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GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

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FATİH AYGENLİ

A THESIS SUBMITTED FOR THE DEGREE OF

MASTER OF SCIENCE

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GEBZE TEKNİK ÜNİVERSİTESİ

FEN BİLİMLERİ ENSTİTÜSÜ

BİYOLOJİK SAAT PROTEİNLERİNİN FLUORESANS PROTEİNLER İLE ETİKETLENMESİ

FATİH AYGENLİ

YÜKSEK LİSANS TEZİ

MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI

DOÇ. DR. NURİ ÖZTÜRK

GEBZE

2019

GEBZE TEKNİK ÜNİVERSİTESİ	**YÜKSEK LİSANS JÜRİ ONAY FORMU**

GTÜ Fen Bilimleri Enstitüsü Yönetim Kurulu'nun ..29./..01../..2019 tarih ve ..2019../..07..... sayılı kararıyla oluşturulan jüri tarafından 20/05/2019 tarihinde tez savunma sınavı yapılan Fatih Aygenli'nin tez çalışması Moleküler Biyoloji ve Genetik Anabilim Dalında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

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...../...../..... tarih ve ...../..... sayılı kararı.

SUMMARY

In most living organisms, biological clock which is an autonomous mechanism that control physiologic activities in daily (circadian) cycle is regulated by the transcription and translational cycle of four proteins. These are not responsible only for biological clock but also for many cellular processes in protein and DNA level to adjust homeostasis between them. Therefore, disruption of biologic clock directly or indirectly related with many metabolic diseases and this has been proven by many studies. This situation clearly shows the potential of to understand the relationship between biological clock and metabolic components about improvement of new drugs and therapeutic agents. Thus, it is important to elaborate the studies that have been preceded in the laboratory which makes them more significant. In this study, it is aimed to develop new tools that give opportunity for real-time imaging of biological clock proteins by using recombinant gene technology (cloning), cell culture and imaging in stably generated U2OS cells. These recombinant proteins will be expressed from their own promoters. In that tool, it is possible to mimicking in vivo endogenous gene expression of the proteins. At the end of the study, it will be obtained new findings about the function and relationship of BMAL1 and Cry1 proteins between other molecules. This study will also demonstrate how biosensor cells can exploit the properties of clock proteins.

Keywords: Biological Clock, Gene Cloning, Fluorescance Probing, Real-Time Imaging.

ÖZET

Canlı organizmaların fizyolojik aktivitelerini günlük (sirkadiyen) döngüde kontrol eden otonom bir mekanizma olan biyolojik saat, temelde dört farklı proteinin transkripsiyon ve translasyon döngüsü ile ayarlanır. Bu proteinler biyolojik saatin yanısıra hücrenin temel fonksiyonlarına protein ve DNA seviyesinde etki ederek hücresel süreçlerin dengeli ve doğru çalışmasını sağlar. Dolayısıyla biyolojik saatin bozulmasının, metabolizmaya bağlı birçok hastalık ile doğrudan yada dolaylı yoldan ilişkilidir ve birçok çalışma ile de desteklenmektedir. Bu durum, sirkadiyen ritim ve metobolik bileşenler arasındaki bağlantıyı anlamanın hastalıklara karşı yeni ilaç ve terapatik ajanlar geliştirilmesindeki potansiyelini açıkça ortaya koymaktadır. Bu amaçla laboratuvarımızda yürütülen çalışmaların, detaylandırılarak daha ileriye taşınması ve daha kararlı bulguların ortaya konulması çok önemlidir. Bu sebeple yapmayı planladığımız çalışmamızda, rekombinant gen teknolojisi (gen klonlama), hücre kültürü ve fluoresans okuma teknikleri kullanılarak belirlenen biyolojik saat genlerinin fluoresans mikroskop ile gerçek zamanlı görüntülemesinin yapılmasına olanak sağlayacak iki farklı hücre ortaya çıkartılacaktır. Bu rekombinant proteinler, kendilerine ait doğal düzenleyeci gen bölgeleri tarafından ifade edilecekler. Bu sistem biyolojik saat proteinlerinin *in vivo* endojen gen anlatımını mimikleyerek hücre içi yerlerinin ve terapatik ajanlar ile olan etkileşimlerinin gözlemlenebilmesine olanak sağlayacaktır. Çalışma sonunda, BMAL1 ve Cry1 proteinlerinin hücre içi fonksiyonlarına ve başka bileşiklerle olan etkileşimlerine dair bulgular ortaya konulacaktır. Bu çalışma, biyosensör hücrelerin biyolojik saat proteinlerine dair özelliklerin incelenmesinde nasıl faydalanabileceğini de ortaya koyacak ve iyi bir altyapı sağlayacaktır.

Anahtar Kelimeler: Biyolojik Saat, Klonlama, Fluoresans Etiketleme, Görüntüleme.

ACKNOWLEDGEMENTS

I would like to thank my thesis supervisor Assoc. Prof. Dr. Nuri ÖZTÜRK for his patience and encouragement. In every trouble, he helps me and examines all process of my project in detail. Also, he has provided a good working atmosphere to complete my all laboratory experiment. I would also like to thank to my jury members Assoc. Prof. Dr. Ayten KANDİLCİ and Assoc. Prof. Dr. Cihan AYDIN for spending their valuable times to evaluate this thesis.

This work has been supported by Research Fund of the Gebze Technical University. Project Number: 2018-A-101-05.

TABLE OF CONTENTS

LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms		Explanations
µg	:	Mikrogram
µl	:	Mikrolitre
APS	:	Amonium persulfate
ARNTL1	:	Aryl hydrocarbon receptor nuclear translocator-like protein-1
CRY1	:	Cryptochrome-1
HEK293T	:	Human embriyonic kidney 293T cells
LB	:	Luria Bertani
PFA	:	Paraformaldehyde
PCR	:	Polymerase chain reaction
Per	:	Period gene
PHY	:	Phytochrome
SDS	:	Sodium dodecyl sulfate
SDS-PAGE	:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TTFL	:	Transcription Translation Feedback Loop
U2OS	:	Human Bone Osteocarcoma Cell Line

LIST of FIGURES

LIST of TABLES

1. INTRODUCTION

This project is mainly about the generation of biosensor cells which can express fluorescence-protein tagged circadian clock proteins BMAL1 and CRY1 from their own promoter like relevant endogenous proteins. By this way, behavior of selected clock proteins and their interactions can be investigated under fluorescent microscopy. Therefore, we can observe the localization of the clock proteins and also their interaction with other metabolics by the help of different fluorescent imaging tools such as time-lapse and live cell imaging by confocal microscopy. By this screening tool, not only clock-related response of clock genes but also their metabolic roles can be studied under different stress conditions or against drugs.

We will have at least two cell lines which express recombinant BMAL1 or CRY proteins in fusion to fluorescent proteins. These recombinant proteins will be under native promoters and therefore will mimic endogenous clock proteins. These cell lines will provide a new tool to screen small chemical or candidate drugs to understand if they affect these clock proteins. We believe that these new tools can be used in future for high throughput screening purposes by our or other research groups.

