DIFFERENTIATION OF HEPATOCYTE LIKE CELLS FROM IMMORTALIZED MOUSE EMBRYONIC FIBROBLASTS HARBORING LARGE T ANTIGEN

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

DIFFERENTIATION OF HEPATOCYTE LIKE CELLS FROM IMMORTALIZED MOUSE EMBRYONIC FIBROBLASTS HARBORING LARGE T ANTIGEN

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Genetic and acquired liver diseases are generally progressive and life threatening with limited curative therapy options. Although organ transplantation is the most potent treatment, number of patients waiting for organ transplant far outnumbers the potential suitable donors. Recently, new alternative methods have been developed to generate functional hepatocytes which can directly be administered to patients. Generating hepatocytes from different cells derived from patient has been one of the most promising alternative. Direct conversion of terminally differentiated cells into hepatocyte like cells has been reported previously. However, hepatocyte differentiation from SV40 Large-T antigen expressing immortalized Mouse Embryonic Fibroblasts has not been reported. To this end, first we have evaluated the effects of individual and combined retroviral expression of liver lineage determining transcription factors: Hnf4α, Foxa2 and Foxa3. Single factor transduced immortal MEFs gave little or no significant epithelial marker expression. These conditions were also insufficient to induce liver specific phenotype. However, combined expression of either Hnf4α+Foxa2 or Hnf4α+Foxa3 have resulted in an increased epithelial and liver specific characteristics such as albumin expression and glycogen storage. To elucidate epigenetic background of this process we genotyped transgenic mouse strains with conditional knockout alleles of histone variants. Histone variant H3.3A conditional knockout immortal MEFs were also infected with Cre expressing retroviral vectors. Our studies indicated that, Large-T antigen immortalized MEFs can be transdifferentiated by using the protocol designated for primary MEFs. Additionally, by isolating and immortalizing genetically determined MEFs, we have established cell lines ready for understanding the roles of histone variants on transdifferentiation.

That will be the foundation of subsequent studies delineating effects of histone variants on hepatocyte differentiation from MEFs.

Keywords: Induced hepatocyte, SV40, Immortal MEF, Hnf4α, Foxa2, Foxa3,direct conversion, H3.3A.

ÖZET

SV40 BÜYÜK T ANTİJENİ İÇEREN İMMORTALİZE FARE EMBRİYONİK FİBROBLASTLARININ HEPATOSİT BENZERİ HÜCRELERE DÖNÜTÜRÜLMESİ

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Edinilmiş ve genetik kaynaklı karaciğer hastalıkları genellikle ilerleyici ve hayati tehlike arz eden özelliktedir. Organ transplantasyonunun en iyi sonuç veren tedavi olarak kabul edilmesine rağmen, hasta sayısı nakile uygun donör sayısını aşmaktadır. Somatik hücrelerin doğrudan indüklenerek hepatosit benzeri hücre elde edilmesi yeni umut vaat eden yöntemlerden biridir ve önceden tanımlanmıştır. Ancak hepatosit benzeri hücrelerin, SV40 büyük T-antijeni ile immortalize edilmiş Fare Embriyonik Fibroblastlardan (FEF) elde edilmesi henüz çalışılmamıştır. Bu amaçla, karaciğerin embriyonik farklılaşmasında rol oynayan Hnf4α, Foxa2 ve Foxa3 transkripsiyon faktörlerinin retroviral vektörlerle ayrı ve farklı kombinasyonlarla ölümsüz FEF hücrelerine verilmesinin etkilerini inceledik. Bir transkripsiyon faktörüyle enfekte edilen FEF hücreleri epitel ve hepatosit hücre karakterlerini moleküler olarak önemli miktarda göstermediler. Ancak, Hnf4α+Foxa2 ya da Hnf4α+Foxa3 kombinasyonu ölümsüz FEF hücrelerinde epitelyal E-cadherin ifadesi; albumin ifadesi ve glikojen depolanması gibi hepatosit karakterlerinin ifadesini uyardığı gözlendi. Bu süreci etkileyen epigenetik mekanizmaların aydınlatılması için, histon varyantlarından koşullu olarak gen ablasyonu yapılabilen transgenik farelerin genotiplemesi yapıldı. Ayrıca, histon varyantı H3.3A geninde koşullu olarak gen ablasyonu yapılabilen ölümsüz FEF hücreleri, Cre rekombinaz genini ifade eden retroviral vektörlerle enfekte edilmiştir. Bu çalışmada Büyük T-antijeni ile immortalize edilmiş FEF hücrelerinin, primer FEF hücreleri için dizayn edilmiş protokolü kullanarak, hepatosit benzeri hücrenin doğrudan elde edilebildiği kanıtlanmıştır. Ayrıca, genetik olarak belirlenmiş hayvanlardan FEF

hücreleri izolasyonu ve immortalizasyonu yaparak, histon varyantlarının hepatosit transdifferensiyasonundaki rolünü anlamak için önemli bir araç elde edilmiştir.

Anahtar sözcükler: İndükte edilmiş hepatosit, SV40, Hnf4α, Foxa2, Foxa3, doğrudan hücre dönüşümü, H3.3A.

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to my family...

Abbreviations

MEF: Mouse Embryonic Fibroblast

ELAD: Extracorporeal Liver Assisting Device

Hnf4α: Hepatocyte Nuclear Factor 4 alpha

Foxa 2: Fork Head Box Protein A 2 (Also known as Hnf 3β)

Foxa 3: Fork Head Box Protein A 3(Also known as Hnf 3γ)

LSPC: Liver Stem/ Progenitor Cell

iPSC: Induced Pluripotent Stem Cell

HSC: Hematopoietic Stem Cell

H3.3: Histone Variant H3.3

TSS: Transcription Start Site

SV40: Simian Virus 40

MSCV: Murine Stem Cell Virus

GFP: Green Fluorescent Protein

EGF: Epidermal Growth Factor

HGF: Hepatocyte Growth Factor

IRES: Internal Ribosomal Entry Site

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Chapter 1

Introduction

1.1 Therapy in end-stage liver diseases

1.1.1 Tissue transplantation

Liver is a multifunctional organ that orchestrates hundreds of physiological process simultaneously such as glycogen storage, xenobiotic detoxification, lipid metabolism and protein secretion [1]. Genetic and acquired liver diseases may be life threatening ; and, most of the time, orthotopic liver transplantation(OLT) is the only therapy option. Liver transplantation centers gives higher priority to patients with end stage liver diseases: chronic liver diseases with hepatocellular carcinoma, cholangiocarcinoma, fulminant liver failure; chronic liver diseases with, portal hypertension, hepatic encephalopathy and hepatopulmonary syndrome [2]. However, number of patients waiting for liver transplants far outnumbers the potential liver donors; and, many patients progress to liver failure and death while waiting [2, 3, 4]. Furthermore, liver transplantation shows moderate tissue compatibility in patients, which decreases the success rate of allogenic liver transplantation [5].

Alternative to whole organ transplantation, cellular therapies such as

transplantation of primary hepatocytes isolated from human livers has been proved to be a potential tool[6]. Moreover, in regenerative medicine, promising methods have been developed to create functional cells of specific organs such as, heart, kidney, pancreas and liver. Such methods mostly based on stem cells, which can give rise to wide variety of functional cells in an organisms[7, 8, 9, 10]. Accordingly, seeking new methods to obtain functional hepatocytes from different sources has been intensively studied.

1.1.2 Cell transplantation

Recent studies on cellular therapy for distinct liver diseases is encouraging. One of the proposed methods is isolation and transplantation of hepatocytes. Although the primary hepatocytes were transplanted to patients and promising results were obtained, isolated hepatocytes are mostly short in number, show varying quality and cannot be expanded *in vitro* [11].

Adult liver harbors bipotential cells that resides in bile terminal ductules (canals of hering). These cells are considered as adult liver stem/progenitor cells (LSPC) which can give rise to both hepatocytes and cholangiocytes. LSP cells are activated when the liver is damaged from various source [11]. Though limited number of LSPCs are found in adult liver, *in vitro* expansion of progenitors has been demonstrated by cell surface marker mediated isolation. Furthermore, these progenitor cells successfully repopulate liver *in vivo* [12].

In 1992, scientists have developed an artificial liver system called Extracorporeal Liver Assisting Device (ELAD) in order to extend life expectancy of patients. ELAD system exploits human C3A cell lines (HepG2 hepatoma derived cell line) to supplement and detoxify patient's blood. This was achieved by perfusion of patient's blood into hollow fiber cartriges containing cultured C3A cells. Cartrige system prevents hepatoma cells to transfuse into patient's bloodstream [13]. Although the clinical trials have resulted increased survival rate and life quality, it is an exhausting therapy course that patients are dependent to perfusion sessions with ELAD.

Hepatocyte-like cells can also be derived from Embryonic Stem Cells (ESCs). Inner cell mass obtained from preimplantation embryos can be expanded in special culture conditions without differentiation. Next, lineage determination through embryoid bodies, endoderm , hepatoblasts and hepatocyte-like states are induced with special conditions [14]. Despite the potential, derivaiton of fertilized embryos are still an ethical issue and during differentiation, hepatocyte yield drops dramatically.

Another potential source of functional hepatocytes are circulating stem cells consisted of Hematopoietic Stem Cells (HSCs) and Mesenchymal Stem Cells (MSCs). Several articles has shown that, upon liver resection, inflammatory stimuli and ischemic damage in liver can induce bone marrow-derived stem cell mobilization [15, 16, 17]. Pilot studies have demonstrated that infusion of autologous MSCs to patients with end-stage liver disease have beneficial effects [18, 19].

In 2006, terminally differentiated mouse somatic cells have been successfully reprogrammed into pluripotent stem cells by using defined transcription factors: Oct4, Sox2, Klf4 and c-Myc. This study started a new era for regenerative medicine, since vast amount of cell types can be achieved by differentiating induced pluripotent stem cells (iPSCs) [20]. In following years, hepatocyte-like cells have been successfully differentiated from human and mouse iPSCs. Human derived iPSCs were differentiated into hepatocytes and successfully transplanted to immunodeficient mouse liver [21]. Furthermore, vascularized liver buds were generated from human iPSCs and showed functional liver characteristics *in vivo* [22].

1.1.3 Transdifferentiation of hepatocyte-like cells

Although iPSCs paved the way through obtaining functional parenchyma cells, reprogramming the cells into pluripotent state followed by differentiation to desired cell phenotype is a time consuming and laboring process. A new method was explored by two different groups in 2011, which aims to directly differentiate

somatic fibroblasts into hepatocyte-like cells [23, 24].

Obtaining hepatocyte-like cell from iPSCs requires two reprogramming steps including: induction of pluripotency from terminally differentiated cells and chemical mediated differentiation into desired cell phenotype. Although this two-step process is already optimized, particularly the latter step shows varying success rate which is deeply affected by: growth factors /chemical substances used in the process and manipulation time [25]. On the contrary, direct induction of terminally differentiated cells into hepatocyte is one single step mediated by transcription factors. Using a single conditioned differentiation medium supplemented with EGF and HGF favor differentiation and proliferation of hepatocyte-like cells.

Direct differentiation of somatic cells requires global change of gene expression. Transcription factors are mostly the key regulators in reprogramming. During liver development many transcription factors involves in differentiation nonexclusively; but, they rather forms stringent and complex combinations to induce liver specific gene expression. There are set of factors playing role in liver development: winged helix family proteins Hnf3α, β and γ (Also called Foxa1, Foxa2 and Foxa3 respectively); Homedomain proteins Hnf1α, Hnf1β; nuclear hormone receptor family Hnf4α, COUP-TFII, LRH-1, FXRα and PXR; Leucine zipper containing factor C/EBPα; Hnf6; zinc finger transcription factors Gata4 and Gata6 [26].

In order to transdifferentiate somtaic cells into hepatocyte like cells, one group from Japan has tested twelve transcription factors some of which were mentinoed above: Hex, Gata4, Gata6, Tbx3, Cebpα, Hnf1α, Hnf1β, Foxa1, Foxa2, Foxa3, Hnf4α and Hnf6. These factors were delivered into Mouse Embryonic Fibroblasts (MEFs) and adult tail tip fibroblasts (TTFs) by using retroviral vectors. By using HGF and EGF supplemented differentiated medium, they have successfully obtained hepatocyte-like cells which exhibit increased liver specific gene expression profile. Retracting transcription factors have resulted that, Hnf4α together with either Foxa1, Foxa2 or Foxa3 constitutes minimum combination required for direct differentiation of somatic MEF cells. These transdifferentiated

cells are so called induced Hepatocyte-Like (iHEP) cells and can be expanded *in vitro* without any genetic instability. Obtained hepatocyte-like cells also rescued fumarylacetoacetate-hydrolase-deficient (Fah -/-) liver failure mouse model [24].

With few exceptions, similar transcription factors were used in direct differentiation protocol by another group in China: Gata4, Foxa1, Foxa2, Foxa3, Hnf1α, Hnf4α, Hnf6, Hlf, Hex, Jarid2, Coup-TF1, Lrh1, Fxr and Pxr. Then, these factors were cloned in lentiviral vectors for gene delivery. Different from experiments mentioned above, primary MEF cells were p19 deleted (p19-/-) which has disrupted p53 pathway and yields expandable iHEP cells. In this set of experiments, scientists reduced number of transcription factors to a final combination of: Hnf1α, Foxa3 and GATA4. Generated iHEP cells are also able to rescue Fah deficient mice [23]. Same group has transdifferentiated human fibroblasts into human induced hepatocyte like cells (hiHEPs) by using three factors in combination: Hnf1α, Hnf4α and Foxa3. Since human derived hepatocytes are mostly not expandable, SV40 large T antigen immortalized fibroblasts were used for unlimited hiHEP culture [27]. This study also proved that induction of hepatocyte like cells from human fibroblasts differs from induction of MEFs in terms of transcription factor combination.

These articles have proven that most potent transcription factor combinations are composed of: Hnf4α, Hnf1α, Foxa1 (Hnf3α), Foxa2 (Hnf3β), Foxa3 (Hnf3γ) and Gata4.[23, 24] Regulation of many hepatocyte specific genes are mediated strongly but not exclusively by Hnf4α, Hnf1β and Hnf1α transcription factors. Especially, Hnf4α alone is indispensible for development that, its deletion leads to growth arrest at gastrulation because of visceral endoderm dysfunction [28]. These factors can also reciprocally stimulate each other by establishing a feed forward loop. Hnf3 factors, which are also called Foxa proteins (Foxa1, 2 and 3) are essential for direct chromatin regulation to facilitate liver specific gene expression(FigureB.2) [26].

