduction to ubiquinol and responsible for ubiquinol transfer to mitochondrial ETC complex III. Heme b may not have any significant role in catalysis (Oyedotun et. al., 2007) but it provides structural support to the mammalian membrane anchorage domain of complex II (Sun et. al., 2005). Among its many cellular roles, is its role as a prosthetic factor for the OXPHOS complexes and essential for SDH complex assembly. SDH mutations involving heme (H127A and SDHC H127Y) resulted in decreased SDHC levels, reduced enzyme activities and complex II assembly malformation (Van Vranken et. al., 2015).

Proton pumping activity of ETC is known to occur in complexes I, III, and IV, but not in complex II. Internal protonation of FAD to $FADH_2$ by SDHA catalysis exists in complex II. Multiple electron transfer pathways interact to facilitate TCA-ETC function. During ubiquinone-semiquinone-ubiquinol reductive reaction, electron transfer to the 3 Fe-S clusters occurs throughout SDH complex to not only cause ubiquinone reduction but also facilitate electron transfer between complex II and complex III (Enriquez et. al., 2014).

The SDH gene is considered a tumor suppressor gene (Gottlieb and Tomlinson, 2005), owing to its renewed role and versatility in cellular function and physiology. Its substrate, succinate an oncometabolite. Mutations in SDH in pathology (familial cancer syndrome) (Gottlieb and Tomlinson, 2005, Tseng et. al., 2018) promoted a metabolic shift into glycolysis driving cellular division. During zebrafish heart regeneration, metabolic reprogramming to glycolytic switch was observed to be essential. This was observed to correlate to a significantly reduced SDH activity (Honkoop et. al., 2019).

2.6.7.1. Succinate and its receptor (SUCNR1/ GPR91)

One major mitochondrial metabolite, succinate, has long been identified as an essential intermediate in the Krebs cycle (Krebs and Johnson,1937), a substrate for oxidation to fumarate via the catalytic action of the subunit A of the SDH enzyme complex. It is known to accumulate in the mitochondria, cytosol and the extracellular matrix (Guo et al., 2020), as a result of energy demand-O_2 supply imbalances (Hoyer and Krier, 1986), especially during ischemic (Chouchani et. al., 2014) and exercise (Lewis et. al., 2010) states. Under some patho-physiologic states, succinate can be produced additionally by other metabolic pathways; malate-aspartate shuttle (Lane and Gardner, 2005) and purine nucleotide shuttle (Lowestein, 1972). Succinate as an essential intermediate metabolite and a signalling molecule, also mediates the gamma-aminobutyric acid (GABA) shunt-TCA cycle intersection (Haas et. al., 2016).

Succinate is therefore a versatile intermediate involved in both signal transduction, metabolism and pseudohypoxia (Nawab et. al., 1984; Adair et al., 1990; Burns and Wilson, 2003; Mills and O'Neill, 2014). Succinate accumulation in inhibiting prolyl hydroxylase (PHD) under normal O_2 availability, may induce HIF-1α activation and stabilization. Angiogenic genes such as the VEGF-containing HRE (hypoxia response elements) succumb to transcription via HIF-1α (Schoors et. al., 2015; Jiménez-Valerioet. al., 2016; Rohlenova et. al., 2018; Du et. al., 2021), an important link between metabolic rewiring and angiogenesis (Kluckova and Tennant, 2018). Succinate-mediated PHD inhibition was also shown to promoted HIF-1α accumulation and activation (Selak et. al., 2005, Selak et. al., 2005).

The succinate receptor GPR91, occurs in numerous tissues such as the liver (stellate cells), heart (cardiomyocytes), kidney, retina and immune system (platelets, dendritic cells, macrophages), as well as several others (Velcicky et. al., 2020). Succinate, acting through the G-protein-coupled receptor (GPR91/SUCNR1), to mediate a range of cellular and biological activities (He et. al., 2004). It activates the liver hepatic stellate cells (Correa et. al., 2007) and hematopoiesis in the bone marrow (Hakak et. al., 2009). Succinate-SUCNR1 axis regulates the cardiomyocyte viability of the heart ventricles (Aguiar et. al., 2010). It causes the release of the kidney's renin (Peti-Peterdi, 2010) while inhibiting lipolysis in

adipose tissue (An et. al., 2021). Succinate via SUCNR1, is known to evidently link tissue metabolism, mitochondrial stress and organ response (van Diepen et. al., 2017).

Numerous studies have also reportedly shown succinate-SUCNR1 involvement in inflammatory and metabolic pathologies (Hollander et. al., 2001; Mills and O'Neill, 2014; Macias-Ceja et. al., 2019; Guo et. al., 2020; MacFarlane et. al., 2020). Succinate-SUCNR1-induces pathologic angiogenesis in normal retinal development and proliferative ischemic retinopathy (Sapieha et al., 2008; Hamel et. al. 2013) tumor angiogenesis (Mu et. al., 2017) and rheumatoid arthritis (Li et. al., 2018).

SUCNR1 cause both proinflammatory and anti-inflammatory effects in immune cells (Krzak et. al., 2021). It amplifies IL-1β release from macrophages (Littlewood-Evans et. al., 2016) and promotes monocyte-derived macrophagic chemotaxis in white adipose tissue (van Diepen et. al., 2017), while decreased (IL10, TLR4 and TLR5) expression and increased TNF-α expression in mononuclear cells of the peripheral blood (Trauelsen et. al., 2017) were observed. Anti-inflammatory effect of SUCNR1 was enhanced via the PKA–CREB–KLF4 pathways (Kieran et. al., 2019).

2.6.8. c-MYC

c-MYC belongs to one of the three well-known proteins of the MYC family. The MYC family is made up of basically: c-MYC, L-MYC and n-MYC genes, and are involved in various cellular functions. All the 3 MYC members belong to basic helix-loop-helix/leucine zipper (bHLH/LZ) class of transcription factors, that via heterodimerization with its binding partner (Max), cause the transcriptional activation or repression of numerous target genes (Grandori et. al., 2000; Mao et. al., 2003; O'Connell et. al., 2003).

c-MYC has been shown to control cell cycle progression, proliferation, growth, adhesion, differentiation, apoptosis and metabolism (Meyer and Penn, 2008; Miller et. al., 2012; Whitfield and Soucek, 2012). c-MYC is known to control cell progression or transition through (G_0-G_1 to S) phase of the cell cycle and regulates expression of genes involved in biosynthesis of ribosomes during cellular proliferation. c-MYC tends to be highly expressed in proliferating cells. Significant decrease in expression levels of c-MYC was seen as proliferating cells progress through the cell cycle till their exit back to the G_0 phase (Grandori et. al., 2000). By virtue of these broad cellular functions, cellular expression of c-MYC is

tightly-regulated. Its dysregulation and overexpression, is known to be a common feature in several human and rodent tumours (Adhikary and Eilers, 2005; Nilsson and Cleveland, 2003). 5-7

Several studies have sought to outline the mechanism of functions of c-MYC. For transcriptional activation of key cell cycle genes such as Cyclin D2, ornithine decarboxylase, and E2F1; c-MYC must bind its partner, Max via the conserved (CACGTG) E box. This molecular binding transcriptionally inhibits multiple genes especially the p21 and p27 genes. Transcription initiator element (Inr) is molecularly complexed to c-MYC-Max with either Sp1 or Miz1 to form the complete inhibition complex (Collier et al., 2000; Fernandez et al., 2003). c-MYC is usually expressed al low cellular levels and is easily broken down. Binding to Max is competitive, so c-MYC is made to compete with other antagonists (Mad, Mnt and Mga). Surprisingly, Max/Mad complexes also employ the E boxes to repress gene expression. On the other hand, Miz1-Inr bonding complexes without c-MYC, are functional activators of transcription (Adhikary and Eilers, 2005).

Similar cellular content of c-MYC protein were measured in both the quiescent and regenerating hepatocytes. Cellular c-MYC expression levels may directly determine the effects of its biological activity (Murphy et. al., 2008). To curb c-MYC expression in quiescent cells, c-MYC is sequestered in the nucleolus. Upon a PHx stimuli, nuclear translocation may occur to activate c-MYC gene expression. Rapidly-dividing fetal hepatocytes also localizes their c-MYC to the nucleus, showing that c-MYC location may be an important variable in cellular proliferation (Sanders and Gruppuso, 2005; 2006).

c-MYC demonstrates distinctive proliferative roles in liver. For example, while c-MYC is necessarily required in adult mouse liver regeneration, postnatal hepatocyte proliferation does not. c-MYC is therefore seen as a key regulator of hepatocyte metabolism and proliferation (Fausto et. al., 1986; Collier et al., 2003). It is expressed quite early in the priming phase of the liver regenerative phase, even as early as the first 30 minutes post-PHx. c-MYC may be possibly be acting as a facilitator of nutrients utilization for the biosynthesis of necessary cellular precursors needed for cell division. In liver regeneration, hepatocyte hypertrophy precedes hepatocyte hyperplasia. Kim et. al., (2000) showed that transiently overexpressing c-MYC in mouse liver caused hepatocyte hypertrophy, rather than hyperplasia. Ribosomal genes and protein synthesis were said to be induced (Kim et. al.,

2000), in order to enhance metabolism for coupled DNA synthesis. Initial transient expression of c-MYC may as well be a part of the mechanisms to rewire the metabolic pathways to meet the cellular energy needs and biosynthesis of essential metabolites. The cell must also prevent their breakdown (catabolism). ther studies have also reported two expression peaks for c-MYC at 2 hrs and 8 hrs respectively (Fausto et al., 1986; sanders et al., 2005).

Prochownik and colleagues reported in a hepatoblastoma study that the mice lacking c-Myc in their livers survived much longer than mice with intact C-MYC. This goes to say that C-MYC support tumour progression but not tumour initiation (Zheng et. al., 2017).

2.6.9. Mitochondrial Aconitase (ACO2)

ACO2 gene encodes for the mitochondrial aconitase (ACO2) enzyme, which is one of the two commonly known enzymes of the aconitase enzyme family; Cytosolic aconitase enzyme (ACO1) and ACO2. ACO2 enzyme, located in the mitochondrial matrix, function basically to catalyse the reversible isomerization of citrate to isocitrate, while generating NADH and $FADH_2$ for ATP and heme biosynthesis (Chen et. al., 2020). ACO2 is therefore, a key player in ATP generation, energy metabolism and mitochondrial biogenesis.

Catalytic conversion citrate to isocitrate is a common trait of both ACO1 and ACO2 enzymes. However, ACO2 has been shown to partake in intermediary metabolism. Interestingly, ACO2 enzyme-encoding ACO2gene is located on the chromosome 22q13.2 (HGNC: HGNC 118), while cytosolic aconitase enzyme-encoding *ACO1* gene resides on human chromosome 9p (Mirel et. al., 1998). ACO2 and ACO1 as well, are both Fe_4-S_4 cubane-structured Fe-S containing proteins. Their catalytic activity of the aconitases requires iron. Iron availability post-transcriptionally regulate ACO2 gene expression (Rouault and Klausner, 1996).

IRPs regulate the mRNA translation of several multifunctional proteins. IRP1 as a key cellular iron sensor via RNA-binding, regulate the post-transcription of some iron-related genes (ferritin, TfR1, ALAS2) and Fe-S containing gene (*ACO2*) (Rouault et al., 1988; Klausner et al., 1993; Kim et al., 1996a). Iron-dependent RNA binding activities of IRP1 can post-transcriptionally regulate the gene expression of *ACO2* via the IRP-IRE regulatory system. IRP1 binds to the IRE on the 5′ end of *ACO2* mRNA to repress the

translation of ACO2 protein in cytosolic iron-depleted states. Polymorphism in ACO2 IRE may affect the IRP-IRE bonding. Based on genetic modifications to the transcription start site, two types of *ACO2* mRNA have been identified in the rat liver: IRE-containing mRNA and non-IRE-containing mRNA (Shen et. al., 2023).

Such iron-dependent IRP1-mediated translational repression of ACO2, may basically be a mechanism to fine-tune regulation of biosynthetic process requiring iron. For example, cellular processes requiring of iron during the rapid proliferation of regenerating hepatocytes (Klausner et al., 1993). Aconitase enzymes are essentially known to control an erythropoietic cell cycle checkpoint, regulated by iron. ACO2 was observed to regulate this iron-regulated checkpoint erythropoietic process via controlling the mitochondrial metabolism-erythropoiesis axis. ACO2 can be oxidative-inactivated, leading to protein accumulation and mitochondrial dysfunction (Bota and Davies, 2002).

Information on expression of ACO2 gene in liver regenerations, after 70% post-PHx is very scarce. However, in a study of adipogenesis model in white adipose tissue, Chen et. al., (2020) showed that ACO2 may regulate mitochondrial metabolism and ATP generation in sustenance of adipogenesis in murine fibroblast (3T3-L1) cells (Chen et. al., 2020).

2.6.10. Glutaredoxin 5 (GLRX5)

Glutaredoxin (GLRX) in conjunction with glutathione S-transferase (GST), are important regulatory enzymes of the cellular redox signalling pathways. GLRX are small thiol-containing disulphide oxidoreductase protein group, structurally belonging to thioredoxin-fold superfamily proteins (Johansson et. al., 2011). Using GSH (reduced glutathione) as the electron donor (Holmgren, 1989), GLRX regulate the redox conditions of cysteine residues.

GLRXs and thioredoxins have been shown to exhibit some common functions, have a closely associated cysteine residue in their active-site sequence (thiol) (Akterin et. al., 2006; Fernandes and Holmgren,2004). Based on the number of thiols in the GLRX, 1-thiol-containing (monothiol/CGFS) and 2-thiol-containing (dithiol [CP/SYC]) GLRXs. Dithiol GLRX plays major roles in catalyzing the reductive reactions of disulphide with glutathiones (protein disulphide). Dithiol proteins were shown to demonstrate cytoprotection against apoptosis and oxidative stress (Lillig et. al., 2008).

As an important molecular player in Fe-S protein biogenesis, GLRX also function to maintain mitochondrial and cytosolic iron states (**Figure 2.13**) (Johansson et. al., 2007). Four types of GLRX (GLRX1, GLRX2, GLRX3, GLRX5) are currently known human. GLRX5 is a single-domain monothiol mitochondrial protein involved in Fe-S cluster biogenesis and heme biosynthesis. It is composed of 150-amino acid and found abundantly in erythroid cells (Rodríguez-Manzaneque et. al., 2002). As a part of the Fe-S biogenetic machinery, GLRLX5 may be required to insert and transfer clusters to acceptor (holo-) proteins (Mühlenhoff et. al., 2003; Vilella et. al., 2004). Previous studies have demonstrated that homodimerization of GLRX5 assembles the Fe_2-S_2 at the cysteine domain (Cys67) of the N-terminal active site (Ye et. al., 2010; Johansson et. al., 2011).

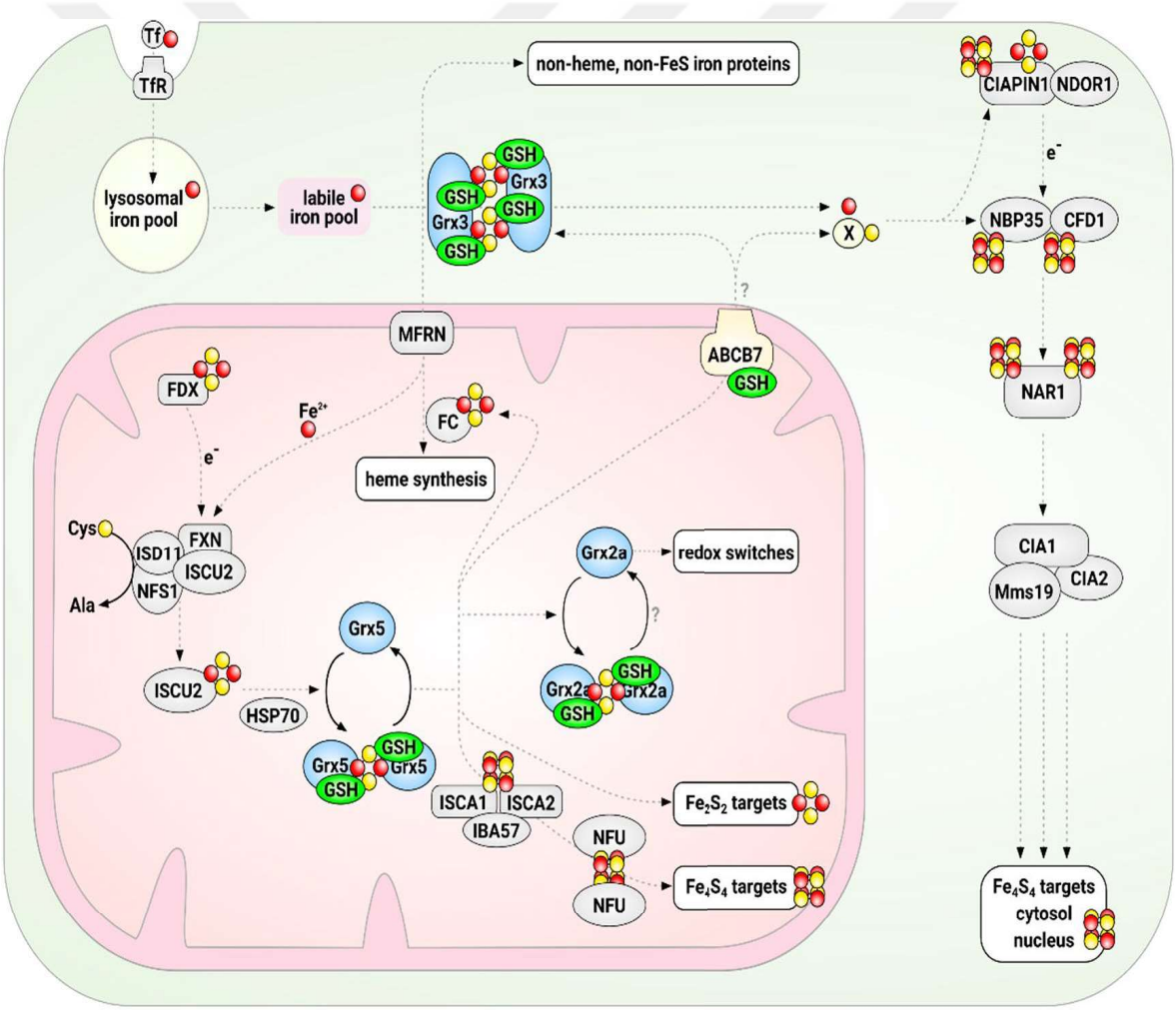

Figure 2.13 Glutaredoxin in Fe-S cluster synthesis and maturation of Fe-S protein (Daniel et. al., 2020).

Using transgenic or knock out animal models, *in vivo* cellular functions of GLRX such as metabolism, angiogenesis, inflammation and fibrosis (Bachschmid et. al., 2010; Anathy et. al., 2018; Gorelenkova et. al., 2016) have been studied. Numerous studies on the role of GLRX5 in various cellular processes. In a study of iron metabolism in a liver fibrosis model, GLRX5 was seen to be a key regulator of iron homeostasis by its down regulation in ALR-deficient mice (Kumar et. al., 2016).

Wingert et. al., (2005) also showed that, blocking GLRX5 function led to the inhibition of ALAS2 mRNA translation via IRP1-mediated mechanisms, causing anaemia in zebrafish. Essentiality of GLRX5 in heme biosynthesis stems from dependence of Fe_2-S_2 cluster-containing FECH enzymes on the functional activities of GLRX5 (Wingert et. al., 2005).

Therefore, this work aimed to study the molecular interactions of Fe-S genes (mitoNEET, IRP1, MINER1, SDHA, ACO2, GLRX5) with partner genes (PGC-1α, HIF-2α, c-MYC, mitoferrin) within a possible gene interaction network, at various time points (0, 6, 12, 24, 48 and 72 hrs) after a 70% partial hepatectomy, using RT-PCR and Western Blotting assays. Biochemical analysis of SUCNR1 with ELISA kit, was done to determine its receptor activity as a function of succinate, in hepatocellular proliferation. It was observed that molecular gene interaction involving Fe-S genes with partner genes, may define key molecular events governing the early acute-intermediate response phase of liver regeneration. This may provide some clues or answers to the molecular basis of cellular functions of Fe-S proteins.

3. MATERIALS AND METHOD

3. Study settings

Our study experiments which involved rat liver tissues were carried out in the Molecular Biology Laboratory of Biology department of Eskisehir Osmangazi university (ESOGU), with ethical approval certificate numbered 823/2021 (**Appendix A**), from the ESOGU animal experiments local ethics committee (HADYEK). The application for ethical approval was done in reference to the predecessor project titled ‘‘The role of ubiquitin signalling pathway on liver regeneration in rats’’ with ethical clearance number (549/2016), from which the frozen rat liver tissues, sera and formaldehyde-preserved tissue slices were used.

The animal experiments were carried out in the Laboratories of ESOGU medical and surgical experimental animals’ application and research centre (TICAM).

3.1. Experimental groups

All animals used in the experiment were obtained from Eskişehir Osmangazi University TICAM. Male Wistar albino rats weighing 250±300g, were used in our experiment. During the experiment, rats were kept alive in polycarbonate transparent cages in automatically adjusted rooms with 12:12 lighting/dark, temperature of (22 ± 2^{o}C) and humidity at (45-50%), while providing standard rat food and tap water.

A total of 52 rats (n=4) randomly selected from among the experimental animals, were used. Experimental animals were arranged into 3 main groups [Control, Sham (SH), partial hepatectomy (PH)] and 13 subgroups (Table 3.1).

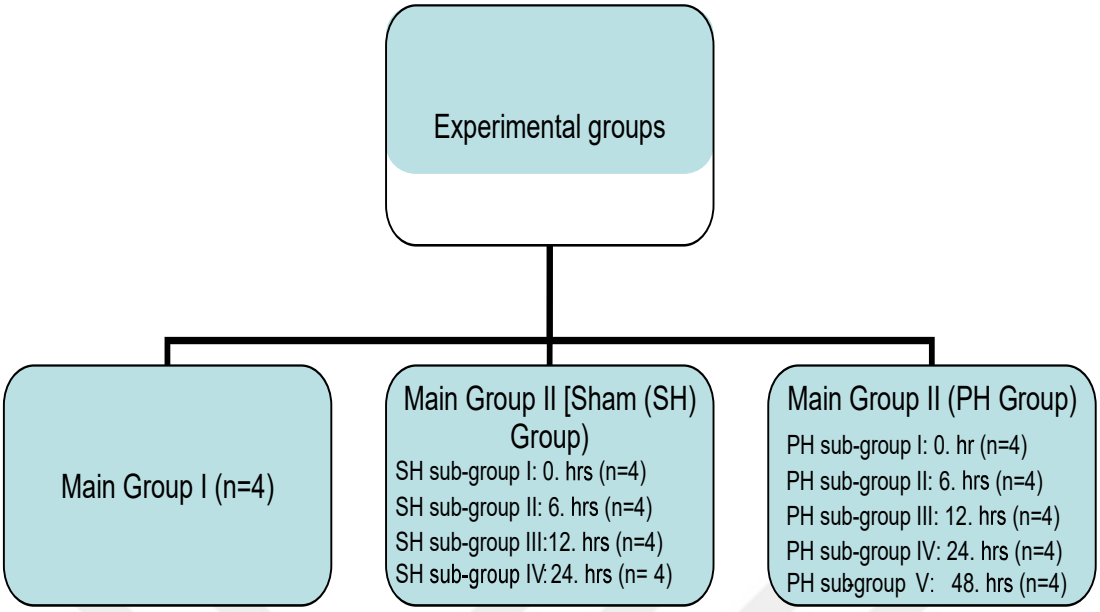

Table 3.1 Designing the groups of the experimental animals

Main Group I (Control Group): No surgical procedure was applied; Liver tissue samples were taken from all rats in this group (n=4) for PCNA immunohistochemical analysis, RT-QPCR test, Western blot analysis and succinate receptor 1(SUCNR1) ELISA assay analysis.

Main Group II (Sham Groups): The rats in this group were sutured by separating the right middle and left lateral lobes of the liver as if they were going to undergo a partial hepatectomy, and the laparotomy area was closed without performing PH. Dissections were performed at the end of the specified time (0, 6, 12, 24, 48, 72 hours). Liver tissue samples were taken for PCNA immunohistochemical analysis, RT- QPCR test, Western blot analysis and succinate receptor 1(SUCNR1) ELISA assay analysis.

Main Group III (PH Group): The left lateral and median lobes, which make up 70% of the livers of the rats in this group, were removed. Dissections were performed at the end of the specified time (0, 6, 12, 24, 48, 72 hours). Liver tissue samples were taken for PCNA immunohistochemical analysis, RT- QPCR test, Western blot analysis and succinate receptor 1(SUCNR1) ELISA assay analysis.

3.2. Anaesthesia and Hepatectomy Procedure

All experimental studies were performed in a sterile environment, using sterile surgical instruments. Considering the possible effects of diurnal hormonal changes on rats, all surgical procedures were performed between 09.00 and 12.00 hours. 10 $mg.kg^{-1}$ xylazine and 70 $mg.kg^{-1}$ ketamine anaesthesia were administered intramuscularly to the animals belonging to the experimental groups.

Rats were fixed in the supine position on a warm and stable dissection table. The surgical application area was cleaned with 70% ethanol, and partial hepatectomy was performed using Higgins and Anderson's (1931) technique (Higgins and Anderson, 1931). Accordingly, the membranes connecting the liver lobes were separated with a 2-3 cm midline incision parallel to the body in the abdomen of the experimental animal. The hepatic artery, vein, and bile ducts of the anterior middle (median) and left lobe, which constitute approximately 68-70% of the liver's mass, were tied with silk thread and cut just above it. Each animal was administered 1 ml of sterile saline into the abdominal cavity to counteract the hypovolemic effects of the fluid lost after surgery. Afterwards, the muscle and skin incisions were sutured separately but continuously with 4/0 silk suture, and the laparotomy area was closed and cleaned with an antiseptic solution.

Each surgically-treated experimental animal was placed separately in a chemically-sterilized, single individual, polycarbonate and transparent cages without any dietary changes and sustained for a specified period of 6, 12, 24, 48, 72 hours based on the group. In rats of the pH0 hour subgroup, intracardiac whole blood was drawn after PH treatment without closing the laparotomy site. All the animals belonging to PH and SH groups, after their specific respective post-PHx period (6, 12, 24, 48, 72 hrs) had all the blood from their heart collected intracardially, under the intramuscular anaesthesia of 10 mg.kg-1 xylazine and 70 mg.kg-1 ketamine.