1.1. The Purpose, Contribution and Content of Thesis

The aim of this project is to label selected clock protein in live cells to follow their behavior in vivo. For this purpose, we provide expression of recombinant fluorescent proteins in fusion to either to BMAL1 or CRY1 proteins in bone osteosarcoma epithelial cells (U2OS). Selected stable cell lines were monitored for 24 hour or longer period in vivo using live cell imaging by fluorescent microscopy. We express these genes under their own promoter to allow the circadian regulation of fluorescent proteins. The fluorescence activity were monitored also a microplate reader to follow the abundance of the relevant recombinant proteins. The cell line are a tool to analyze the behavior of clock protein under normal or under stress conditions

as in the presence of small chemicals interacting or affecting the circadian clock proteins or DNA damage. This study will reveal how this biosensor cells can be exploited to study the properties of clock proteins over a circadian day such as changes in subcellular localization or protein stability.

Detailed literature data concerning the mammalian circadian clock, biosensor cells and fluorescent imaging systems is given in chapter 2 while materials and methods that had been used during the study is broadly explained in chapter 3 and 4. In the following chapter which is chapter 5, the results are represented by figures and quantitative analysis. Finally, the thesis will be exploring by providing appropriate conclusions and discussions in chapter 6.

2. LITERATURE REVIEW

2.1. Biological Rhythms

2.1.1. Circadian Clock

Most living systems have adapted to the Earth's rotation with an ongoing adaptation. Therefore, biological processes have a spatiotemporal regulation for the maintenance of cellular function. Biological rhythms that confer daily rhythmicity on many biochemical and physiological functions are an integral part of the temporal regulation of biological processes. In many organisms, biological mechanisms oscillate under the effect of rhythmic daily 24-hours light and dark cycle which is called circadian rhythms. Circadian is known as "circa-diem" in Latin language and means "around a day". These rhythms in physiology and behavior are phylogenetically ancient, so it is possible to observe in both prokaryotic and eukaryotic organism, even in plants. Although timing of biological processes is all coordinated with this rhythm, it is not triggered only with environmental photoperiod but also endogenous timekeeping mechanism called circadian clock [1]. This situation was proven by an experiment done by a French speleologist named Michel Siffre. As a result of the research called Caveman, some internal rhythms such as temperature or urinary electrolyte cycle of the body do not depend on external cues. Therefore, there is an internal rhythm independently working from external rhythm. Circadian clock is one well conserved genetic program that helps organisms with sustaining daily oscillations in gene expression and protein activity in order to keep metabolism and physiological events in balance; and its aberrations has been strongly linked with physical and mental problems such as sleep disorders, diabetes, heart disease, depression and cancer [2].

Circadian Clock is an autonomous mechanism that generates daily rhythms even in the absence of environmental cues (zeitgebers) such as light or magnetic field of the Earth. These daily rhythms are produced in cellular level and form a network that

involves cells, tissues, organs and organisms. In mammals, suprachiasmatic nucleus (SCN) is the pacemaker of the clock that is intimately linked with the molecular basis of cellular timekeeping and behavioral rhythms of the body [3]. SCN which adjusts the tempo of body life is found in hypothalamus in the brain. It builds up from 20.000 neurons that display a hierarchical and multi-oscillator system in rats and mice. The output signals are distributed from here to both different brain regions and peripheral organs. For instance, the photic information is reached to SCN passing through the retina and synchronizes the neuronal cellular clock which means initialization of the internal day. Therefore, time cue signals are delivered to the whole body cells from there [4].

Up to now, tremendous number of structural and biochemical studies about the major components of circadian clock have ben enlightened its mechanism. However, there are still many uncertain pieces on the puzzle. Thus, many of investigation on it have been also conducted to improve these data and make it clear.

2.1.2. TTFL: Transcription Translation Feedback Loop

Biological clock which biochemically, physiologically and behavioral impacts many regulatory systems and pathways controls the mechanisms by regulating 10% of the gene expression and 20% of protein functions in mammals. Circadian systems, an autonomous oscillation are highly conserved in many organisms with an internal molecular mechanism which maintains the timing processes even in the absence of any external cue [5]. Although there have been some alterations in daily oscillation of the clock in different organisms like mammals, fungi, plants and bacteria through the evolution process, the core circadian clock has highly conserved. In cyanobacteria, the mechanism is known as Post-Translational Oscillator (PTO) composed of KaiA, KaiB and KaiC protein while in mammals and fungi, the core clock working in 24-hour-period is the Transcription-Translation Feedback Loop (TTFL) consists of negative and positive arms. There are two proteins in each arm [6]. Circadian Locomoter Output Cycles Protein Kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1; also known as MOP3,

JAP3, or ARNT3) are members of positive arm while negative arm consists of PERIOD (*Per1* & *Per2*) and CRYPTOCHROME (*Cry1* & *Cry2*) genes. Expressions of negative arm genes are provided by positive arm proteins while they activate the transcription repressor of positive arm proteins [7]. Negative arm proteins cannot be expressed due to absence of positive arm proteins and also, they cannot repress the transcription of positive arm proteins. Thus, positive arm proteins can be expressed again. As a result of this, a negative feedback loop in almost 24-hours-rhythm occurs between two arms. There are three control elements of the clock on DNA which are E-Box (morning-time), D-Box (Day-time) and Rev-Erb/ROR-binding element or RRE (night-time elements) [8].

During the E-box (E/E') mediated transcriptional program, CLOCK and BMAL1 proteins make a heterodimer transcription factor called "CLOCK/BMAL1" which has a basic helix-loop-helix formation. The main role of this DNA-binding heterodimeric transcription activator is providing the expression of *Per* and *Cry* genes by binding their PAS (period-ARNT-single-minded) domains. The name "PAS" comes from signaling protein-related amino acid domains which are Per, Arnt and Sim (Hefti MH et al, 2004). CLOCK/BMAL1 heterodimeric complex targets E-Box cis-element which includes promoter regions of clock-controlled output genes *Per* and *Cry*. Therefore, negative arm genes begin to express by the help of positive arm members [9]. After the synthesis of PER and CRY proteins, they localize in cytoplasm to heterodimerize with each other. Then, heterodimerized PER/CRY complex translocate to the nucleus and contact with CLOCK/BMAL1 complex. By this way, PER/CRY complex inhibits their own further transcription activations. After that, PER and CRY proteins are degraded via proteasomes after their ubiquitination. Their degradation leads to removal of suppression on BMAL1 and CLOCK transcription which gets start the clock again. By this way, an oscillation pattern is appeared in 24-hour periodicity [10].