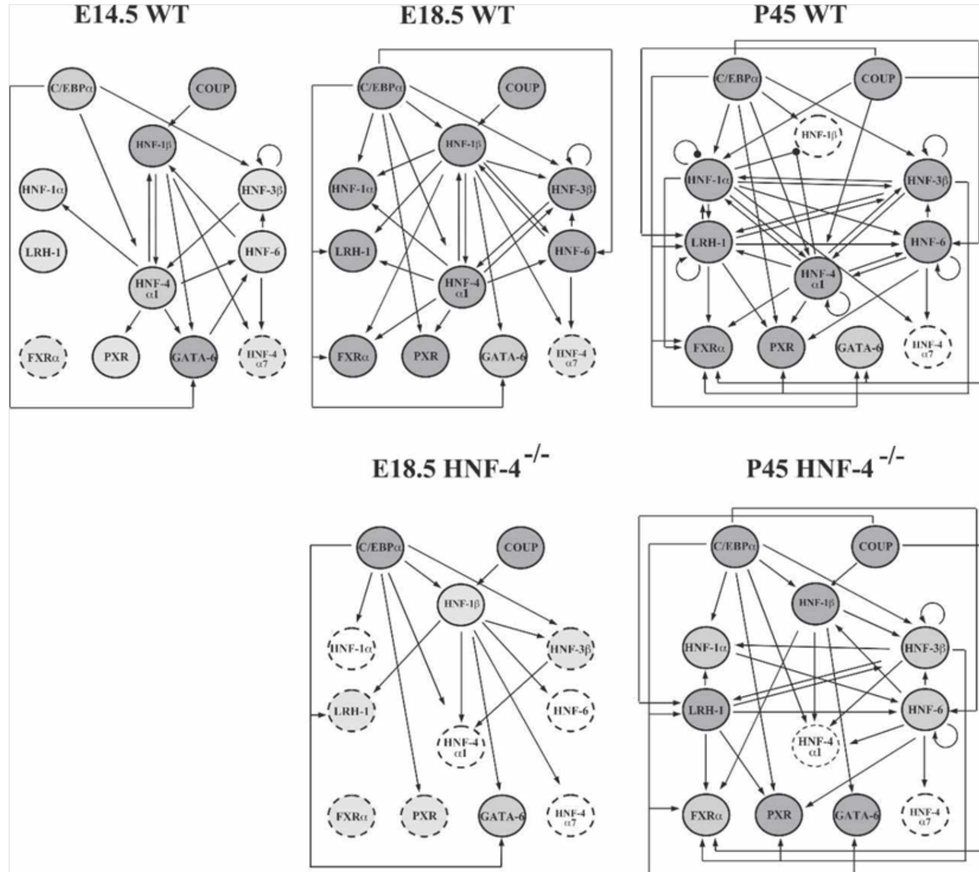


Figure 1.1: Interaction of liver enriched transcription factors in embryonic development and maturity. Effects of Hnf4α deletion in development and adulthood is represented in lower diagrams (adapted from Kyrmizi et. al, Genes & Development, 2006)[26]

In addition to the the developmental roles, given transcription factors are essential for normal liver function in adults. For example, deletion of Hnf4α and Hnf1α factors cause glycogen storage failure in mice. Epithelial phenotype is also severely disrupted in Hnf4α deleted mice. In adult mice, although Foxa3 null mice show decreased hepatic Glut2 expression, Foxa1 knockout mice and Foxa2 fl/fl conditional knockout mice show no severe phenotypes except hypoglycemia [29]. Besides the direct actions of transcription factors, a complex crosstalk between individual transcription factors have different phenotypic effect on cells. During direct differentiation, only stringent combination of transcription factor meet minimum criteria for hepatocyte-like phenotype [24, 23, 27].

In summary, specific transcription factors should be delivered to the cells with stringent combination to induce a liver specific function, just as in iPSC generation process. However, required criteria for direct induction of hepatocytes can be crudely represented as two major steps: 1- relaxation of chromatin structure by a liver lineage transcription factor which can modulate chromatin such as Foxa proteins.; 2- a key hepatocyte master regulator transcription factor for activating liver specific gene expression such as Hnf4α.

1.1.4 Cellular immortalization in reprogramming

Immortalization of primary or early passage of cells without differentiation or disruption of phenotype is an invaluable tool for functional studies. Many human or rodent derived cells have been successfully immortalized [30]. There are two most prominent ways of immortalization: 1- blocking of p53, pRb pathway to promote cell cycle progression; 2- activating telomerase which results in elongation of telomeric repeats. Increased telomeric sequences protects cells from crisis caused by shortened telomeres. While inactivation of p53 and pRb pathway is enough to immortalize most of the rodent tissues, additional telomerase activation is required for human derived cells due to lack of hTERT activity in many tissues [31].

A widely used immortalization method is inactivation of p53 and pRb pathway by SV40 large T-antigen expression. SV40 is an abbreviation for Simian Virus 40 which contains three elements: 17K T-antigen, small t-antigen and large T-Antigen. Large T-antigen can bind and inactivate heatshock chaperone 70 (hc70), Rb family tumor suppressor proteins (pRb, p107 and p130) and tumor suppressor p53 (Figure1.2). Large T-antigen can be transiently or permanently delivered to the desired cells. Many rodent cells can be immortalized merely by overexpression of Large T-antigen [32].

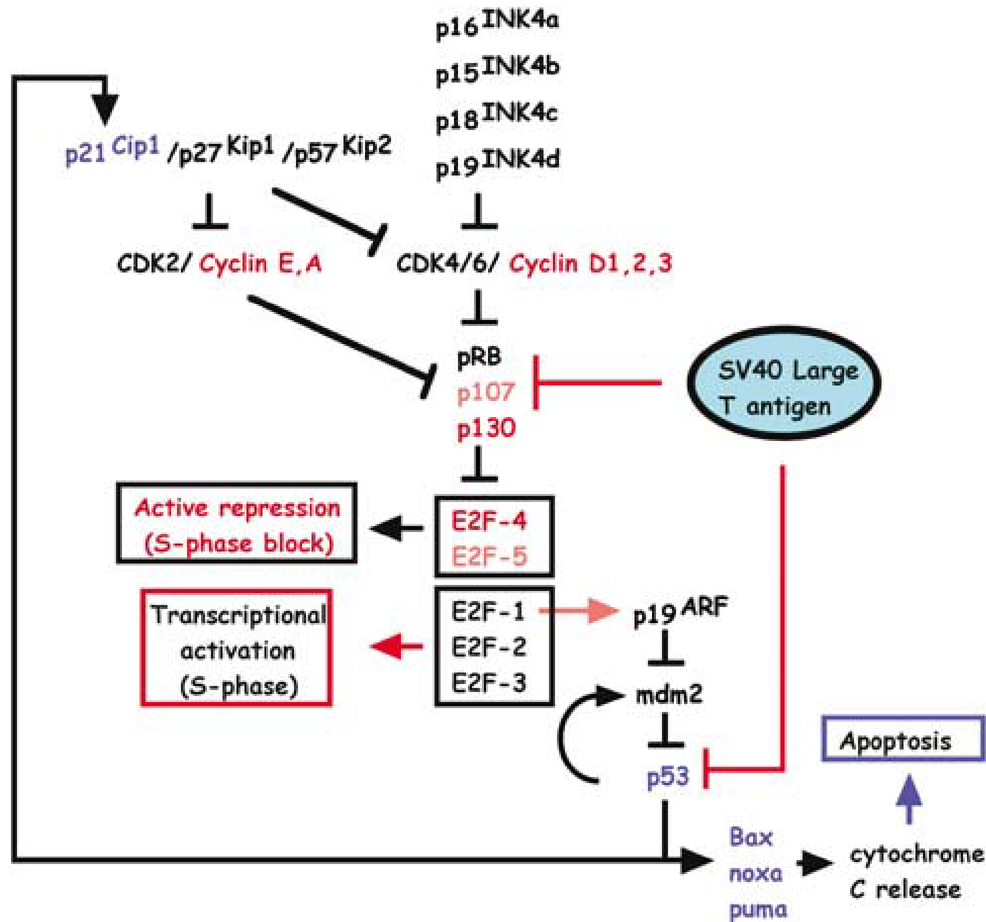


Figure 1.2: Effects of SV40 large T antigen in intracellular pathways (adapted from Ahuja et. al, Nature, 2005)[32]

Inhibition of p53 pathway has additional benefits on reprogramming other than immortalization of the cells. Inventors of iPSCs, Shinya Yamanaka and his colleagues have explored that heterozygote and homozygote deletion of p53 increase iPSC colonies from MEFs by up to 10 and 40 folds respectively even in the absence of c-Myc factor. [33] Similarly, p53 sh-RNA expression was shown to significantly increase numbers of hiPSC colonies derived from human fibroblasts. However in the same study, ectopic expression of hTERT did not yielded increased number iPSC colonies [34]. In another study, transient SV40 large T-antigen over expression in peripheral mononuclear cells increased iPSC colonies and reduced reprogramming duration [35].

During cellular differentiation, pRb also plays critical roles. In addition to

gate-keeper role on restriction point of cell cycle, it can also regulates chromatin structure by distinct mechanisms: 1- direct recruitment of co-repressors when bound to gene promoters; 2- direct interaction of polycomb group (PcG) repressor proteins; 3-genomewide regulation and maintenance of heterochromatin structure. Deletion of pRb cause inhibition of differentiation, and leads to cellular expansion and tumorigenesis [36].

Besides the elimination of p53 and pRb, mouse epithelial cells were shown to be expanded when p19 is deleted [37]. In the same study, mouse hepatocytes derived from p19 knockout mice (p19-/-) were cultured without losing genetic stability and cell characteristics. These cells also successfully populated in liver without tumor formation. Same knockout model was utilized in direct differentiation studies that, differentiated cells were expandable *in vitro* [23]. p19 acts as a p53 activator by inhibiting the Mdm2, a p53 inhibitor.

All studies indicates that, during reprogramming, p53, pRb and p19 tumor suppressor proteins mostly acts as a barrier on dedifferentiation process. This results encouraged us to inspect the effects of p53/pRb immortalization on direct differentiation. To best of our knowledge, there is no study aiming identification of phenotypinc effect of disrupted p53/pRb mouse cells on direct hepatocyte differentiation.

1.2 Cellular reprogramming and epigenetics

1.2.1 Regulation of cellular differentiation by epigenetic mechanisms

Cellular reprogramming can be simply considered as alterations in gene expression pattern which is controlled mainly by two protein families: 1- Transcription factors, which physically interacts with genomic DNA and directly modulates gene expression patterns by affecting transcription machinery; 2- epigenetic

regulators which affect gene expression patterns in various ways such as histone modifications and DNA metylation [38, 39]. These two concepts can be best explained with generation of induced Pluripotent Stem Cells (iPSCs). Terminally differentiated somatic cells are forced to change their gene expression pattern by ectopic expression of four transcription factors Oct4, Sox2, Klf4 and c-Myc. After the induction, chromatin architecture switches to more "open" state, which potentially exposes numerous regulatory sites of various lineage determining genes. Both ESCs and iPSCs are more abundant for active chromatin marks, such as modified histones; H3K4Me3 and H3K9Ac; and, global DNA hypomethylation.

Contrarily, terminally differentiated somatic cells show prevalent heterochromatin status accompanied by repressive chromatin marks such as H3K27Me3. Thus, induction of pluripotency from terminally differentiated cells has been intensively studied to elucidate the complex relationship between defined transcription factors (Oct4, Sox2, c-Myc and Klf-4) and epigenetic status of induced cells [40]. To this end, potential epigenetic barriers acting in differentiated-pluripotent transition have been revealed including H3K9me3/2, H3K27me3, insufficient histone acetylation on H3K9 and DNA hypermethylation [40].

Generation of iPSCs aims to establish a chromatin state which allows to apply any cell differentiation protocol. However, induced pluripotent stem cells show varying levels of epigenetic memory inherited from cells which undergo dedifferentiation. Attempts to erase the memory of target cells can affect the yield and quality of iPSCs [40, 41]. For example, in an sh-RNA mediated loss of function study, DOT1L, a H3K79 methyl transferase, has been discovered as a specific barrier to iPSC formation [42].

Besides generation of iPSCs, direct differentiation of somatic cells require profound epigenetic alterations in order to repress genes peculiar to original cells and to activate genes related with desired cell types. In this concept, global epigenetic modifiers have been shown to affect transdifferentiation susceptibility of cells [43]. In order to increase fibroblast responsiveness to Wnt3a for osteogenesis differentiation, scientists have used 5'-aza-dC and trichostatin-A which are inhibitor of DNMT and inhibitor of HDAC respectively.

1.2.2 Epigenetic regulations on liver specific gene expression and lineage determination

Complex functions in liver mainly regulated by liver enriched transcription factors during development and in maturity. Recruitment and activation of these factors require cooperation with histone modifiers. Liver enriched Hnf4α, for example, can interact with CBP, SRC1 and p300, which leads to increased transcriptional activity of Hnf4α [44]. Another striking example is Hnf3 (FoxA) family transcription factors. These factors can directly affect conformation of chromatin structures of liver specific genes such as Albumin and Alpha Fetoprotein and upregulate their expression without recruiting any coactivator. To to this, FoxA factors turns heterochromatic domains into open nucleosomal state which allows other factors to bind DNA [45].

Global epigenetic modifiers also affects liver specific gene expression in differentiated cell. For example, hepatoma cell lines treated with either 5-aza-2'-deoxycytidine (5-aza-dC, a histone methylase inhibitor) or Trichostatin A (histone deacetylase inhibitor) resulted in increased xenobiotic mechanism related gene expressions [46]. Liver enriched transcription factors can also be regulated by global epigenetic modifiers. As an example, inhibition of HDAC in hepatoma cells induces growth arrest and leads to upregulation of liver specific transcription factors C/EBPα, HNF1α, HNF3α, HNF3β and HNF4α [47]. Differentiation of pluripotent embryonic stem cells (ESCs) and mesenchymal stem cells into liver specific phenotype can be facilitated by inhibiting the HDACs and DNMTs [48].

1.2.3 Histone variants and reprogramming

Histone variants are noncanonical histone molecules which share varying level of homology with canonical histones: H1, H2A, H2B, H3 and H4 (Figure 1.3).Histone variants are recruited to nucleosomes by chromatin remodeling complexes and replace with their matching canonical histone molecules (e.g H3.3 variant replace with H3) [49]. Identified histone variant specific chaperones guide

these remodeling complexes to where a nucleosme exchange is required. Unlike the canonical histones, most of the variants can be dynamically deposited to chromatin structure in a replication independent manner. Therefore, vast number of studies have been ongoing to elucidate roles of histone variants in dynamic chromatin structure. Some of the identified functions of histone variants are activation/repression of gene transcription, DNA damage repair, chromosome segregation and chromosome inactivation [50].