3.3. Liver Regeneration rate

During the PH procedure, the wet weights of the median lobe and left lateral lobe, making up about 68-70% of the liver were measured on a precision scale, and thus the total weight of the removed liver during hepatectomy was calculated. At the end of 0, 6, 12, 24,

48, and 72 hours, the regenerated liver pieces removed during dissection were also weighed. Regeneration rates were calculated by placing the formula below;

Liver Regeneration (%) = 100 x [(A-B) /C]

3.4. Succinate receptor (SUCNR1) ELISA assay

Measurement of succinate receptor concentration in the serum was performed with the Rat Glutathione ELISA measurement kit purchased from Bioassay Technology Laboratory (E1267Ra, Bioassay Technology Laboratory, Shanghai, China). The ELISA kit plate came pre-coated with Rat SUCNR1 antibody.

All the necessary reagents, standard solutions and harvested serum were prepared at room temperature according to manufacturer's instructions.

50ul standard solution was initially added to the standard wells. Because there is biotinylated antibody in the standard solution, antibody is not added to the standard wells. 40ul of sample serum was added to the sample wells. And then 10ul of the biotinylated Rat SUCNR1 antibody to bind the SUCNR1 in the sample. 50ul streptavidin-HRP was added and made to bound the biotinylated SUCNR1 antibody in the sample well. The plate was covered with a sealer and incubated in the dark for 60 minutes at 37°C. After incubation, unbound streptavidin-HRP is washed away serially with a wash buffer. Substrate solution is then added and colour develops in proportion to the amount of Rat SUCNR1. The reactions were then terminated by addition of the acidic stop solution and absorbance measured at 450 nm.

Absorbance reading was done with the Chromate 4300 brand ELISA optical device reader (Awareness Technology, Inc. Martin Hwy. Palm City, USA) and absorbance recorded as optical density in the unit of nm. Data were calculated using 4-parameter logistic regression (R2 value: 0.997).

3.5. PCNA immunohistochemical test procedure

Liver tissue samples previously preserved in a jar of formaldehyde, were removed and prepared for the PCNA procedure.

- The samples were first passed through a series of xylene and alcohol concentrations respectively in a serial fashion. (5 min xylene - 5 min xylene - 1 min alcohol 96% -1 min alcohol 80% - 1 min alcohol 70% - 1 min alcohol 50%), after which it is finally passed through distilled water. Prior to this procedure, slides are incubated at 60°C for 2 hours.
- A mixture of 20 ml of H_2O_2 + 180 ml of distilled water is prepared and sample made to stand in it for 10 min.
- Wash in distilled water for 2-3 minutes.
- Citrate buffer is prepared (75ml citrate buffer+675ml distilled water) and boiled in microwave for 15 minutes. NOTE: Were it to be in a pressure cooker, it is set to 120°C and then left to stand for 5 minutes to cool.
- The longer it is in the microwave, the longer it should stay outside for 15 minutes and cool.
- It is kept in distilled water for 3 minutes, then drawn with a marker. Then washed with 2-3 min with 1X PBS. It is treated with 2% BSA for 15 minutes.
- 1st antibody is prepared with 1X PBS is applied overnight at +4°C.
- Wash with 1X PBS for 2x3 minutes.
- Secondary antibodies are added. Because it is diluted, it is made to wait for maximum 3 hrs at room temperature.
- ABC kit is prepared in 1X PBS and made to stand for 15 minutes at room temperature
- 2x3 min wash with 1X PBS
- The prepared ABC kit is placed on the slides (room temperature for 1 hour).
- Wash with 1X PBS for 2-3 min.
- Chromogen is dripped on it (8 minutes) and stained with Mayers Hematoxylin as counterstain (2 min).

Table 3.2 Equipment and products used in PCNA immunohistochemical test application

Microtome	ThermoShandon
Slide	Thermo Menzel- Glasser Polysine Slides Lot: 0889
Oven	Mecan Technic
XYLENE	A.D.R Group Cat: 129007
Alcohol	% 96 Ethanol
Hydrogen Peroxide	Riedel-de Haen %35 Lot: 1130A
Citrate Buffer	SCYTEK Lot: 44833
PBS	BBI Life Sciences Lot: EA17KA9844
Primary antibody	PCNA Antibody (F-2): sc-25280
Secondary Antibody	HRP Goat Anti-Mouse IgG (H+L) AB-clonal Cat: AS003
Albumin	Bio Shop Lot: 8E54929
ABC Kit	Vectastain PK-6100
AEC Chromogen / Substrate Kit	SCYTEK Lot: 50373
Haematoxylin Mayer	Biognost Lot: HEMM-21/16
Consul-Mount Adhesive	Thermo Scientific Lot: 214586

3.6. Real Time PCR (RT-PCR) test protocol

RT-PCR were performed with 4 biological replicates in each subgroup. RT-PCR primers of 10 genes belonging to the mitochondrial (iron-sulphur) biogenesis with β-actin as the control gene used in the study, were obtained from Sentebiolab company, Ankara, Turkey. The primers used in this study are therefore given in Table 3.3a.

At the time of use, primers were diluted according to the manufacturer's instructions as outlined in the data sheet. The primers were prepared to a stock of 10 μM.

Table 3.3 Base chains of used Primers' and their length

Primer's name	Base chains (BCs)	Length BCs
ACO 1 F	CTT GTC GCT GTT GGG GTA CT	20
ACO 1 R	TCC ACC TCC TGG ATC TCA TC	20
ACO 2 F	AGT ATG GTG TGG GCT TCT GG	20
ACO2 R	AAT CAC CTT GGG ACA CTT CG	20
C-MYC F	CGA GCT GAA GCG TAG CTT TT	20
C-MYC R	CTC GCC GTT TCC TCA GTA AG	20
GLRX5 F	ATC CTT CCC CCT TTC TGC TA	20
GLRX5 R	CTC AGT GCT ATG GTG GCT GA	20
MFRN F	GCC TGA ACG TGA TGA TGA TG	20
MFRN R	TCG CTG TTT CAC CAAC TTC TG	20
MINER 1 F	TCT TCC CGA AGA AGA AGC AA	20
MINER 1 R	GGA TGA GAG GAC CCA CGT TA	20
MITONEET F	GAC AAC GTG GGA CCT CTG AT	20
MITONEET R	CAC GAT GTT TCA ATG GCA AG	20
PPARGC1A F	TTC AGG AGC TGG ATG GCT TG	20
PPARGC1A R	GGG CAG CAC ACT CTA TGT CA	20
SDHA F	AGC CTC AAG TTC GGG AAA GG	20
SDHA R	CAC AGT GCA ATG ACA CCA CG	20
HIF2A F	GGG CAG ATC TAA CAC GCC TT	20
HIF2A R	TCT GAT TGC CCA CAG ACC AC	20

Table 3.4 Equipment and products used in RT-PCR

	Brand	CAT No
RNA extraction kit	A.B.T.™ Blood/Tissue RNA Purification Kit for Leukaemia	I04-01-10 100 preps
Beta Mercapto-ethanol	M3148 Sigma-Aldrich 2-Mercaptoethanol	CAS Number 60-24-2
Proteinase K	Thermo Scientific™ Proteinase K, recombinant, PCR grade	EO0491
Chloroform Isoamyl	Merck Isoamyl Alcohol	516726
Nuclease-Free ultra-distilled water	Biochrome AG Ultra-Pure Water, Sterile, Suitable for HPLC	L0015-BC
cDNA kit	High-Capacity cDNA Reverse Transcription Kit	4368814
Nanodrop	Thermo Scientific™ NanoDrop™ 2000/2000c Spectrophotometers	ND-2000
PCR	Applied Biosystems™ MiniAmp™ Thermal Cycler	A37834
Real Time PCR Master Mix	Thermo Scientific™ Maxima SYBR Green/ROX qPCR Master Mix (2X)	K0221
Real Time PCR	Applied Biosystems™ 7500 Real-Time PCR System	4376600
Primer	100 nmol HPLC Grade	
Centrifuge	Neofuge 13R-Refrigerated Centrifuge-Heal Force Bio	
Water	Thermo Scientific™ Water, Nuclease-free, Molecular Biology Grade, Ultrapure	7732-18-5
PBS	Gibco™ PBS, pH 7.2	20012027

3.6.1. RNA Isolation

Lysis Buffer (for 1 sample): (Buffer L-500 µl; 14.3M Beta mercaptoethanol 10 µl

- 200 µl of PBS (Ca^{2+}/Mg^{2+} free) was added to 10-20 mg of the liver tissue. 10 µl Proteinase K (20mg/ml), 500 µl lysis buffer and 60 mg zirconium bead added and homogenized.
- The homogenate was transferred to a new 2 ml centrifuge tube, and incubated for 3 min at room temperature.
- 500 µl of Buffer P was added again, vortex well and incubated for 3 minutes at room temperature.
- 200 µl of Chloroform isoamylalcohol mixture (49:1) was added to the mixture. The tube was shakingly mixed in an upside-down fashion and left for another 2 minutes at room temperature.
- The mixture was centrifuged at 12000 xg for 15 minutes at 4°C. After which approximately 600 µl of the supernatant was transferred to a new tube.
- Approximately 800 µl of 1.25x ethanol (96-100%) was added and mixed by pipetting.
- 700 µl of the lysate is transferred to the column and centrifuged at 12000 x g for 20 seconds, at room temperature.
- The tube is poured out and the column placed back into the tube. This step is repeated until all lysates are transferred to the column.
- 700 µl of wash buffer1 solution is added to the column, centrifuged at 12000 x g for 20 seconds at room temperature. The tube is poured out and the column placed back.
- 600 µl of Wash Buffer 2 solution was added to the column, centrifuged at 12000 x g for 20 seconds at room temperature, the tube is poured out and the column placed back again.
- The column is centrifuged again for 1 minute at maximum speed and the column is placed on a 1,5 ml Eppendorf tube.
- 50-100 µl of nuclease-free, ultra-distilled water or DEPC water kept at 65-70 ^{0}C is added to the centre of the column, centrifuged at 12000 x g for 1 minute at room temperature.
- RNA is used immediately or stored at -80°C until used.

3.6.2. cDNA Synthesis

The RNAs obtained were measured in the nanodrop device. Since the values measured in the device are in ng/µl, the reaction is pegged at 20 ng, and necessary calculations made. For each sample;

$$\text{Calculation} = \frac{1\mu l \text{ (measured value on the device) ng} \times 1000ng}{\text{X= 1000/ (measured value on the device) µl}}$$

Table 3.5 Standard reaction of cDNA

cDNA Standard Reaction (for 1 sample)	
10X RT Buffer	2 µl
25X dNTP Mix (100 mM)	0.8µl
10X RT Random Primers	2 µl
MultiScribe™ Reverse Transcriptase	1 µl
Nuclease-free H_2O	4,2 µl
	Total = 10 µl

Based on one standard reaction measure, the individual sample number was also multiplied by the total sample number. The prepared mix is placed into a 1.5 mL Eppendorf tube and evenly distributed into the wells. Since the reaction mix per 1 sample is 10 µl in total, the RNA of each sample is added to subtract the total volume from 20 µl. And the remaining topped up with quantities of water.

The temperature and time values were then entered into the device according to the data sheet:

Settings	Step 1	Step 2	Step 3	Step 4
Temp.	25°C	37°C	85°C	4°C
Time	5 minutes	120 minutes	10 minutes	∞

3.6.3. Preparation of the standard reaction plates for RT-PCR procedure

The plate, which contains gene primers belonging to Fe-S biogenesis and other related genes. In preparing the standard reaction plate, SYBR Green Master Mix (Cat. No. K0221, Thermo Scientific) was prepared using the kit protocol.

Table 3.6 Standard Reaction Plate (reagents required for a well/sample)

Reactive	**volume**
Water (RNase free)	6 µl
PCR Primer	2 µl
Master Mix	10 µl
cDNA	2 µl
Total	**20 µl**

- Samples were prepared and distributed into the various well. The RT-PCR procedure was carried out per the manufacturer's instruction.
- RT-PCR analyses were performed on the Step OnePlus™ Real-Time PCR device (Thermo Fisher Scientific, USA).
- RT-PCR steps for SAC B primer: 5min at 95°C; 15s at 95°C, 30s at 60°C, 30s at 72°C (Amplification), 5min at 95°C; 15s at 95°C; 30s at 60°C; 30s at 72°C (Melting) for 40 cycles.

3.7. Western Blot procedure

3.7.1. Preparation of samples

Samples' concentrations were initially adjusted, according to the formula (M1.V1 = M2.V2). Required solutions were also reached after calculation.

Table 3.7 Amount of solution required for a sample

LDS sample buffer	2,5 µl
Sample reducing agent	1 µl
Sample	Calculated amount - µl
Distilled water	6,5 µl - (sample) µl
Total	10 µl

3.7.2. Incubation

- Prepared tubes were incubated at 70 °C for 10 minutes.
- At the end of the incubation, the tubes were kept on ice or at +4 °C for a while.

3.7.3. Execution

- Bolt™ 4-12% Bis-Tris Plus Gel is prepared for use.
- The proteins are made to move in Running Buffer 1X solution.
- The first well contains marker is placed in the first well. Proteins were moved for approximately 30-35 minutes at 200 volts.

3.7.4. Transfer to membrane

- Thermo Fisher Scientific IBlot 2 Dry Blotting System was used for the transfer process.

Gel

- The gel was rinsed in a pure-water filled tub, in order to remove it from its reservoir. The upper cover was slowly lifted with the help of a spatula till separated completely.

iBlot™ 2 Transfer Stacks

- Using the sandwich-shaped membrane transfer system (IBlot™ 2 Transfer Stacks) of the IBlot 2 device, the gel-to-membrane transfer process was performed at 20 volts in 7 minutes. After which, the blocking solution is prepared.
- In preparing the blocking solution from iBind™ Flex Solution Kit;

A mixture of 40 ml distilled water + 10 ml 5X Buffer + 500 µl 100X additive solution was prepared.

- The gel was discarded after the transfer process. The gel image on the transfer paper was cut out and placed into the prepared blocking solution. It is kept for 5 minutes.

3.7.5. Antibody Administration

- Antibody administration is performed on the Invitrogen™ iBind™ Flex Western instrument. For this, solutions suitable for the device were used. Solutions of the antibody, washing and blocking mixtures were prepared from these chemicals.
- The binding process is completed in a short period of like 2.5 hours for the first and secondary antibodies on the unique iBind™ Flex card.

Table 3.8 Used antibodies

Antibodies Used		
Name of antibody	origin	Purchase source
β-Actin Antibody (N-21) (42 KDa)	Rabbit	sc-130656, Santa Cruz Biotechnology
HIF-2α (EPAS)	Rabbit pAb	A7353, ABclonal Technology
MitoNEET (CISD1) (15 KDa)	Rabbit pAb	A10317, ABclonal Technology
DMT-1	Rabbit pAb	A10231, ABclonal Technology
IRP1	Mouse mAb	sc-166022, Santa Cruz Biotechnology

Antibody Preparation

- Per the number of antibodies to be used, 2 ml each was prepared from the blocking solution and transferred into 2.5 falcon tubes. Pipetting is done.
- Wet the iBind™ Flex card with the blocking solution (10ml). Another 1 ml of the same solution is added to the place where we will put the membrane. When the membrane is placed on the card, it is put upside down. It is fixed by going over it with a roller.
- It is left in the iBind™ Flex device for 3 hours. ECL solution is then prepared.

ECL Solution Preparation

- 1 ml of Luminol/Enhancer solution + 1 ml of Peroxidase solution
- The membrane removed from iBind™ Flex is placed in the solution.

3.7.6. Image Capturing

- A chemiluminescence imaging system was used for imaging. Since the basis of this imaging system is the chemical luminol, a secondary antibody-specific substrate (ECL) is used. ECL solution was prepared in a in 1:1 ratio. The membrane was incubated in the dark for 5 minutes prior to the commencement of the imaging process.
- The membrane was placed on the table in the Genbox device and picture taken with a software program on the PC.

Table 3.9 Numbering of sample in qubit measurements

Numbering of sample in qubit measurements									
Groups	control	SH0	SH6	SH12	SH48	PH0	PH6	PH12	PH48
Qubit measurement	30.6 µg/µl	35.8 µg/µl	39.6 µg/µl	36.8 µg/µl	36.2 µg/µl	33.6 µg/µl	38.6 µg/µl	34.4 µg/µl	31.2 µg/µl

Ladder Used: Invitrogen SeeBlue Plus 2 Prestained Standard.

3.8. Statistical analysis of obtained results

Results obtained from the various biochemical (ELISA), immunohistochemical (PCNA) and molecular biology (RT-PCR and Western Blot) methodologies were analysed using GraphPad Prism 9 statistical package. In data analysis of gene expression based on the RT-PCR, Livak's method of comparing relative changes in threshold cycles (ΔCt) was calculated and fold values ($2^{-\Delta\Delta Ct}$) derived as a measure of activity of gene expression. For analysis of protein expression based on the Western Blot, signals of the antibody-tagged proteins were detected in terms of optical density. One-way ANOVA with Tukey's multi-

comparison test was used in analysing statistically significant inter- and intra- groups gene and protein expressional patterns.

Same was also done for the ELISA test. Relative absorbances of the receptor protein in all the groups, were read and recorded. Obtained results were analysed using one-way ANOVA with Sidak multi-comparison test was used in analysing statistically significant inter- and intra- groups gene and protein expressional patterns.

In the PCNA test, images captured under the microscope were analysed for their staining patterns, as Brownish red or red-coloured/PCNA (+) or Blue-coloured/PCNA (-). The number of cells were analysed in terms of 1000 cells.

4. RESULTS AND DISCUSSION

4.1. Rate of liver regeneration

After a PHx, the liver must initiate tissue repair and regeneration process to increase its mass to body weight ratio, for a functional homeostasis. G_0 phase hepatocytes begin well-regulated entry into the cell cycle to divide once or twice, after they return back to the quiescent stage. Hepatocyte proliferation is stimulated from the first hours after 70% PHx (Fausto, 2000). DNA synthesis is commenced at the 14th hour, reaching its peak at the 24th hour. The remnant liver is almost doubled at the 48 hour and liver regeneration is completed within a week, after which hepatocytes growth is arrested (Wang et al., 2009; Toydemir et al., 2015).

In our study, rats that had their liver resected over (0, 6, 12, 24, 48 and 72 hrs) were sacrificed and their liver harvested for measurement of regained weight (rate of liver regeneration). Regeneration rates of the liver after PHx based on regained weights in this study, have been reported in our previous project titled "The Role of Ubiquitin Signaling Pathway in Liver Regeneration in Rats" and published as (Yaylaci and Canbek, 2023).

Liver regrowth was considered in terms of two cluster periods (**Figure 4.1**); pre-replicative regrowth (PH0, PH6, PH12) and replicative regrowth (PH24, PH48, PH72) respectively. No significant statistical differences were observed between early PHx (PH0, PH6, PH12) hour groups. However, in comparison to the late PHx (PH24, PH48, PH72) hour groups, significant difference was observed ($p \leq 0.05$) against the early PHx hour groups. Even though the regeneration rates were observed to have increased respectively with increasing PHx time (0, 6, 12, 24, 48, 72), no significant difference was found. The highest liver regeneration rate was seen in the PH72 group and significantly differed from all the other PH groups ($p \leq 0.05$). The findings were supported by literature. And that rate of regeneration increases generally with increasing time.

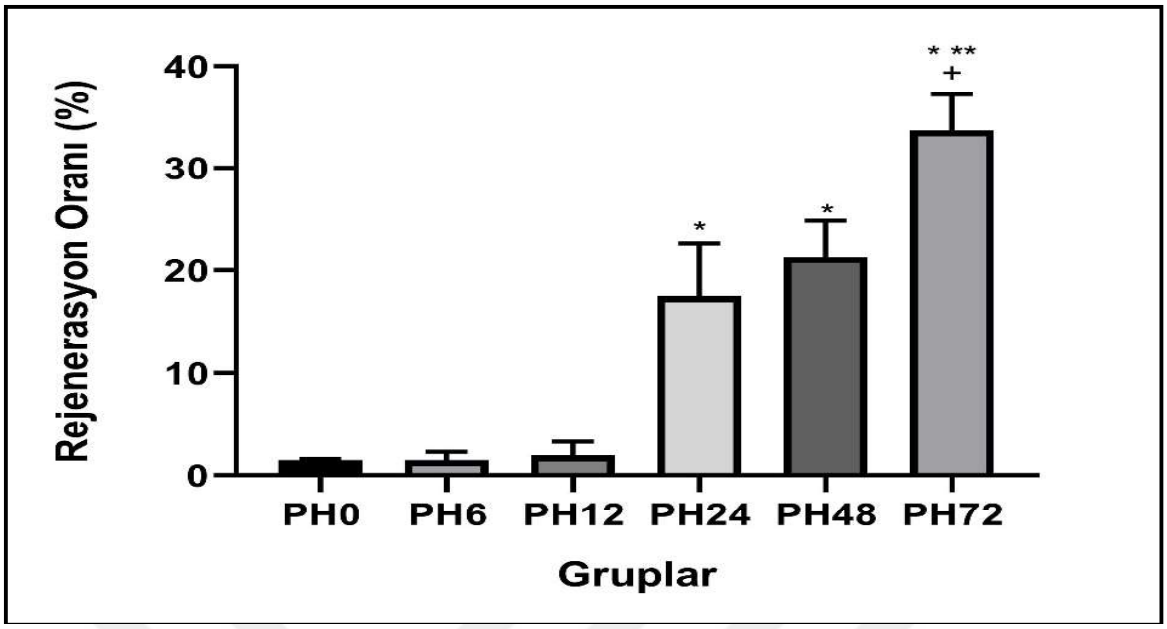

Figure 4.1 Rate of liver regeneration (taken from Yaylaci and Canbek, 2023)

4.2. Proliferating Cell Nuclear Antigen (PCNA) immunohistochemistry

Calculating the liver weight ratios may not be enough in estimation of regeneration rate, and must therefore be supported with other molecular analytical parameter such as the protein (Ki67 or PCNA) immunohistochemistry (Assy and Minuk, 1997).

In this study, PCNA immunohistochemistry was employed to supplement the liver regeneration rates calculated. PCNA is one of the most preferred immunohistochemical method as an endogenous G_1-S histologic cellular proliferation marker. PCNA is a well-conserved 36 KDa nuclear protein, acting as an auxiliary DNA polymerase delta protein and playing a major dual regulation role in DNA synthesis and cell proliferation (Assy et. al., 1998). PCNA is very essential for DNA replication and nucleotide excision DNA repair (NER). PCNA is known to be synthesized during the early G_1-S cell cycle phase and drops to immunohistochemically undetectable levels during mitosis (Wang et. al., 1997).

Tissue sections immunohistochemically-labelled for PCNA proteins were examined under a light microscope and various PCNA-stained cells counted. A careful analysis of the staining section showed PCNA positive cells to be red-brown coloured nuclei, while negative cells had a blue-coloured cells. The control and sham groups (SH0, SH6, SH12, SH24, SH48, SH72) were observed to have very few or no PCNA positive hepatocytes (**Figure 4.2**). PH groups were determined to have an increased number of PCNA positive

cells. PCNA positive hepatocytes observed in the PH groups were seen to be quite high for PH6, PH12 and PH24 hour groups, and cells at various mitotic stages also encountered in the PH48 and PH72. The highest number of PCNA positive cells were observed in the PH24, at a time period peak DNA synthesis is known to be achieved. Statistically, PH24 in comparison to SH0, SH6, SH48 and SH72, showed significance (**) but against SH24 (*), was not as significant as in the other SH groups. But within the PH groups, PH0 vs PH24 (*) was the only significant comparison.

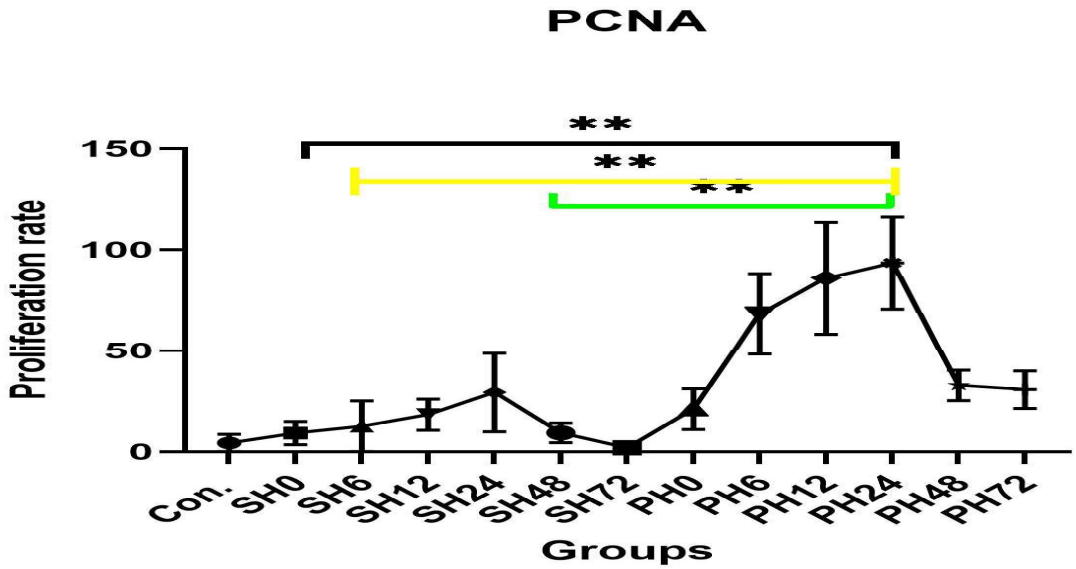

Figure 4.2 PCNA expression

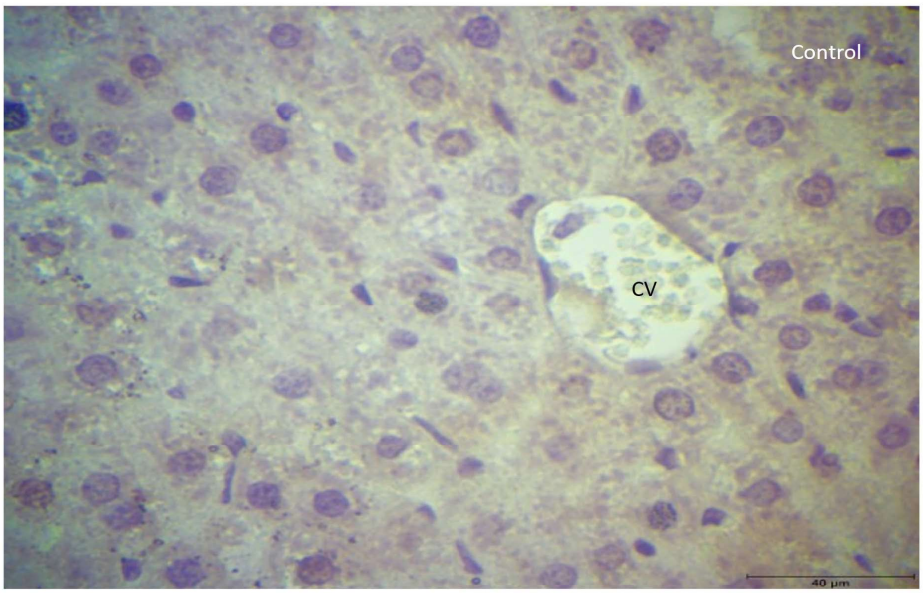

Figure 4.3 PCNA immunohistochemistry staining of a liver section, (control group).