Throughout the REV-ERB/ROR-binding element related transcriptional program, there is another feedback loop between orphan nuclear receptors and BMAL1. The retinoid-related orphan receptors which are RORα,-β, -γ and orphan nuclear receptor REV-Erbα/REV-Erbβ have E-Box on their promoters which lead their transcription in a

24-hour (circadian) cycle via binding of CLOCK/BMAL1 heterodimer complex. Expressed RORα and REV-Erbα behave as positive and negative regulators of transcription of BMAL1 respectively [11]. RORE known as ROR response elements is a specific DNA sequence and recognized by RORs. They are in a competition for binding the RORE found in the *BMAL1* promoter. The winner of the competition can be differing from tissue to tissue. Moreover, there are some other data claimed that REV-Erbα negatively regulates CLOCK gene by binding its intron [12]. Thus, the oscillation of REV-ERBα/RORα in SCN creates a secondary feedback loop within the circadian clock.

Furthermore, the other important genes for circadian clock are D-Box regulators which are DBP (albumin gene D-site binding protein) and E4BP4 (Nfil39). Their expressions are caused by CLOCK/BMAL1 heterodimer complex. DBP is one of the proteins that have PAR-domain basic leucine zipper (PAR bZip) like TEF and HLF. It has different roles in different body tissues such as liver and kidney. It increases the expression of circadian clock genes by binding their D-Box while E4BP4 decreases. DBP and E4BP4 found at the same cycle but regulate it in favor of opposite. DBP positively and E4BP4 negatively arrange the period of the clock proteins. Particularly, *Per2* is affected by these proteins. On the other hand, E4BP4 has interaction with CRY1 and PER2. This also influences the period of them. Besides internal clock, DBP and E4BP4 are significant for downstream of the clock. Their interaction with other proteins and impacts on diseases are unclear [13].

The importance of TTFL has increased in everyday with the improvement of high-throughput analysis techniques and results that taken from these kinds of analysis. Thus, many unknown interactions and relationships between the clock and members of other pathways have been appeared. Today, it is well-known that all of components of the clock have also different metabolic roles and behaviors in the cell aside from circadian clock. For example, an organ-based study that performed by RNA-seq and DNA arrays acquaints the maintenance of 43% of all protein coding genes in circadian rhythms [14]. What is more, E-Box binding ability of CLOCK/BMAL1 complex results in transcriptionally regulation of not only circadian clock genes but also many

different genes in number of groups in a circadian rhythm. Many of these are investigated by Chip-Seq analysis of BMAL1 and CLOCK which supplies genome-wide DNA binding sites. As a result of one of these studies represents that more than 2000 sites in mouse genome is in attention of BMAL1. Although it binds on mainly with core circadian clock and associated genes' sequences, transcriptions of some genes in carbohydrate and lipid metabolisms are also related with it (Rey G. et al., 2011, PLOS Biology). On another study claims that canonical E-box are not only sequence allows BMAL1/CLOCK binding. They have an ability to bind also six different non-canonical regions on genome [15]. All these studies clearly shows that the complex transcriptionally regulates circadian clock related or unrelated many genes in an oscillation.

In this perspective, it has been started to disclose that some metabolic disorders are frequently associated with mutations in clock genes explained by altered expression of these transcriptional regulators that affect key metabolic factors in the cell. Therefore, it is important to examine the linkage between circadian rhythmicity and the optimal functioning of relevant metabolic processes for improving new drugs against disorders.

2.1.2.1. Positive Arm Gene: BMAL1

One of the PAS domain transcriptional factor that acts as a regulator of circadian clock is BMAL1 which is also known as Mop3 and ARNTL. BHLHE5, BMAL, BMAL1C, JAP3, PASD3, and TIC are less commonly known aliases of it. Bmal1 gene is found on p15 band of the 11th chromosome. The gene contains 25 exons [16].

According to X-ray crystallographic analysis, BMAL1 protein consisting of 387 amino acids includes one basic helix loop helix (bHLH) domain and two PAS domains which are PASA and PASB. bHLH is used for DNA binding while PAS domains implicate on dimerization of CLOCK/BMAL1 complex by attaching to PAS domains of CLOCK protein. There is also a flexible loop (L1) which provides a connection between bHLH (N-term) and PASA (C-term). L1 of BMAL1 shows an unstable feature with its

approximately 15 residues. However, L1 of CLOCK protein has almost 30 residues. Thus, CLOCK/BMAL1 dimerization represents an asymmetric structure.

CLOCK/BMAL1 dimerization is formed from hydrophobic interactions of three different domains with each other. Firstly, binding of PASA subunits of both BMAL1 and CLOCK proteins provides a connection that Ile317 residue has a crucial role in it. Secondly, PASB subunits of both BMAL1 and CLOCK proteins allow another dimerization site between them. Trp284 on α-helix of PASB of clock and Trp427 on β-sheets of PASB of BMAL1 are important for this dimerization site. Both interactions from PASA and PASB are symmetric. Finally, connection between bHLH domains of both proteins leads stabilization of the dimerization with four leucine residues found on the domains [17].

Another important point is the structural dynamics of BMAL1 which are N-terminus, C-terminus of it. Firstly, the most effective site of BMAL1 protein for the maintenance of molecular mechanism of circadian clock is C-terminus of the protein. Cells that have C-terminal mutant BMAL1 proteins represent loss of circadian function because studies show that both repressive and inducible regulation of it are allowed by the C-terminus (Haiyan Xu et al., 2015). Also, the same study shows that only mPER2 protein attaches to C-terminal mutant BMAL1 protein. These results suggest that C-terminus is essential for survival of circadian oscillation [18]. Secondly, N-terminus of BMAL1 protein provides a site for nuclear localization signals (NLS) while PAS domain provides nuclear export signals (NES).

Furthermore, attaching of bHLH domains each other creates α-helix forks which are essential for DNA binding. This complex recognizes the E-Box (CACGTG) on DNA by the bHLH domain and binds. This results in transcriptionally regulation of 43% genes in a circadian pattern. This is because interaction of DNA with the complex proceeds in a cycle due to the posttranslational regulation of them. Phosphorylation one of the well-known modifications regulates the stability and localization of proteins. Both phosphorylated and non-phosphorylated CLOCK proteins accumulate in nucleus while just non-phosphorylated found in cytosol at CT 6 in mouse liver. Moreover, only

phosphorylated CLOCK is observed in nucleus while both forms can be seen in cytosol at CT 18. However, phosphorylated BMAL1 protein is abundant in nucleus at these times even though each forms of it are observed in both nucleus and cytoplasm. What is more, after immunoprecipitation of CLOCK protein, non-phosphorylated BMAL1 proteins are observed even so it commonly stick to phosphorylated forms of it. Besides there is no proven effect of phosphorylation of DNA binding ability of CLOCK, it is suggested that phosphorylation of CLOCK is important for CLOCK/BMAL1 dimerization.