Histone H2A.Z is a highly conserved variant showing 60% of homology with core histone H2A [51]. It replaces H2A core histone in replication independent manner by two remodeling complexes: SWR-C and INO80. H2A.Z can mediate gene activation and gene silencing upon deposition into chromatin [50]. Same variant also protects euchromatin from being invaded by spread of heterochromatin [52]. One of the most significant roles of H2A.Z is its ability to poise gene for expression by localizin in promoter region. Nucleosomes in poised genes can easily be replaced by transcription machinery upon activatio of gene [50]. Another very interesting role attributed to H2A.Z is its cooperation with Foxa2 to generate nucleosome depleted regions in order to facilitate endoderm lineage determination in ESCs [53].

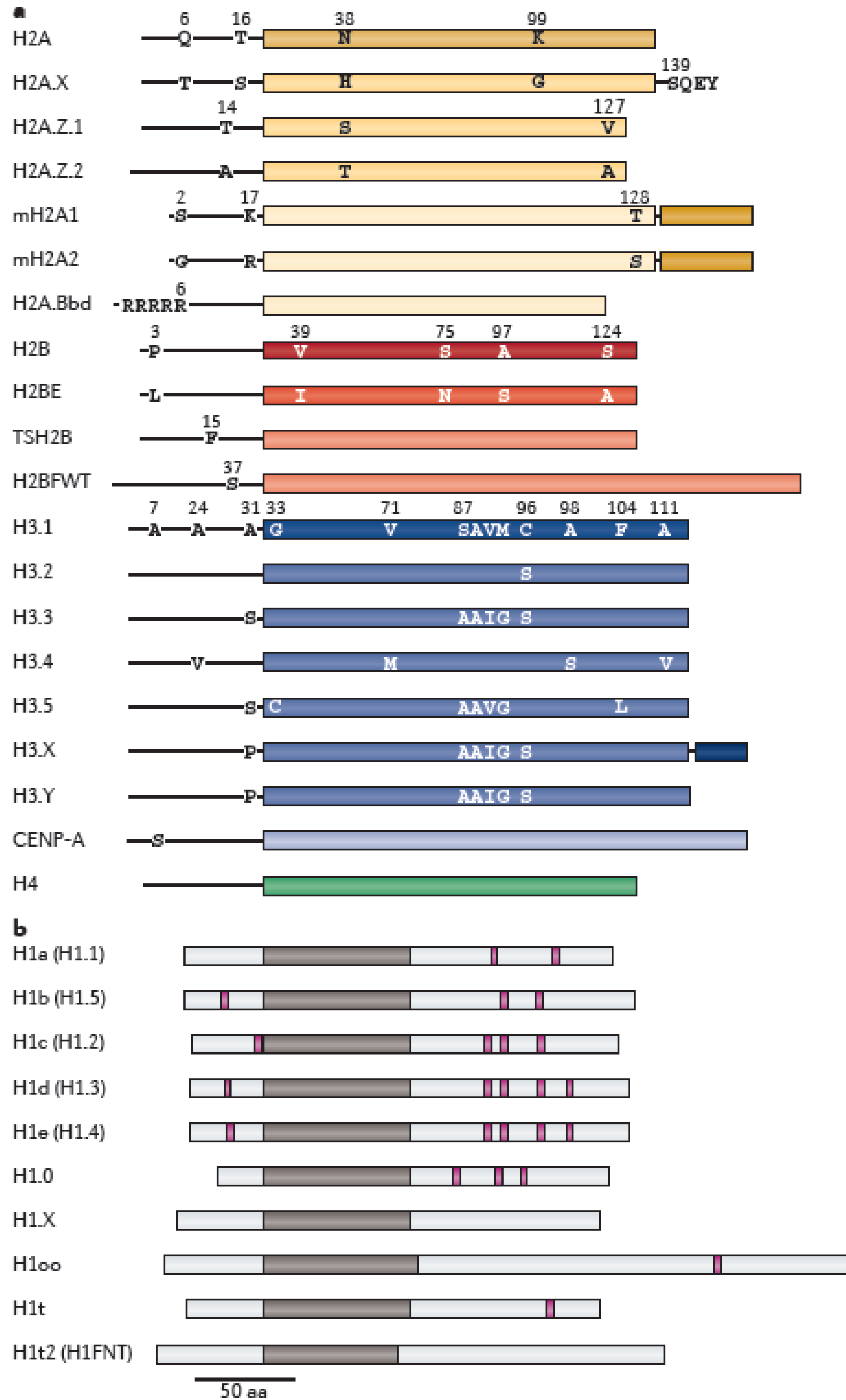


Figure 1.3: Histone variants and difference from their canonical histone counterparts. (adapted from Maze et. al, Nature, 2014)[49]

Another highly conserved histone variant H3.3 differs only four amino acids from H3 core histon [50]. Two different genes, H3.3A and H3.3B codes for variant H3.3 protein. Deposition of H3.3 variants into genome is mediated by specific chaperones HIRA and Daxx.[54] Although several roles were attributed to H3.3, a well characterized function is activation of gene expression by H3.3 deposition into Transcription Start Sites (TSSs) of actively transcribed gene promoter (figure 1.4) [55]. Since embryonic development is tightly regulated in transcriptional level, active deposition of H3.3 indicates a crucial role in developmental process. This concept was proven in several studies. Deletion of H3.3B in mice causes severe developmental retardation, chromosome segregation defects and infertility [56]. In another report, loss of H3.3 has been shown to cause over-condensation and mis-segregation of chromatin [57]. In regenerative medicine, mRNA of maternal H3.3 has been found to have an indispensible role in reprogramming of cells with Somatic Cell Nuclear Transfer (SCNT) technology [58]. In a report, H3.3 has been shown to protect epigenetic memory of active gene states, and mutant H3.3 is related with decresed epigenetic memory [59].

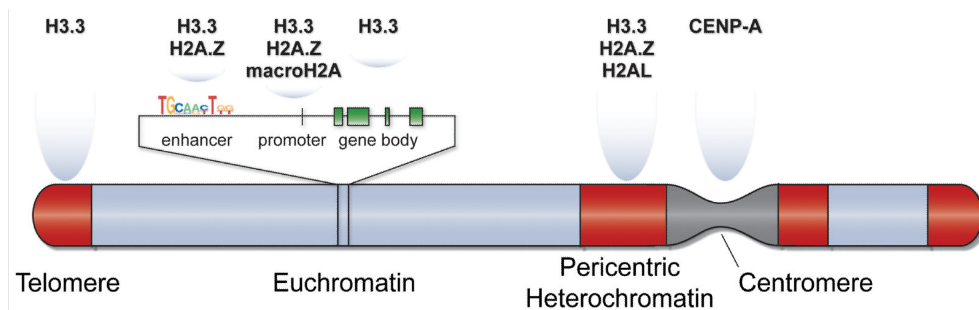


Figure 1.4: Positioning of histone variants on chromosome structure. Different chromosomal regions are occupied with distinct histone variants. E.g CENP-A is a variant of H3 and predominantly found in centromeres to guide chromosome segregation. (adapted from Banaszynski et. al,Developmental Cell, 2010)

Histone variant macroH2A is another conserved histone variant whose N-terminal region shares 60% of homology with canonical H2A. C-terminal domain of mH2A contains a "macro" globular domain sized 30kDa. Function of mH2A on chromosome X inactivation is one of the best identified functions [60]. Regulation of genomic imprinting mediated by heterochromatin structure has been strongly associated with mH2A [61]. In recent years several studies showed that histone

variants also play critical roles in cellular reprogramming and differentiation. For instance, histone mH2A.2, acts as a barrier between differentiated and pluripotent state of cells [62]. Furthermore, deletion of mH2A results in disrupted differentiation patterns in human ESCs [63].

All of these works together indicates potential important roles for histone variants in reprogramming which is still largely undiscovered. Future studies focusing on how these variants take role in differentiation and transdifferentiation would be a great leap for regenerative medicine.

1.3 Specific aims

Our studies constitutes the initial phase of a framework program aiming the establishment of a hepatocellular therapy program based on the use of hepatocytes obtained by direct differentiation of fibroblasts.

The specific aims of this master thesis were the following:

1- *Establishment of a hepatocyte transdifferentiation protocol using SV40 Large T-antigen immortalized mouse embryonic fibroblasts*

The reasons for adopting this procedure are as stated: first, immortalized cells provide unlimited supply for reprogramming and differentiation; secondly, although p19 -/- MEFs has been utilized in direct hepatocyte differentiation, effects of both pRb and p53 deletion has not been evaluated for MEF differentiation into hepatocytes; finally, it is also unknown whether Large T-antigen immortalized MEFs can be successfully transdifferentiated into hepatocyte like cells. Therefore we decided to focus on this approach by adopting previously described system. Three transcription factors, Hnf4α, Foxa2 and Foxa3 has been transduced into MEF cells separately, and in combination of Hnf4α+Foxa2 and Hnf4α+Foxa3.[24] To best of our knowledge, same system has not been tested on SV40 immortalized MEFs previously.

2- *Preliminary work aiming at obtaining mouse models to study role of histone variants in fibroblast-to-hepatocyte transdifferentiation*

Since roles of histone variants on differentiation is largely unknown, we aimed at obtaining appropriate mouse models lacking major histone varaints. These variants are H2AZ.1, H2AZ.2, H3.3A, H3.3B, mH2A.1 and mH2A.2. Except mH2A.1, all other mice strains carries inducible knockout allele provided by LoxP sequences which flanks critical exons. mH2A.1 mice are permanently knockout. Knockout of variants is likely to affect MEF-to-hepatocyte differentiation positively or negatively.

Chapter 2

Materials & Methods

2.1 Materials

2.1.1 Genes and plasmids

The retroviral vectors carrying the transcription factor coding sequence were ordered from Addgene. These pGCDNsam-IRES-GFP vectors carry retroviral 5' and 3' long terminal repeats flanking the insert gene and Internal Ribosomal Entry Site sequence followed by GFP coding sequence. Retroviral plasmids were submitted by Atsushi Suzuki.[64] The genes coding for transcription factors were: mouse HNF4A, FOXA2 and FOXA3.

The Cre expressing retroviral MSCV-CreERT2-Puro vector was kindly provided provided from Institut Albert Bonniot-FRANCE. Retroviral vector carries 5' and 3' long terminal repeats (LTRs) of Mouse Stem Cell Virus lacking Gag-Pol and Env gene which are required for competent viral partical production. CreERT2 gene is inserted between two LTRs and codes a fusion protein Cre flanked by two estrogen responsive peptides; ERT.

2.1.2 Cells used in reprogramming

Immortal MEF cells were provided in two genotypes: Wild Type (Wt) and H3.3A F/F. Particular exons of histone variant alleles H3f3a and H3f3b are flanked by LoxP sequence. Cre induction in H3.3A MEFs leads to exon excision and gene knockout subsequently. There are also other SV40 immortalized MEFs with followed genotypes: H2AZ.1 F/F, H2AZ.2 F/F, H2AZ.1& H2AZ.2 F/F, H3.3A F/F, H3.3B F/F, H3.3A -/- and H3.3B -/-. These cells are immortal but do not contain Cre-recombinase expression.

Phoneix 293 cells were provided from Institut Albert Bonniot-FRANCE. Retroviral plasmids are lack of Gag-Pol and Env gene, which are required for production of complete viral particles. Phoneix 293 is Hek293 derived cell line stably transduced by Gag-Pol and Vesicular Stomatitis Virus-G envelope glicoprotein (VSV-G). Transfection only with retroviral vector is enough to produce replication deficient viral particles.

To verify correct protein expression of retroviral plasmid with western blot, Hek293 cells was used for transient transfection protocol.

2.1.3 Cell culture

Immortal MEFs are cultured in Dulbecco's Modified Eagle's Medium (DMEM) low glucose supplemented with final concentration of; 10% FBS, 2mM L-Glutamine, penicillin/streptomycin 25 g/ml and non-essential amino acids. After viral transduction of MEFs , cells cultured in DMEM:F12 (50:50) medium supplemented with 10% FBS, 2mM L-Glutamine, 25 g/ml penicillin/streptomycin, Non-essential amino acids, 1 g/ml insulin, 1 M dexamethasone, 10mM nicotinamide, 50 M β-mercaptoethanol, 20 ng/ml hepatocyte growth factor and 20 ng/ml epidermal growth factor. 0,05% Trypsin used for trypsinization. For transfection of Hek 293 and Phoneix 293 cells, Poly-Ethyleneimine (PEI) was used with the ratio of 1:3 (g DNA: g PEI). The

10cm plates were coated with poly-l-lysine 100 g/ml for phoneix 293 cell culture. To culture reprogrammed cells, rat tail collagen type I, 5 ug/ml was used for coating of 6 well plate.

2.1.4 Primers

The primers used in genotyping is listed below as table 2.1.

Table 2.1: Primer pairs used to identify genotypes of the transgenic mouse colonies

Allele	Primer
H2AZ1 LoxP F	GCTACCATTGCTGGTGGTGGTATGTCA
H2AZ1 LoxP R	TGTGTGGGATGACACCTAGGAGAGGG
H2AZ1 Knockout F	CGTCGGAGCTTCAGCACGGTCC
H2AZ1 Knockout R	TGTGTGGGATGACACCTAGGAGAGGG
H2AZ.2 LoxP F	CCACGTAATGAGATCCAGTGCCCT
H2AZ.2 LoxP R	CATGCACCTGCCATTATGTCTGGTA
H3.3A LoxP F	TTTGCAGACGTTTCTAATTTCTACT
H3.3A LoxP R	ATATCGGATTCAACTAAAACATAAC
H3.3A Knockout F	TTTGCAGACGTTTCTAATTTCTACT
H3.3A Knockout R	AAATGCCCCACCACTGCCCAGC
H3.3B LoxP F	TCCTCATTCTACCACATGTTCA
H3.3B LoxP R	TCAATCTAGGCCTAAGACCAAA
H3.3B Knockout F	CTGCCCGTTCTGCTCGCCGATT
H3.3B Knockout R	TCAATCTAGGCCTAAGACCAAA
mH2A.1 knockout 1	CCACCACACCCAAGCCATAGTGCC
mH2A.1 knockout 2	GTCACAGGATGAAATGTGCGAAGC
mH2A.1 knockout 3	GCTGGACGTAAACTCCTCTTCAGAC
mH2A.2 LoxP F	TTCCACACAGCTACTGAGATGTGCC
mH2A.2 LoxP R	TCAGCACAGGGGCTCAAAATACCAG
ROSA26 LacZ 1	AAAGTCGCTCTGAGTTGTTAT
ROSA26 LacZ 2	GCGAAGAGTTTGTCCTCAACC
ROSA26 LacZ 3	GGACCGGGAGAAATGGAT
TTR::Cre F	CCTGGAAAATGCTTCTGTCCGTTTGCC
TTR::Cre R	GAGTTGATAGCTGGCTGGTGGCAGATG
Internal Control F	AGAGGGTCAGCTGAGCAAAA
Internal Control R	GCTGGGTAAGGCTGAAAGTG

2.1.5 Antibodies

Primary antibodies were: α-mouse Hnf4α (Mouse monoclonal, abcam), α-mouse Foxa2 (Rabbit polyclonal, abcam), α-mouse Foxa3 (Rabbit monoclonal, abcam), α-mouse Serum Albumin (Rabbit Polyclonal, abcam), α-mouse E-cadherin (Mouse monoclonal, abcam), α-mouse Vimentin (Rabbit monoclonal, abcam). Secondary antibodies were: α-mouse HRP, α-rabbit HRP, α-mouse Alexa 568, α-mouse Alexa 488, α-rabbit Alexa 568, α-rabbit Alexa 488.