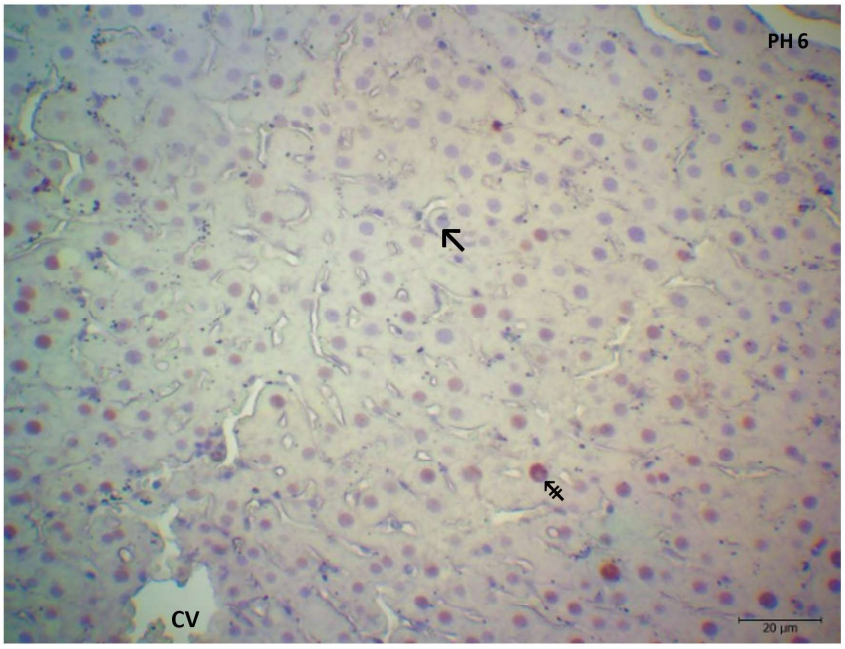

Figure 4.4 PCNA immunohistochemistry staining of a liver section of PH6 group depicting positive PCNA (↟) cells and negative PCNA cells (↑).

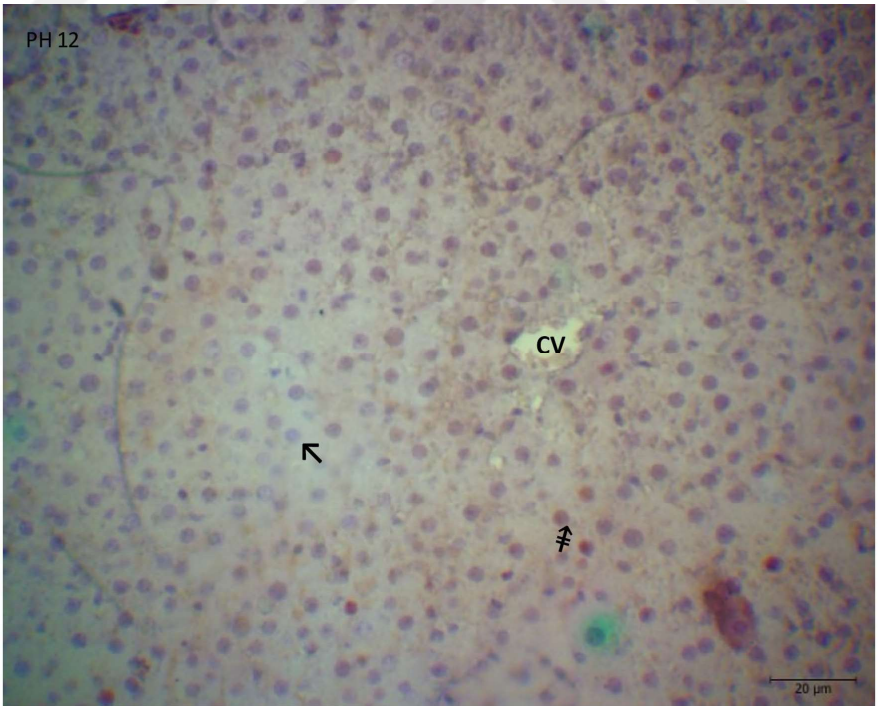

Figure 4.5 PCNA immunohistochemistry staining of a liver section of PH12 group depicting central vein (CV), positive PCNA (↟) cells and negative PCNA cells (↑).

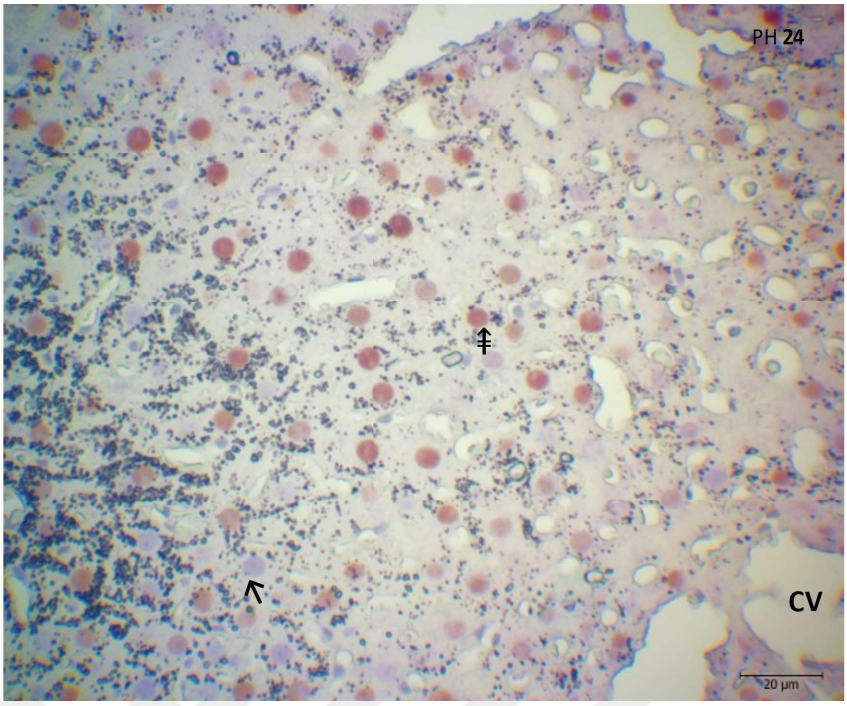

Figure 4.6 PCNA immunohistochemistry staining of a liver section of PH24 group depicting central vein (CV), positive PCNA (⇞) cells (red colour) and negative PCNA (↑) cells (Blue colour)

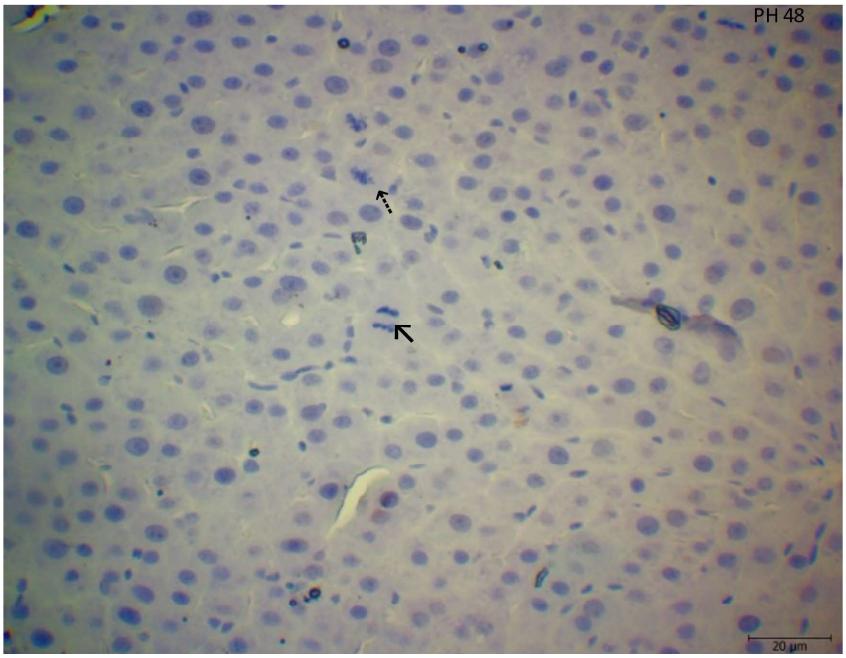

Figure 4.7 PCNA immunohistochemistry staining of a liver section of PH48 group depicting positive PCNA (⇞) cells and cells in late prophase (↑).

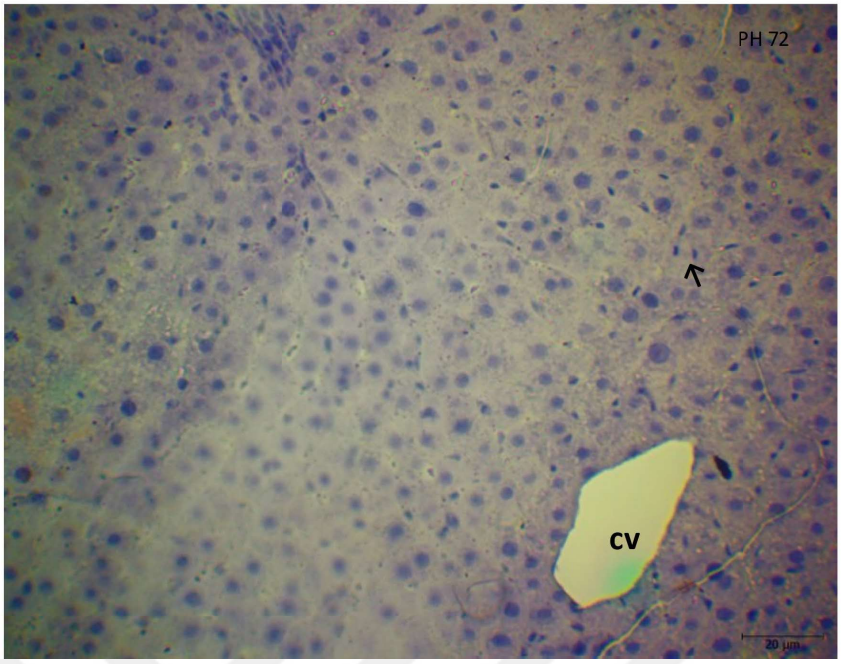

Figure 4.8 PCNA immunohistochemistry staining of a liver section of PH72 group depicting Kupffer cells (↑).

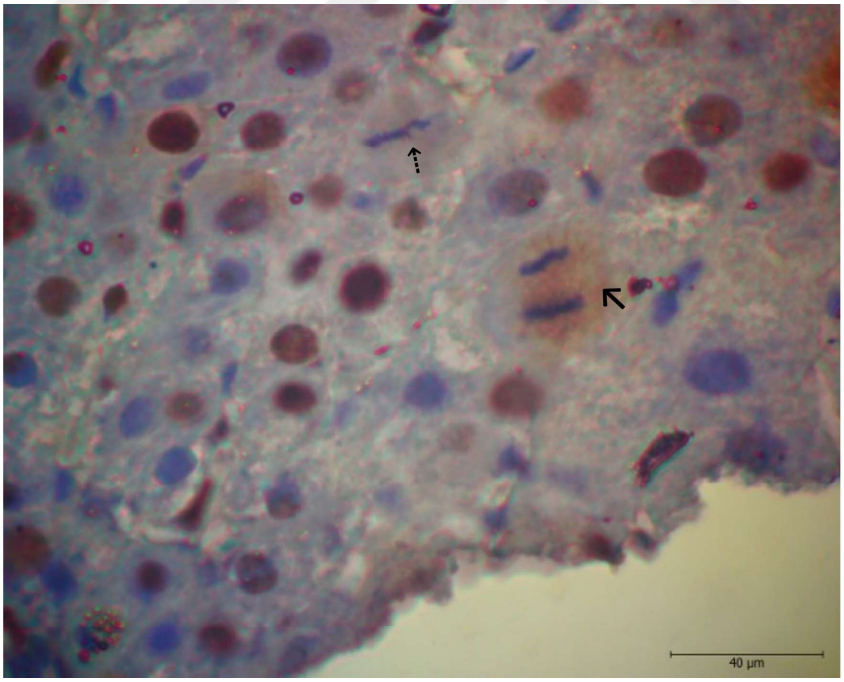

Figure 4.9 PCNA immunohistochemistry staining of a liver section of PH72 group depicting cells in anaphase (⇡) and late anaphase (↑).

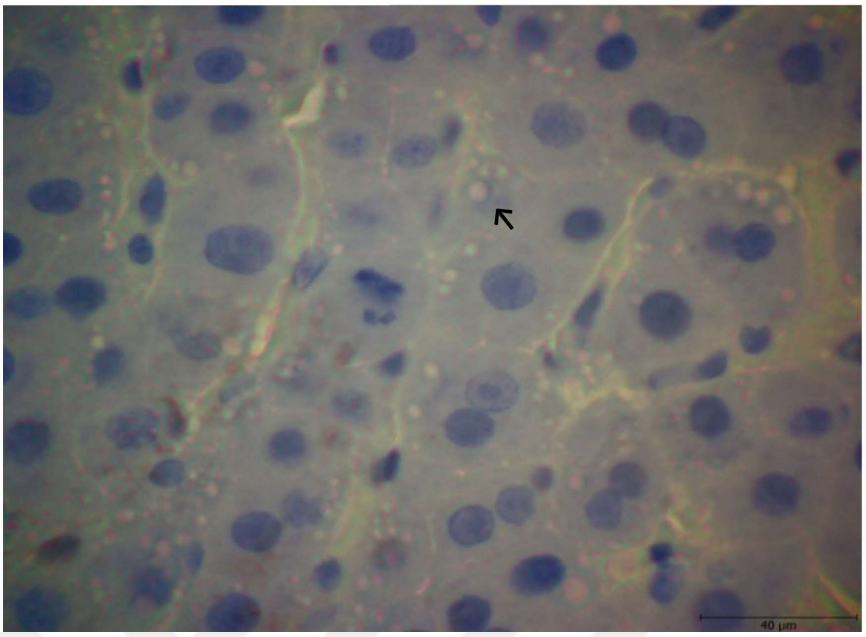

Figure 4.10 PCNA immunohistochemistry staining of a liver section of PH48 group depicting vacuolization in cells (↑), one of the key signs of liver regeneration.

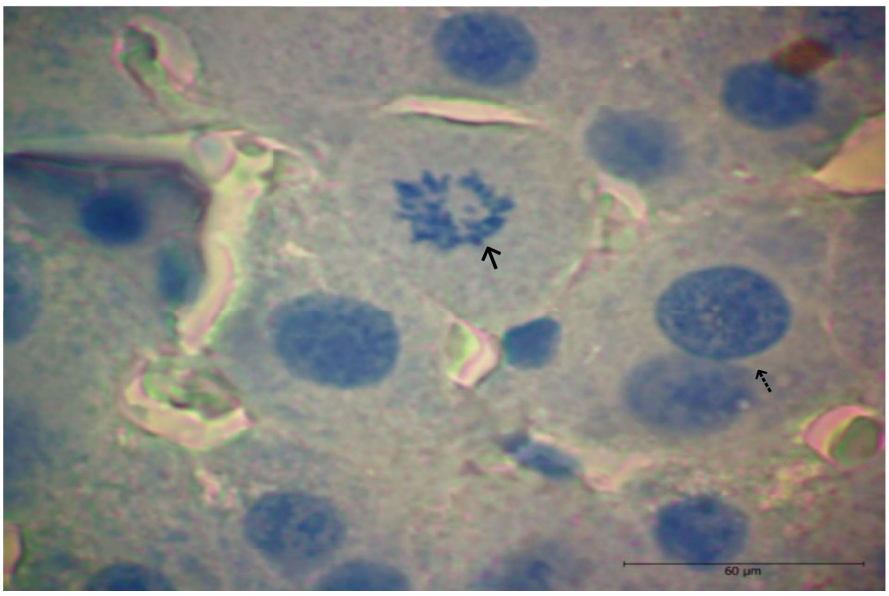

Figure 4.11 PCNA immunohistochemistry staining of a liver section of PH72 group depicting cells in MNGHs (↟) and multi-nucleated mega hepatocyte (↑).

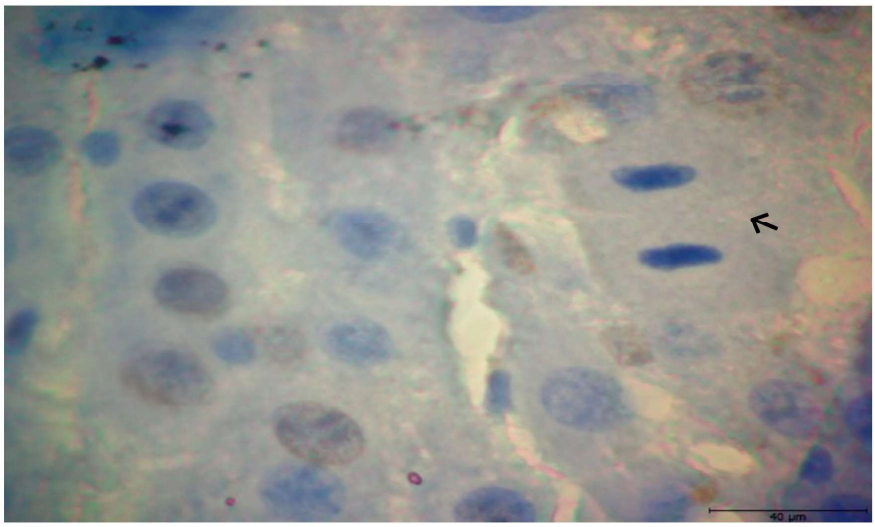

Figure 4.12 PCNA immunohistochemistry staining of a liver section of PH48 group depicting cells in cytokinesis (↑).

4.3. RT-PCR and Western Blot results analysis

Change in threshold cycle or delta threshold cycle (ΔCt and ΔΔCt methods) (Livak method) were used to calculate gene expression differences in RT-PCR data analysis. In this analytic method, reference gene such as (β-actin) whose expression is accepted as unchanged under any circumstances, was used. In this way, its use may minimize the differential biases resulting from the preparatory processes of RNA isolation and cDNA (cloned DNA) synthesis. (Mazza and Mazzette, 2014).

Genome software may be used in selection of the appropriate reference gene (Alizai et al., 2016). The most stable gene for control gene expression in our study was determined to be β-Actin. The geometric mean of this gene was taken (Rychtrmoc et al., 2012) and normalization was performed by finding ΔCt. The gene formula (ΔCt sample−ΔCtref) was used to calculate the (ΔΔCt) value. Fold values were calculated by converting the ΔΔCt values to $2^{-\Delta\Delta Ct}$ values (Tables: 4.1, 4.2 and 4.3; Appendixes: B and C) (Livak and Schmittgen, 2001; Alizai et al., 2016; Yu et. al., 2016). Those with a fold value of ≤0.5 and a fold value of ≥2 was considered significant (Fujimoto et al., 2012). Differences in gene expression between groups were evaluated using the GraphPad 9 program, and those with a p value less than or equal to 0.05 ($p \leq 0.05$) were considered statistically significant.

Table 4.1 Fold values ($2^{-\Delta\Delta Ct}$) Green representing fold value change ≥2 (gene upregulation) and red is for fold value change ≤0.5 indicating downregulation.

Groups vs Target genes	Fold change ($2^{-\Delta\Delta Ct}$) up-regulated (↑) and down-regulated (↓) gene expression						
	CON	SH0	SH6	SH12	SH24	SH48	SH72
MitoNEET	1.0	0.91	3.2	1.9	0.99	1.5	2.3
HIF-2α	0.63	0.17	0.35	0.86	0.082	0.23	5.2
ACO1/ IRP1	1.07	0.83	0.28	0.32	0.39	0.48	0.33
Mitoferrin	1.0	0.84	1.8	1.3	1.8	1.9	2.0
MINER1	0.92	0.89	1.3	1.3	0.95	0.87	0.61
SDHA	1.1	0.91	0.77	1.2	0.80	1.2	0.98
CMYC	1.1	0.46	1.5	0.67	0.26	0.26	1.4

Table 4.2 Fold values ($2^{-\Delta\Delta Ct}$) Green representing fold value change ≥2 (gene upregulation) and red is for fold value change ≤0.5 indicating downregulation.

Gruplar Genler	Fold change ($2^{-\Delta\Delta Ct}$) upregulated expression (↑) and downregulated expression (↓) genler					
	PH0	PH6	PH12	PH24	PH48	PH72
MitoNEET	7.8	3.5	2.5	1.6	0.85	1.2
HIF-2α	5.1	4.3	1.8	0.40	0.20	0.27
ACO1/ IRP1	0.003	1.20	0.41	0.64	0.52	0.59
Mitoferrin	0.21	0.74	0.62	1.1	0.79	0.69
MINER1	0.098	0.55	0.61	1.6	0.96	0.46
SDHA	0.00072	0.63	0.43	0.60	0.52	0.40
CMYC	5.9	4.0	2.2	1.0	0.34	0.64

Table 4.3 ΔCt values of genes in all the experimental groups (Mean ± SD)

GENES		Mean of ΔCt values ± SD					
		0	6	12	24	48	72
PGC-1α	SH	0.76 ± 0.40	0.60 ± 0.077	0.38 ± 0.25	0.31 ± 0.35	0.86 ± 0.47	0.42 ± 0.50
	PH	0.11 ± 0.0093	0.97 ± 0.27	0.43 ± 0.18	0.16 ± 0.062	0.20 ± 0.055	0.18 ± 0.069
MitoNEET	SH	0.91 ± 0.15	3.2 ± 1.0	1.9 ± 0.33	0.99 ± 0.13	1.5 ± 0.81	2.3 ± 1.1
	PH	7.8 ± 1.1	3.5 ± 0.61	2.5 ± 0.060	1.6 ± 0.45	0.85 ± 0.24	1.2 ± 0.29
HIF-2α	SH	0.17 ± 0.12	0.35 ± 0.20	0.86 ± 0.21	0.082 ± 0.045	0.23 ± 0.24	5.2 ± 0.63
	PH	5.1 ± 2.3	4.3 ± 0.30	1.8 ± 1.2	0.40 ± 0.29	0.20 ± 0.084	0.27 ± 0.025
ACO1-IRP1	SH	-0.65 ± 1.7	2.1 ± 0.78	1.7 ± 0.24	1.7 ± 1.1	1.2 ± 0.46	1.1 ± 0.99
	PH	5.5 ± 4.8	-0.84 ± 0.56	3.4 ± 2.9	0.79 ± 0.46	1.1 ± 0.50	0.081 ± 1.6
Mitoferrin	SH	0.84 ± 0.15	1.8 ± 0.39	1.3 ± 0.067	1.8 ± 0.48	1.9 ± 0.28	2.0 ± 0.49
	PH	0.21 ± 0.08	0.74 ± 0.34	0.62 ± 0.26	1.1 ± 0.12	0.79 ± 0.10	0.69 ± 0.32
MINER1	SH	0.89 ± 0.19	1.3 ± 0.17	1.3 ± 0.19	0.95 ± 0.26	0.87 ± 0.097	0.61 ± 0.42
	PH	0.098 ± 0.05	0.55 ± 0.18	0.61 ± 0.38	1.6±0.37	0.96±0.31	0.46 ± 0.20
SDHA	SH	0.91 ± 0.39	0.77 ± 0.29	1.2 ± 0.11	0.80 ± 0.15	1.2 ± 0.17	0.98 ± 0.14
	PH	0.00072 ± 7.9e-005	0.63 ± 0.27	0.43 ± 0.36	0.60 ± 0.19	0.52 ± 0.10	0.40 ± 0.057
c-MYC	SH	0.46±0.13	1.5 ± 0.75	0.67±0.49	0.26±0.13	0.26±0.11	1.4±0.64
	PH	5.9 ± 2.0	4.0 ± 1.2	2.2±0.72	1.0±0.088	0.34±0.19	0.64±0.19
GLRX5	SH	0.036 ± 0.026	0.10±0.15	0.061±0.061	0.092±0.15	0.013±0.0091	0.051±0.044
	PH	0.16 ± 0.16	0.0093 ± 0.0021	0.22±0.29	0.0032 ± 0.0006	0.0061 ± 0.0014	0.0079 ± 0.0062
ACO2	SH	8.5 ± 3.3	6.2 ± 4.2	3.0 ± 3.9	5.6 ± 2.6	4.7 ± 5.8	5.4 ± 2.3
	PH	7.1 ± 1.2	6.1 ± 1.4	7.5 ± 0.60	6.4 ± 5.7	8.6 ± 1.8	12 ± 1.5

4.4. Gene and protein expression

Research studies are still ongoing to unravel both the early stimulating events and the late terminating processes, characterizing the molecular basis of gene interactions during liver regeneration process. Exceptionality of liver regenerative capacity may be well known. However, proximal events stimulating the process and the distal signals terminating the liver regeneration process are still unknown. Such processing signals may define the general principles governing the hepatic regenerative response. And include tight regulation of specific extracellular and intracellular signals required during normal liver regeneration (Eming et al., 2014; Fan et. al., 2016). These signals and many other complex cellular events occur at the molecular level in liver regeneration (Chen et. al., 2010). This complex chain of events that occur in liver regeneration defines our motives for this thesis.