Circadian clock has an effective molecular mechanism due to its association with many kinds of pathways in cell. It regulates almost 40% of whole cell proteins. Disruption or loss of circadian function leads irreversible results for the cell even for the tissue. It is well-known that BMAL1 is an essential component for mammalian circadian clock. Lack of BMAL1 in mice leads loss of function in circadian clock. While these studies represent the importance of BMAL1 for circadian mechanism, another studies show the role of BMAL1 in both mental and physical diseases such as sleep disorders, cancer, early aging and depression. At the beginning, BMAL1 which is an E-Box transcription factor has a huge downstream and large network. For example, BMAL1 involves cartilage gene regulation due to its relationship between the SIRT1, NAD^+-dependent histone deacetylase. Also, life span of *Bmal1*$^{-/-}$ mice decreases when compare the wild type (*Bmal1*$^{+/+}$) profile. These mice show an early aging phenotype. In another study, it claims that mass of bone in mice does not growth well in the absence of *Bmal1* [19].

2.1.2.2. Negative Arm Gene: Cryptochrome

Photolyases/cryptochromes (PHR/CRY) is an enormous family which includes members that share the same structure but have tremendously different functions. These molecules are photoactive and can be observed through kingdoms from archaebacterial to mammals. The cyclobutane pyrimidine dimer called CPD photolyases, the “6–4” pyrimidine-pyrimidine adduct photolyases and the cryptochromes known as CRY are three main groups of protein found in the family [20].

Photolyase (PHR) enzymes was discovered by Claud S. Rupert, a physicist. Then, Professor Aziz Sancar, a 2015 Nobel Laureate in Chemistry had been investigated its gene in *Escherichia coli* and exhibited its ability to repair DNA thymidine dimers (T-T) caused by ultraviolet (UV) light from sun. It uses photon energy from low wavelength of the sunlight (350-500nm (blue)) for this function. Therefore, they are also called light-dependent DNA Repair Enzymes. This repair mechanism is crucial for survival of microorganisms in early Earth since lack of ozone layer allowed UV light to reach directly on the surface of the Earth.

3. MATERIALS

3.1. General Kits and Reagent

Table 3.1: List of kits and reagents

Nucleospin Plasmid Kit	740588.50, Macherey Nagel, Germany
PCR clean-up Gel extraction Kit	740609.50, Macherey Nagel , Germany
Protease Inhibitor Coctail Tablets	S8830-2TAB, Sigma-Aldrich, USA
Protein Molecular Weight Marker	P7712S, New England Biolabs,USA
Chemiluminescent HRP Substrate	Thermo, Femto, Germany
DNA Ladder	N3232S,New England Biolabs (NEB),USA
DNA Loading Dye	B7021S, New England Biolabs (NEB),USA

3.2. Buffers and Solutions

Tris-Buffered Saline (TBS): Tris-Base: 20mM, NaCl: 150mM, adjust pH to 7.6. Tris-Borate-EDTA (TBE): Tris-Base: 0.9M, Boric Acid: 0.89M, 40mL of EDTA: 0.5M (pH:8.0) SDS-PAGE Running Buffer: 250mM Tris-Base (pH: 8.3), 1.90 M Glycine, 1% SDS (20g). Phosphate Buffer Saline (PBS): BE17-517Q, Lonza, Belgium. Separating Gel Buffer: Tris-Base: 1.5M (pH: 8.8). Stacking Gel Buffer: Tris-Base: 1M (pH: 6.8). Laemni Buffer (4X): Tris-Base: 277.8mM (pH:6.8), Glycerol:44.4mL, SDS: 4.4%, Bromophenol blue: 0.02%. Comassie R250: Comassie R250 dye: 0.005% (w/v), Methanol (99.9%): 1:2 (v/v), Acetic Acid: 1:10 (v/v), ddH_2O: 40%. Destaining Solution: Methanol (99.9%): 2:5 (v/v), Acetic Acid: 1:10 (v/v), ddH_2O: 1:2. RIPA Buffer (5X): Tris-Base: 250mM, NaCl:750mM, 8% iGEPAL, 0.5% SDS, 1% NP40.

3.3. Antibodies

Table 3.2: List of Antibodies

Name	**Concentration**	**Source (Cat. No.)**
Anti – β-actin	1:5000	ThermoFisher (#15G5A11/E2)
Anti – GFP	1:1000	Santacruz (#sc9996)
Anti – Mouse, HRP	1:5000	Cell Signalling Technology (#7076)
Anti – Rabbit, HRP	1:5000	Cell Signalling Technology (#7074)
Anti – BMAL1	1:2000	Bethyl Lab (#A302-616A)
Anti – CRY1	1:2000	Bethyl Lab (#A302-614A)

3.4. Oligonucleotide Sequences

3.4.1. Cloning of *Bmal1*

Forward Primer: 5'- *AGCT^GTACAAG*ATGATTAATATAGAAAGCA -3'

Reverse Primer: 5'- *AAAGC^GGCCGC*TTACAGCGGCCATGGCAAGTCACT -3'

3.4.2. Cloning of *mVenus*

Forward Primer:

5'– *TGGCAAACAGCTATTAT^GGGTATTATGGGT*ATGGTGAGCAAGGGCGAG – 3'

Reverse Primer:

5' – *ACCCATAATACCCATAA^TAGCTGTTTGCCA*CTTGTACAGCTCGTCC – 3'

4. METHODS

4.1. Molecular Cloning

4.1.1. Digestion of DNA with Restriction Enzymes

A DNA substrate (plasmid) was cut with two selected restriction enzymes from its specific sites. In a 1.5 mL tube, the reaction was set up with 1µg/µL of DNA, 5µL from purchased reaction buffer (10X) as it was 1X in the mixture, 1µL (10 units) from each enzyme and bovine serum albumin (BSA) if it was recommended by manufacturer. The mixture was filled up to 50µL with nuclease-free water. The reaction was mixed and centrifuged gently and then, it had been incubated at appropriate temperature of used enzymes for 1 or 2 hours.

4.1.2. Agarose Gel Electrophoresis

Digested and PCR amplified DNAs were investigated with agarose gel electrophoresis. Concentration of the gel depends on the investigated fragments can be 1 or 2% (w/v). For 1% gel, 500mg of agarose was solved in 50mL Tris-Borate-EDTA (TBE) buffer by boiling in microwave. 1µL of redsafe or promosafe was added into the mixture and then, it dropped into a cassette. After freezing, the tank was filled with 1X-TBE Buffer. Then, samples were loaded after mixing with 6x-loading dye with its final concentration of 1x. 1kb or 100bp marker was also loaded. Gel was run at 80V for 20 minutes. The image of the gel was taken from Biorad-ChemiDoc Imaging Systems.

4.1.3. Ligation of Inserts into Plasmids

During the preparation of a recombinant DNA, interested fragment called insert DNA is inserted into the target site on the vector by ligation. After cutting the vector

and insert from certain sites by relevant restriction enzymes, blunt or sticky ends were appears. In ligation, these ends were bound together by phosphodiester bonds. T4 DNA ligase was used for ligation. The enzyme requires ATP which is supplied by the T4 Ligase Buffer. Generally, 10ul of reaction was set up. It contains 3ul of plasmid vector, 4ul of insert DNA, 2ul of 10X Buffer and 1ul of T4 ligase. The reaction was incubated overnight at 4^{o}C. Then, it was amplified for further conformation analysis of cloning by using transformation method.