2.1.6 Periodic Acid Schiff Staining (PAS)

Periodic Acid Schiff (PAS) staining kit (abcam) used for detection of polysaccharides in tissue and cell. Kit contains: Periodic Acid solution, Schiff's solution, Eosin, Bluing Reagent and Light Green Solution.

2.2 Methods

2.2.1 Plasmid amplification

The plasmids ordered from Addgene arrived as bacterial stabs in conditioned LB agar. These stabs were streaked on fresh LB agar which contains ampicillin 100g/ml, and incubated at 37 C° overnight. Next day, single colonies were picked and incubated in LB medium containing ampicillin 100 g/ml at 37 C° in shaker. The next day, plasmids were isolated by using midiprep kit (Machery-Nagel).

2.2.2 Western blot

Total protein was isolated from transiently transfected HEK293 cells by using RIPA buffer supplemented with Protease inhibitor mix, Sodium Orthovanadate and Sodium Fluoride. Protein concentrations were calculated with BCA assay kit. 30 g protein from each samples were loaded on SDS-PolyAcrylamide Gel with stacking (pH 6,8) 8%, resolving (pH 8,8) 10% density. Samples were run 30 minutes in stacking gel at 80 V ; and, 2 hours in resolving gel at 110V. Gel was then transferred to PVDF membrane 2 hours with wet transfer at 400 mA. For blocking, membranes were incubated with 10% Milk powder dissolved in 0,05% TBS-T, 1 hour at room temperature. Primary antibodies α-Hnf4α, α-Foxa2, α-Foxa3 and E-cadherin were diluted with 1:1000 concentration in blocking and membranes were incubated with primary antibody overnight at +4 C° . Secondary antibodies α-Mouse and α-Rabbit were diluted in blocking medium at 1:5000 and membranes were incubated with secondary antibody 1 hour at room temperature. After each incubation, membranes were washed with 0,05% TBS-T three times. After last wash, ECL kit solutions; Luminol and Phenol were mixed with 1:1 ratio and put on membrane for 5 minutes in dark. Membrane was then visualized under chemiluminescent detector for 30 seconds, 1 minute and 5 minutes.

2.2.3 Retrovirus production

Before Phoneix 293 cells were plated, 10 cm plates were coated with Poly-l-lysine. Phoneix 293 cells were cultured under standard cell culture conditions (DMEM, 10% FBS) and seeded 80% confluency on a 10cm plate the day before plasmid transfection. At the day of transfection, 293 Phoneix cells reach around 90-100% confluency, which increase the yield of viral particle. 10 g retroviral expression vectors (pGCDNsam-Hnf4α, Foxa2, Foxa3 and MSCV-CreERT2) and 2 g VSV-G envelope plasmid was added in 1ml Opti-MEM, serum reduced culture medium. According to the ratio of DNA:PEI, 36 g PEI was added to the DNA-OptiMEM mixture and vortexed. After 15 minutes, DNA-PEI-OptiMEM mixture was given to the Phoneix 293 cells under standard culture conditions (Figure2.1). After 24 hours of transfection, the medium was replaced by 5ml of fresh medium. After 48 hours of transfection, the GFP expression was checked under inverted fluorescent microscope. Existence of GFP indicated successful transfection. In the same day (after 48 hours of transfection) supernatant was collected and stored in -20 as aliquots.

2.2.4 Viral delivery of genes

Immortal MEF cells were cultured under standard cell culture conditions (DMEM, 10% FBS). One day before viral transduction, cells were seeded on 12 well plates at 20% confluency. Just prior to viral introduction, hexadimethrine bromide (Polybrene) was added to viral supernatant with final concentration of 4 g/ml. 500 l each supernatant was combined with polybrene and added to wells (Figure2.1). Hnf4α, Foxa2, Foxa3 only and CreERT2 wells received only regarding plasmids. Hnf4α+Foxa2 and Hnf4α+Foxa3 wells received 500 l of each vectors (total 1000 l). This protocol serially repeated in every 2 hours, total three times. Two hours after last infection, fresh DMEM with 10% FBS was added to the wells. 24 hours after last infection medium was replaced by hepatocyte differentiation medium without growth factors. Cell medium was replaced with fresh medium.

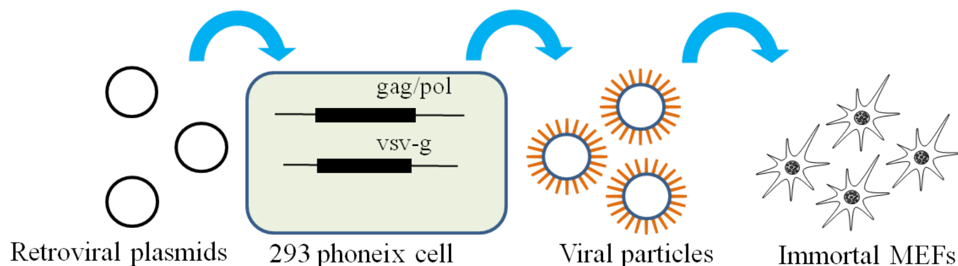


Figure 2.1: Virus production and transduction protocol

2.2.5 Culture of iHep cells and colony selection

Before transduced cells were replated, 6 well plates were coated with collagen type I. One week after viral transduction, reprogrammed cells were replated into collagen coated 6 well plates at 1/20 and 1/200 concentrations in order to observe single colony formation and colony selection. At this stage, differentiation medium was supplemented with EGF and HGF. After first replating, colony forming cells were observed under inverted brightfield microscope. Colony selection was performed according to cells morphology. In heterogenous cell population, epithelial-like cells were considered as differentiated and marked. After medium is drawn, single colonies were selected with pipette trypsinization and replating to cover slipped, collagen type-I coated 12 well plate.

2.2.6 Mouse colony formation

All strains were transferred from Grenoble-FRANCE and Lyon-FRANCE in September 2013. In animal house of Bilkent University-Ankara, there are six transgenic mouse strains all of which are conditionally or permanently knockout for histone variants: H2AZ.1, H2AZ.2, H3.3A, H3.3B, mH2A.1 and mH2A.2. Except mH2A.1 all strains carry transgenic LoxP sequences which flanks critical exons of regarding genes. mH2A.1 knockout mouse strain represent permanently deleted gene of mH2A.1 (Table 2.2).

There is one more transgenic mouse strain which carries ectopic Cre gene under

the control of ”Transthyretin (TTR)” promoter, which is found exclusively in liver and plexus of the brain. Cre gene is constantly expressed in liver but cannot perform a recombination due to structural modification of Cre protein. Its expression is controlled by ”murine estrogen receptors(MER)” sequences flanking Cre gene; and, when expressed, Cre translocates into the nucleus in the presence of 4-OHT (hydroxytamoxifen). Same straine carries LacZ expression under the control of ubiquitous ROSA26 promoter. Expression is absent in normal conditions, since this transgenic allele has a stop cassette flanked by LoxP sequences, only active Cre recombinase can induce LacZ expression. Therefore, cre expression can be traced in this strains.

Table 2.2: List of mouse transgenic mouse strains and available transgenic alleles

Transgenic Strain Name	Allele
H2AZ.1	Flox, knockout, wild type
H2AZ.2	Flox, wild type
H3.3A	Flox, knockout, wild type
H3.3B	Flox, knockout, wild type
mH2A.1	knockout, wild type
mH2A.2	Flox
TTR::CreTAM, ROSA26	Cre+, R26R+, R26R wild type

Name of the mouse strains and alleles in gene pool. Flox means LoxP sequence flanking regarding gene (Flanking LoxP). Kockout is deleted allele. Cre+ gives qualitative result of genotyping, since Cre insertion into mouse genome is not targeted and cannot be detected homozygosity with single PCR. R26R represents transgene for ROSA26-Lox-STOP-Lox-LacZ construct.

2.2.7 Mouse breeding

Breeding was applied as crossing of mice inside the strain. Crossing procedure is as followed: one male to two-three female are placed in the same cage. Three weeks after the copulation plug is observed, female mice give births. Maximum ten days after birth, tail-finger samples are collected from infant mice for genotyping. One month after birth, litters are weaned and separated to

different cages. If there is a reproductive problem in the colonies, wild type strains are crossed with transgenic strains to expand genetic pool and reproductive capacity.

2.2.8 Genomic DNA extraction from mouse tissues

Tail samples are incubated in Tail Buffer and Proteinase K in 55 C° overnight (shorter for cell suspension). Saturated NaCl solution is added to the samples to precipitate proteins. After centrifugation, DNA is precipitated with isopropanol, 70% Alcohol and 100% alcohol sequentially. After alcohol is dried, DNA pellet is reconstituted with sterile, ultra pure water. DNA concentration is measured with NanoDrop (Thermo Scientific).

2.2.9 Genotyping with PCR

Genotyping analyses are performed by conventional Polymerase Chain Reaction(PCR) on genomic DNA. All LoxP containing alleles are genotyped with CRE40 program and steps are as followed: Initial Denaturation: 95 C°, 3 min. First 2 Cycle: Denat, 95 C°, 1 min; Anneal 58 C°, 1 min; Extension, 72 C°, 1 min. Next, 38 cycle as follows: : Denat, 95 C°, 30 sec; Anneal 58 C°, 30 sec; Extension, 72 C°, 30 sec. Final extension; 72 C°, 3min. Next, samples were cooled down to 10 C°.

For R26R alleles "ROSA" program is used: Initial Denaturation: 95 C°, 3 min. 32 Cycle; Denat, 95 C°, 30sec; Anneal 65 C°, 1 min; Extension, 72 C°, 2 min. Final extension; 72 C° 10min. Next, samples were cooled down to 10 C°.

For permanently knockout alleles "LONG" program is used: Initial Denaturation: 95 C°, 3 min. 32 Cycle; Denat, 95 C°, 30sec; Anneal 58 C°, 1 min; Extension, 72 C°, 2 min. Final extension; 72 C° 10min. Next, samples were cooled down to 10 C°.

PCR products are run on 1,5% Agarose gel at 80V for 40 minutes. Safe Green (abm good) is utilized for DNA visualization.

In gel electrophoresis, LoxP containing allele gives approximately 150-200bp higher bands compared to Wild Type alleles. Permanently knockout allele primers target complete exone from both sides. Thus, PCR product giving around 1500bp for complete exon yields 500bp for deleted version. For ROSA strain, lighter band, which is 340bp gives mutant allele while 650bp gives wildt type allele.

2.2.10 Purification of MEFs and immortalization

H2AZ.1 F/F, H2AZ.2 F/F, H2AZ.1& H2AZ.2 F/F, H3.3A F/F, H3.3B F/F, H3.3A -/- and H3.3B -/- genotyped male and Female mice are placed in a cage and every day copulation plug is checked. The day plug is detected, considered as day zero. Thirteen days after, embryos (E13) are removed from female mouse under anesthesia. Head, internal organs and feet of embryos discarded. Remaining body is cut into small pieces. In a falcon tube, pieces are incubated with trypsin in +4 C° . Next day, suspension is incubated at 37 C° for 10-20 minutes. Culture medium and DNaseI is added and incubated at 37 C° . Cells are centrifuged and passaged in culture medium. At P1, MEFs are frozen for further process. To immortalize MEF cells, SV40 Large T antigen containing retroviral vectors are delivered to the Wild Type MEFs and cells were selected with Zeocin.

2.2.11 Inducible Cre-LoxP system in MEFs

Immortal MEF cells with Wt and H3.3A genotype are transduced with Cre-ERT2 containing retroviral supernatants. Next, cells are cultured with puromycin for selection of genes. Cre-ERT2 fused protein is constantly expressed in host genome; however, it can only be induced with hydroxytamoxifen (4-OHT).

2.2.12 Immunofluorescent staining

Cells seeded on 12 well plate were fixed with 4% paraformaldehyde 5 minutes. Cells were washed with 1X PBS three times. Fixed cells were permeabilized with 0,3% TritonX(in PBS) for 5 minutes and washed with 1X PBS three times. Blocking was performed with 10%FBS in 0,1% PBS-T (Tween) 1 hour in room temperature. Primary antibodies were diluted in blocking solution in following concentrations: E-Cadherin, 1:200; Vimentin, 1:200; Serum albumin, 1:50; and, cells were incubated overnight at +4 C°. Primary incubation is ended by washing with 0,1% PBS-T three times. All secondary antibodies were used in 1:500 dilution in blocking medium and cells were incubated 1 hour in room temperature. After washing with 0,1% PBS-T three times, 1/10.000 DAPI, diluted in ddH2O, was used to stain nuclei. Finally coverslips were mounted on slides with mounting medium.

2.2.13 Periodic Acid Schiff's staining (PAS)

Staining protocol was applied as indicated in Abcam's manual. Cells were immersed in periodic acid solution for 5 minutes then washed with distilled water. Cells immersed in Schiff's solution for 15 minutes then washed with tap and distilled water. Coverslips then stained with hematoxylin for 30 seconds then washed with tap and distilled water. Bluing reagent was applied 30 seconds then washed with distilled water. Light green solution was applied for 2 minutes then washed with distilled water. Cells dehydrated with graded alcohols, finally with xylene and mounted with entellan on slides.