The 70% partial hepatectomy model is an important model for understanding the initiation, progression, and termination of tissue growth in liver regeneration (Kurinna and Barton, 2011). It provided us the opportunity to study molecular interaction of Fe-S genes with other cellular genes intracellularly, as a perfect model for *in vivo* cell biology studies (Hu et. al., 2014). Characterization, purification and oxygen lability of Fe-S clusters, make studying it outside of its natural ecology challenging. The cellular roles of several Fe-S genes are still unknown or debatable (Ferecatu et. al., 2014).

During liver regeneration after PH in rats, hepatocytes are said to divide at most twice and return to the G_0 phase. The entry of cells from the G_0 phase to the G_1 phase after PHx, occurs 4-6 hrs after PHx (Ozeki and Tsukamoto, 1999; Fausto, 2000). In the study of Xu et al., (2005), cells were reported to have entered into the S phase 12 hrs after PHx (Xu et. al., 2005). DNA replication is said to peak initially at 24 hrs after PHx (Starkel et al. 2005) and later at 30-32 hrs. The G_2 phase lasts 2-4 hours and is approximately 36-48 hrs PHx (Xu et. al., 2005). Cells were then shown to enter mitosis every hour (Steer, 1995; Fausto, 2000; Kurinna and Barton 2011).

Mitochondrial oxidative phosphorylation is known to fuel the liver regenerative process, and the energy status of the liver is known to be associated with post-hepatectomy liver function and clinical outcome (Ozawa et. al., 1982; Satoh et. al., 1996; Mann et. al., 2002; Alexandrino et al., 2016). Koyama et. al., (1998) also reported an increased

mitochondrial DNA and RNA metabolism within the remnant liver in the early hours post-PHx, as well as increased expression of several enzymes involved in energy metabolism, such as the ETC enzyme cytochrome oxidase (COX) (Koyama et. al., 1998). This may result in an overall increase in the energy-producing capacity of the liver in the first 2-4 hrs after PHx (Nagino et. al., 1989).

Mitochondrial quality and quantity in cells are affected by the balance of formation of new mitochondria (biogenesis) and destruction of diseased mitochondria (mitophagy) (Stotland and Gotlieb, 2015). Fe-S genes and proteins may be an essential component of this dynamic mitochondrial process.

Our aim was to explore for the first time in liver regeneration;

i. The possible molecular interactions of Fe-S genes (mitoNEET, Miner1, SDHA, ACO1/IRP1, ACO2, GLRX5,) with associated genes (HIF-2α, c-MYC, mitoferrin) and a mitochondrial biogenesis-related gene (PGC-1α), at various time points (0, 6, 12, 24, 48, 72) after PHx.
ii. To further understand mechanisms behind the cellular, molecular and physiological functions of Fe-S genes and proteins.
iii. The probable molecular basis of Fe-S biogenesis as an essential factor of mitochondrial biogenesis in the liver regenerative process at various time points (0, 6, 12, 24, 48, 72), after partial hepatectomy.

In our study, Fe-S genes with high gene expression and statistically significant ($p \leq 0.05$) were found to be (mitoNEET****; ACO1***; MINER1*; SDHA*) and associated genes (HIF-2α****; mitoferrin***; and c-MYC****). Gene expressions were determined to be statistically more significant in the PH groups, except for mitoferrin. Expressional patterns of the genes: upregulation, downregulation, statistical significance and inter-group are as given in (Figure 4.13; Table 4.4) Genes were upregulated or downregulated.

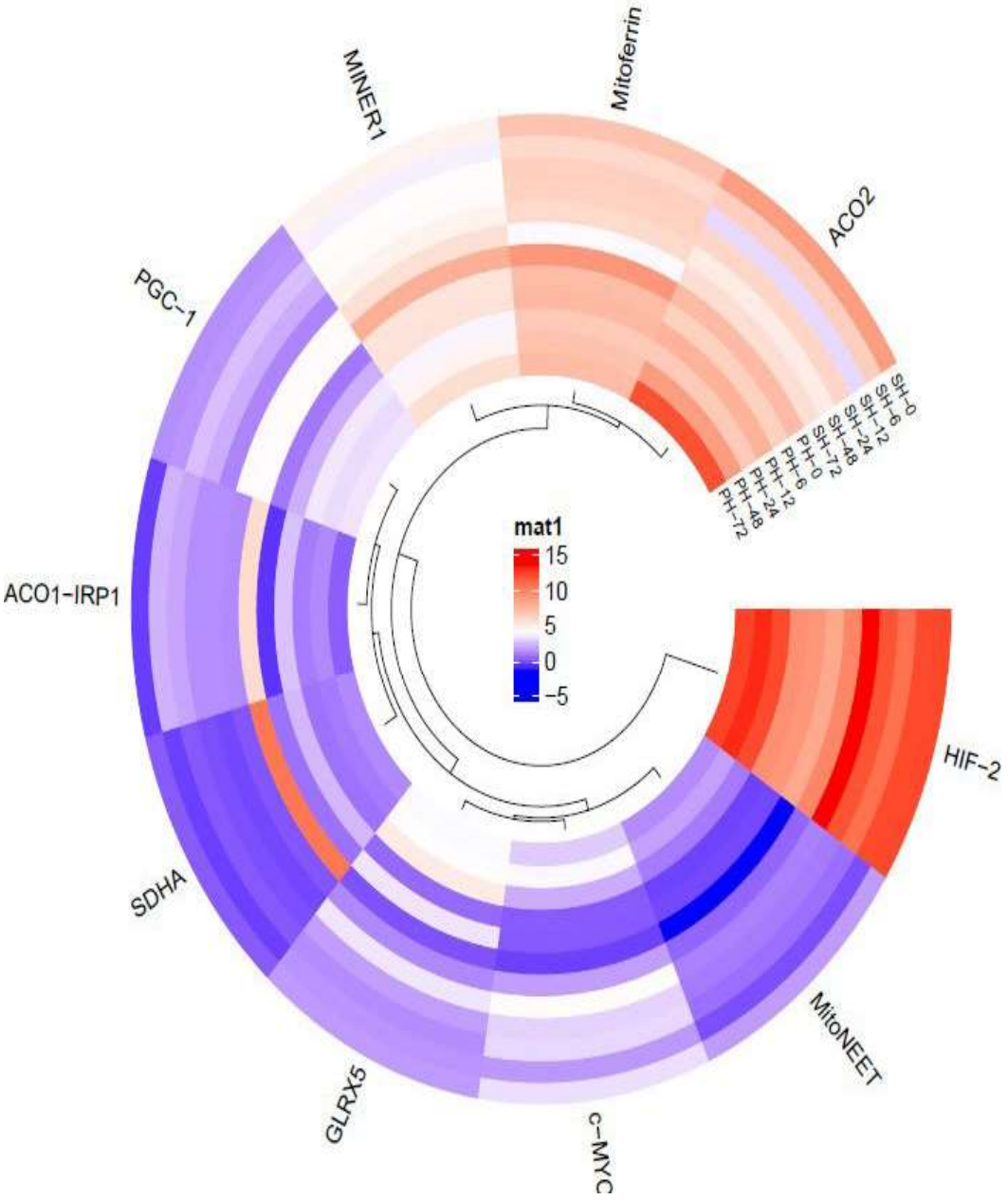

Figure 4.13 Circular cluster heatmap of differentially expression patterns of some Fe-S-containing genes with their interacting genes in a liver regeneration model (Generated from https://www.bioinformatics.com.cn/en, a free online platform for data analysis and visualization)

Table 4.4 Comparative analysis of inter-groups gene expressions

Genes	COMPARATIVE STUDIES OF INTER-GROUPS GENE EXPRESSIONS					
	SH0 vs PH0	SH6 vs PH6	SH12 vs PH12	SH24 vs PH24	SH48 vs PH48	SH72 vs PH72
PGC-1α	0.2202	0.9999	> 0.9999	> 0.9999	0.1954	0.9950
MitoNEET	< 0.001 (****)	< 0.99999	0.9803	0.9860	0.9619	0.4992
HIF-2α	< 0.0001 (****)	< 0.0001 (****)	0.9479	> 0.9999	> 0.9999	< 0.0001 (****)
ACO1-IRP1	0.1180	< 0.0001 (****)	> 0.9999	0.9984	> 0.9999	0.9984
Mitoferrin	0.3232	0.0084 (**)	0.2484	0.1408	0.0050 (**)	0.0004 (***)
MINER1	0.0259 (*)	0.0446 (*)	0.1231	0.1231	> 0.9999	0.9999
SDHA	0.0332 (*)	> 0.9999	0.6637	0.9997	0.3255	0.4886
c-MYC	< 0.0001 (****)	0.0210 (*)	0.4402	0.9871	> 0.9999	0.9876
GLRX5	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
ACO2	> 0.9999	0.9994	0.9683	> 0.9999	> 0.9999	> 0.9999

- PGC-1α: (PH0-PH6) ~ (**p= 0.0325,** *)
- MINER1: ([PH0-PH24 ~ (**p value= < 0.0001,** ****); PH6-PH24 ~ (**p value= 0.0010,** ***); PH24-PH72 ~ (**p value= 0.003,** ***)]

According to the expressional patterns and observed significance, our genes of interest were observed to be most active in the early and intermediate (priming and proliferation) phases of the liver regenerative process.

The possibility of high iron demand in the regenerating hepatocytes, could only mean another iron carrier protein may have been preferred over mitoferrin. In determining the protein expression, DMT-1 (in place of mitoferrin), mitoNEET (CISD1), IRP 1 (ACO1) and

HIF-2α (EPAS) with statistically significant gene expression ($p \leq 0.05$), were selected for Western Blotting study.

It is common knowledge that hepatocyte DNA replication begins approximately 12 hours after PH and normally peaks at 24 hours. However, there is a second peak at 36-66 hours in rats. In this study, 10 time points during liver regeneration are determined as 3 phases; 0-6 hours (early phase), 12-72 hours (intermediate phase), when hepatocytes are activated and G_0/G_1 transition occurs. 120-168 hours when cell proliferation occurs and regeneration ends (late phase) (Wang and Xu, 2011).

Previous work has demonstrated that the acute-phase response dominates during the first 18 h after PH, but from that point on, expressions of genes related to the growth of the liver become increasingly important, and the synthesis of acute-phase plasma proteins is gradually reduced (Milland et. al., 1990).

In our study, when we looked at the literature and carefully examined the genes that were found to be significant as a result of RT-PCR array analysis, it was determined that they were related to the triggering of signal molecules involved in metabolic changes, angiogenesis, cell signalling and iron metabolism, and mechanisms of mitochondrial biogenesis, in the early (initiation and proliferation) phase of liver regeneration.

4.4.1. PGC-1α

Expression of PGC-1α as a mitochondrial biogenesis-related gene, was evaluated according to 0, 6, 12, 24, 48, 72 hours and compared with the SH groups. It was determined no significant difference exists between the SH and PH (**Figure 4.14**). It is worth mentioning that the statistical data show a significant different was witnessed between PH0 and PH6, within the PH groups ($p \leq 0.05$; ($p = 0.0325$, *)) (**Table 4.4**; **Appendix C**). Our findings are similar to that of Wang et. al., (2008).

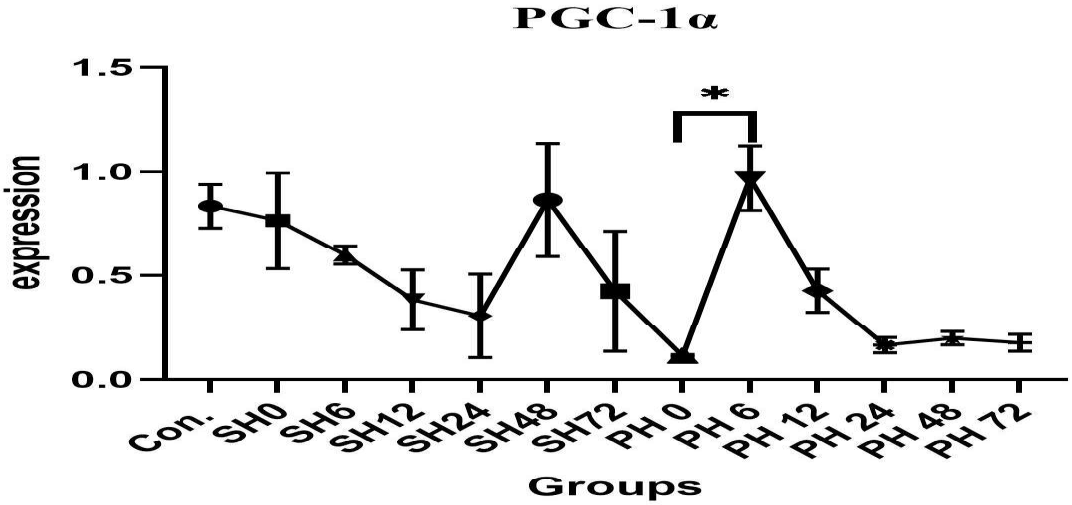

Figure 4.14 PGC-1α gene expression

Wang et. al., (2008) reported a significantly increased PGC-1α induction after a PHx, with maximum gene and mRNA expression observed at the (2 and 4) hrs (Wang et. al., 2008). Our results may be similar, as we observed a very sharp and significant rise in PGC-1α expression between 0-6 hrs, an acute response phase of the liver regenerative process. SH groups showed a decreasing PGC-1α expressional pattern as time increases, except the SH48 group.

Moreover, in a different study analysing muscle regeneration days after post injury (dpi), Beltra et. al., (2022) reported an increased PGC-1α expression was strongly seen in the initial stages of muscle regeneration at 7 and 10 dpi before returning to normality between 13-49 dpi. This may represent a PGC-1α-mediated activation of mitochondrial biogenesis in the early phase of muscle regeneration, to restore their metabolic identity (Beltra et. al., 2022).

PGC-1α is a known key regulator of mitochondrial biogenesis and energy metabolism. As a transcriptional co-activator of PPAR genes family, PGC-1α is usually abundantly expressed in highly energy-dependent tissues such as musculoskeletal, brain, cardiac and renal tissues, but quite lowly-expressed in the liver and white adipose tissue (WAT) in the fed state (Puigserver et. al., 1998). Upon a physiological stimulus such as fasting and exercise, PGC-1α is induced to meet high energy requirements of such stimuli (Gureev et. al., 2019). Its induction regulates several genes involved in energy metabolism pathways (TCA, ETC, OXPHOS) and co-induction of oestrogen-related receptor α (ERRα)

(Hatazawa et. al., 2012). This stimulates mitochondrial biogenesis via mitochondrial transcription factor A-regulated mtDNA replication and transcription (Dillon et. al., 2012).

PGC-1α is stimulated by glucagon and glucocorticoids (Yoon et. al., 2001). Upon PGC-1α stimulation, hepatic gluconeogenesis, glycogenolysis and fatty acid oxidative metabolism in the liver to are activated to increase glucose contents (Herzig et. al., 2001; Leone et. al., 2005; Lin et. al., 2004). These processes were shown to activate key gluconeogenic genes such as glucose-6-phosphatase (G-6-Pase) and phosphoenolpyruvate carboxykinase (PEPCK), activate fatty acid β-oxidation and regulate heme biosynthesis (Koo et. al., 2004; Wang et. al., 2008).

However, because the dimensions of metabolic demands and nature of energy-demanding cellular processes of liver regeneration after a PHx are different from that of a physiological fasting, PGC-1α was observed to be sharply activated in the early priming phase (2-4 hrs) after a partial hepatectomy (Wang et. al., 2008). This dramatic induction may be reflective of the need to immediately rewire metabolism for production of necessary cell building materials (ATP, phospholipids, nucleotides, ribonucleotides, amino acids) to support the compensatory hepatic regrowth. And to restore glucose homeostasis.

In our study, comparative expressional pattern of PGC-1α induction in the SH and PH was observed to be non-specific. Nonetheless, the intra-group expression dynamics of PGC-1α in PH leaves much to be desired. Unlike in the SH groups, a significant sharp rise in PGC-1αexpression at (0-6) hrs could be observed and this was very similar to existing literature (Wang et. al., 2008). PGC-1α induction was reported to be both transcriptionally and post-transcriptionally regulated by multiple factors. Neither the PHx-induced stress nor metabolic stress may be enough to trigger PGC-1α induction. Just as it is in liver regeneration, no one single factor is enough to trigger the liver regeneration process, it has become very necessary to research the various parameters involved in PGC-1α induction and its possible role in linking mitochondrial biogenesis to metabolic rewiring in the early phase of the liver regenerative.

AMPK-mediated PGC-1α phosphorylation and (Jäger et al.,2007) and cGMP-mediated PGC-1α activation (Nisoli et al., 2003), are known to start the mitochondrial biogenesis. Increasing levels of nitric oxide was seen to upregulate cGMP and thus PGC-1α

activation (Nisoli et al., 2003). Increasing nitric oxide activate TNF-α, an important component of the cytokine signalling involved in the early molecular events of liver regeneration (Kiseleva et. al., 2021). This further indicates the need to pursue not only the molecular interactions of PGC-1α in cellular proliferation but mitochondrial dynamics and biogenesis in whole.

4.4.2. MitoNEET (CISD1) gene and protein expression

MitoNEET gene expression, was evaluated according to 0, 6, 12, 24, 48, 72 hours and compared with SH groups. It was determined that MitoNEET expression peaked in PH0 (fold=7.8), PH6 (fold=3.5) and PH12 (fold=2.5) groups and showed a significant difference. There was no difference between the SH and PH 24, 48, 72 hrs groups (**Figure 4.15**).

In Western blot analysis of MitoNEET; MitoNEET protein content was high in PH0, PH6 and PH12 groups; In the PH48 group, the amount of protein was found to be low (**Figure 4.16 and 4.17**).

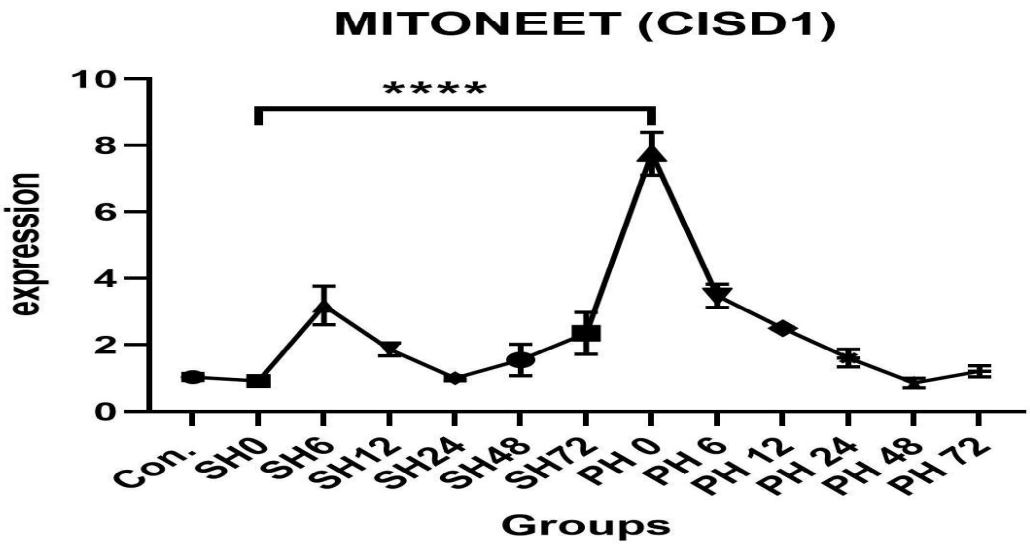

Figure 4.15 MitoNEET (CISD1) gene expression

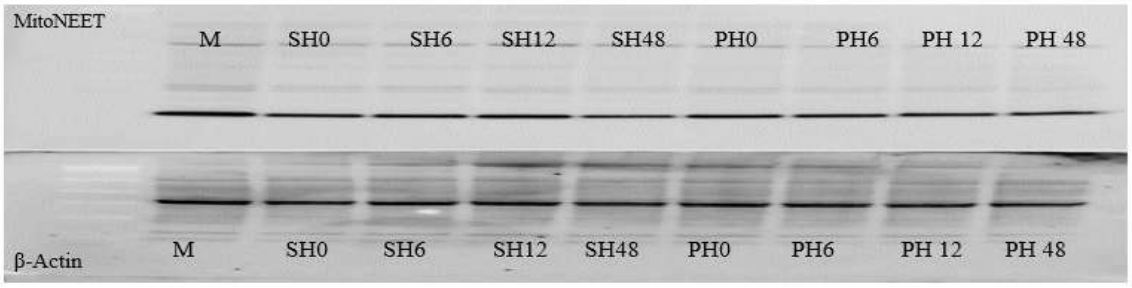

Figure 4.16 protein expression bands of MitoNEET (CISD1)

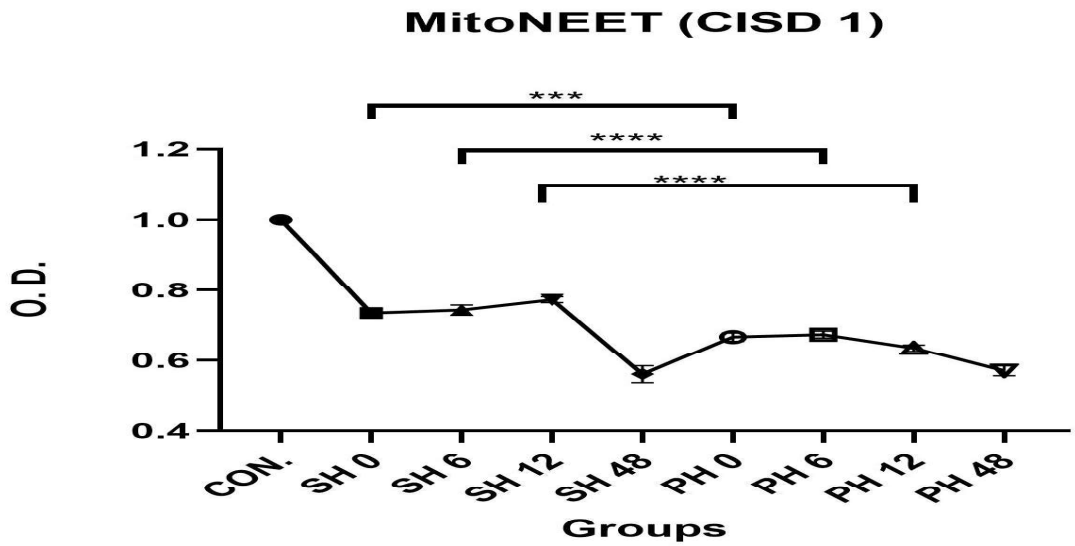

Figure 4.17 Graphical depiction of MitoNEET (CISD1) protein expression

Studies on gene and protein expression of mitoNEET in liver regeneration after a 70% PHx is very scarce. Gene and protein expression of mitoNEET in such a non-drug, non-genetically manipulated rat model used in our study, may be the first to report on mitoNEET expression post-PHx.

MitoNEET has been reported in other model of tissue repair and renewal. In a study of spinal cord injury (SCI) repair and renewal, pioglitazone was given at 15 min or 3 hrs post-SCI. Pioglitazone directly interacting with mitoNEET, showed significant neuroprotection via sustenance of a functional mitochondrial respiration 24 hrs after SCI. Pioglitazone-mitoNEET interactions after SCI, may help sustain a functional mitochondrial homeostatic balance (Yonutas et. al., 2020).

Also, in white adipose tissue (WAT) model, Kusminski et. al., (2012) showed that via reduced mitochondrial oxidative capacity and regulation of mitochondrial matrix iron in

WAT, mitoNEET activated PPARγ and increased adiponectin production. This caused massive WAT expansion and improved sensitivity of hepatic cells to insulin. MitoNEET causes massive adipose tissue expansion and improves hepatic insulin sensitivity. MitoNEET-overexpressing adipocytes were also seen to significantly increase intracellular uptake and storage of lipids, thus increasing tissue mass (Kusminski et. al., 2012).

Vernay et. al., (2017) further demonstrated the cellular loss of mitoNEET resulted in decreased mitochondrial volume and thus decreased cellular respiration. This established the essential roles of mitoNEET in regulation of a functional mitochondrial homeostasis and lipid metabolism (Vernay et al., 2017).

This may indicate that cellular levels of mitoNEET are regulated to meet changing metabolic demands of the cells during cellular proliferation. Changing mitoNEET levels is now considered a characteristic hallmark of metabolically challenged tissues (Kusminski et. al., 2012), such as the regenerating hepatocytes.

Initiation of liver regeneration is tightly-bound to the priming phase of the liver regenerative process. Central to those molecular processes is the expression of the ALR protein, which is immediately produced post-PHx. ALR is reportedly involved in post-injury hepatocyte regeneration (Polimeno et al., 2011), and offered hepatoprotection (Liu et al., 2019) as well as renal protection (Huang et al., 2018). ALR may also play important molecular roles in cardiac development (Dabir et al., 2013) and maintains embryonic stem cells (Todd et al., 2010).

ALR is a predominantly mitochondrial sulfhydryl oxidase protein expressed in the liver, kidney, brain, etc. It is made up of three (3) isoforms: mitochondrial long (full-length) isoform, cytoplasmic short isoform and long form ALR. The mitochondrial full-length form ALR is precisely known to interact with mitoNEET and relocates it to the mitochondrial intermembrane space. Thus, mitoNEET is released and molecular processes leading to hepatocyte proliferation initiated (Nalesnik et. al., 2017; Ibrahim and Weiss, 2019). Cytoplasmic short-form ALR elevates IL-6-sensitivity in hepatocytes via induction of STAT3 phosphorylation (Li et al., 2002; Ibrahim and Weiss, 2019). Increased expression of the other long form ALR is to reduce liver damage in pathologic conditions. Long form ALR

functions to decrease cellular levels of Ca^{2+} and provides protection against ER and oxidative stress (Ibrahim and Weiss, 2019).