4.1.4. Transformation to Competent Bacteria

Transformation is one of the gene transfer process in which entrance of naked exogenous DNA into organism. Competent cells are suitable cells for transformation due to their ability to take up extracellular DNA from its environment. These cells can be made competent with different methods. Chemically-competent cells are made competent by treating them with $CaCl_2$ solution under low temperature. Negative phospholipid heads on membrane and negative phosphate groups on DNA backbone repel each other, so DNA cannot close to the cell. $CaCl_2$ ions make these negative charges neutral. Cells were incubated into $CaCl_2$ bath in ice for a long time and then, they were heated at 42C for 90 seconds. Heat shock is a mechanical method. Heat opens the pores on the membrane of the cells, so plasmid or DNA can enter the cells easily. At the end of the procedure, cells were aliquoated and stored at -80^{o}C.

In this study, *Escherichia Coli* (DH5α) competent strain was used for transformation. When they were used for transformation, an aliquoted cells taken from -80^{o}C was thawed on ice. If it is ligated plasmid, half of whole volume, usually 5uL, were mixed with approximately 50-60uL of competent cells in another autoclaved eppendorf tube near to fire. However, mixing 1ul of DNA (50-100ng/uL) with 100uL of competent bacteria might be enough for amplification. After this, the mixture was incubated for 30 minutes on ice. Then, it was heated at 42^{o}C for 1 minute and incubated on ice for 1.5 minute respectively. Liquid Luria Broth (LB) was added on the

tube as the final volume will be 1mL. Cells were incubated on shaker at 37°C and 200rpm for 1 hour to growh enough amount of bacteria. Also, in this period, they express resistance protein in a sufficient level. After incubation, cells containing x antibiotic resistant gene were seed on solid agar plate having x antibiotic by spreading method near the fire. Whole ligated cells were obtained as a pellet after centrifuge at 13000rpm for 30 minutes. On the other hand, seeding of 100uL from amlified plasmid culture is enough for growing. Spreaded cells onto plates were incubated at 37°C for 16 hours. At the 2nd day, single colonies were selected and these colonies were separately grown in a falcon having 5mL LB medium containing x antibiotic after taking from the plate with the tip of 100ul micropipette. These cells were incubated on shaker at 37°C and 200rpm for 16-18hours. At the 3rd day, colonies were obtained after the centrifuge at 4000rpm for 10 minutes. Supernatant was wasted and the pellet is our bacteria that have interested plasmid.

4.1.5. Plasmid Isolation

Macherey-Nagel Nucleospin Plasmid Isolation Kit was used for plasmid isolation. The kit was prepared according to alkaline lysis method which is one of the commonly used procedures for circular DNA isolation. In this method, SDS destructs cell membrane and NaOH breaks down the cell wall and linear DNA. Then, naked plasmid DNA was obtained by NucleoSpin Plasmid Column. After washing steps for removal of chemicals, plasmids were eluted by using elution buffer. Purity and concentration of the eluted samples were analyzed by using NanoDrop Spectrophotometer. OD260 value gives the nucleic acid concentration while OD280 value gives amount of protein into the sample. OD260/OD280 ratio between the value of 1.8-2.0 means pure DNA.

4.1.6. PCR Amplification of Constructs with Cloning Primers

PCR was used for amplification of interested gene sequence that found between the two segments on the vector. Primers, DNA template, buffer, dNTP,

polymerase and water are the essentials of the reaction. Firstly, primers were designed by using online software such as ncbi-primer designing tool or primer3 according to interested gene sequence. Ordered primers (powder) were centrifuged to obtain all of them. Then, they were prepared by dissolving inside the molecular biology grade – Tris-EDTA (TE) buffer. For example, the primer is 25.5 nmol, it must be solved in 255uL TE buffer, so the final concentration became 100uM. Also, forward and reverse primers might be mixed to generate 10uM primer mixture. Secondly, ThermoFisher Scientific Platinum *Pfx* DNA polymerase was used. The reaction was set up from 50mM of $MgSO_4$ (final concentration: 1mM), 10uM of dNTP mixture (final concentration: 0.3mM), 10uM of primer mixture (final concentration: 0.3uM), 10X of *Pfx* Buffer (final concentration: 1X), DNA template (final concentration: 20ng/ul) and 0.2uL of *Pfx* polymerase. Finally, the volume was completed to 20ul or 50ul with DNase-free dH_2O. The PCR condition was 94^oC for 5 minutes during initial denaturation. Denaturation, annealing and elongation was done at 94^oC (15sec), 60^oC (30sec), 68^oC (1min) respectively in 33 cycles. Final elongation was subsequently occurred at 68^oC for 5 minutes. At the end of the reaction, the temperature was automatically decreased at 4^oC. On the other hand, gradient PCR was also performed at different annealing temperatures such as 55, 57.5, 60, 62.5 and 60^oC. By this way, the most efficient annealing temperature was optimized. After the PCR, 2ul of the product was checked by agarose gel.

4.2. Generation of Constructs

4.2.1. *pLenti.III.P(Bmal1).mCerulean.Bmal1.HA*

To generate a stable cell have an ability to express Cerulean tagged Bmal1 from its natural promoter, the construct is designed. At the first step, CMV promoter in pLenti-III-HA was removed by restriction enzymes. Then, P[Bmal1] was cloned into the CMV promoterless pLenti-III-HA vector. At the second step, PCR amplified Bmal1 gene was put downstream of *Cerulean* gene in mCerulean.N1 plasmid. Finally, Cerulean-

Bmal1 was obtained from gel digestion and cloned into pLenti-III-P[Bmal1]-HA construct.

4.2.1.1. *pLenti.III.P[Bmal1].HA*

CMV promoter region of pLenti.III.HA (ABM- Applied Biological Materials Inc.) was removed by digesting with BstBI (ThermoFisher) at sites 2292 and 2422 and SmaI (ThermoFisher) at site 3070. Fragment around 7400bp was excised from the gel. The end product was designated as mCerulean-N1.Bmal1. The end product was designated as promoterless.pLenti.III.HA. This construct was also used for advancing other studies in laboratory besides this study.

The promoter region of mouse Bmal1 (P[Bmal1]-541 bp) was digested from pGL3-Bmal1-Dluc (Trey K.Sato et al. 2004) by using SacI and XhoI (New England BioLabs). 541 bp – length fragment was excised from the gel. Sticky ends of both promoter region and promoterless.pLenti.III.HA were converted to blunt end by using Klenow Fragment (New England BioLabs). Promoterless pLenti.III.HA was dephosphorylated of by FastAP alkaline phosphatase (ThermoFisher) and P[Bmal1] was phosphorylated by T4 polynucleotide kinase-pnk (New England BioLabs). Then, fragments were ligated by T4 ligase (ThermoFisher). The resulting vector was designated as pLenti.III.P[Bmal1].HA. BamHI restriction enzyme was used for the analysis. The insert, P[Bmal1], and promoterless pLenti-III-HA has only one BamHI recognition site. However, due to the presence of blunt ends in both vector and insert, it is normal that there are bands in different levels. Clones that gave band at 130bp have correct array.