Chapter 3

Results

3.1 Direct induction of hepatocyte-like cells from immortalized Mouse Embryonic Fibroblast

3.1.1 Verification of retroviral plasmid gene expression

The retroviral vector plasmids were transformed into competent bacterial DH5α strain and amplified in antibiotic resistant medium and purified with midiprep kit. These plasmids were previously published, and they contain GCsap retroviral vector sequence comprising modified Long Terminal Repeats(LTRs) of Murine Stem Cell Virus(MSCV) [24, 64]. The vectors carry Hnf4α, Foxa2 and Foxa3 open reading frames for expression, which is followed by an Internal Ribosomal Entry Site (IRES) and a Green Fluorescent Protein (GFP) coding sequence. Hek293 cells were transfected with given plasmids and western blot was performed. Hnf4α, Foxa2 and Foxa3 proteins were detected at 53 kDa, 52k Da and 37 kDa respectively as seen in Figure 3.1. This result indicates that the plasmids are intact and able to express correct proteins in transient transfection protocol.

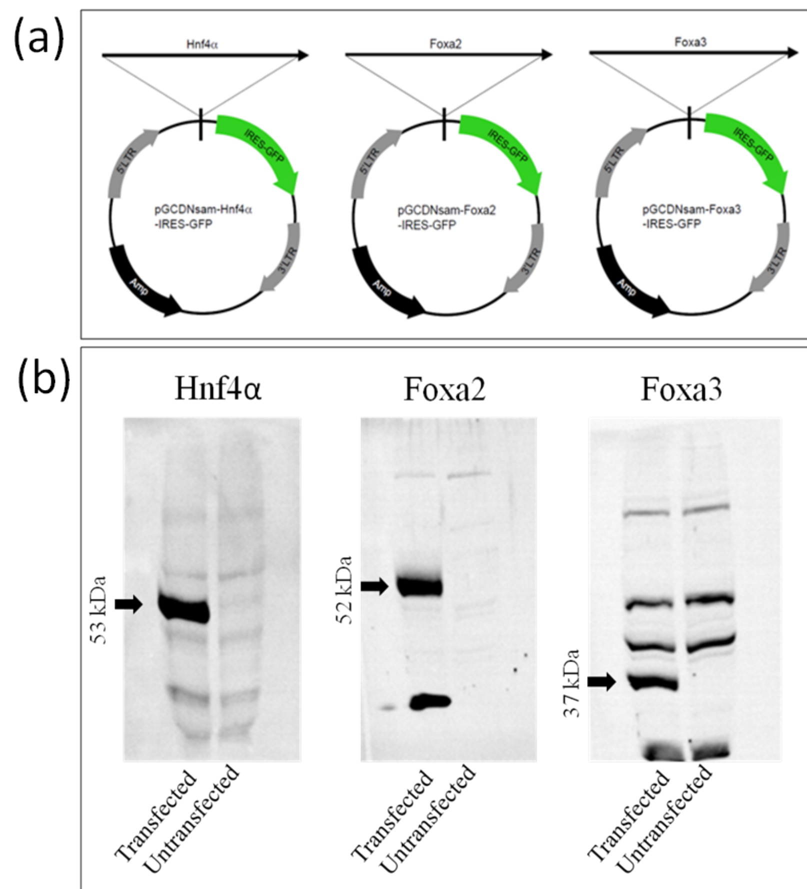


Figure 3.1: Plasmid maps and plasmid verification by western blot. a: pGCDNsam vectors are retroviral plasmids carrying open reading frame of Hnf4α, Foxa2 or Foxa3 genes followed by a IRES-GFP sequence. b: Western blot results of Hek293 cell transfection with regarding vectors. Black arrows indicate expected band sizes.

3.1.2 Viral delivery of transcription factors

Immortal MEF cells were cultured in 12 well plates and transduced serially three times with viral supernatants of Hnf4α, Foxa2 and Foxa3. Viral transduction of MEF cells with Hnf4α+Foxa2 or Hnf4α+Foxa3 factors has been previously shown to give rise to iHEP cells [24]. Same transcription factor combinations were adopted and additionally we transduced MEFs with single gene, Hnf4α, Foxa2 and Foxa3. Combined transduction was performed simply by mixing viral supernatants 1:1 ratio. 24 hours after infection, cell medium was changed to

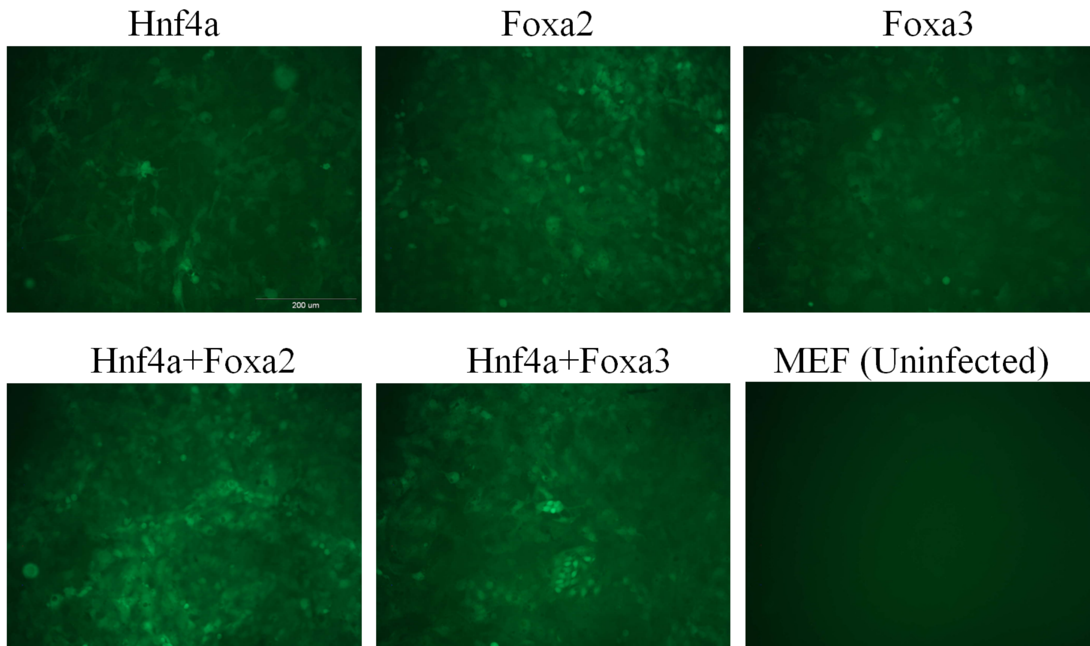


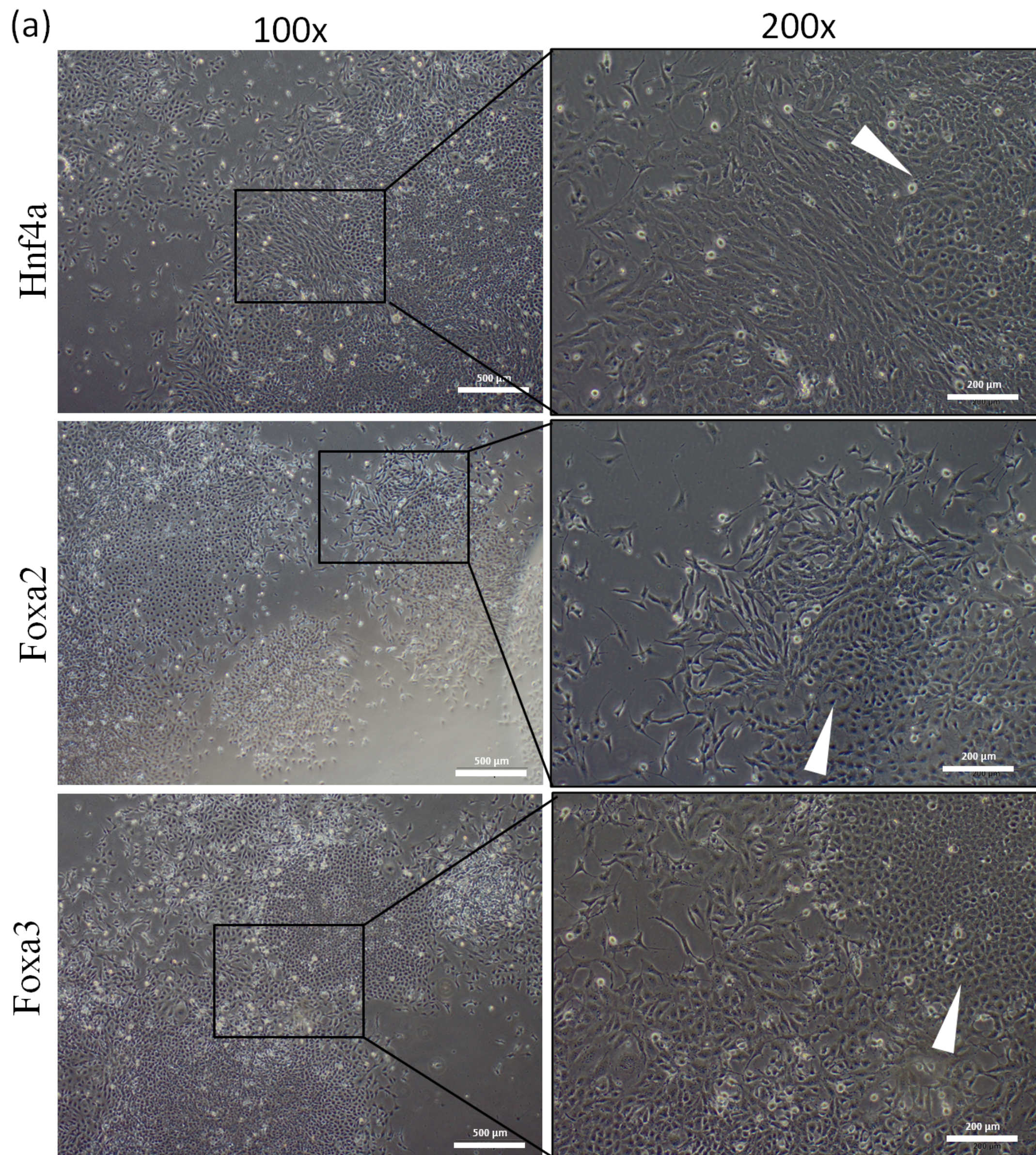
Figure 3.2: Evaluation of viral transduction efficiency by GFP expression. Each picture shows GFP expression of transuduced MEF cells and uninfected MEF cells.

differentiation medium without HGF and EGF. Two days after viral delivery, infection level was evaluated by GFP expression. Most of cells were GFP positive with varying levels of fluorescence. Transduced cells were cultured one week under differentiation conditions. After one week, cells were highly confluent and expressed GFP as in Figure 3.2.

3.1.3 Morphological changes of transduced MEFs

One week after viral transduction, cells were replated on collagen-I coated plates. From this point, cells were cultured with EGF and HGF supplemented differentiation medium. One week after first passage, epithelial-like colonies formed in all transduced cells (Figure3.3). In these wells, MEFs extensively lost their spindle-shaped morphology and gained a polygonal-shaped cytoplasmic organization. Moreover, loose cell-to-cell adhesion and lack of cell organization in MEFs transformed into a more densely organized colonies. Cells

showing morphological changes were detected in clusters surrounded by larger spindle-shaped cells.



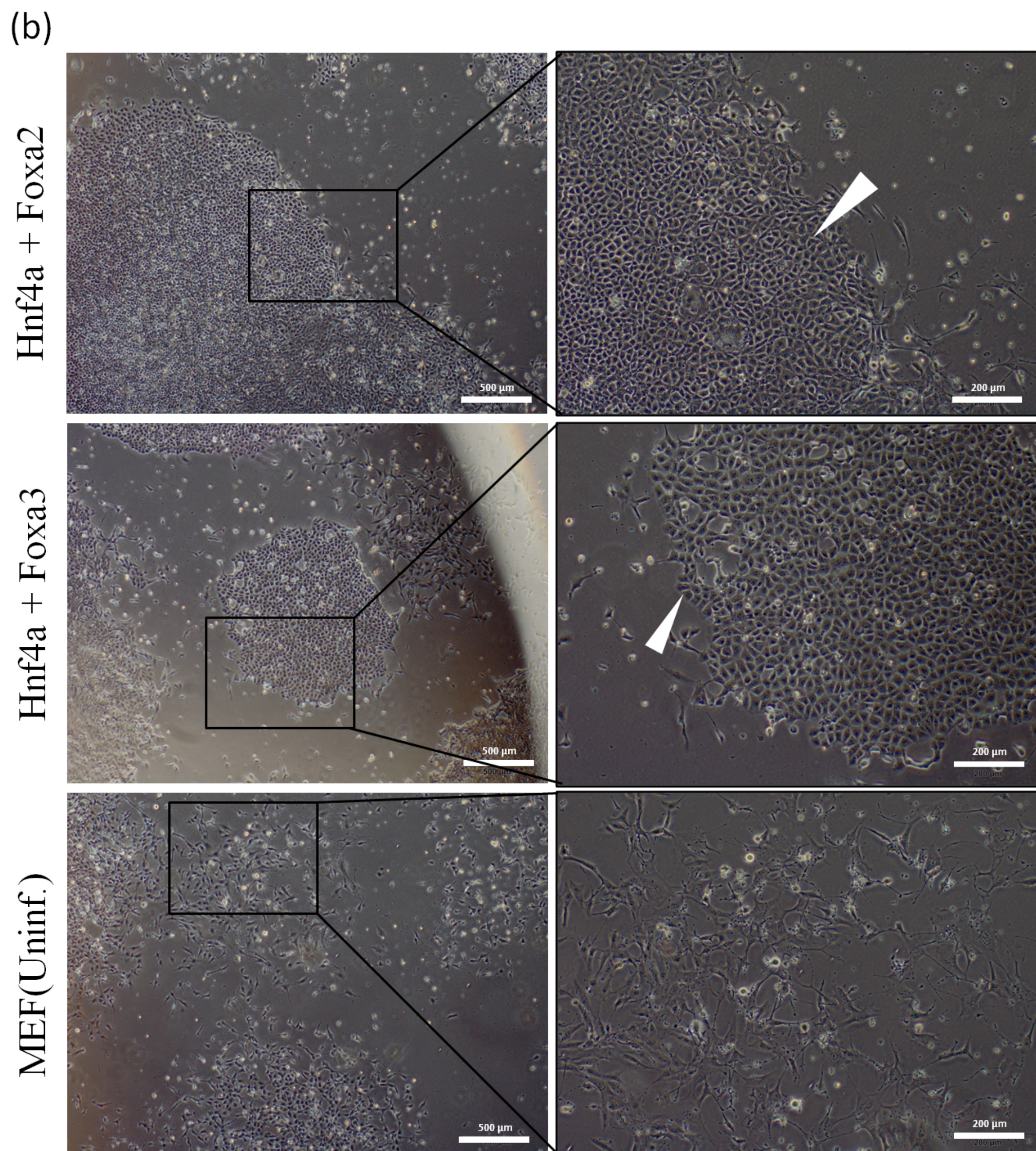


Figure 3.3: Morphological changes of MEFs. a: Cells infected with single Hnf4α, Foxa2 and foxa3 factors shows epithelial like colonies. Note that, cells in the colonies are polygonal shaped and tightly attached each other. b: Hnf4α+Foxa2 and Hnf4α+Foxa3 combinations also show densely packed colony morphology. Also note that the colonies are larger and well demarcated from untransformed spindle-shaped cells. Arrowheads show the colonial structures. Scale bars: 100x column: 500m, in 200x column 200 m.