Following a 70% PHx, increasing ALR concentration and mitoNEET release may activate a chain of molecular events, including production of signal molecules, cell signalling, cell-to-cell interactions, functional mitochondrial dynamics, iron and ROS metabolism. ALR via induction of Kupffer cells, may cause an increasing release of IL-6, TNF-α and NOS. ALR-mediated STAT3 phosphorylation may elicit the IL-6/STAT3 signalling, a key signalling pathway known to initiate liver regeneration post-PHx (Ozen and Uyanoglu, 2018). Increasing ALR-mitoNEET-mediated MAPK, PI3K/AKT, and TNF-activated NF-kB signalling pathways, may have been amplified to sustain the proliferative signals to the regenerating hepatocytes.

Mitochondrial oxidative phosphorylation powers the liver regenerative process. This could result in increased mitochondrial ROS and stress production. Mitochondrial ROS production was seen to be reduced significantly in mitoNEET-overexpressed mice. However, absence of mitoNEET increased oxidative phosphorylation and ETC events (Wiley et. al., 2007; Furihata et. al., 2018), leading to mitochondrial dysfunction (Taminelli et. al., 2008). Thus, molecular interactions of ALR-mitoNEET may function in ensuring not only a functional mitochondrial dynamic (respiratory capacity), but also a tight-regulated balance of redox and iron metabolism (Kusminski et. al., 2012).

High iron affinity in the regenerating hepatocytes would drive an increased iron mobilization. MitoNEET is considered a potent regulator of cellular iron homeostasis and must activate the IRP1 (ACO1) for the mobilization and regulation of cellular iron (Ferecatu et al., 2014). In a molecular network, mitoNEET was seen to interact with glutathione reductase, a redox regulator (Landry et al., 2015) and glutamate dehydrogenase 1, a metabolic enzyme and insulin regulator (Roberts et al., 2013), to couple redox-metabolism axis to iron metabolism. In regenerating hepatocytes, it can be inferred therefore that hepatocyte proliferation may be linked to both Fe-S-dependence and iron-dependence.

A possible molecular mitoNEET-PARKIN association (Kusminski et al., 2016), may function to regulate mitochondrial stability and activating the mitophagy pathway

(Kusminski et al., 2012), especially the Parkin-mediated pathway (Villa et. al., 2018). Mitophagy has not been previously reported in any study of liver regeneration mechanisms.

4.4.3. HIF-2α gene and protein expression

Gene expression of HIF-2α, as a major gene of the HIF protein family, was evaluated according to 0, 6, 12, 24, 48 and 72 hrs in the PH groups compared with the SH groups. It was determined that HIF-2α gene was significant expressed in PH0 and PH6 groups. There was no difference in expression at 24 and 48 hrs between the SH and PH groups. But a very high expression was noticed at SH72 compared to PH72, of which we had no explanations (**Figure 4.18**).

Gene expressions of both HIF-1α and HIF-2α have been previously reported. HIF-1α was expressed in the earliest minutes of liver regeneration, peaking at 1 hr and continuously expressed till 3 hrs. HIF-2α was seen much later, at the 3rd hr till the 6th hr (Weisener et. al., 2003). Our findings seem similar to the previously reported results.

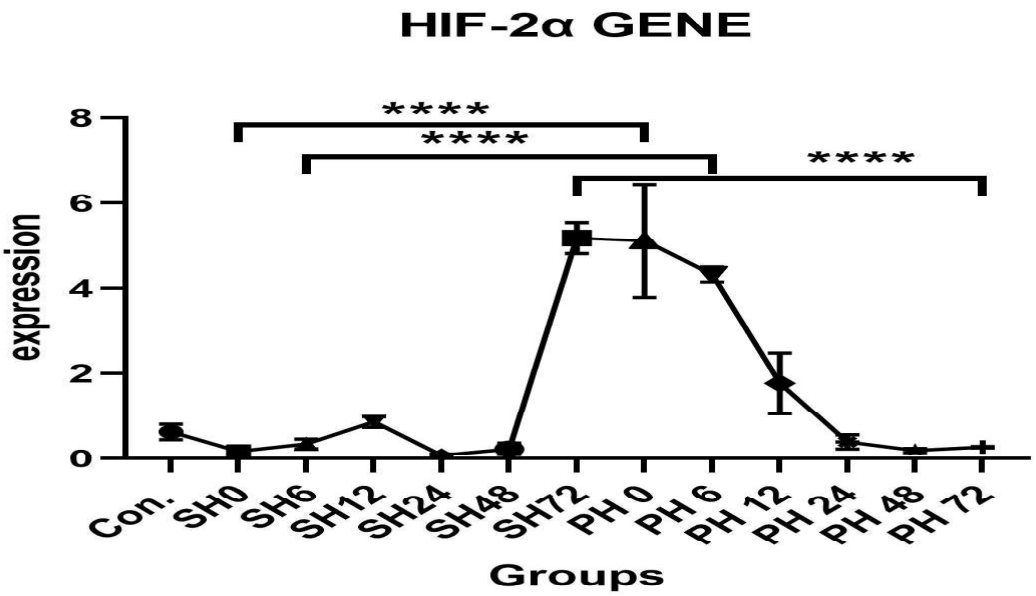

Figure 4.18 HIF-2α gene expression

In Western blot analysis of HIF-2α; HIF-2α protein expression was found to be significantly expressed at PH6 and PH48. Expression of the HIF-2α protein was found to be sustained within a certain expression plateau (PH0-PH12), before declining to the lowest

expression at PH48 (Fig. 4.1, Fig. 4.43c.). Similar results were reported have been reported (Weisener et. al., 2003).

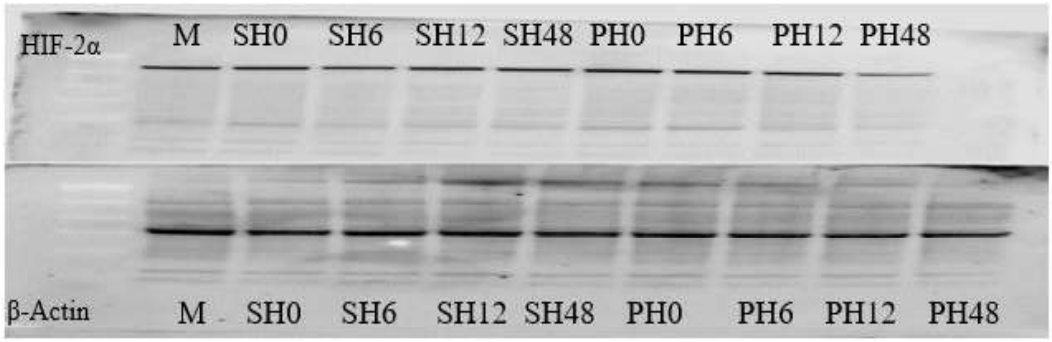

Figure 4.19 Protein expression bands belonging to HIF-2α and β-actin

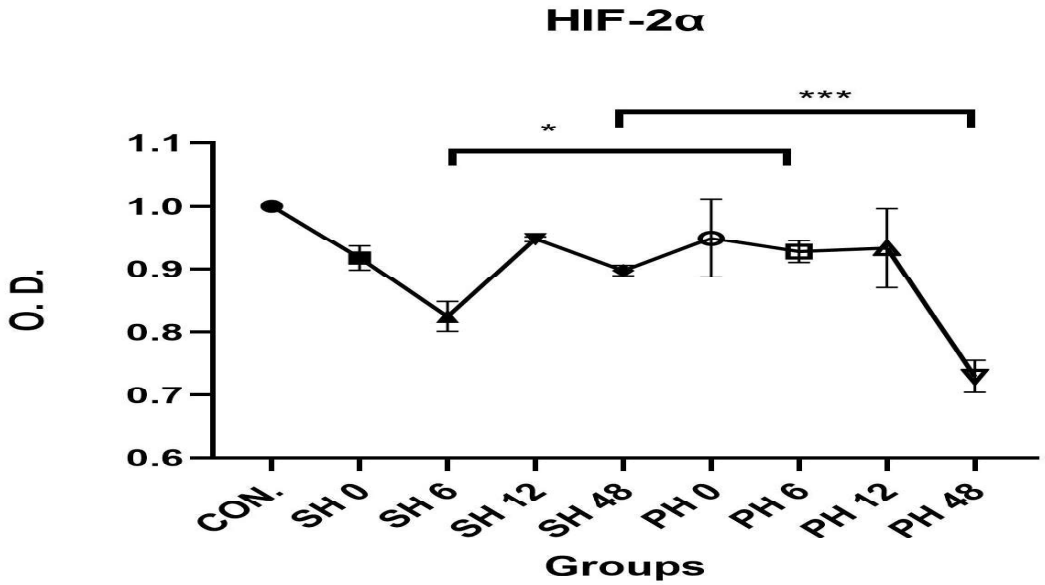

Figure 4.20 Graphical depiction of HIF-2α protein expression

In a different regeneration model using the house gecko tail, Titta et. al., (2019) also showed a peak HIF-1α mRNA expression on day 1 with high tissue counts of erythrocyte and leucocytes, while HIF-2α mRNA expression was observed to reach its peak on day 3, with active cellular proliferation, migration and differentiation. In an overlapping manner, as HIF-1α expression decreases, HIF-2α mRNA expression and overall cellular activity were observed to increase (Titta et. al., 2019). Similarly, they further reported that a more gradual but steady increase usually characterizes HIF-2α mRNA expression.

Primarily, *in vivo* HIF-2α regulation is via mechanisms of post-translational processing. Although only liver HIF-2a mRNA was fairly observed to increase, changes in HIF-2α mRNA abundance may not necessarily lead to HIF-2α induction. Whether the total abundance of HIF-2a mRNA in certain tissues is of importance for the ability to induce HIF-2a protein is less clear (Weisener et. al., 2003). A lot of factors interplay to induce HIF-2α in different tissues (**Figure 4.21**). However, O_2 tensions in the liver and erythropoietin were known to decrease from the peripheral tissues towards the liver lobule (Koury et. al., 1991). In a similar manner, this distinct zonation concentrates HIF-2α signals towards the central vein of the liver tissue. This is an established connection between induction of HIF-2α and oxygen gradients in the liver tissue (Koury et. al., 1991; Weisener et. al., 2003)

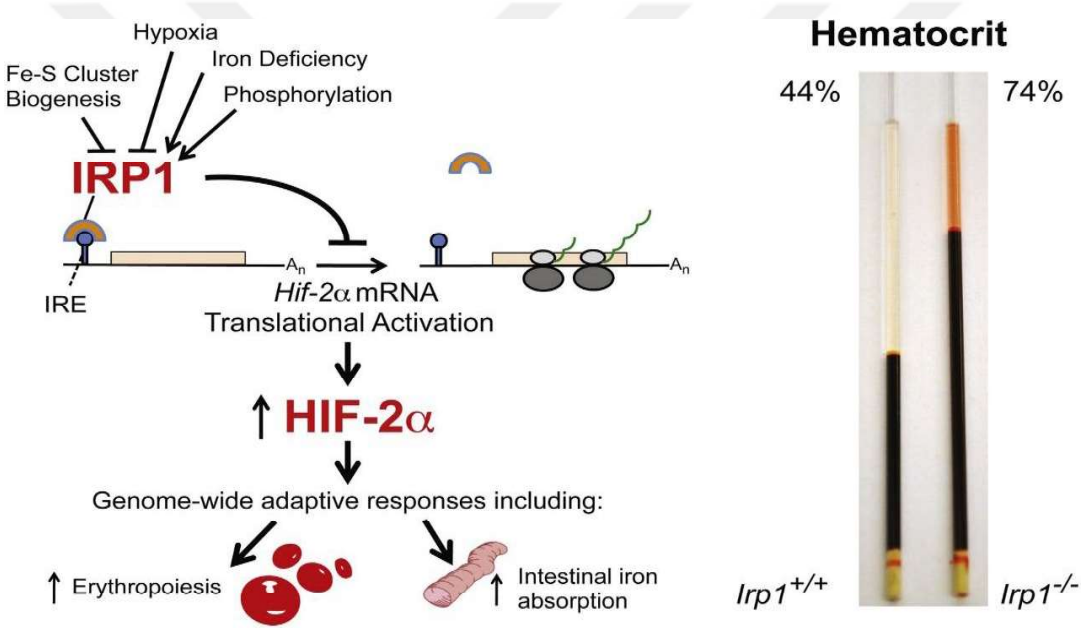

Figure 4.21 HIF-2α post-translational processing (Anderson et. al., 2013)

Hepatocytes are known to upregulate both HIF-1α and HIF-2α in state of tissue hypoxic pressure, such as in regenerating hepatocytes. HIF-1α and HIF-2α are thus expressed variably and overlap in functions. HIF-1α is transiently induced till 3 hrs, while HIF-2α induction was reportedly prolonged up to 6 hrs (Weisener et. al., 2003).

Immediately after the 70% partial hepatectomy section, 70% loss of the liver tissue may be evaluated in terms of 70% loss of tissue oxygen, glycogen, glucose, iron and all other contents. This loss may result in transient tissue hypoxic condition, amplified by tissue vascular (hepatic portal) pressure, redox state and other bio (-chemical/-physical) factors.

These may induce post-transcriptional regulation of HIF-α transcription factors (HIF-1α and HIF-2α), and (pVHL and PHD) inhibition via gene regulation (Maxwell et. al., 1999; Yu et. al., 2001).

After the PHx, HIF-1α may have been initially activated in response to PHx-induced hypoxia and redox state. Because of the time design (0, 6, 12, 24, 48, 72) in our study, we could only observe HIF-2α induction between (0-6) hrs, similar to the reported observation (Weisener et. al., 2003). In line with numerous studies, we assume that increasing O_2 and iron content after the PHx and the resulting redox mechanism may be controlled on a gene level to post-transcriptionally regulate HIF-2α.

Hepatocytes must mount a coordinated cellular response to the transient PHx-activated hypoxia and other related factors. HIF-1α must necessarily be activated. On the cellular and genetic level, deletion of HIF-1α (Tajima et. al., 2009) delays initial processes of liver regeneration while inhibition of pVHL and PHD (Mollenhauer et. al., 2012) accelerates hepatocyte cell cycle.

HIF-1α is both present in the cytoplasm and nucleus (Stroka et. al., 2001) but HIF-2α only accumulates in the nucleus (Weisener et. al., 2003). Hypoxic stimulation may change this nuclear translocation, affecting HIF-2α posttranscriptional induction. Therefore, Stroka et. al., (2001) opined that irrespective of the vascular pressure, *in vivo* tissue hypoxic response is regulated post-transcriptionally (Stroka et. al., 2001).

After a PHx, the resulting transient physiologic hypoxia in the liver may initially activate HIF-1α. Approximately 6 percent oxygen concentration (~6% O_2) state considered to be highly hypoxic was shown to be needed in HIF-1α activation (Weisener et. al., 1998).

As oxygen is rushed to the liver and increasing the overall O_2 content, HIF-2α may then be induced at much higher oxygen concentrations (Rosenberger et. al., 2002). The increasing O_2 and iron concentrations via redox mechanisms, may facilitate HIF-2α binding to DNA (Lando et. al., 2000).

MitoNEET as an essential player in regulation of mitochondrial iron homeostasis and cellular redox mechanism, can cause IRP1 activation (Ferecatu et. al., 2014). IRP1 via binding to IRE domain of HIF-2α mRNA, can induce HIF-2α (Hentze et. al., 2013).

Molecular binding of ALR-mitoNEET during the early phase of the liver regenerative process, may further induce HIF-2α via IRP1-mediated activation, leading to a possible molecular interaction involving ALR-mitoNEET-IRP1-HIF-2α complex.

This molecular interaction may play a role in activation of several HIF-2α-dependent cellular processes, especially erythropoietin production (and erythropoiesis). HIF-2α-mediated activation of VEGF (Hu et al., 2003), TGF-α (Gunaratnam et. al., 2006), cyclin D1 (Baba et. al., 2006) and oct4 (Covello et. al., 2006), may also be affected (Patel and Simon, 2008). Owing to these molecular interactions, HIF-2α can activate the MAPK/ERK pathway (Zhu et. al., 2016). And together with the ALR-mitoNEET complex, they may amplify the activated signalling pathways, and thus sustain the liver regenerative drive.

Excessive iron demand, reactive oxygen derivatives (ROS) and cellular proliferation in rapidly proliferating cells are highly intertwined and interdependent (Jones, 2008). ALR-mitoNEET are also important redox players in regulating mitochondrial ROS metabolism. In a study of adult HIF-2α-null mice, Scortegagna et. al., (2003) suggested that HIF-2α may regulate expression of antioxidant enzymes (Scortegagna et. al., 2003). This novel function may further support a molecular complex of ALR-mitoNEET-HIF-2α-IRP1. Considering the fact that haemoglobin-mediated O_2 transport in erythrocytes is iron-dependent, erythropoiesis may also be bound to iron-dependence. IRP1-mediated HIF-2α induction is thus linked to the erythropoietic-iron homeostasis balance. mitoNEET-mediated IRP1 activation coupled to IRP1-mediated activation of HIF-2α, may provide further a possibility ALR-mitoNEET-HIF-2α-IRP1 molecular interactions.

HIF-2α-mediated expression of c-MYC (Gordon et. al., 2007) has been demonstrated in several types, to promote cyclin D2 and E2F1 expression. This facilitates cell cycle progression. Zhang et. al., (2007) observed in a VHL-deficient renal cell carcinoma that reduced activity in c-MYC expression resulted in decreased amounts of DNA and consumed oxygen in mitochondria (Zhang et. al., 2007). Hepatic lipid metabolism *in vivo* was showed to be predominantly regulated by HIF-2α (Qu et al., 2011), a major metabolic adaptative mechanism to hypoxic conditions (Rankin et. al., 2009). Therefore, HIF-2α-c-MYC axis may be coupling cellular metabolism to cell cycle progression in rapidly regenerating hepatocytes.

In probable regulatory molecular network, ALR-mitoNEET-HIF-2α-IRP1 complex may interact to possibly enhance c-MYC-mediated expression of important cell cycle genes and proteins, linked to cellular metabolic biosynthesis needed in liver regenerative process. This links progression of cell cycle to increasing oxygen concentrations for mitochondrial oxidative phosphorylation process and rising iron availability to DNA/RNA metabolism, to support synthesis of essential metabolites and cellular precursors of amino acids, phospholipids, nucleotides and many others.

Expressional patterns of HIF-2α gene and protein in our study, were as previously reported (Weisener et. al., 2003). As it is in literature, we can possibly state that HIF-2α functioned to promote the growth signals of the early priming phase, sustaining the whole liver regenerative process. Therefore HIF-2α may be inferred both as a promoter and sustainer of the liver regenerative process.

However, in the regulation of HIF-2α by IRP1 as a Fe-S protein, oxygen-liability of Fe-S proteins, hypoxia, and Fe-S biogenesis, may all be possibly intertwined in a tightly-regulated molecular interactions to induce HIF-2α.

4.4.4. IRP1 (ACO1) gene and protein expression

ACO1/IRP1 is a dual functional gene, as a cytosolic aconitase enzyme and master cellular iron regulator. Gene expression of ACO1/IRP1 was evaluated according to 0, 6, 12, 24, 48, 72 hours in SH and PH groups. It was observed that IRP1 expression rose sharply, with highest expression at PH6 ($p \leq 0.05$), and showed a significant difference. There was no difference in 0, 12, 24, 48, and 72 hrs between the SH and PH groups (**Figure 4.22**).

In Western blot analysis of IRP protein content was high in PH6, PH12 and PH48 groups; In the PH48 group, it was observed that the amount of protein peaked (Figure: 4.23 and 4.24). The SH groups showed a declining expression while an increasing expression trend was observed in the PH groups. An unexplained high mRNA expression was seen to be induced in the control group. Similar results have been reported (Sheikh et. al., 2007; Mollbrink et. al., 2012).

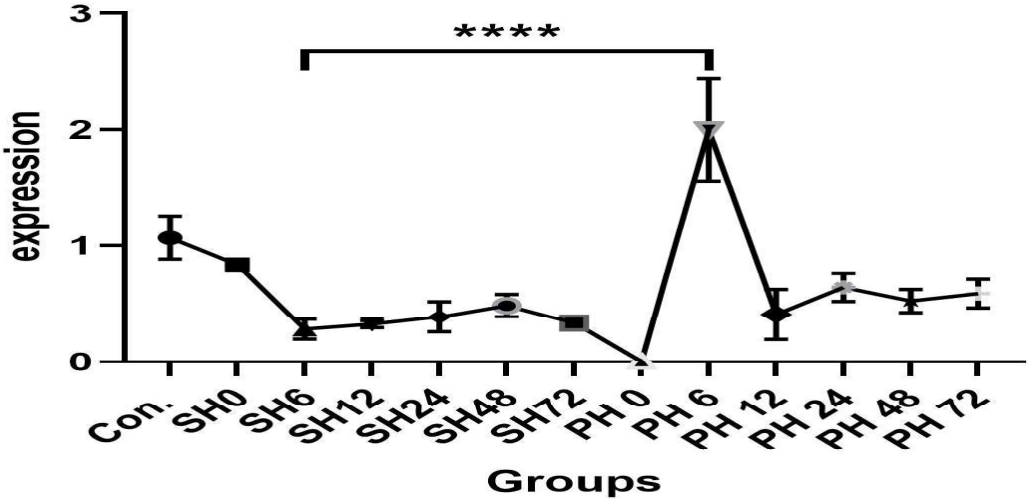

Figure 4.22 IRP1/ACO1 Gene expression

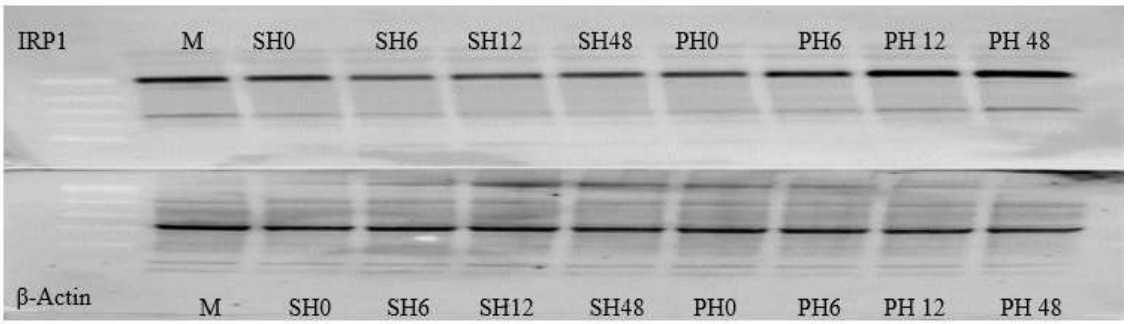

Figure 4.23 Protein expression bands of IRP1 and β-actin

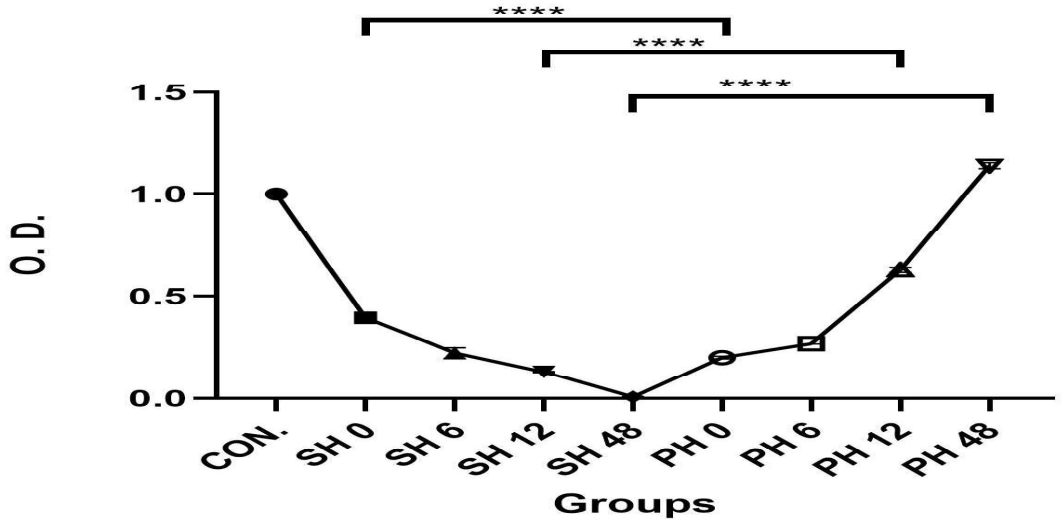

Figure 4.24 Graph of IRP1 protein expression

Sheikh et. al., (2007) reported increased iron demand and altered IRP expression in fast proliferating regenerating hepatocytes and neoplastic cells. This increased IRP expression and iron metabolism is directly related to the resulting IL-6-mediated inflammation (Sheikh et. al., 2007). After PHx, IL-6 phosphorylation is an important process of the IL-6-STAT3 signalling pathway, a key initiator of liver regenerative process required for the proper control of metabolic functions (Cornell et. al., 1990; Schmidt-Arras and Rose-John, 2016).

Mammals have developed complex mechanisms to maintain appropriate iron concentrations *in vivo*. Iron homeostasis in mammals is mainly regulated by a series of interconnected regulatory systems; these are: hepcidin-ferroportin (FPN1)-mediated regulation of serum iron levels, iron-regulating proteins (IRPs)/iron-sensitive element (IRE)-mediated intracellular regulation of iron homeostasis, and hypoxia-inducible factor-2α (HIF-2α)-mediated transcriptional regulation.

In the early hours of a liver regenerative process, IRP1 activation in response to the high iron demand, elicits the expression of iron carrier receptor proteins such as TfR or DMT-1 while repressing the iron storage proteins (Cairo and Pietrangelo, 1994; Graziadei et. al., 1997). IRPs via IRE mRNA-IRP system, can then regulate transcriptionally the expression of target genes involved in iron metabolism (Kuhn, 2014). This essential regulatory system of cellular iron homeostasis is key to maintaining a functional liver homeostasis. IRP1-IRE system may function to post-translationally regulate HIF-2α and DMT-1 (Wilkinson and Pantopoulos, 2013).