4.2.1.2. *mCerulean.N1.Bmal1*

PCR amplified mouse Bmal1 gene was cloned into mCerulean.N1 plasmid (Addgene, #54758). For this purpose, the full length coding sequence of mouse Bmal1 (1786 bp) was firstly amplified from MCS-BMAL1-Linker-BioID2HA (previously

generated in our laboratory) using PCR with the following primers: Forward primer which contains BsrGI recognition site (5'-*AGCT-GTACAAG*ATGATTAATATAGAAAGCA-3') and Reverse Primer which contains NotI recognition site (5'-*AAAGC-GGCCGC*TTACAGCGGCCATGGCAAGTCACT-3'). The gradient PCR was done with Q5 polymerase and the reaction was started with denaturation at 94°C for 5 minutes. Then, the cycle reaction which was completed in 33 repeats was performed at 94°C for 15 seconds. Annealing was done for 10 seconds but the temperature altered from well to well. 62.5°C, 60 °C, 57.5 °C and 55 °C were used for the annealing temperatures. The elongation was done at 68°C for 1 minute. After 33 cycles, final elongation was done at 68°C for 5 minutes. PCR product was removed from the gel and restricted with BsrGI (New England BioLabs) and NotI (ThermoFisher) enzymes. mCerulean.N1 plasmid was also digested with the same enzymes. After the ligation, four clonies were selected and analysed by both restriction digestion analysis and fluorescence imaging. First, selected colonies was digested with AgeI and NotI. These enzymes recognize upstream of mCerulean and downstream of mouse *Bmal1* gene sequences respectively. Thus, the final product was the combination of mCerulean and *mBmal1* which was about 2700bp.

4.2.1.3. *pLenti.III.P(Bmal1).mCerulean.Bmal1.HA*

Plasmids that produced in section 4.2.1.1 and 5.2.1.2 were used. pLenti-III-P[Bmal1]-HA and mCerulean.N1-Bmal1 were digested with AgeI and XbaI (New England BioLabs) sites. mCerulean.Bmal1 sequence (2511 bp) was cloned into pLenti.III.P[Bmal1].HA downstream of P[Bmal1] to obtain pLenti.III.P(Bmal1).mCerulean.Bmal1.HA . In this plasmid, mCerulean.Bmal1 expression is regulated by the promoter region of mouse Bmal1 (P[Bmal1]).

The confirmation of the cloning was firstly done by restriction analysis. 6 colonies were selected after ligation and checked by digesting with BsrGI (New England BioLabs). The enzyme recognizes the plasmid from 2 sites which are 171bp and 3624bp. After digestion process, samples were run in agarose gel. As a result of

the analysis, the band at 3463bp was expected from positive colonies. Also, there must be another band at the upper side of the gel. This is the other side of the construct.

4.2.2. pMU2-P[Cry1].)-(Cry1 intron 336).mVenus.Cry1 Plasmid

Cloning of pMU2-P[Cry1].)-(Cry1 intron 336).mVenus.Cry1 was started with PCR amplification of *mVenus* gene as shown in figure 5.10 and continue with digestion of both mVenus fragment and pMU2-P[Cry1]-Cry1 intron336)-Cry1 plasmid. After digestion, both fragments were ligated.

The full length coding sequence of mVenus (747 bp) was amplified from mVenus C1 (Addgene, #27794) using PCR with the following primers: Forward primer and reverse primer which contain recognition site of PI-PspI (5'-*TGGCAAACAGCTATTAT-GGGTATTATGGGT*ATGGTGAGCAAGGGCGAG-3') and (5'-*ACCCATAATACCCATAA-TAGCTGTTTGCCA*CTTGTACAGCTCGTCC-3') respectively. Pfx DNA polymerase (ThermoFisher # 11708039) was used. The reaction was started with denaturation at 94^{o}C for 5 minutes.

Then, the cycle reaction which was completed in 33 repeats was performed at 94^{o}C for 15 seconds. Annealing was done for 10 seconds at 67 ^{o}C. The elongation was done at 68^{o}C for 1 minute. After 33 cycles, final elongation was done at 68^{o}C for 5 minutes. PCR product was removed from the gel and restricted with PI-PspI (New England BioLabs) enzyme. pMU2-P[Cry1]-Cry1 intron336)-Cry1 was also cut with the same enzyme. Then, the ligation was done.

PCR product was restricted with PI-PspI (New England BioLabs) enzyme and then, cloned into pMU2-P[Cry1].)-(Cry1 intron 336).Cry1 (Ukai-Tadenuma, M. et al., 2011) immediately upstream of mouse Cry1 gene. Final product was pMU2-P[Cry1].)-(Cry1 intron 336).mVenus.Cry1. The confirmation of selected colonies was done by restriction analysis, fluorescence imaging and westernblotting. After that, positive colonies were maintained.

4.3. Cell Culture

4.3.1. Maintenance of the Cell Lines

U2OS human bone osteosarcoma epithelial cells and stable cell that were generated from them were grown and maintained in Dulbecco's Modified Essential Medium (DMEM) with high glucose level (4.5g/L), 10% Fetal Bovine Serum (FBS), 1% Streptomycin (PS) and 1% of nonessential amino acids (NEA) incubated in humidified incubator containing 5% CO_2 at 37^oC.

After the confluency of HEP3B cells reached up to 70%, passaging was made. Medium was wasted. Cells were washed with PBS. Cells were removed from the surface by 0.05% trypsin-EDTA and incubated at 37^oC. Deattached cells were observed under microscope and medium was put into the flask as twice volume of the trypsin. All material into the flask was put into a falcon. Cells were counted by hemocytometer. The falcon was centrifuged for 5 minutes at 300G. Supernatant was removed. After pellet was solved with medium, cells were seeded into culture flask by diluting 1:6 or 1:5 and incubated at 37^oC, 5% CO_2.