Epithelial-like cell characteristics are best observed in Hnf4α+Foxa2 and Hnf4α+Foxa3 transduced wells, since number of the epithelial like colonies were higher and these colonies were finely sequestered from mesenchymal-like cells. Furthermore, Hnf4α+Foxa2 and Hnf4α+Foxa3 colonies showed epithelial basal membrane-like organization on colony edges (Figure3.3). At this stage colonies were picked from Hnf4α+Foxa2 and Hnf4α+Foxa3, and replated into collagen coated plates.

These results indicate that all transductions have a significant effect on mesenchymal cell morphological characteristics. In the reference study, epithelial colonies were first observed within 3 weeks after first passage. However, we first detected epithelial shaped colonies as early as one week after replating [24]. Although these results were promising, morphological changes alone cannot be interpreted as complete differentiation of MEFs. Epithelial characteristics should be further characterized with molecular markers.

3.1.4 Epithelial characterization of transduced MEFs

Epithelial cells have diverse functions in organism and show special type of organization. Adherent junction is the main adhesion type in epithelial cells. On the contrary, mesenchymal cells are highly motile and express cytoskeleton markers predominantly. As the cells gain epithelial characteristics, mesenchymal markers decrease and epithelial markers increase. E-cadherin is an essential transmembrane protein which predominantly found in adherent junctions. Its extracellular domain is self-associate which allows two cells to attach; and, this adhesion type is highly expressed ib epithelial cell. Therefore E-cadherin is a reliable epithelial marker [65]. Conversly, vimentin, a cytoskeleton marker, is highly expressed in mesenchymal cells; and, its expression is absent or strongly diminished in epithelial cells. Thus, in differentiation studies, epithelial characteristics can be monitored by changes in E-cadherin and vimentin levels.

In our study, induced cells were co-immunostained with both vimentin and E-cadherin antibodies and labeled with fluorescent secondary antibodies. While

the uninfected MEF cells showed intact vimentin expression, barely diminished vimentin expression was observed in Hnf4α-only and Foxa3-only transduced MEFs. Vimentin expression is further diminished in Foxa2-only, Hnf4α+Foxa2 and Hnf4α+Foxa3 transduced MEFs (Figure3.4).

On the contrary to mesenchymal marker expression, E-cadherin was not observed in expression in Hnf4α-only, Foxa2-only, Foxa3-only and uninfected MEFs. However, Hnf4α+Foxa2 and, to a lesser degree, Hnf4α+Foxa3 co-infected MEFs showed strong E-cadherin expression which is localized to cell-to-cell junction boundaries (Figure3.4).

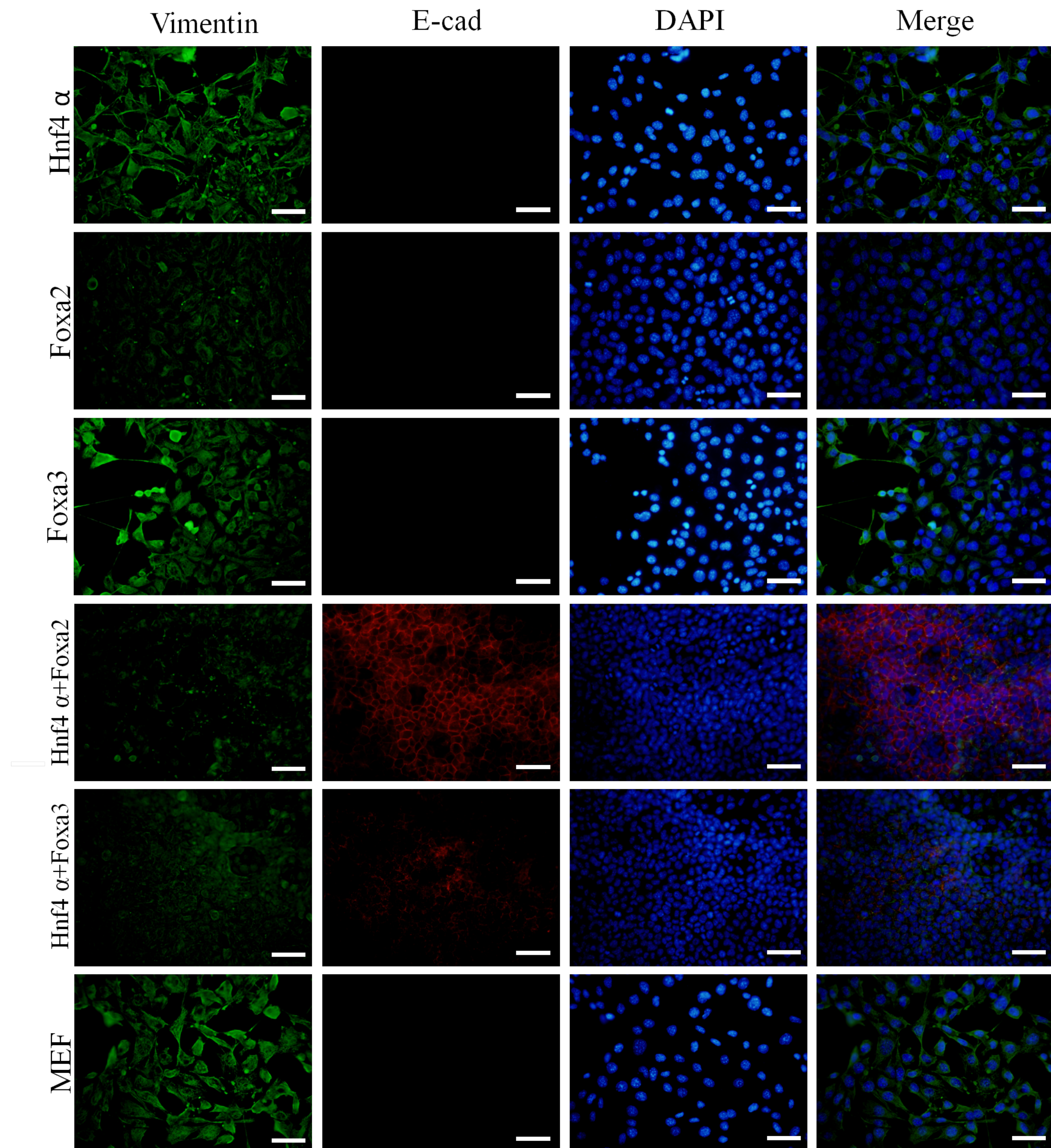


Figure 3.4: Immunofluorescent staining of epithelial and mesenchymal marker in transformed cells. Mesenchymal marker vimentin expression is barely diminished in Hnf4α and Foxa3 only transduced MEFs. However, combined infected cells with, Hnf4α+Foxa2 and Hnf4α+Foxa3 shows greater decrease in vimentin. Epithelial marker E-cadherin is almost completely absent in Hnf4α, Foxa2 and Foxa3 only infected cells. Contrarily, high levels of E-cadherin can be observed in Hnf4α+Foxa2 infected cells, and to a lesser degree in Hnf4α+Foxa3cells. Note that cells showing high E-cadherin levels are densely contact each other in clusters. Scale bars: 100m.

E-cadherin expression was also tested with western blot analysis. Confirming the immunofluorescence data, highest E-cadherin expression was observed in Hnf4α+Foxa2, and to a lesser extend in Hnf4α+Foxa3 transduced immortal MEFs (Figure3.5). Interestingly, Foxa3 only infected MEFs showed slight increase in E-cadherin expression, which was not confirmed in immunostaining experiment. Vimentin expressions were inversely correlated with E-cadherin also in western blot analysis. However, the decrease in vimentin was very low and best observed in Hnf4α+Foxa2 infected MEFs. These results suggest that, infection

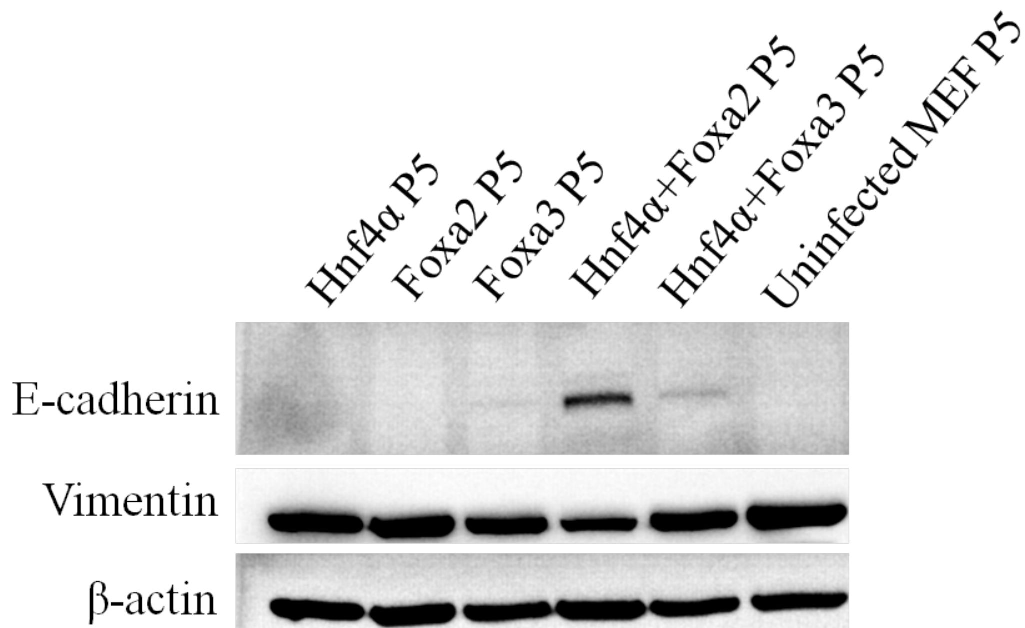


Figure 3.5: Western blot analysis of E-cadherin and vimentin expression. Only co-infected MEFs shows highest E-cadherin expression. Loss of vimentin is best observed in Hnf4α+Foxa2 infected MEFs. P5, passage number.

of SV40 immortalized MEFs with single transcription factor may induce loss of mesenchymal characters to a limited degree as evidenced by immunofluorescence and western blot analysis. Immunofluorescence shown slight decrease in vimentin for Foxa2-only infection ; but, it is not enough to express epithelial specific E-cadherin marker. Co-infection of MEFs with Hnf4α+Foxa2 and Hnf4α+Foxa3 combination could have diminished the vimentin and induced E-cadherin, which

is best observed in immunofluorescence. The reason why vimentin is barely diminished in western blot compared to immunostaining can be best explained by heterogenous differentiation status of cells. In summary, the highest mesenchymal to epithelial transition phenotype was observed in Hnf4α+Foxa2 combination.

3.1.5 Identification of hepatocyte specific markers

Mature hepatocytes are responsible for expression and secretion of blood serum albumin into blood. This unique function has been utilized as a hepatic differentiation marker [66]. Thus, induced MEF's were immunostained with anti-mouse serum albumin after fifth passage.

Hnf4α, Foxa2, Foxa3-only infected MEFs were negative for albumin fluorescence. However, Hnf4α+Foxa2 and Hnf4α+Foxa3 co-infection resulted in albumin positive islets (Figure3.6). Although strong albumin expression was observed in islets, the overall albumin positive cells were not widespread over the field.

Correlated with previous report, this results indicate that the immortalized MEF cells can only be differentiated into hepatocyte-like cells with Hnf4 together with either Foxa2 or Foxa3 transcription factors [24]. Transduction solely with Hnf4, Foxa2 or Foxa3 is not enough to induce liver specific protein expression even in the conditioned hepatocyte medium. Albumin positive cells in small clusters suggest a incomplete differentiation process for rest of the cells in colonies.