The findings of Mollbrink et. al., (2012), in which increased expression of IRE mRNAs of TfR1, HIF-2α and DMT1 in PH groups, as an acute priming phase response, similar to our findings, may be indicative of iron metabolic signalling need (Mollbrink et. al., 2012). Xue et. al., (2016) in a mouse model of colorectal tumourigenesis, reported that HIF-2α-mediated DMT-1 induction may cause iron-cyclin dependent kinase 1 (Fe-CDK1) interaction, leading to activation of JAK-STAT3 signalling pathways (Xue et. al., 2016)

At this juncture, IRP1-HIF-2α-DMT-1 molecular interaction may therefore be a functional regulator of iron uptake coupled to the IL-6/JAK/STAT3. This may support the proper metabolic rewiring needed to prime the regenerating hepatocytes.

Prior to these interactions, is the known activation of IRP1 and HIF-2α by mitoNEET. The ALR-mitoNEET functions not only to control the ROS and iron metabolism but also activates other signalling pathways such as IL-6/STAT3 and MAPK (Zhu et. al., 2016). Nuclear translocation of the holo-IRP1 is requiring of a functional mitoNEET with glycogen branching enzyme 1, as a special regulator. MitoNEET performs two functions: cluster transfer and metabolic link. IRP1-GBE1 binding is been linked to glycogen synthase (Huynh et. al., 2019). Even though, Hernandez-Gallardo and Missirlis, (2020) reported no change in glycogen contents between the wild-type and $IRP1^{-/-}$ mouse livers (Hernandez-Gallardo and Missirlis, 2020), molecular complex of ALR-mitoNEET-IRP1- HIF-2α-DMT-1 may interact to link glycogen metabolism-iron metabolism.

Iron metabolism-related cellular processes may link iron availability to erythropoiesis. This means IRP1- HIF-2α binding may be key to hepatic erythropoietin activation (Rankin et. al., 2007), adding up to the possible molecular functions of this complex interactions.

4.4.5. Mitoferrin gene and DMT-1 protein expression

Gene expression of mitoferrin, involved in mitochondrial iron transport and metabolism, was evaluated according to 0, 6, 12, 24, 48, 72 hours, compared with SH groups. It was determined that mitoferrin expression significantly peaked in SH6, SH48 and SH72 ($p \leq 0.05$), compared to the PH. There was no statistically significant difference at 0, 12 and 24 of the SH and PH groups (**Figure 4.25**). An unusually high mitoferrin expression was observed in the SH groups over the PH groups.

Seguin et. al., (2019) in a 48-houred 70% PHx, reported a contrasting result in which higher mitoferrin gene expressions were seen in the PH groups than the SH (Seguin et. al., 2019).

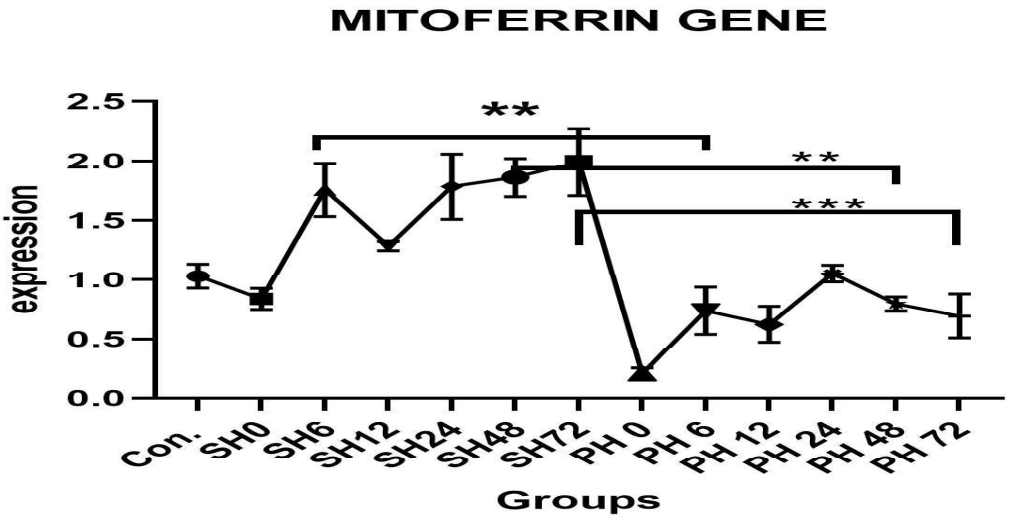

Figure 4.25 Mitoferrin (MFRN) Gene expression

Our results were unexpected, owing to the fact that rapidly proliferating cells have a higher iron requirement (Bomford et. al., 1984) to regulate major cellular processes such as DNA synthesis, Fe-S biogenesis, erythropoiesis, mitochondrial dynamics and Fe-S biogenesis (Ali et. al., 2022). Based our results, we proposed therefore the expression of a different iron carrier gene or protein over the mitoferrin in the regenerating hepatocytes during cellular iron uptake.

Liver regeneration is therefore highly iron-dependent. Iron taken into the hepatocytes, are either transferrin-bound iron (TBI) or non-transferrin-bound iron (NTBI) via DMT-1 or DMT-1-mediated ‘‘kiss and run’’, iron-bound endosomes and VDAC (Upadhyay and Agarwal, 2019). The iron is then delivered to the necessary organelles, especially to mitochondria via mitoferrin, for Fe-S biogenesis and heme biosynthesis. How mitoferrin mediates this delivery of iron to the mitochondria is still unknown (Lane et al., 2015). Mechanisms of mitoferrin’s transcriptional, translational and post-translational regulations in terms of cellular and mitochondrial iron homeostasis, are yet to be revealed (Ali et. al., 2022).

Two iron carriers may be considered the likely candidates: transferrin receptor 1 or DMT-1. Mitoferrin and DMT-1 were shown to share similarity in the amino acid residues of t, responsible for gliding iron across the mitochondria (Brazolotto et. al., 2014). DMT-1 may also be involved in the mitochondrial-endosomal inter-organellar iron transfer (Ali et.

al., 2022). Thus, our reason for selection of DMT-1, for the analysis of protein expression using Western Blot method.

Protein expression of DMT-1 was found to be significant in PH0 PH6 and PH12 groups ($p \leq 0.05$); It was found that the amount of DMT-1 protein decreased with increasing time till the lowest expression at the PH48 (**Figure 4.26; 4.27**).

Mollbrink et. al., (2012) reported that after a 70 % PHx, DMT-1 was seen to commence rising at 4 hrs and peaked at 16 hrs in the PH group (Mollbrink et. al., 2012). they also reported no changes in DMT-1 levels in SH, except for a decreased DMT-1 at 48 hrs (Mollbrink et. al., 2012). This was similar to our findings.

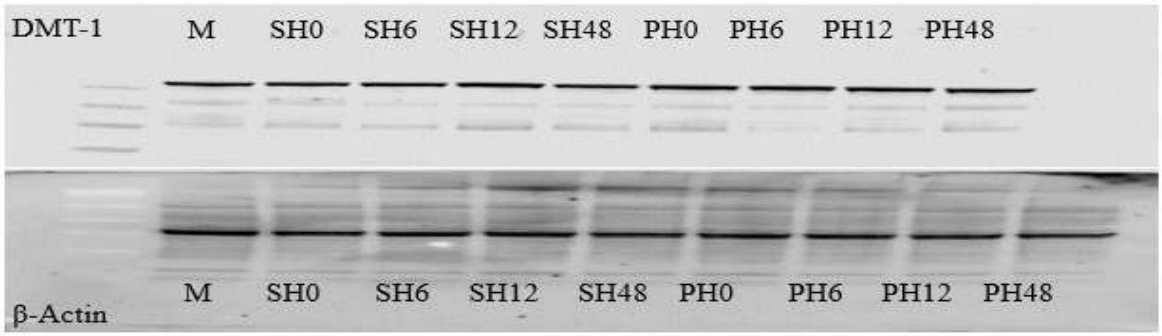

Figure 4.26 protein expression bands of DMT-1 and β-actin

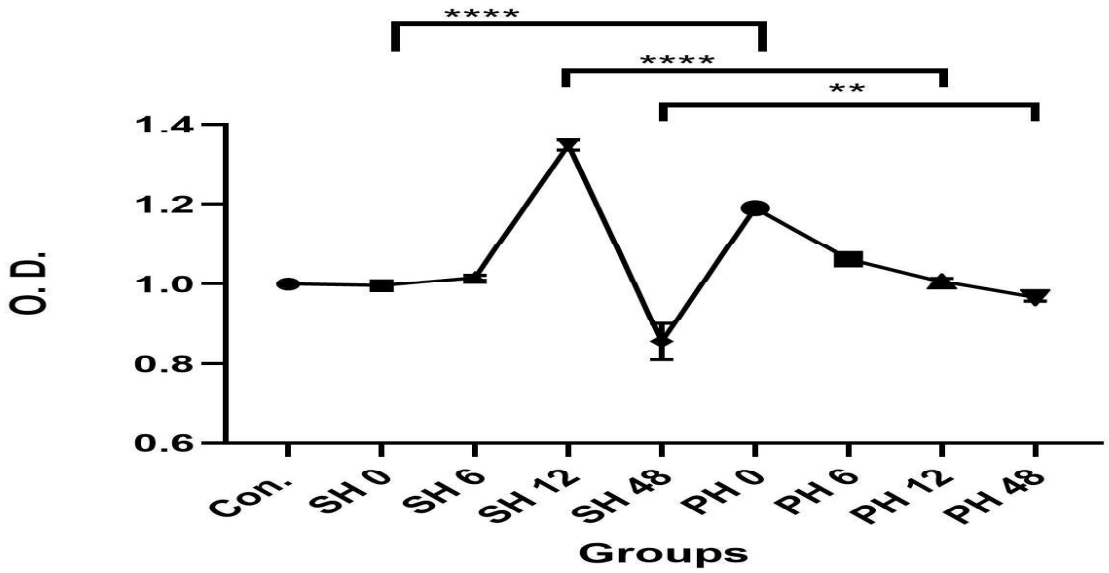

Figure 4.27 Graph of DMT-1 protein expression

Increase in mRNA expression of DMT-1 (or TfR1) during the acute liver regenerative phase was shown to be post-transcriptionally regulated by IRP (IRE-IRP) (Cairo and Pietrangelo, 1994; Graziadei et. al., 1997). The 3′ UTR-containing IRE may act to stabilize the mRNA. Thus, cell-specific IRE/IRP system may regulate DMT-1 post-transcription (Gunshin et al., 2001), and also depending in part on the exon sequence, alternatively transcribed upstream of the 5′ end DMT-1 mRNA (Hubert and Hentze, 2002).

4.4.6. MINER1 (CISD2) gene expression

Gene expression of MINER1 was evaluated according to 0, 6, 12, 24, 48, 72 hours in PH groups, compared to SH groups. It was determined that MINER1 expression was significantly higher in SH0 and SH6 groups ($p \leq 0.05$), compared to PH. There was no difference between SH and PH 0, 12, 24, 48 and 72 groups (**Figure 4.28**). But in the PH groups, two peaks; first peak at PH6 and a second and highest peak was observed in PH24. This was found to be significant in comparison to all the PH groups [PH0-PH24 ~ (p value= < 0.0001, ****); PH6-PH24 ~ (p value= 0.0010, ***); PH24-PH72 ~ (p value= 0.003, ***)].

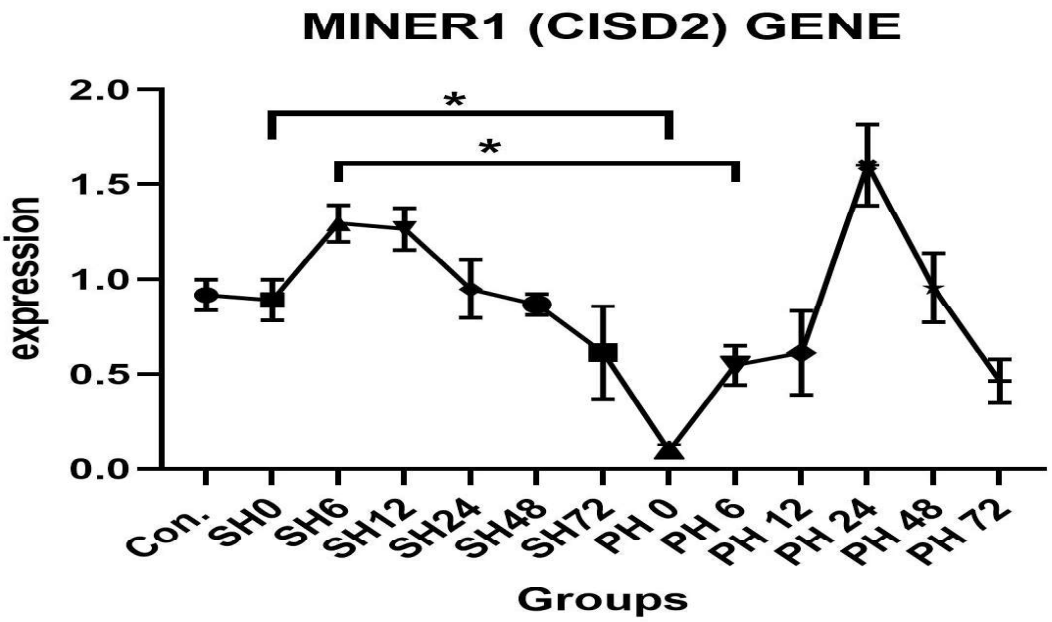

Figure 4.28 MINER1 Gene expression

It has been observed that both mitoNEET and MINER1 are highly expressed in rapidly proliferating cells such as cancer cells (Li et. al., 2021). Like mitoNEET, MINER1 plays an important role in ROS metabolism and iron regulation. The SH groups were only

given stress. So, they are likely to express stress-related and ROS genes (MINER1, mitoNEET, antioxidant genes). The stress in the PH group is a function-related stress, as the rush to proliferate is activated in the PH groups; endoplasmic stress, DNA replication fork stress, mitochondrial dynamic-related stress also may be activated.

In the PH groups, the initiating functions of ALR-mitoNEET communication, is known to protect against oxidative stress and endoplasmic reticulum stress by decreasing Ca^{2+} level (Ibrahim and Weiss, 2019). This may have direct repressive effect on MINER1, as an important Ca^{2+} regulator in the cell. Though opposing as they may seem, an ALR-mitoNEET-MINER1 communication plays an important role in ROS and iron metabolism. So, a molecular interaction of ALR, mitoNEET and MINER1 can be established to not only regulate ROS but probably activate the ROS signalling mechanisms for a more successful initiation phase.

Also, MINER1 plays a fundamental role in regulation of intracellular calcium homeostasis, Bcl-2-activated autophagy, apoptosis, mitochondrial dynamics and in development of neurons (Salem et al., 2012). Ca^{2+} signalling is known to function in regulation of cellular secretion, metabolism and differentiation (Huerta-Bahena et al., 1983). Some studies in hypercalcaemic rats, have showed the important role of intracellular Ca^{2+} in liver regeneration (Marti et al., 1995; Picard et. al., 2000). This may in part be due to role of Ca^{2+} signals in cell secretion and apoptosis.

During the progression phase of liver regeneration, proliferative mechanisms or signals such as growth factor signalling are needed to maintain the hepatocyte progress through the cell cycle. Molecular players such as the epidermal growth factor (EGF) and hepatocyte growth factor (HGF) via the MAPK-ERK signalling axis, can phosphorylate and activate *myc*, *fos* and *jun*, to promote cellular division (Katz et. al., 2007; Roskoski, 2012). Ca^{2+} signalling may be essentially mediating cellular secretion of growth factors, and thus HGF and EGF signalling (Mine et. al., 1991). Cell-cycle genes acting as inhibitors of apoptosis such as (Bcl-2, Bcl-X, and GADD45), have been previously observed to be up-regulated and implicated in priming of hepatocytes (Fausto, 2000; Su et. al., 2002). MINER1, a major intracellular Ca^{2+} regulator and molecular partner of Bcl-2-related cellular process.

Sun et. al., (2021) showed that in a mouse model of corneal regeneration via regulating the intracellular Ca^{2+} homeostasis and mitochondrial function, MINER1 (or CISD2) contributed to healing corneal wounds and maintenance of corneal epithelial integrity. Essentiality of MINER1 in corneal epithelial regeneration was demonstrated (Sun et. al., 2021). A neuroblastoma model also shows that MINER1 deficiency arrested cell cycle at G1, inducing neuroblastoma cell differentiation. MINER1 knockdown was reported to cause down-regulation of several cyclins and cyclin-dependent kinases (CDKs) (Li et. al., 2021).

Without the administration of any drug or genetic manipulation, this may be the first time a MINER1 gene expression is being reported after a 70% PHx. A careful analysis of the expression pattern of MINER1 in our study showed, it may be more of an early intermediate gene in the liver regenerative process.

4.4.7. SDHA gene expression

Gene expression SDHA, as the subunit A of the mitochondrial marker (SDH) enzyme in the Krebs cycle, was evaluated in SH and PH according to 0, 6, 12, 24, 48, 72 hours. It was determined that there was a significant difference in PH0 groups ($p \leq 0.05$), and a very sharp increase in gene expression between PH0 and PH6. There was no difference between SH and PH 6, 12, 24, 48 and 72 groups (**Figure 4.29**).

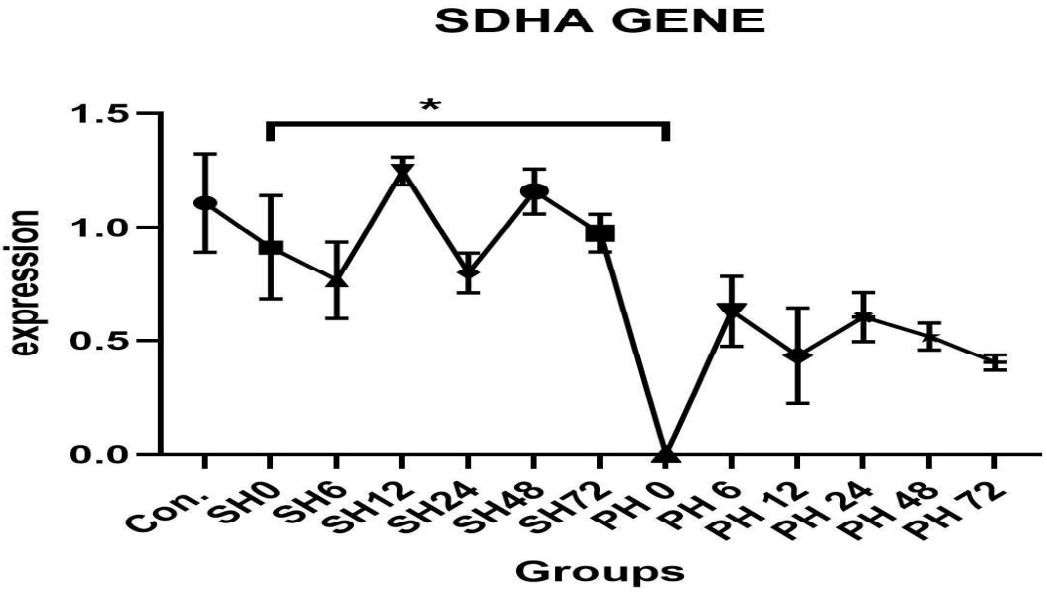

Figure 4.29 SDHA gene expression

Due to the versatility of its renewed role in cellular function and physiology, the SDH gene is been recently considered a tumour suppressor gene as a ‘molecular check’ on uncontrolled cellular proliferation. Interestingly its substrate, succinate is a known oncometabolite. Studies on the SDHA gene expression in liver regeneration after PHx is still lacking. This may be the first of its kind.

However, in other models of tissue (muscle) regeneration, Spelat et. al., (2022) assessed muscle regeneration at (7, 10 and 13) days post injury (dpi). And reported levels of mitochondrial SDHA proteins were only seen with improved definition at 13 dpi but not in (7 and 10) dpi, as the regeneration progresses (Spelat et. al., 2022). This study contrast with our observation

SDHA as a flavoprotein, catalyses the oxidation of its substrate (succinate) to fumarate. Succinate is also a known oncometabolite and essential intermediate metabolite involved in a variety of cellular processes such inflammatory response, HIF-1α activation and VEGF release, metabolic rewiring-angiogenesis axis, PHD inhibition. Several studies (Schoors et. al., 2015; Jiménez-Valerioet. al., 2016; Rohlenova et. al., 2018; Du et. al., 2021), have shown that VEGF-containing HRE may be activated via HIF-1α-mediated transcription. Succinate via its receptor (SUCNR1) is known to link between tissue metabolism, mitochondrial stress and organ response (van Diepen et. al., 2017).

The initial base line expression of SDHA gene after PHx, may reflect a cellular mechanism to permit succinate-SUCNR1 molecular action in hepatocyte response to stimuli, metabolic rewiring, HIF-1α activation via PHD inhibition and VEGF release. among others. As the hepatocytes responds to the stimuli and initiating mechanism mechanisms are established, succinate as an oncometabolite may immediately be oxidized to fumarate. This helps prevent the liver regenerative process from becoming tumorigenic. Additionally, a decreasing c-MYC gene expression observed in (Figure 4.9.), may fit perfectly in this interaction. Both succinate and c-MYC may confer oncogenic abilities while SDHA is tumour-suppressive. A SDHA gene expression together with c-MYC and succinate, may act in opposing manner but towards an overall regenerative capacity.

4.4.8. c-MYC gene expression

c-MYC gene expression in SH and PH groups was comparatively analysed at 0, 6, 12, 24, 48 and 72 hrs. c-MYC gene was seen to be significantly expressed in PH0 and PH6 groups ($p \leq 0.05$), with its highest expression at the PH 0. A steady but gradual fall in c-MYC gene expression in PH groups with increasing time (6, 12, 24, 48 hrs) was observed, hitting its lowest expression at the 48 hrs (**Figure 4.30**). There was no significant difference in c-MYC gene expression in SH and PH at 12, 24, 48 and 72 hrs.

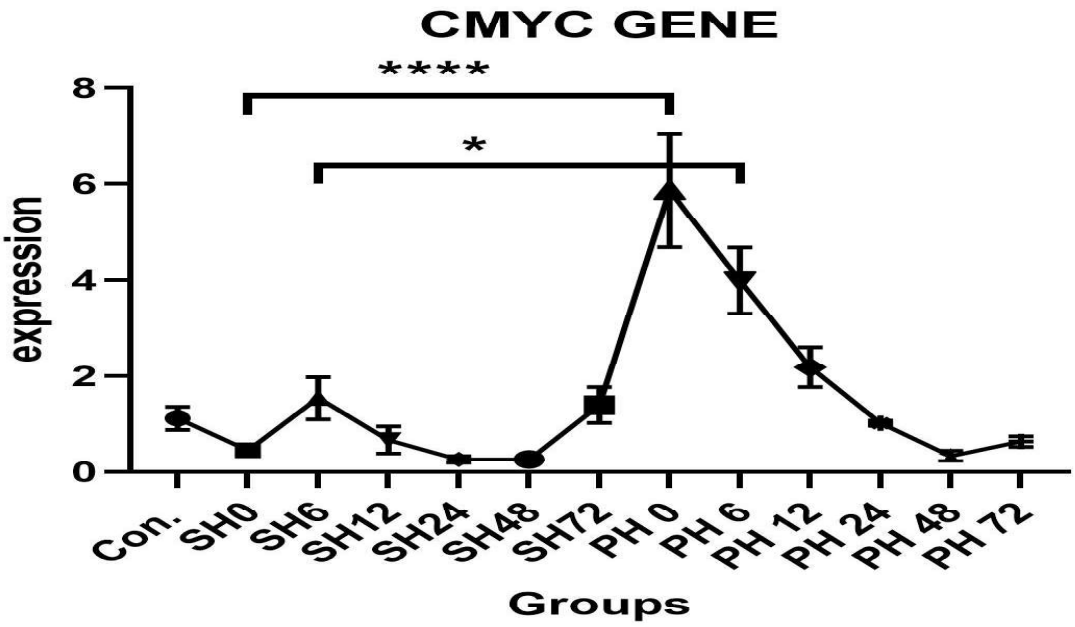

Figure 4.30 c-MYC gene expression

As an immediate early gene, c-MYC is known to regulate key transcriptional responses of cell cycle progression, guiding hepatocyte's transition from G_0/G_1 to S phase. These transcriptional responses may include metabolism, growth and cellular proliferation (Miller et. al., 2012; Whitfield and Soucek, 2012). This might explain why adult mouse liver regeneration requires c-MYC.

Previous studies have reported a rapid increase in c-MYC prior to DNA synthesis in the first 30 minutes following PHx, reaching its highest level in 2 hrs and succeeded by a second (2^{nd}) peak at 8hrs (Fausto et. al., 1986; Sanders et al., 2005). Another study reported only the first peak of c-MYC expression at 2 hrs before declining to its low at the 4 hrs (Morello et. al., 1990). These findings contrast with ours.

However, Kim et. al., (2000) reported that irrespective of significant decrease in c-MYC, cellular decision in favour of hepatocyte growth, may occur purposely for induction of ribosomal (protein) biosynthesis (Kim et. al., 2000). HIF-2α-mediated c-MYC expression **(Figure 4.31**) (Patel and Simon, 2008)., rather than regulating expression of cell cycle genes such as cyclin D2 and E2F1, may have preferred initially metabolic growth. In liver regeneration, hepatocyte hypertrophy precedes hyperplasia of the hepatocytes (Kiseleva et. al., 2021). This cellular decision may have selected c-MYC gene expression, in favour of cellular metabolism for utilization of nutrient and production of cellular precursors for hepatocyte hypertrophy, rather than rapid proliferation (Kim et. al., 2000).