4.3.2. Transfection of Mammalian Cells

Purified plasmid DNAs were introduced into mammalian, U2OS and HEK293T cells. Polyethylenimine (PEI) which is a cationic polymer can easily form a complex with negatively charged DNA by binding and covering it. By the complex, DNA can be introduced into the cell like endocytosis. U2OS and HEK293T cells were seeded in 6-well plate as will being 60% confluency the next day. In 2nd day, 75uL of Tris-Buffer (ph: NaCl vs..) was put inside the eppendorf tube. Then, an appropriate amount of DNA (2-3ug) was mixed with PEI in a volume with 3-fold of the weight of DNA that was put inside the tube. The ratio was DNA (w) : PEI (v) = 1:3. For instance, 4uL and 2ug of DNA which has 0.5ug/ul concentration was mixed with 6ul of PEI. After gently mixing of solution with fingers, it was incubated at room temperature for 20 minutes. In this

step, PEI is linking with DNA. After incubation, 1mL of medium was gently added on the mixture and the whole volume inside the tube was poured on the cells. Cells were incubated with PEI:DNA complex for 24 hours. Then, cells were gently washed with 1X PBS and continued to growth in newly added DMEM without DNA or PEI. After 48 hours from transfections, cells were harvested for western blotting or investigated under microscopy to check whether the expression exists. PEI is important for the efficiency of transfection. It can affect from freeze-thaw process, so it has to be stored with aliquots.

4.3.3. Stable Cell Line Generation

Stable cells can carry an inserted gene in their genome. Thus, they have an ability to express an inserted gene for a long time. Although there are different ways to generate a stable cell line, in this study, pLenti.P(Bmal1).mCerulean.Bmal1.HA plasmid was inserted into U2OS cell by using lentivirus while P(Cry1).intron336.mVenus.mCry1 plasmid was inserted by limiting dilution as explained at 4.2.3.1 and 4.2.3.3.

4.3.3.1. Lentivirus Production

A subset of retrovirus, lentivirus can incubate in their host for a long time. In this period, they are introduced their genetic material into the host cell genome. Therefore, they can maintain into there for a long time. This ability makes them very important for stable cell generation. Therefore, lentivirus packaging systems have been improved. In this system, three different plasmids which are envelope, packaging and transfer are used. Packaging plasmid has gag (retroviral core protein), pol (reverse polymerase), rev (RRE which provides nuclear export) while envelope plasmids has envelope proteins. Also, the third one is transfer plasmids, replication-deficient lentiviral particles (pLenti.III.HA in the study) used for cloning of interested DNA sequence into it. By this method, all requirements for lentivirus production have been supplied and deficient particle is become producible. Produced viruses are found in

medium of the transfected cells. These plasmids are co-transfected in a certain amount into the pre-seeded HEK293T cells into 6-well plate which has high transfection efficiency. The difference of co-transfection from the transfection, amount of PEI is dependent on total weight of whole plasmids. In our system, 1ug of 2A and pLenti.P(Bmal1).mCerulean.Bmal1.HA plasmid, 500ng of 2B and 7.5uL of PEI were used. After 24h, medium was changed. Then, cells were incubated for 3 or 4 days at the same medium. Medium was collected and centrifuged at 5000rpm for 5 minutes to remove cells. At the end, lentiviruses has P(Bmal1).mCerulean.Bmal1 are found in supernatant. Lentiviruses can be stored at -80 for maximum 2 weeks. Their working efficiency have decreased in a meantime. Therefore, they have to be used immediately for the transfection.

4.3.3.2. Lentiviral Transduction

This is another method for gene delivery to mammalian cells. In this technique, 7ug/ul of polybrene was mixed with lentivirus solution to improve their efficiency during integration of their genome into host. In the study, lentiviruses including P(Bmal1).mCerulean.Bmal1 sequence was used and incubated with U2OS cells for 24 hours. Then, cells were incubated in fresh medium for 3-4 days. After the incubation, cells can be spread or directly, incubate with mammalian selection antibiotics that its resistance gene found into the lentivirus. By this way, cells has lentivirus genome can survive while others died. Cells were incubated with 2ug/ml of puromycin in this study for 3 or 4 days. After antibiotic treatment, limiting dilution was done with living cells.

4.3.3.3. Limiting Dilution

Lentivirus transfection generates a heterogeneous cell population after puromycin treatment. Hence a monoclonal stable cell line was obtained by limiting dilution. First, 100uL of medium was put into each well of 96-well plate. Transfected cells in 6-well plate were passaged after 3 or 4 days from puromycin treatment. 100uL

of cells were seeded into just one well of 96-well plate. Then, 100ul from the well was taken and added onto the next well. Then it continues to the last well. By this way, cells were diluted in 1:2 ratio. After 24 hours, medium was changed with the medium that had 2ug/ml of puromycin. After 2 or 3 weeks, only one cell begins to grow up and generate a homogenous cell group into the last well. The group was split out from the plate and seeded into one well of 24-wells plate. Then, it was transferred into 6-well plate for screening with westernblotting and confocal imaging. Therefore, the cell line was checked whether it can express the interested gene. Positive clones were selected and used for further studies.

4.4. Western Blotting

4.4.1. Quantitation of Total Protein

Bicinchoninic Acid Protein Assay (BCA Assay) had been performed for determination of protein concentration in the sample. This is a quantitative assay. Cysteine, tyrosine, and tryptophan which are amino acids found in protein structure trigger the copper reduction inside the chemical supplied by the assay with biuret reaction (Cu^{+2} to Cu^{+1}). As a result of the reaction, the liquid becomes purple by bicinchoninic acid. Firstly, standardization of the kit was done by different concentration of bovine serum albumin (BSA) and standard curve was obtained according to the results. Whole results that taken from the samples were analyzed according to the equation obtained from standardization. However, the assay was performed by mixing the working reagent and diluted protein samples into 96-well plate. Working reagent was prepared using reagent A and reagent B provided in the kit with a ratio of A:B=1:20. This working reagent was pipetted into the microplate wells (200µl/well). Then, each sample was 1:10 diluted with RIPA buffer used during lysis. 10µL of diluted-sample was put into wells that had 200µL of working reagent. Only one set was applied for each sample. 96-well plate was incubated at 37°C for 30 minutes

and then, the plate was measured at 562 nm in spectrophotometer. The amount of proteins in the sample was calculated by standard curve and absorbance values, so almost equals amount of proteins were loaded for each sample during SDS-page.

4.4.2. Sample Preparation

According the results of BCA assay, volumes of samples were determined as each of samples has appropriate amount of proteins. Radioimmunoprecipitation (RIPA), 5X laemni buffer (277.8mM of Tris-HCl (pH:6.8), 44.4% of glycerol, 4.4% of SDS and 0.02% Bromophenol Blue) was added into each sample as will being 1X in final concentration. 10% of beta mercaptoethanol was added into 5X laemni buffer before using it. These processes were done on ice. Then, samples were boiled at 95^{o}C for 10 minutes. Vortex and mini-centrifuge were done after boiling and samples were loaded. Laemni buffer were mixed with samples and cause easily falling down of sample into the wells. Also, the samples can be observed while they are running.

Table 4.3: Amount of chemicals for preparing of stacking and separating gels in commonly used concentrations.