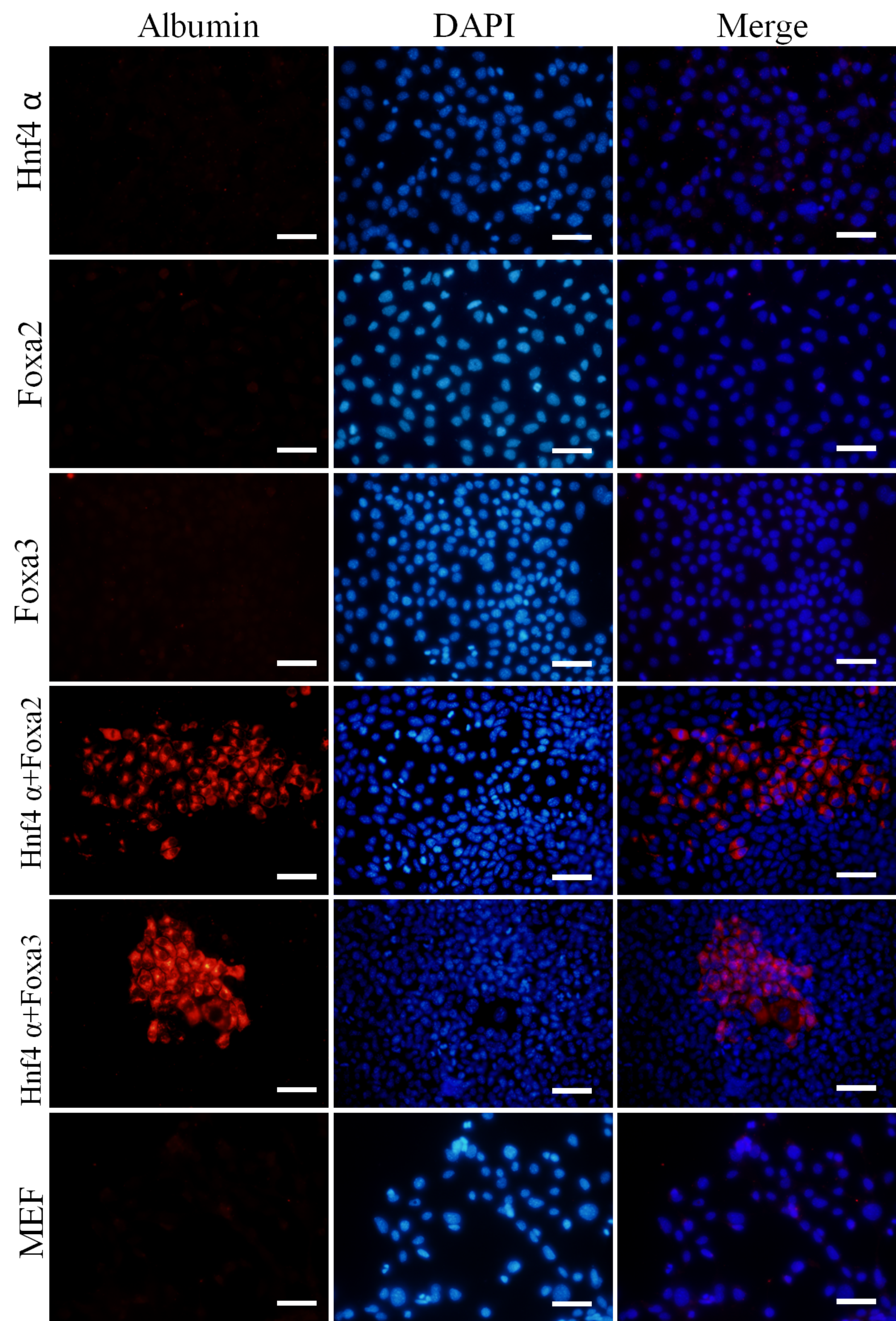


Figure 3.6: Immunofluorescent staining of albumin in transduced MEFs. Except co-infection with Hnf4α+Foxa2 and Hnf4α+Foxa3 combination, no Albumin positive cell was observed. Note that albumin positive cells in Hnf4α+Foxa2 and Hnf4α+Foxa3 infected cells show clusters.

3.1.6 Glycogen storage of induced MEFs

Although albumin expression represent a liver specific function, activation of a single is not sufficient to identify a functional metabolism of the hepatocytes. One of the major physiological role of liver is to deposit glucose in form of glycogen polymers. Conversely, the blood glucose levels can be regulated in hepatocytes by breaking down of the glycogen into glucose and release into bloodstream [67]. Besides, process of glycogen storage from circulating glucose requires organization of complex gene expression pattern peculiar to hepatocytes. Therefore, glucose uptake and storage in the form of glycogen is considered as one of the main criteria in obtaining hepatocytes.

Periodic Acid Schiff's (PAS) staining method shows polysaccharides in fixed tissues and is used to show glycogen storage in differentiated hepatocytes [12, 27, 23, 68]. In our study, we have performed Periodic Acid Schiff's staining in order to visualize glycogen storage of induced hepatocytes.

After passage four, induced MEFs were evaluated for glycogen storage levels. PAS staining gives acidic cytoplasmic magenta color which contrast with blue basic stain of cytoplasm and nucleus. The cell clusters stained with magenta colored cytoplasm were visible at lower magnifications. Foxa2-only, Foxa3-only and uninfected immortal MEF cells showed no PAS staining. However, Hnf4α+Foxa2 and Hnf4α+Foxa3 infected MEFs were strongly stained with magenta color. Hnf4α+Foxa3 transduced cells showed slightly less staining than Hnf4α+Foxa2 (Figure3.7). Furthermore, cytoplasm of HNF4α-only transduced cells were stained with PAS very slightly compared to fibroblasts, which is visible under 400x magnification.

Correlated with epithelial characterization results,positive PAS staining in Hnf4+Foxa2 and Hnf4 +Foxa3 co-infected immortal MEFs suggest that double factor transduced cells show glycogen storage. Additionally, single transcription factors were insufficient to stimulate molecular pathways regarding glycogen storage except Hnf4α. MEFs transfected only with Hnf4α gave very light PAS staining in several cells (Figure3.7). This result was not surprising, since previous

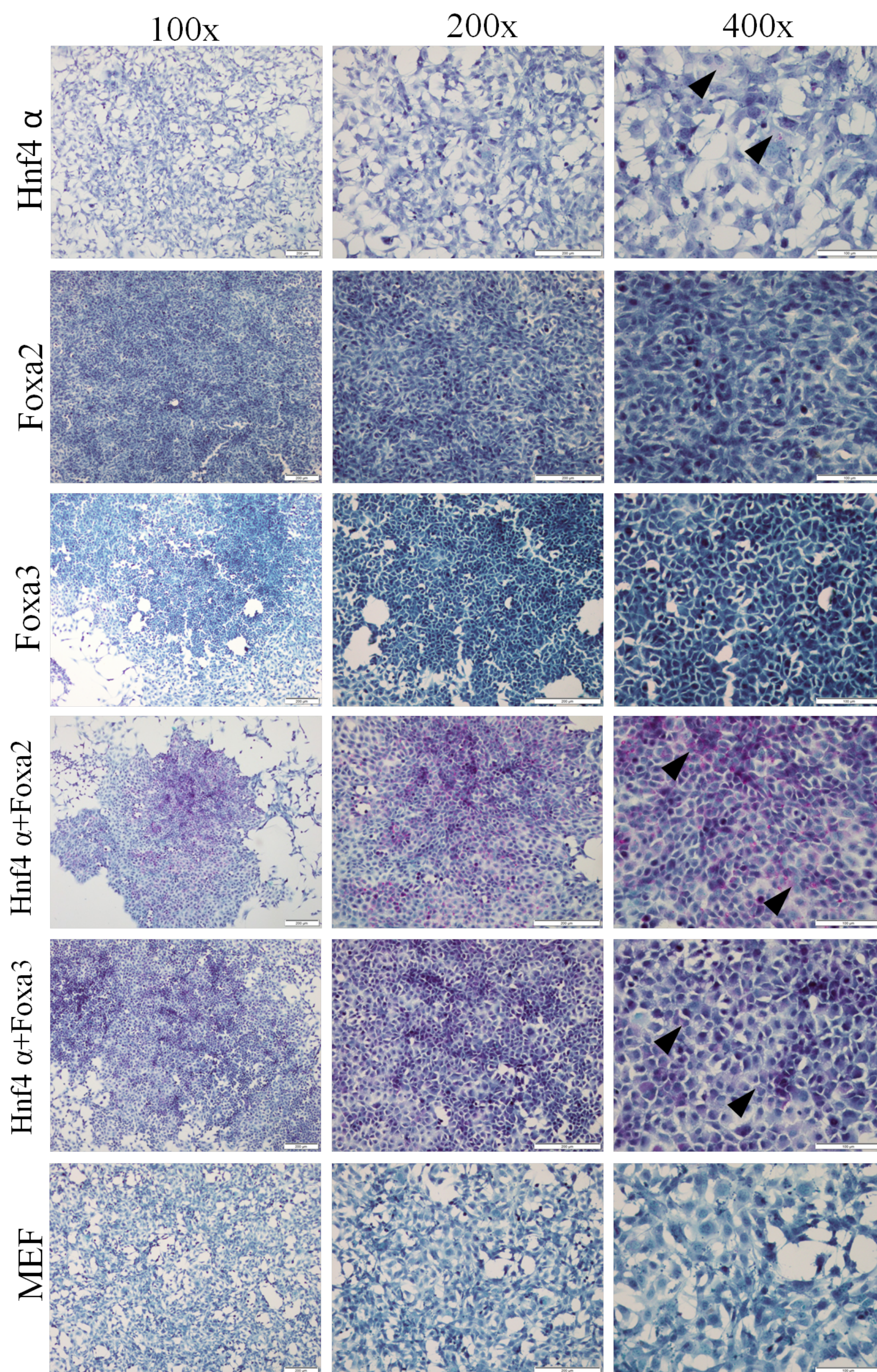


Figure 3.7: PAS staining of induced MEFs. Unlike the single transcription factor infected MEFs, , Hnf4α+Foxa2 and , Hnf4α+Foxa3 shows extensive PAS staining at lower magnification (100x and 200x column). At higher magnifications, cytoplasmic PAS staining in individual cells are visible particularly in H+F2, H+F3 and Hnfα only infected cells. Black arrowheads show cytoplasmic PAS stain. Scalebars: 200 m in 100x and 200x column; 100 m in 400x column.

data shown that deletion of Hnf4α affects glycogen storage related genes and causes lack of PAS staining. [69] In literature, effects of Foxa3 on development and function of the liver was reported [70, 71]. Deletion of Foxa3 resulted no abnormal liver development and its expression is compensated by Foxa1 and Foxa2 proteins. However, its deletion disrupts Glut2 expression in adult mice. Absence of glycogen stain after single Foxa3 overexpression indicates it does not play a direct role on glycogen storage. Although the Foxa2 is indispensible for healthy liver development, its overexpression leads to glycogen storage defects [72]. Consistent with these data, overexpression of Foxa2 alone leads no glycogen storage in MEFs.

In conclusion, overexpression of individual transcription factors cannot dramatically affect glycogen storage. However, co-infection of Hnf4α together with either Foxa2 or Foxa3 positively regulates glucose uptake and storage in the form of glycogen. Thus, combination of these factors are able to finely orchestrate a complex energy metabolism, which is a characteristic for functional hepatocyte.

3.2 3.2 Preliminary results on genetically modified mice

3.2.1 Mouse colonies and genotyping

Mouse strains were provided as mentioned in methods sections. Most of these mice have functioning gene in their genome; but, a critical exon is flanked by LoxP sites which can be deleted with Cre recombination. One exception is mH2A.1 strains that, these animals permanently knockout for this gene.

All genotyping procedure was performed by conventional PCR method on genomic DNA. Primers targets specific genomic sequences in each strain. Conditionally knockout strains contain LoxP sequences flanking a critical exon. A primer pair flanking one of the loxP sequence can amplify 100-200 bp sequence more than wild type allele, which can be detected as a band shift in agarose gel electrophoresis (Figure3.8). If there is a permanent knockout allele of these gene, a primer pair flanking whole exon is used for genotyping. Cre primers target directly the gene body. In ROSA26 strains, transgenic gene gives smaller bands compared to wt allele which can be also detected in gel electrophoresis.

Mouse genotyping results are preliminary data which will be utilized in future in vitro and in vivo studies. Especially, primary cell lines derived from these strains would be an invaluable tool for direct differentiation of hepatocyte.

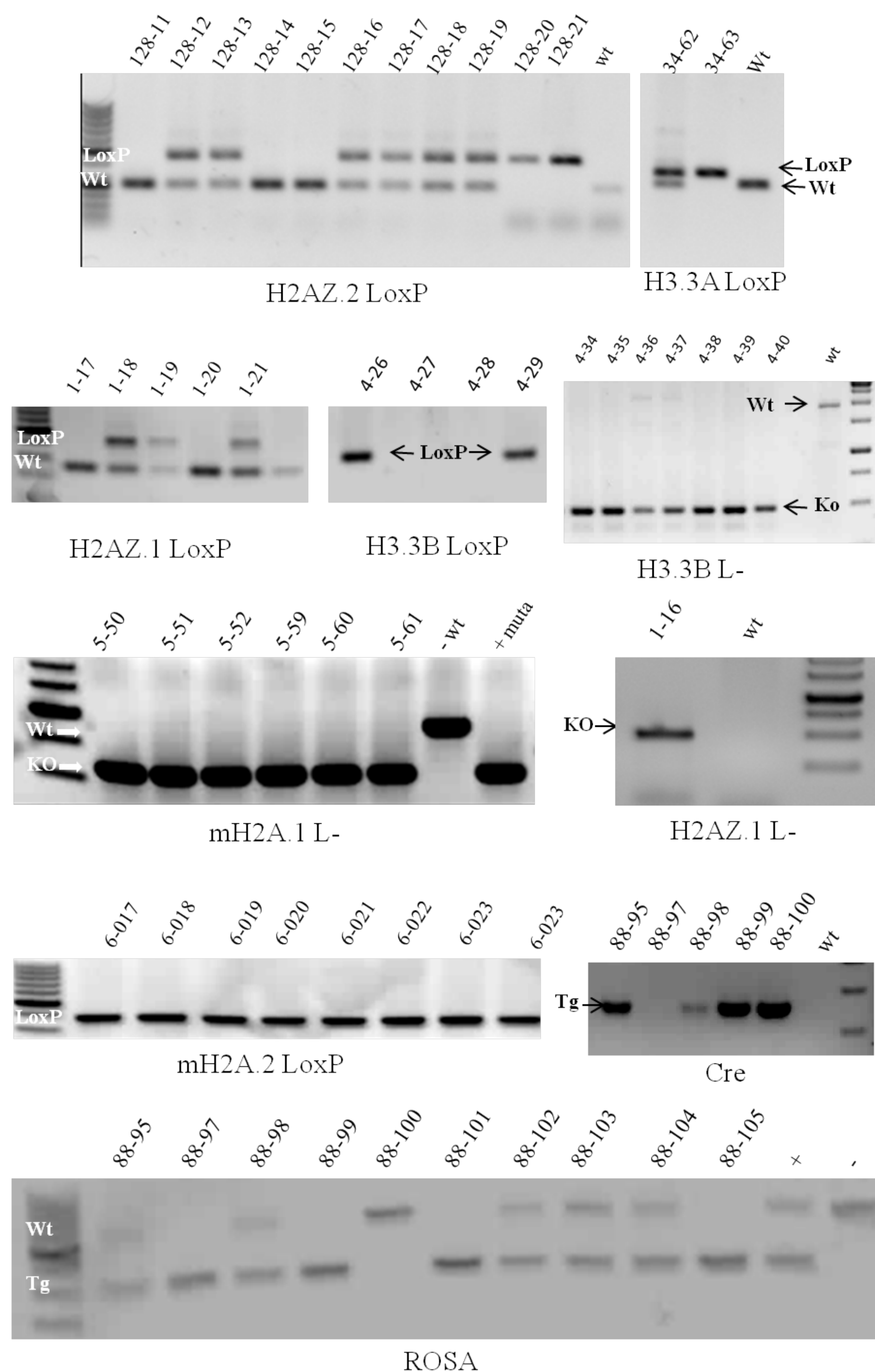


Figure 3.8: Gel electrophoresis images of genotyping. Note that LoxP genotyping results 100-200 bp band shift compared to wild type. On the contrary, ROSA strain has lower size bands for transgenic allele compared to wild type. Wt, wild type; KO, knockout; L-, knockout allele. Each number above the gel picture represents identity of an animal.