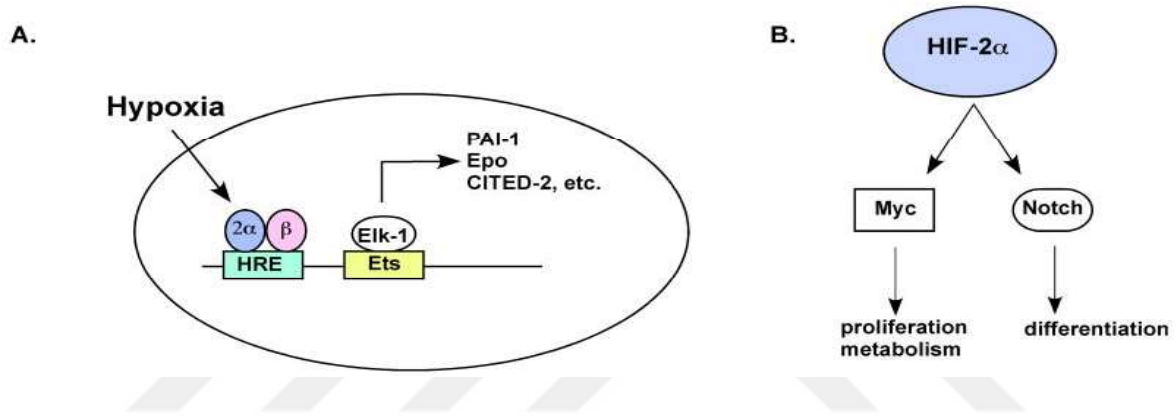

Figure 4.31 HIF-2α-mediated activation of c-MYC expression (Patel and Simon, 2008)

In a functional liver regeneration model, pro-proliferative and anti-proliferative factors are at a tightly-regulated balance. c-MYC as a proto-oncogene, may be complemented in action by HIF-2α and succinate-SUCNR1, as an oncometabolite. On the other hand, SDHA, as a tumour suppressor gene may act together with VHL, a known transcriptional inhibitor of HIF-2α and tumour suppressor gene. The net effect is a promotion of the overall regenerative capacity. HIF-2α-c-MYC and IRP1-SDH cross-communications may generally have interacted to link production of precursor products and ATP, to iron incorporation into DNA/RNA precursors, and thus hepatocyte hypertrophy before hyperplasia.

A decreasing c-MYC as observed in our study, may promote induction of p53-mediated apoptosis. How such a relationship would fit into our study design (molecular interactions) will be a topic for future research. However, transcriptional inhibition of c-MYC in hypoxia by both isoforms of HIF-α via HIF-α-mediated Mxi1 regulation and

competitive HIF-α-Max binding (Corn et. al., 2005; Zhang et. al., 2007) may have also played a role in the decreased c-MYC expression of our study.

HIF-1α, via p21 gene upregulation (Koshiji et. al., 2004) or Mxi1-independent proteolytic c-MYC breakdown (Zhang et. al., 2007), may also have functioned to inhibit c-MYC expression in the early phase of the liver regenerative process.

4.4.9. ACO2 gene expression

ACO2 gene expression in SH and PH groups was comparatively analysed at 0, 6, 12, 24, 48 and 72 hrs. There was no significantly difference in ACO2 gene expression in 0, 6, 12, 24, 48 and 72 hrs of both SH and PH (**Figure 4.32**). ACO2 gene was observed to be repressed in our study.

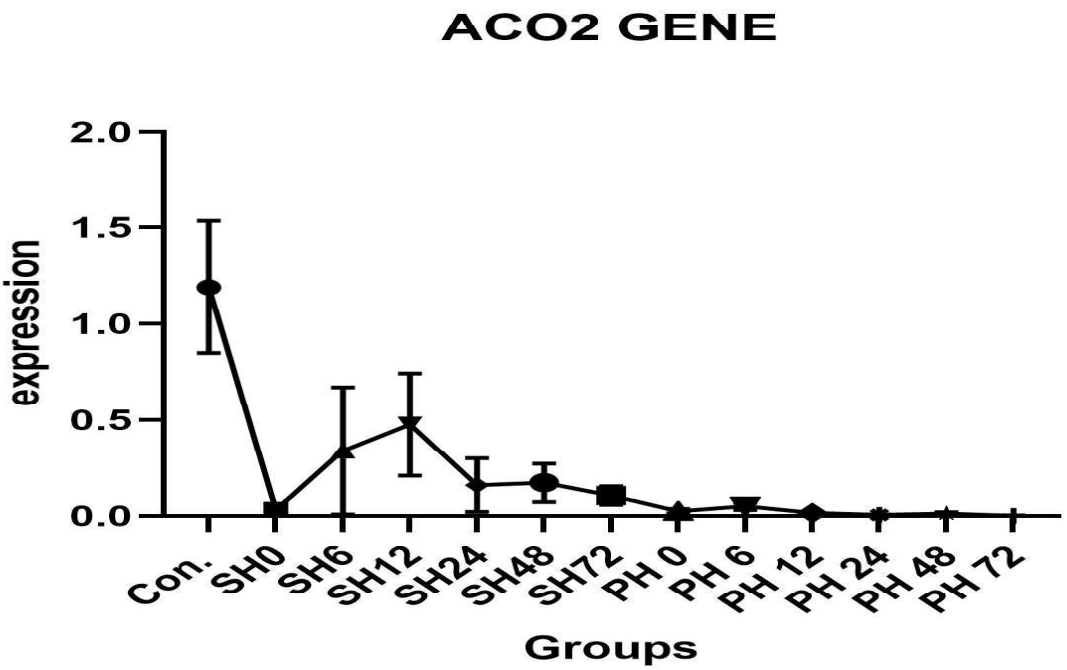

Figure 4.32 ACO2 gene expression

In the context of our study design, information on ACO2 gene expression in liver regenerations after 70% post-PHx, may be scarce. In a study of adipogenesis in white adipose tissue, Chen et. al., (2020) showed that increased ACO2 expression drive adipogenesis via regulating mitochondrial metabolism and ATP synthesis in murine fibroblast (3T3-L1) cells (Chen et. al., 2020).

Martelli et al., (2015) had previously shown that induction of ACO2 was correlated to increased IRP1 activity in frataxin-deficient mice. Frataxin is the sole iron transfer carrier protein in Fe-S biogenetic machinery. Genes of the ETC complex encoded by the nuclear genome such as (Ndufab 1, SDHB, Cox2 and Cox4) and other Krebs cycle genes such as citrate synthase and isocitrate dehydrogenase, were shown to be fairly expressed (Martelli et al., 2015).

Erythroblasts and neurons possess abundant deposits of the IRP2 proteins, regulated by F-box/LRR-repeat protein 5 (FBXL5)-dependent proteasomal degradation. In maintenance of intracellular iron homeostasis, IRE-IRP2 bindings in erythroblasts and neuronal tissues was observed to increase TfR1 and DMT1, while reducing storage (ferritin), export (Ferroportin 1) and utilization (ACO2 and eALAS) of iron (Zhang et. al., 2014). This is in similarity to the assumed functions of IRP1-IRE binding observed in our study and several others (Kuhn, 2014). IRP1 may have bound to the IRE of ACO2 mRNA, to repress its expression.

PHx-induced oxidative stress can disintegrate Fe-S clusters of many Fe-S proteins such as the ACO2 (Oktay et. al., 2007). ACO2 serves as the most sensitive mitochondrial biomarker enzyme for oxidative stress, disintegrating more faster than even the Fe-S cluster centers of complex I and II (Bulteau et. al., 2003). This PHx-stressed ACO2 inactivation may be both reversible or irreversible (Drapier and Hibbs, 1986).

4.4.10. GLRX5 gene expression

GLRX5 gene expression in SH and PH groups was comparatively analysed at 0, 6, 12, 24, 48 and 72 hrs. compared with the SH groups. There was no significant difference in the expression of GLRX5 gene in SH and PH groups at 0, 6, 12, 24, 48 and 72 hrs (**Figure 4.33**). GLRX5 gene was thus repressed in our study.

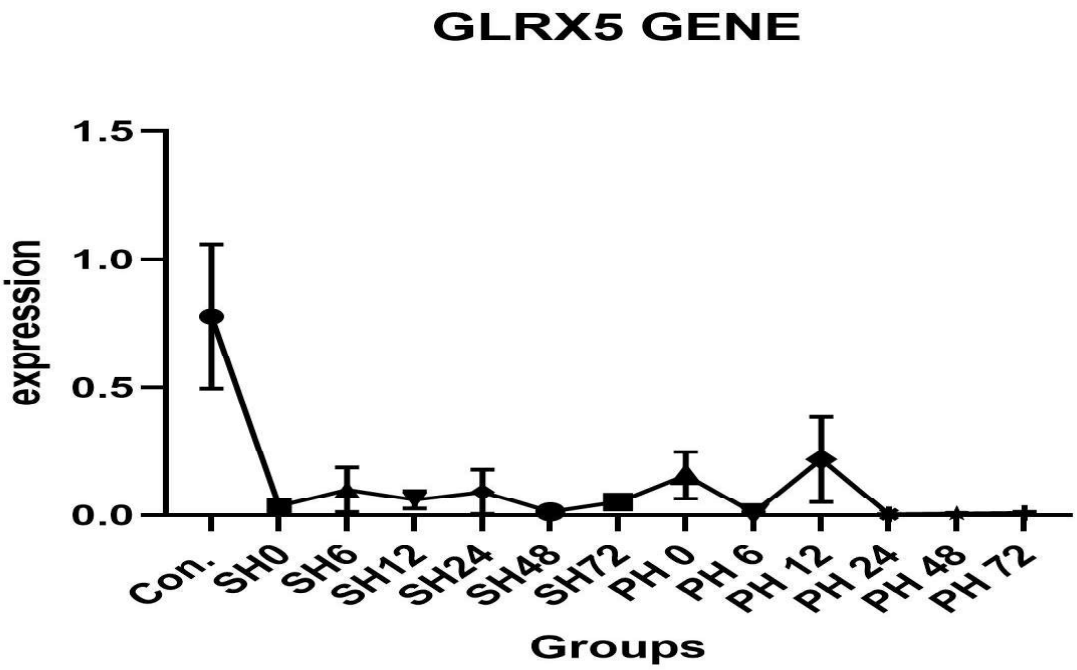

Figure 4.33 GLRX5 gene expression

Previous studies on GLRX5 expression after PHx is very rare. Thus, little or no information exist in literature. However, in a study of iron metabolism during liver fibrosis progression, Kumar et. al., (2016) reported a decreased GLRX5 expression in ALR-deficient mice, indicating that GLRX5 may be a major player in cellular iron homeostasis and redox metabolism (Kumar et. al., 2016).

GLRX5, abundantly found in the liver, is known to be directly linked to hepatic iron homeostasis (Wingert et. al., 2005). Inhibition or repression of functional GLRX5, may result in Fe-S biogenetic insufficiency leading to IRP1 activation (Beilschmidt and Puccio, 2014). IRP1 activation leads to expression of TfR, DMT1 and other iron carrier genes (Ozbek, 2010). These cellular mechanisms may support iron mobilization and promotes intracellular iron uptake during the rapid hepatocyte proliferation, post-PHx.

In our study, GLRX5 repression may have been a cellular decision to activate IRP1. GLRX5 is considered a possible Fe-S cluster transfer protein to holo-proteins (Wingert et. al., 2005; Beilschmidt and Puccio, 2014), but mitoNEET has been reported to transfer clusters to both MINER1 and IRP1 (Ferecatu et. al., 2014; Karmi et. al., 2017). MitoNEET may therefore be a possible functional alternative to GLRX5.

4.5. Measurement of serum rat SUCNR1 levels

Measurement of expression levels of serum succinate receptor 1 (SUCNR1) was performed using Bioassay Technology Laboratory Rat Glutathione ELISA kit (E1267Ra, Bioassay Technology Laboratory, Shanghai, China). Absorbance was read as optical density, with the Chromate 4300 brand ELISA reader. Concentration results in the unit of ng/ml (**Table 4.5**), were then calculated using 4-parameter logistic regression and R^2 value determined as 0.9954 (**Figure 4.34**).

Table 4.5 Mean SUCNR1 Concentration (ELISA analysis)

Groups	Mean SUCNR1 concentrations ± SD (ng/mL)
Control	7.4 ± 0.60
SH0	7.4 ± 5.1
SH6	8.2 ± 2.3
SH12	9.1 ± 2.1
SH24	12 ± 4.1
SH48	15 ± 5.0
SH72	12 ± 0.88
PH0	9.9 ± 3.7
PH6	14 ± 2.7
PH12	13 ± 0.66
PH24	13 ± 2.1
PH48	13 ± 1.9
PH72	16 ± 4.3

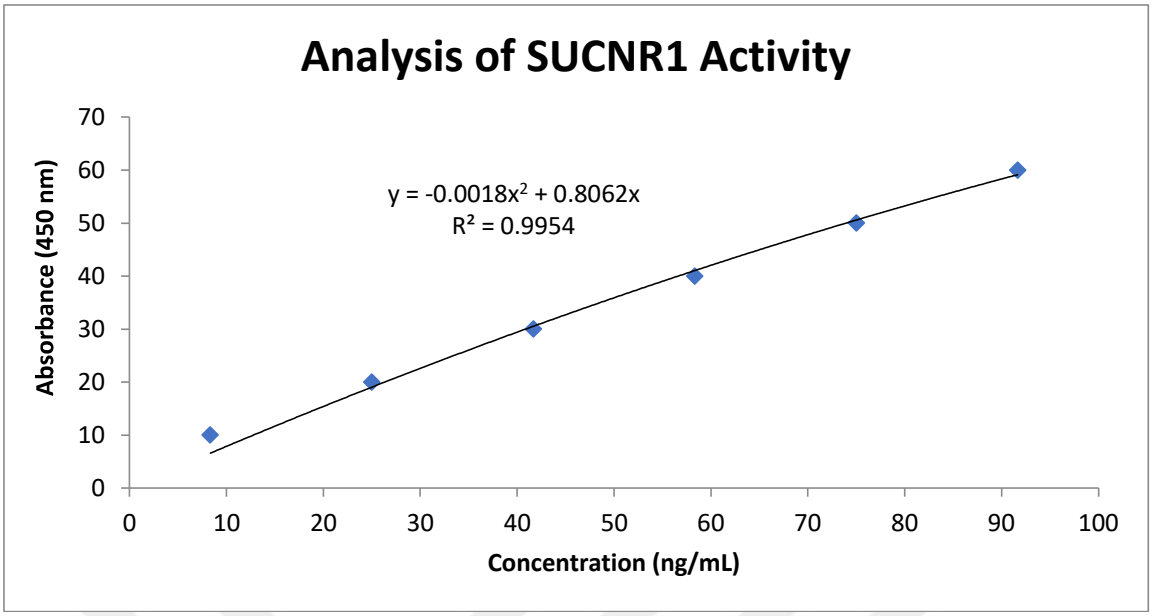

Figure 4.34 Expression of SUCNR1 activity (Standard curve)

Also known as GPR91 (G-protein-coupled receptor 91), SUCNR1 was found to be significantly expressed (p=0.0020, **) in both the SH and PH groups. Evaluation of SUCNR1 activity in PH groups, was comparatively analysed at 0, 6, 12, 24, 48 and 72 hrs, compared with the SH. SUCNR1 was found to be significantly expressed in PH6 (p=0.0415), compared to SH6 (**Figure 4.35**). Expressional pattern of SUCNR1 activity (**Figure 4.36**) indicated increased receptor activities in 6, 24, and 72 hrs in both SH and PH groups

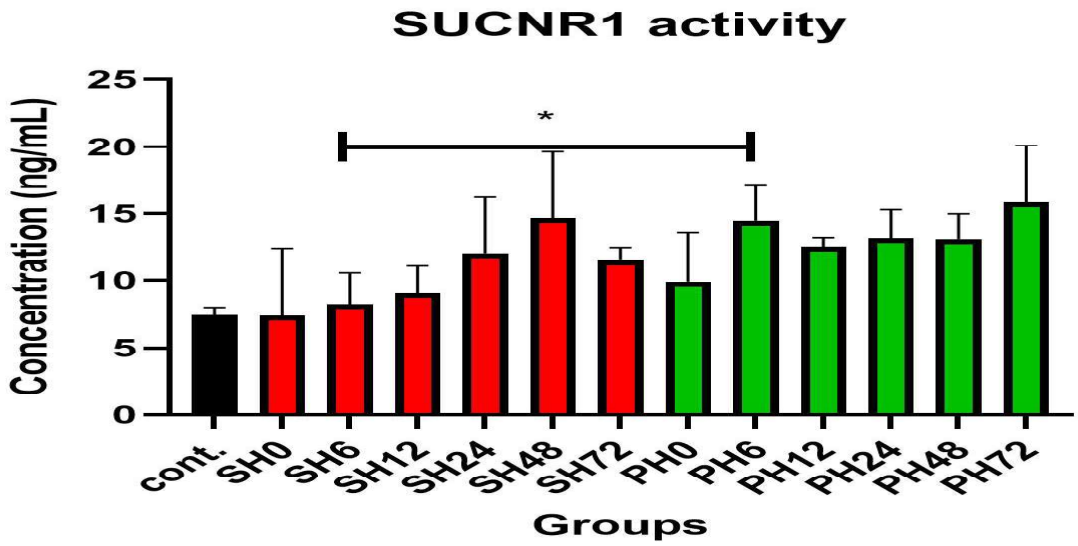

Figure 4.35 Graph of ELISA analysis of SUCNR1 expressions

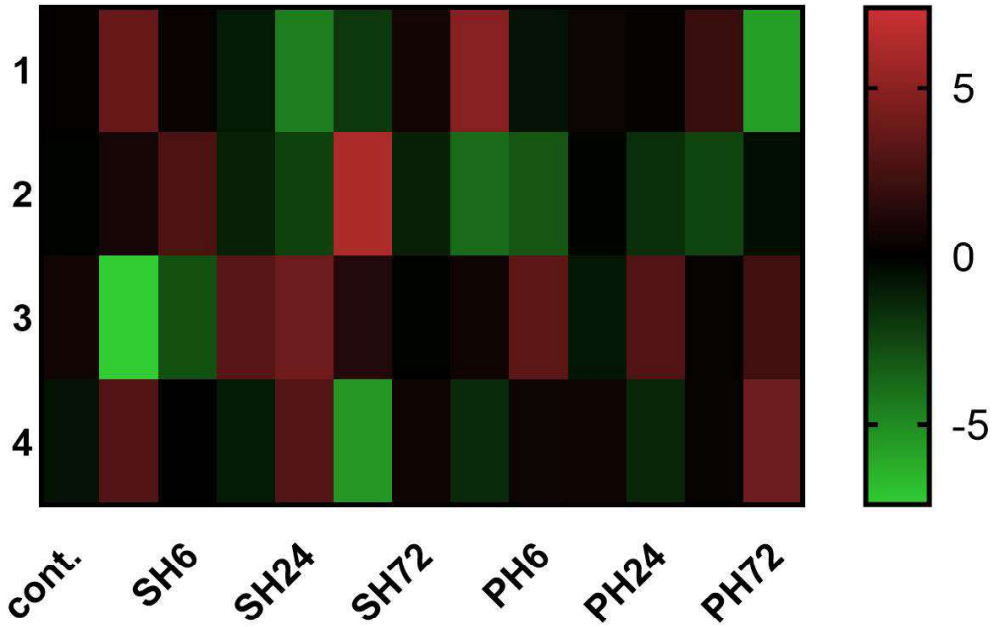

Figure 4.36 Heat map plot: Ordinary one-way ANOVA of SUCNR1 ELISA (Generated from GraphPad Prism 9)

High metabolic demands and/or hypoxic conditions are known to cause a sharp increase in succinate concentrations (Sapieha et. al., 2008; Hamel et. al., 2014). After a partial hepatectomy procedure, transient O_2 supply insufficiency in relation to increasing metabolic energy demand (Hoyer and Krier, 1986), may induce a metabolically stressed state. Metabolic stress was previously observed in ischaemia (Chouchani et. al., 2014) and exercise (Lewis et. al., 2010). This can result in metabolic stress-induced succinate accumulation in the mitochondria, cytosol and the extracellular matrix (Guo et al., 2020).

Succinate is considered an oncometabolite, with the ability to promote tumourigenesis (Mu et. al., 2017). Concentrations of intracellular succinate, as a key signal molecule and essential intermediate metabolite of the mitochondrial TCA, is thus tightly regulated by SDH. SDH via catalytic oxidation, transforms succinate to fumarate (Dalla Pozza et. al., 2020; Bandara meat. al., 2021). Electrons from this catalytic oxidation, are then channelled into ETC to reduce ubiquinone to ubiquinol (Cho, 2018). Therefore, SDH is affect by oxygen unavailability and thus succinate accumulation (Ariza et. al., 2012).

SUCNR1 is subcellularly located on ER (Sanchez et. al., 2022) and highly expressed in liver hepatic stellate cell (HSC), cardiomyocytes, immune cells, renal cells and several others (Mills and O'Neill, 2014). Succinate, acting through SUCNR1, mediate a range of cellular and biological activities (He et. al., 2004). Cellular role of the succinate-SUCNR1

complex in liver tissue, was first described by Michael H. Nathanson and colleagues. Succinate-SUCNR1 was shown to activate quiescent rats HSC and an ischaemic blockage of liver's portal flow caused a 14-fold increase in succinate release, activating HSC (Correa et. al., 2007).

Very few studies on the molecular roles of succinate-SUCNR1 in physiological liver regeneration exist. By inference, the importance of succinate-SUCNR1 complex in liver regeneration, has been reported (Mabuchi et. al., 2004). HSC activation via succinate-SUCNR1, was seen to be an essential molecular event of the early liver regenerative phase (Mabuchi et. al., 2004). Transdifferentiation of activated HSC into myofibroblasts, drives ECM remodelling and cytokine secretion. ECM remodelling and cytokines secretion initiates the liver regeneration process (Shang et. al., 2016).

Succinate-SUCNR1 is also reportedly involved in regulation of pseudohypoxia, prolyl hydroxylase inhibition, HIF-lα activation and VEGF production (Mills and O'Neill, 2014; Kluckova and Tennant, 2018). Functions of succinate-SUCNR1 via the G-coupled protein receptor (GCPR) signalling transduction pathway in liver (Yang and Zhang, 2021) have also been reported.

In our study, significant SDHA expression ($p= 0.0332$) was seen in SH0 compared to PH0. SDHA expression in PH0, was initially seen at the baseline (approximately zero line), after which a sharp rise was witnessed at PH6. This may have been caused by the low oxygen concentration in the remnant liver, resulting in decreased SDHA activity. Low SDHA activity may therefore have resulted in transient succinate accumulation. Succinate-SUCNR1 could have played a role in the initial molecular events of metabolic rewiring, ECM remodelling, cytokines, HIFs activation, VEGF production, signalling events and others. Succinate as an oncometabolite, together with c-MYC as a proto-oncogene, may complement each other to amplify their pro-stimulatory effects of the early liver regenerative processes.

Low O_2 concentration may change SUCNR1 glycosylation and subcellularly translocate SUCNR1, subsequently altering the downstream gene expression (Sanchez et. al., 2022). At the endoplasmic reticulum (ER) or the ER-mitochondrial junctions is the

MINER1 gene, an important component of our study plan. Functional molecular interaction of a possible MINER1-SUCNR1-succinate complex in cellular proliferation, is unknown.

In our study, a significant expression of SUCNR1 (p= 0.0415, *) and MINER1 gene (p= 0.0446, *) at PH6, may indicate intracellular calcium homeostatic-related cellular roles. SUCNR1 via GCPR signalling pathway, was shown to regulate intracellular Ca^{2+} levels in kidney cells, resulting in activation of MAPK-ERK1/2 signalling (Robben et. al., 2009). Also, MINER1 acts similarly to activate cell signalling pathways via intracellular Ca^{2+} homeostasis (Huerta-Bahena et al., 1983; Salem et. al., 2012).

Succinate-SUCNR1 have been shown to functionally couple tissue metabolism to mitochondrial stress and organ response (Van Diepen et. al., 2017). Other studies have also shown the metabolic rewiring and angiogenesis link as an important component (Schoors et. al., 2015; Jiménez-Valerioet et. al., 2016; Rohlenova et. al., 2018; Du et. al., 2021).

More studies are needed to decipher the possible molecular interactions of MINER1-SUCNR1 complex as two ER calcium-related genes. Our assumption in this work, may only be a tip of the iceberg.

5. CONCLUSION AND RECOMMENDATIONS

Biological processes such as the liver regeneration may be defined by tightly-regulated interactions of different molecular players, acting either in unity (and complementary) or opposing. But the cumulative effect is towards executing a particular molecular action(s). Expression of different genes at the same time or in sync, may indicate a fine-tuned interactions or relationships between these genes.

In this study, mitoNEET**** gene expression; ACO1***; mitoferrin***; Miner1*; SDHA* and related HIF-2α**** and C-MYC**** genes were found to be statistically significant ($p \leq 0.05$). Compared to the PH, mitoferrin gene proved more significant in the SH group. Given that, rapid proliferating cells such as the regenerating hepatocytes have an unusually high iron affinity and the fact that several iron transporters such as the transferrin, DMT-1 may have been utilized, DMT-1 was selected for protein analysis. Other significantly expressed genes: mitoNEET, IRP 1 (ACO1) and HIF-2α and β-actin were also analysed for their mRNA expression. Protein of mitoNEET, IRP1 (ACO1), HIF-2α and DMT-1 were expressed significantly ($p \leq 0.05$) in the PH group than the SH.

The nature of gene and protein expression in our study, showed two possible basic design patterns of gene interaction network. These molecular interactions consist of both pro-stimulatory and anti-stimulatory well-crafted and concerted effort to drive hepatocyte proliferation during the liver regeneration process after PHx.

MitoNEET-MINER1-IRP1-DMT1-HIF-2α; all work complementarily to regulate various aspects of cellular functions.

I. ROS and O_2 metabolism, iron metabolism
II. vasculogenesis and metabolic adaptation to hypoxia
III. gene regulation, activation of cell signalling pathways and release of mitogenic factors such as cytokines, nitric oxide, VEGF, EPO and others.

C-MYC-SDHA-succinate-SUCNR1; may work opposingly in a gene interaction network but as a whole system, to drive various aspects of cellular functions in cell signalling, metabolism, proliferation, hypoxia and VEGF release.

Our genes of interest were generally expressed during the early (priming phase) and middle (proliferation) phases of liver regeneration. Fundamentally, the Fe-S genes can be viewed at the angle of both metabolism and proliferation. Fe-S genes are functionally linked to Fe-S biogenesis and Fe-S biogenesis, an essentiality of mitochondrial biogenesis.

Therefore, mitochondrial biogenesis and the resulting changes in mitochondrial dynamics, may as well be the molecular drivers underlying metabolic rewiring, prior to tissue repair and regeneration. Mitochondrial biogenesis increases mitochondrial mass, in anticipation of increased energy and metabolic demands. This demand could be evaluated on two terms: ATP production and biosynthesis of essential metabolites for efficient cell division. To build a cell, macromolecular precursor materials must be synthesized. For example;

i. phospholipid precursors need to be synthesized for the phospholipid membrane component
ii. amino acids, especially glutamate or glutamine
iii. nucleotides
iv. energy (ATP) production and ATP/ADP-P_i (inorganic phosphate) ratio's maintenance

Therefore, cell division can be said to be metabolite-ATP-dependent. This can as well be thought of as a form of metabolic rewiring, similar to metabolic rewiring hallmark of cancer. Functional liver mass threshold once reached will set in the negative regulatory feedback of liver regeneration, to terminate the process.

In tumourigenesis, prior to tumour initiation,

i. matrix metalloproteinase (MMP)-mediated extracellular matrix (ECM) remodelling to support cell migration from the cell ECM (anoikis shut-down)
ii. hypoxia, VEGF production and blood vessel development to meet increasing O_2 and metabolic demands
iii. The pressure and increasing O_2 tension within the disorganized vasculature of the tumour is a stimulus for gene expression (VEGF, PHD, VHL)

iv. activation of endothelial, immune cells and other secretory cells for all autocrine and paracrine secretions

v. Growth factors and activation of cell signalling

Prior to the priming phase of liver regeneration after PHx, the initial stimuli of haemodynamic and portal pressure changes and rising uPA (urokinase plasminogen), initiates hepatocytes into the regenerative mode. The priming phase of the liver regeneration phase is equally known to be characterised by a MMP-mediated ECM remodelling and degradation of fibrinogen; immune cell and Kupffer release of necessary cytokines, growth factors production and cell signalling.

A 70% PHx takes away not only the two-thirds of the liver tissue, but also two-thirds of the organ's ATP, O_2, glucose, glycogen, lipids and several others. Such a shock would probably mean diverting all the body's glucose and oxygen to the liver, to prevent system failure. Failure to manage such molecular shocks and factors, may lead to insufficient liver regeneration. Heat shock protein 70 (Hsp70) and related proteins especially, are considered key players in the initiation of the liver regeneration process as a major inducer of TNF-α, an important requirement of early liver regenerative phase. MitoNEET and MINER1 proteins are actively involved in iron, ROS, O_2 and redox metabolism. By virtue of its association with ALR, mitoNEET may be considered an early phase gene. MitoNEET in conjunction with Hsp70 chaperone, may function to absorb these shocks.

Hepatocytes with their high mitochondria turnover, make a move to increase mitochondrial mass, preferring mitochondrial fusion for a robust metabolic efficiency. These mitochondrial dynamics would probably activate PGC-1α in concert with mitofusin and OPA proteins. PGC-1α besides being a mitochondrial biogenetic protein, is also a ROS and metabolic gene. PGC-1α regulates mitochondrial biogenesis and hepatic lipid metabolism. PGC-1α together with the Nrf-1α, would control the excessive ROS production by the mitochondrial biogenetic machinery. MitoNEET as a critical regulator of lipid and iron homeostasis, may act together with PGC-1α-Nrf-1α. This association may amplify the hepatic lipid metabolism, driving metabolic rewiring and possibly coupling mitochondrial biogenesis to cell proliferation. Thus, the possible foundation of a novel cellular bioenergetics in liver regeneration. A possible first of the most important metabolic choices, is the preference for glycogen (or lipid metabolism) over glucose during the early

regenerative process and utilization of fatty acids (FAs) and β-oxidation for energy production. This may be a crafted plan to maximize biosynthesis of ATP, phospholipids and other key macromolecular as key cell precursors.

Lipids would produce more ATP than glucose. The hepatic lipid metabolism may seem the most plausible cellular decision, in the hepatocyte's quest to decide its survival fate to regenerate without forfeiting its unique metabolic functions. The regenerating liver must carry out its function of xenobiotic metabolism and regain its original mass (regeneration) at the same time. Regeneration as critical as it is, the liver would also institute critical molecular events and interactions to regrow its original functional state.

Technically, the priming phase may also be considered basically as a phase of metabolic and bioenergetic rewiring. It therefore makes sense that after 70% PHx, hepatocyte hyperproliferation (hyperplasia) in the liver regeneration process is preceded by hepatocyte hypertrophy. Hepatocyte hypertrophy enables enlargement of cells. This cellular enlargement may necessarily support cellular bio-accumulation of needed nutrients, energy and all required metabolites in preparation for cell division. Mitochondria at this point, also select mitochondrial fusion over fission, not simply for metabolic and energy efficiency but for mitochondrial repair of impaired mtDNA proteins. This ensures defective mitochondria and mtDNA proteins do not pose any threats to cell division process.

Our genes of interest like most other Fe-S genes and proteins, may be considered primarily metabolic genes but their active cellular roles in DNA/RNA metabolism, can only mean Fe-S biogenesis links metabolism to cellular proliferation.

ALR-mediated MitoNEET release at the earliest phase of liver regeneration may be the stimuli for metabolic rewiring and proliferation, as well as partaking in ROS and iron metabolism. Following PHx, increased ALR expression may phosphorylates STAT3 and thus promotes hepatocytes response to IL-6. ALR also induces in Kupffer cells the production of IL-6, TNF-α and NOS while inhibiting apoptotic stimulus and natural killer (NK) activity. Cumulative production of these cytokines by these molecular interactions of cells (hepatocytes, Kupffer, NK) and genes/proteins (ALR-mitoNEET, IL-6, TNF-α, NOS, STAT3) may activate the cytokine signalling, STAT3 (IL-6), NF-κB (TNF-α) and MAPK (ALR) pathway signalling. This drives the mature hepatocytes selected for the regenerative

process to start the proliferation process. Tissue hypoxia and other stimuli elicit VEGF production by the hepatocytes and endothelial cells. VEGF supports blood vessel formation. Several other mitogenic release is synchronously started. ALR-mitoNEET cross-communication with other molecular players, complementarily function to initiate liver regeneration because no one stimulus or molecular player can sufficiently commence the regeneration processes.

Rapid proliferation requires high iron amounts. Iron is need for not only cellular proliferation, but DNA synthesis, Fe-S biogenesis, mitochondrial function, erythropoiesis and others. Thus, IRP1 is activated for iron mobilization. IRP1 activation from ACO1 releases extra iron. Lack of aconitase function in the cell would drive the biochemical conversion of citrate to fat droplets, which may aid the general hepatic lipid metabolism. Fat accumulation is a key feature of NAFLD (non-alcoholic liver diseases). IRP1 may repress iron storage (eg. hepcidin) while enhancing the activities of iron carriers (such as the transferrin, mitoferrin, DMT1) to promote increased iron uptake by cells. Meanwhile, repression of hepcidin activity by IRP1 is equally known to cause IL-6 release, further supporting sustained cell signalling processes. IRP1 may couple iron metabolism-O_2 sensing mechanism to HIF-2α. IRP1 activity would readily activate HIF-2α, over HIF-1α. Transfer of Fe_2-S_2 cluster from mitoNEET is needed to repair the oxidatively damaged IRP1, as well as assist in ROS metabolism. A functional interaction of ALR-mitoNEET-IRP1-DMT1 may exist to manage ROS and iron metabolism, in addition to IRP1 repair.

The early phase is characterised by a relatively hypoxic environment. Hypoxic microenvironment would activate HIF-1α, as a promoter of initiation of liver regeneration. Further, HIF-1α may activate generally glycolytic enzymes in support of the early regeneration phase. With relatively increasing O_2 and ROS content, in addition to IRP1 preference for HIF-2α, HIF-1α mRNA is stabilized and inactivated. HIF-2α selectively cause VEGF production for a sustained angiogenic process and cyclinD1 in support of cell cycle entry and progress. Increasing process of tissue regrowth requires developing blood vessels to meet rising demand of oxygen, nutrients and other essential products. IRP1-HIF-2α activity link iron metabolism to O_2 sensing mechanisms to provide more support to the erythropoietic process. Moreover, tissue hypoxic stress and the complex metabolic pathways employed in liver regeneration, may lead to succinate accumulation. Succinate is a known signal molecule and oncometabolite. Succinate may function to support the proliferation

process. Succinate-GPR91 signalling may act to open up several other signalling via the G-proteins coupled receptors (GPRCs) signalling. Ras, Raf and other small proteins all act in the general cell signalling pathways; for example, Ras-MAPK signalling. At this juncture, ALR-mitoNEET-DMT1-IRP1-HIF-2α molecular interaction may be envisaged to regulate ROS and iron metabolism, cell signalling, angiogenesis, IRP1 repair, erythropoiesis, cellular proliferation and others.

MINER1 as a major player within the mitochondrial-endoplasmic reticulum (ERMES), functions basically to regulate the Ca^{2+} homeostasis, apoptosis and autophagy. MINER1 via controlling intracellular Ca^{2+} signals may promote cellular secretion of growth factors, while utilizing its interaction with Bcl-2 to inhibit apoptosis during this pro-proliferative phase. The molecular interactions of (MINER1-Bcl-2 or MINER1-CAPN2) may therefore be considered important in hepatocyte proliferation. MINER1 also contribute to the iron and ROS metabolic regulation. It can possibly interact with the ALR-mitoNEET-IRP1-HIF-2α-DMT1 molecular complex as MINER1-Bcl-2 or MINER1-CAPN2, controlling the ROS and functional stress (ER stress, DNA replication fork stress, mitochondrial dynamic-related stress) to ensure mitochondrial function is maintained while apoptosis is inhibited. SUCNR1 is also an ER membrane-based protein, also involved in intracellular Ca^{2+} regulation. Both MINER1 and SUCNR1 may play an indirect role in cellular secretion of growth factors, such as VEGF via Ca^{2+} signalling. MINER1-SUCNR1 may be a functional Ca^{2+} balance between mitogenic secretion, hepatocyte proliferation and apoptosis. MINER1-SUCNR1 in our gene interaction complex, may be intracellular Ca^{2+} signalling hepatocyte. Intracellular Ca^{2+} levels may be lowered ALR, in suppression of ER stress to block apoptosis during liver damage. A molecular interaction network of ALR-MINER1-SUCNR1 interaction may therefore fine-tune intracellular Ca^{2+} concentrations, to the levels required of an efficient successful regenerative process.

At a glance, c-MYC, a proto-oncogene and SDHA as a tumour suppressor may be considered to function antagonistically, but both work towards a cumulative liver regenerative capacity. c-MYC and SDHA may be considered important metabolic mediators. c-MYC a proto-oncogene, in sync with HIF-2α-SUCNR1-succinate activity, may have utilized cellular metabolism to support hepatocyte proliferation. SDHA, a subunit of the SDH complex, a mitochondrial enzyme marker, also utilizes the TCA cycle to convert succinate to fumarate, aiding in energy metabolism. SDHA may have interacted with VHL,

another tumour suppressor, in its function. c-MYC-HIF-2α-SUCNR1-succinate and SDHA-VHL complexes in a tightly-regulated gene interactive network, can be seen to be complementary but opposing in enhancing the overall regenerative capacity. One is guided by the critical roles of known tumour genes such as (pRb1, p53, Hippo and Pten) in metabolism and development, and as such the general tissue homeostasis. SDHA as a suppressor, must suppress positively, to support the overall liver regenerative capacity, while the c-MYC must also support a controlled proliferation within the limit of liver regeneration.

Careful analysis of some molecular interactions of Fe-S genes or protein and their associated counterpart have also shown that they may play unique roles in inter-organellar communication; PGC-1α-Nrf-1α (probable nuclear-mitochondrial) and MINER1-SUCNR1 (ER-mitochondrial).

A complex system of molecular interactions of genes, proteins, signal molecules and organelles may function in a well-crafted efficient system to drive the liver regeneration process or hepatocyte proliferation. These complex molecular interactions trigger signal molecules at various liver regeneration phase to effect metabolic changes, angiogenesis, cell signalling and iron metabolism, and mitochondrial biogenesis. Mechanisms of these interactions can be seen to underlie important molecular events of liver regeneration, seeking for answers. Molecular basis of the functions of some proteins such as NEET (mitoNEET and MINER1) are still debated. By their molecular interactions, some clear inferences can be made on the possible molecular basis of their cellular functions.

This study for the first time, therefore sought to not only unravel the functional role Fe-S protein biogenesis in liver regeneration but also the complex nature of molecular interactions governing the cellular functions of Fe-S proteins with other associated gene partners in a gene interaction network. These molecular interactions may form the basis of their cellular functions in hepatocellular proliferation. These molecular interactions may govern a cellular fate decision of delicately balancing formation and destruction Fe-S biogenesis during hepatocellular proliferation to meet critical cellular needs.

5.1. RECOMMENDATIONS

Mitochondrial biology can be said to be relatively young and gaining increasing important in molecular and cellular biology. Mitochondrial metabolism lies at the centre of the general cellular metabolism. Much have been studied concerning mitochondrial biology in cancer. Mitochondria are both metabolic and signalling hubs. In an almost perfect system like the liver regeneration system;

Mitochondrial dynamics and biogenesis, their machinery components and the molecular players including the fundamental mechanisms of molecular further research.

Hepatocytes are primarily metabolic hubs. Liver has structurally and anatomically been defined. And the heterogeneity of hepatocytes, based on zonation and its resulting metabolic allocations well known. But how these hepato-dynamics interplay to initiate or sustain the regenerative process is still unknown. Further researches on these dynamics and metabolism in general during this phase of rapid hepatocyte proliferation must be studied.

Fe-S biogenesis as an essentiality of mitochondrial biogenesis in liver regeneration is little known. Fe-S proteins governs a lot of cellular processes especially DNA/RNA metabolism. DNA repair proteins, helicases, polymerases, primases and several others, are Fe-S proteins. DNA synthesis and repair is very indispensable to the whole liver regeneration process. A comprehensible study on DNA metabolism during liver regeneration is much needed.

Fe-S biogenesis usually occurs *de novo* in the mitochondria and is the primary source of cluster for cytosolic Fe-S and nuclear Fe-S biogenesis. This may imply a unique inter-organellar communication which is definitely in need of further research especially during proliferation.

Mitochondrial and Fe-S biogenesis also means energy production. For mitochondria to be functional, an intact OXPHOS and ETC systems must be in place. Fe-S biogenesis may function in that regard. Energy is indispensable for the highly energy-dependent liver regeneration process, fuelled by the mitochondrial oxidative phosphorylation. Energy

metabolism and cellular bioenergetic adaptation during liver regeneration, clearly seek more study.

Fe-S proteins are active players in redox sensing, ROS regulation, iron and oxygen metabolism. They tend to be oxygen-labile, inferring that they break down in the presence of oxygen. The body needs oxygen in every sense. It is therefore important that the physiological redox behaviours of Fe-S proteins and how they utilise those unique abilities to evade oxidative breakdown, is definitely a topic worth researching.

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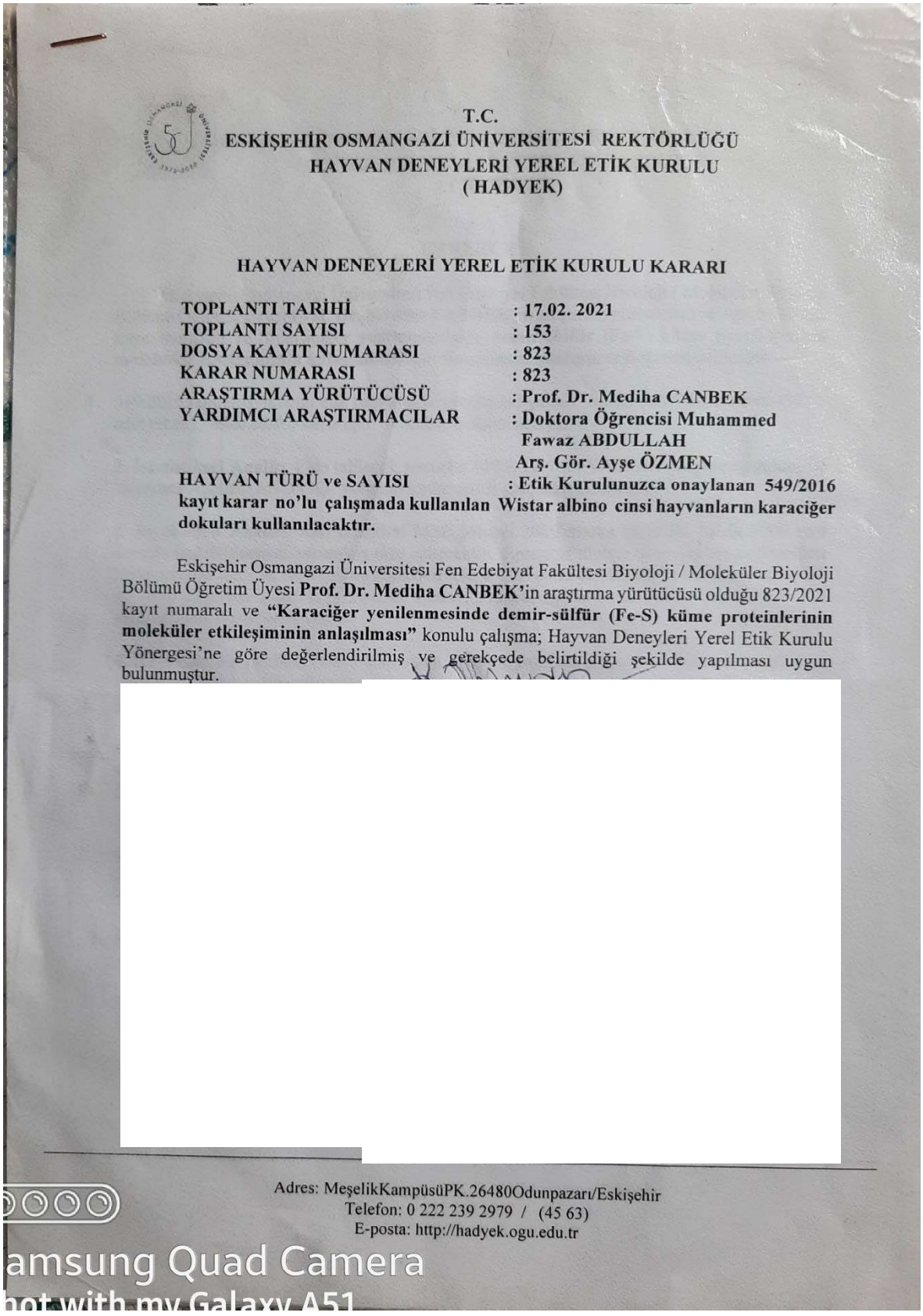
APPENDIX-A



T.C.
ESKİŞEHİR OSMANGAZİ ÜNİVERSİTESİ REKTÖRLÜĞÜ
HAYVAN DENEYLERİ YEREL ETİK KURULU
(HADYEK)

HAYVAN DENEYLERİ YEREL ETİK KURULU KARARI

TOPLANTI TARİHİ	: 17.02. 2021
TOPLANTI SAYISI	: 153
DOSYA KAYIT NUMARASI	: 823
KARAR NUMARASI	: 823
ARAŞTIRMA YÜRÜTÜCÜSÜ	: Prof. Dr. Mediha CANBEK
YARDIMCI ARAŞTIRMACILAR	: Doktora Öğrencisi Muhammed Fawaz ABDULLAH Arş. Gör. Ayşe ÖZMEN
HAYVAN TÜRÜ ve SAYISI	: Etik Kurulunuzca onaylanan 549/2016 kayıt karar no'lu çalışmada kullanılan Wistar albino cinsi hayvanların karaciğer dokuları kullanılacaktır.

Eskişehir Osmangazi Üniversitesi Fen Edebiyat Fakültesi Biyoloji / Moleküler Biyoloji Bölümü Öğretim Üyesi **Prof. Dr. Mediha CANBEK'**in araştırma yürütücüsü olduğu 823/2021 kayıt numaralı ve **"Karaciğer yenilenmesinde demir-sülfür (Fe-S) küme proteinlerinin moleküler etkileşiminin anlaşılması"** konulu çalışma; Hayvan Deneyleri Yerel Etik Kurulu Yönergesi'ne göre değerlendirilmiş ve gerekçede belirtildiği şekilde yapılması uygun bulunmuştur.

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APPENDIX-B

Table 4.1. Fold values ($2^{-\Delta\Delta Ct}$) (Fold values). Green representing fold value change ≥2 (gene upregulation) and red is for fold value change ≤0.5 indicating downregulation.

GROUPS vs TARGET GENE	Fold change ($2^{-\Delta\Delta Ct}$) Expressions showing upregulated gene in red (↑) and downregulated genes in red (↓)												
	CON	SH0	SH6	SH12	SH24	SH48	SH72	PH0	PH6	PH12	PH24	PH48	PH72
ACO1	1.07	0.83	0.28	0.32	0.39	0.48	0.33	0.003	1.20	0.41	0.64	0.52	0.59
ACO2	1.2	0.03	0.34	0.48	0.16	0.17	0.11	0.02	0.05	0.02	0.006	0.01	0.001
GLRX5	0.78	0.04	0.10	0.06	0.09	0.01	0.05	0.16	0.009	0.22	0.003	0.006	0.008
c-MYC	1.1	0.46	1.5	0.67	0.26	0.26	1.4	5.9	4.0	2.2	1.0	0.34	0.64
MFRN	1.0	0.84	1.8	1.3	1.8	1.9	2.0	0.21	0.74	0.62	1.1	0.79	0.69
MINER1	0.92	0.89	1.3	1.3	0.95	0.87	0.61	0.098	0.55	0.61	1.6	0.96	0.46
MNEET	1.0	0.91	3.2	1.9	0.99	1.5	2.3	7.8	3.5	2.5	1.6	0.85	1.2
HIF-2α	0.63	0.17	0.35	0.86	0.082	0.23	5.2	5.1	4.3	1.8	0.40	0.20	0.27
PGC-1α	0.83	0.76	0.60	0.38	0.31	0.86	0.42	0.11	0.97	0.43	0.16	0.20	0.18
SDHA	1.1	0.91	0.77	1.2	0.80	1.2	0.98	0.00072	0.63	0.43	0.60	0.52	0.40

APPENDIX-C

Table 4.3c. ΔCt Values and inter-genes comparative studies

GENES		ΔCt VALUES ± SD						COMPARATIVE STUDIES OF INTER-GROUPS GENE EXPRESSIONS					
		0	6	12	24	48	72	SH 0 vs PH 0	SH 6 vs PH 6	SH 12 vs PH 12	SH 24 vs PH 24	SH 48 vs PH 48	SH 72 vs PH 72
PGC-1α	SH	0.76±0.40	0.60±0.077	0.38±0.25	0.31±0.35	0.86±0.47	0.42±0.50	0.2202	0.9999	>0.9999	>0.9999	0.1954	0.9950
	PH	0.11±0.0093	0.97±0.27	0.43±0.18	0.16±0.062	0.20±0.055	0.18±0.069						
MitoNEET	SH	0.91±0.15	3.2±1.0	1.9±0.33	0.99±0.13	1.5±0.81	2.3±1.1	< 0.001 (****)	<0.99999	0.9803	0.9860	0.9619	0.4992
	PH	7.8±1.1	3.5±0.61	2.5±0.060	1.6±0.45	0.85±0.24	1.2±0.29						
HIF-2α	SH	0.17±0.12	0.35±0.20	0.86±0.21	0.082±0.045	0.23±0.24	5.2±0.63	<0.0001 (****)	< 0.0001 (****)	0.9479	> 0.9999	>0.9999	<0.0001 (****)
	PH	5.1±2.3	4.3±0.30	1.8±1.2	0.40±0.29	0.20±0.084	0.27±0.025						
ACO1-IRP1	SH	-0.65±1.7	2.1±0.78	1.7±0.24	1.7±1.1	1.2±0.46	1.1±0.99	0.1180	< 0.0001 (****)	> 0.9999	0.9984	>0.9999	0.9984
	PH	5.5±4.8	-0.84±0.56	3.4±2.9	0.79±0.46	1.1±0.50	0.081±1.6						
Mitoferrin	SH	0.84±0.15	1.8±0.39	1.3±0.067	1.8±0.48	1.9±0.28	2.0±0.49	0.3232	0.0084 (**)	0.2484	0.1408	0.0050 (**)	0.0004 (***)
	PH	0.21±0.083	0.74±0.34	0.62±0.26	1.1±0.12	0.79±0.10	0.69±0.32						
MINER1	SH	0.89±0.19	1.3±0.17	1.3±0.19	0.95±0.26	0.87±0.097	0.61±0.42	0.0259 (*)	0.0446 (*)	0.1231	0.1231	>0.9999	0.9999
	PH	0.098±0.051	0.55±0.18	0.61±0.38	1.6±0.37	0.96±0.31	0.46±0.20						
SDHA	SH	0.91±0.39	0.77±0.29	1.2±0.11	0.80±0.15	1.2±0.17	0.98±0.14	0.0332 (*)	> 0.9999	0.6637	0.9997	0.3255	0.4886
	PH	0.00072± 7.9e-005	0.63±0.27	0.43±0.36	0.60±0.19	0.52±0.10	0.40±0.057						
c-MYC	SH	0.46±0.13	1.5±0.75	0.67±0.49	0.26±0.13	0.26±0.11	1.4±0.64	<0.0001 (****)	0.0210 (*)	0.4402	0.9871	>0.9999	0.9876
	PH	5.9±2.0	4.0±1.2	2.2±0.72	1.0±0.088	0.34±0.19	0.64±0.19						
GLRX5	SH	0.036±0.026	0.10±0.15	0.061±0.061	0.092±0.15	0.013±0.0091	0.051±0.044	>0.9999	> 0.9999	> 0.9999	> 0.9999	>0.9999	>0.9999
	PH	0.16±0.16	0.0093±0.0024	0.22±0.29	0.0032±0.00061	0.0061±0.0014	0.0079±0.0062						
ACO2	SH	8.5±3.3	6.2±4.2	3.0±3.9	5.6±2.6	4.7±5.8	5.4±2.3	>0.9999	0.9994	0.9683	> 0.9999	>0.9999	>0.9999
	PH	7.1±1.2	6.1±1.4	7.5±0.60	6.4±5.7	8.6±1.8	12±1.5						

- PGC-1α: (PH0-PH6) ~ (**p= 0.0325,** *)

MINER1: ([PH0-PH24 ~ (**p value= < 0.0001,** ****); PH6-PH24 ~ (**p value= 0.0010,** ***); PH24-PH72 ~ (**p value= 0.003,** ***)]