3.2.2 Infection of H3.3A F/F MEFs with Cre-ERT2 expressing retroviral vector

In order to show effects of histone variants, primary MEFs were isolated from H3.3A F/F, H3.3B F/F, H3.3A -/-, H3.3B -/-, H2AZ.1 F/F, H2AZ.2 F/F and H2AZ1/H2AZ2 F/F mouse strains. These cells were infected with retroviral vector carrying SV40 large T-antigen open reading frame sequence, and cells were selected with zeocin. In first trial we continued our study with immortal H3.3A F/F MEFs.

To excise LoxP flanked exon using Cre mediated recombination in future studies, we have used Cre-ERT2 expressing retroviral vectors. The open reading frame is composed of Cre recombinase gene which is fused to a modified estrogen receptor protein. Under normal conditions, Cre is expressed continuously, but cannot translocate to nucleus. In case of 4-Hydroxytamoxifen (4-OHT) administration, Cre translocates to nucleus and initiates recombination by excising exon between two LoxP sites. Vector also contains mammalian puromycin resistance selection marker. We infected transgenic cells with retroviral vectors and selected with puromycin.

We evaluated the integration of viral gene into MEF genome by using conventional PCR method. Genomic DNA was extracted from both wild type and transgenic MEFs. LoxP sites in mutant MEFs makes about 170 bp difference compared to wild type as seen in (Figure3.9), lower panel. The presence of Cre was seen at 650 bp in gel electrophoresis.

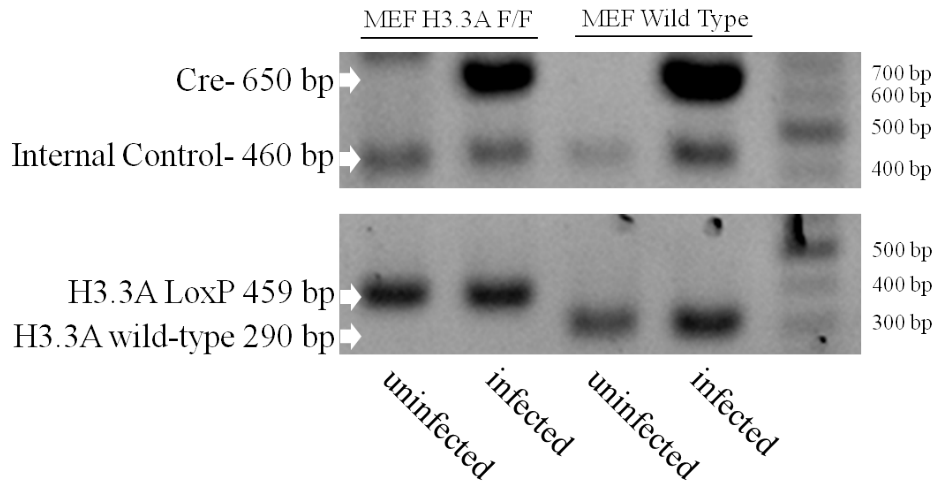


Figure 3.9: Conventional PCR from genomic DNA. Lower panel shows genotyping of MEFs. Mutant MEFs show about 170 bp difference caused by LoxP sequence. In (Figure3.9), upper panel shows presence of Cre gene on the genome (650 bp). Second primer pair was used as an internal control.

In this set of experiments, primary Mouse Embryonic Fibroblasts isolated from mice were immortalized and Cre expressing vectors successfully delivered to one of these strains.

Chapter 4

Discussion & Conclusion

Direct differentiation of hepatocyte-like cells from mouse and human fibroblast cells was previously published [21, 24, 23, 27]. All methods are based on over expression of liver lineage specific transcription factors. One of these reports, scientists have utilized Hnf4α transcription factor together with either Foxa1, Foxa2 or Foxa3 to directly differentiate primary mouse embryonic fibroblasts into hepatocytes [24]. However, same combination of factors have not been tested on SV40 immortalized MEFs. Although transdifferentiation from p19 knockout MEFs was mentioned somewhere else, there is no information about how knockout of both p53 and pRb pathways affect MEF to hepatocyte transition.

In our study, we have isolated and frozen viral supernatants, which may decrease transduction efficiency. Additionally, serially infections were repeated only three times. These infection conditions were suboptimal compared to the reference article, where scientists have used concentrated viral supernatant and infected cells six to eight times. But, we successfully infected immortal MEFs as shown in figure3.2. More significantly, we have obtained morphologically differentiated cells as early as one week after replating of transduced immortal MEFs. However, same morphological chances from primary MEFs occur in at least three weeks [24]. This difference can be mainly due to suppressed p53 and pRb pathway, inactivation of which facilitates dedifferentiation of cells [33].

Although Hnf4α+Foxa2 and Hnf4α+Foxa3 has been proven to be sufficient for transdifferentiation, single gene expressions for each factor has not been evaluated in SV40 immortalized MEFs. Hnf4α is one of the master regulators in liver development and its deletion has a lethal phenotype in mice[28]. Immortal MEFs which are infected only with Hnf4α shows limited change in morphology. However, absence of epithelial marker E-cadherin and absence of albumin expression (figure 3.4 and figure 3.6) indicates an inadequate differentiation stage. Contrarily, in previous study, Hnf4α only infection was shown to induce epithelial-like characteristics in mouse fibroblast cell line NIH-3T3, such as gaining polygonal shape and E-cadherin expression [69]. This contradiction may be due to infection efficiency and ectopic expression levels introduced to fibroblasts. Hnf4α is also a key glucose metabolism regulator in liver and its absence in adult animals leads to several glycogen storage deficiency phenotype [29]. Consistently, overexpression of Hnf4α showed little storage of glycogen in our study.

Hnf3 factors (also named as Foxa factors) belong to winged helix protein family and have important functions in both developing and adult liver. Both Foxa1 (Hnf3α) and Foxa2 (Hnf3β) deletion gives lethal phenotypes in mice [73, 74]. However, in literature, deletion of Foxa3 was shown to have no severe phenotype on liver function, which is compensated largely by other liver specific transcription factors [70].

Foxa2 expression is involved in endoderm derived organ development such as liver, pancreas and lungs [75, 76, 77]. In a previous report, ectopic expression of Foxa2 alone was proven to be insufficient to induce E-cadherin expression; but, its deletion is essential for epithelial to mesenchymal transition [78]. Consistent with these data, in our study, Foxa2-only infections yielded cells which shows neither epithelial nor hepatocyte marker. Furthermore, absence of glycogen staining in Foxa2-only infected cells correlates with a previous report which shows that, over expression of Foxa2 leads to glycogen storage defects [72]. Similarly, ectopic expression only with Foxa3 in MEFs resulted morphological changes without any further epithelial or hepatocyte specific phenotype.

Besides the transcriptional activation role of Hnf3 (Foxa) family factors, they can exclusively modify heterochromatin structure with a direct interaction. This action creates nucleosome free regions locally which facilitates other liver enriched factors to bind this exposed DNA region [48]. In our study, indeed we overexpressed Foxa2-only and Foxa3-only factors in immortal MEFs. However, lack of differentiation in molecular level indicates that, even if these factors can directly modulate chromatin structure upon binding, additional factors are required to differentiate immortal MEFs.

Expression of transcription factors in combination of Hnf4α+Foxa2 and Hnf4α+Foxa3 has shown transition from mesenchymal to epithelial phenotype. As early as one week after replating, finely demarcated and well growing colonies were formed in Hnf4α+Foxa2 and Hnf4α+Foxa3 infected MEFs. This data was further supported with increased E-cadherin and decreased vimentin expressions by immunofluorescence. With western blot analyses, increased E-cadherin expression levels were verified in Hnf4α+Foxa2 and Hnf4α+Foxa3 infected cells; however, only slight decrease in vimentin expression was observed in these cells. This could be explained by heterogeneity in differentiation levels, since some MEFs may resist to lose mesenchymal characteristics during differentiation. Additionally, results indicated that, infection with two factors of Hnf4α+Foxa2 has resulted in higher levels of epithelial molecular markers than Hnf4α+Foxa3, which is also proven by immunofluorescence and western blot analyses.

Similar changes were also visible in determination of hepatocyte phenotype with specific function. In glucose uptake experiment, PAS staining results showed that, Hnf4α+Foxa2 and Hnf4α+Foxa3 combinations yielded highest levels of glycogen storage. As in epithelial marker staining experiment, Hnf4α+Foxa2 gives more intensive PAS staining compared to Hnf4α+Foxa3.

Albumin production and secretion is the one of the unique functions of the liver. Immunofluorescence analysis showed only Hnf4α+Foxa2 and Hnf4α+Foxa3 infected MEFs are albumin positive. Although we have not quantified the albumin expression levels with flow cytometry, albumin positive cells were clustered containing few cells. There is no significant difference between

these two combinations in terms of albumin levels. Sparse albumin positive clusters indicates that only a subpopulation of cells have undergone complete differentiation. Decreased reprogramming efficiency could be a potential answer, since we have repeated viral transduction only three times. This may cause insufficient gene expression and eventually decreased albumin levels. Another potential reason could be the immortal nature of MEFs. Since pRb deletion has been associated with decreased differentiation potential of the cells [36]. In one transdifferentiation study, immortalization of human fibroblasts with SV40 Large T-antigen lead to decreased expression of albumin in later passages [27].

Here we used a protocol designed for non-immortalized MEFs into large T antigen immortalized model. To best of our knowledge there is not study which differentiate pRb and p53 ablated MEFs into hepatocyte like cells with this method. The most significant difference was that, time required for differentiation was decreased in our method using Large T antigen immortalized MEFs.

In second course of the study, we have bred mice strains different genetic background. Except the one mouse strain with permanent knockout for mH2A.1, other strains (H2AZ.1, H2AZ.2, H3.3A, H3.3B, mH2A.2) carry konditional knockout alleles with LoxP. Another strain carries Cre gene expression under the control of TTR promoter; and, the same strain carries R26R-LacZ allele for tracking Cre activity. There are well established SV40 large T-antigen immortalized MEF cells isolated from mice strains: H3.3A F/F, H3.3A -/-, H3.3B F/F, H3.3B -/-, H2AZ.1 F/F, H2AZ.2 F/F and H2AZ.1/H2AZ.2 F/F. Among these, we transduced H3.3A F/F cells with controllable Cre expressing retroviral vector. Genotyping results indicate that cells contain integrated Cre gene and it is ready for induction of recombination with 4-OHT (hydroxytamoxifen) [79]. To understand effects of histone variants in hepatocyte transdifferentiation process, this conditional knockout cell strain is a invaluable tool.

Despite the recent discoveries, role of histone variants are still largely unknown. Growing amount of articles are defining new roles in reprogramming process. For example, scientists have discovered that, variant H3.3 has a crucial role in establishing epigenetic memory in differentiated cells. Its deletion has been found

to be beneficial for iPSC reprogramming [41]. It would be interesting to see the effects of H3.3 deletion during direct hepatocyte induction. H3.3 ablation is likely to contribute this process by helping cells to ”forget” what cell type they transdifferentiating from.

Direct regulatory effects of Hnf3 factors on chromatin structure strongly implies a potential relationship with histone variants. One study highly supports this hypothesis, since cooperation of H2AZ and Foxa2 has been found to create nucleosome free regions. Furthermore, together they contribute the endoderm lineage determination [53]. Deletion of H2AZ during hepatocyte reprogramming would cause a resistence for differentiation.

Chapter 5

Future Perspectives

First of all, naive wild type MEF culture should be used as control in order to elucidate effects of SV40 large-T antigen on differentiation.

In this study, we established a direct hepatocyte induction protocol derived from Large T-Antigen immortalized Mouse Embryonic Fibroblasts. However, efficiency of viral transduction; E-cadherin and Albumin expression has not been quantified. As a next step, viral transduction efficiency should be calculated by Fluorescence-Activated Cell Sorting. After cells are transduced, sequential differentiation steps occur during differentiation. Using FACS technique to count vimentin, E-cadherin and albumin positive cells would give us an idea how this stepwise events progress.

To identify success of hepatocyte differentiation, comprehensive gene expression profile would be carried out by Q-PCR with the same protocol. Same quantification should be also performed on endogenous and exogenous expression levels of given transcription factors.

To establish a method to detect differentiated cells earlier, Cre-ROSA-LacZ system can be utilized. Cre transgenic mouse strain expresses Cre recombinase protein under the control of Transthyretin promoter, a liver specific gene. Mouse embryonic fibroblasts derived from this strain can be immortalized and used for

transdifferentiation studies. As the cells start to gain liver gene expression profile, TTR controlled Cre would mediate the excision of stop cassette from ROSA-LacZ system and differentiated cells can be detected by X-gal staining. With this method, quantification of efficiency may become much easier.

There are several mouse strains which can be used as a source of MEFs and different type of somatic cells. Besides the genotyped animals, there are well established genetically defined immortalized MEF cell lines (H3.3A F/F, H3.3B F/F, H2AZ.1 F/F, H2AZ.2 F/F and H2AZ.1& H2AZ.2 F/F). As previously mentioned, Cre-ERT2 retroviral system has been utilized to control knockout of H3.3A F/F MEFs. Same system would be used for other immortalized cells due to its convenient inducibility. There is also another Cre expressing method which uses Adenoviral system for Cre delivery. This system has been successfully used by taking advantage of non integrating cargo and strong infection potency of adenoviral vectors [80]. Before advancing to gene knockout studies, Adeno-Cre system should also be tested for proper Cre expression on primary and immortal cell lines.

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Appendix A

Standard Solutions and Buffers

10X PBS

- 80 g NaCl
- g KCl
- 8,01g $Na2HPO4.2H2O$
- 2g $KH2PO4$
- 1 liter $ddH2O$ pH: 7,4
- Working solution (1XPBS) was prepared by diluting 10XPBS by 10 times

50X TAE Buffer

- 2M Tris Base (242 g)
- 57,1 ml Glacial Acetic Acid
- 50mM EDTA
- Add to 1 lt by $ddH2O$

- Working solution (1XTAE) prepared by diluting 50XTAE.

TAIL BUFFER

- 1% SDS
- 0.1 M NaCl
- 0.1M EDTA
- 0.05M Tris(pH8.0)

10X TBS

- 60.6 g Tris Base
- 87.6 g NaCl
- 1M HCl
- Adjust pH to 7,6 and fill up to 1 Liter ddH2O
- Depending on the procedure, it is diluted to 1X TBS and added either Tween 20 or Triton-X.

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