	Stacking (5%)	**Separating (12%)**	**Separating (10%)**	**Separating (8%)**	**Separating (6%)**
dH_2O (mL)	2.92	4.3	4.8	5.3	5.8
Stacking Buffer (pH:6.8) (mL)	0.5	-	-	-	-
Separating Buffer(pH: 8.8)	-	2.5 mL	2.5	2.5	2.5
Acrylamide - 40% (mL)	0.5	3	2.5	2	1.5
SDS - 10% (uL)	40	100	100	100	100
APS - 10% (uL)	40	100	100	100	100
TEMED (uL)	4	4	4 µl	6 µl	6 µl
TOTAL (mL)	4	10	10	10	10

Furthermore, cells can be lysed directly in 1.5X Lysis Buffer in another method. Cell pallets were directly mixed with appropriate amount of the buffer. Cells would be lysed in a few minutes and protein samples can be immediately loaded into gel after boiling at 95^{o}C for 10 minutes. The buffer has 1.5X laemni buffer and 10% Beta-Mercaptoethanol. Due to color change and SDS inside the buffer, protein quantitation cannot be done in this protocol.

4.4.3. SDS-PAGE Gel Preparation

Glasses were put onto gel cassette as thin glass being forward. One grey rubber was put onto each cassette part of the gel assembler and gel cassette was also put onto them. There were two different parts in the gel: Separating and Stacking. Gels were prepared as shown in table 4.3.

Ammonium persulfate which is called APS is initiator of polymerization by helps of tetramethylethylenediamine which is called TEMED. TEMED increases the polymerization rate, so after adding these two components, polymerization of the gel begins and gel solidify in a short time. Therefore, APS and TEMED were added just before the gel was dropped into cassette. Separating gel must be at the bottom of the gel, so firstly, it was put into the hole that found between the 2 glasses. Separating gel did not pass the green line onto the cassette. There might be bubbles onto the gel. A few amount of isopropanol was pour onto gel in the glasses for cleaning and making the gel smooth. It becomes solid after 5-10 minutes. After it solidified, it was washed with dH_2O three times. Then dry the empty areas into glass by using whatman paper. Stacking gel was prepared and put onto the solid separating gel until it run over the cassette and comb was put onto the stacking gel. If gels were not used immediately, they were wrapped with paper first and wet with running buffer and wrapped again with cling film and then, store at 4^{o}C for a week. After the comb was taken off, gels were washed gently with dH_2O to remove bubbles into wells. Then, they were put at the cassette for the running.

4.4.4. SDS-PAGE Gel Electrophoresis

Glass that had the gel was placed onto tank. There were 2 places to put gels in running cassette, so if only one gel would be run, a plastic glass have to be used for closing both side of the electrode assembly. Gels were put into the electrode assembly. Then, running cassette was also put into the tank. The tank and the running cassette was filled with 1X Running buffer diluted from 10X running buffer (2%SDS, 248mM Tris-HCl (MW: 121.14 g/mol), 1.22M Glycine (MW: 75.07g/mol)). Samples were loaded with equal concentration after boiling of them at 95^{o}C for 10 minutes. Voltage was adjusted as to be constant during the running. Gels were run at 80W and after they passed the separating gel, voltage were increased to 120W. After all samples exited from the gel, running was stopped. Gel was taken out from the glasses. Stacking part was cut off and separating part was washed with dH_2O. Then, the gel can be transferred to the membrane as explained in part 4.3.5 or stained with comassie blue (60.5uM of Comassie R250 dye, 50% of Methanol, 10% of Acetic Acid and 40% of dH_2O). Gels were incubated with the dye for 16 hours on shaker, then the nonspecific dye was removed by using destaining solution (40% of Methanol, 10% of Acetic Acid and 50% of dH_2O). After enough washing steps, protein bands on the gel can be imaged with ChemidocTM XRS+ system using without any reagent.

4.4.5. Protein Transfer from the Gel to the Membrane

To blot proteins with antibodies in later, proteins were transfered to the nitrocellulose membrane from the gel by using sem-dry system. Memberane and whatman papers were incubated for a short time inside transfer buffer including 20% Methanol and 1X running buffer (0.1% SDS). Two amount of whatman papers were placed in two sides of the sandwich. Current goes from (-) to (+) side, so membrane has to be close to (+) side. Transfer was done at 12V for 60 minutes. After transfer, gel was dyed with comassie blue to check the transfer process while the membrane was dyed

with ponceau-S (50% ddH_2O, 5% Acetic Acid). After capture the image of ponceau-S stained membrane, the dye was removed by using TBS-T solution.

4.4.6. Blocking and Antibody Incubation

To avoid membrane from non-specifically bindings, blocking is done after transfer process. BSA or milk powder can be used for blocking. The membrane was incubated with %5 of milk powder solved in TBS-T buffer for minimum 60 minutes and it can be left in blocking solution for a weekend. If membrane leave in the solution overweekend, 100mM of sodium azide (NaN_3) which is a toxic material and bacteriostatic agent has to put into solution to prevent any bacteria contamination on membrane. However, it is effective on just gram (-) bacteria because gram (+) bacteria has resistance against it. After blocking step, the membrane was incubated with primary antibody overnight at 4^oC. The next day, membrane was washed with TBST for three times and then, HRP-conjugated secondary antibody (anti-mouse or anti-rabbit or anti-goat were 1:5000 diluted in TBST) was added and incubated for 1 hour at room temperature on shaker. Membrane was washed again with TBST three times.

Membrane was visualized with Femto ECL Substrate by imaging with ChemidocTM XRS+ system. 25 images were taken in 300 seconds. Quantitation of images was done by using Image Lab Software 3.0. Normalization was done by using housekeeping proteins such as b-actin or calnexin as a loading control.

4.5. Imaging

4.5.1. Subcellular Localization Analysis with Confocal Microscope

$6x10^4$ of cells were seeded in 6-well plate that contained autoclaved coverslip. After an overnight incubation, cells were treated with 200uM Dexamethasone for 2 hours. Then, medium that have dexamethasone was removed and cells were grown in normal DMEM. After determined time period, cells were fixed by 4% Paraformaldehyde

(pH: 7.4) incubation for 10 minutes. After washing three times with 1X PBS buffer and they were stained with DAPI (1:1000 in PBS) for 5 minutes. After washing three times with 1X PBS, coverslip was inverted and put on slide that has mounting medium on it. Zeiss LSM 880 was used. It supplies 4X, 10X, 20X, 40X, 40X-Oil, 63X-Oil objectives. Zen 2.1 software was used for measuring the overlap coefficients.

4.5.2. Fluorescence Imaging

$6x10^4$ of cells were growth on microscope cover glasses (circle, 24mm). After an overnight incubation, cells were treated with 200uM Dexamethasone for 2 hours. Then, medium that have dexamethasone was removed and cells were grown in normal DMEM. After determined time period, glasses were put on chamber and add 1200ul atmospheric medium on it. Then, cells were stayed on 37^oC plate of the microscope and visualized with Leica DMI8 fluorescence microscopy.

5. RESULTS

The major aim of this project is to label selected clock protein in live cells to follow their behavior in live cells. For this purpose, we generate two different stable cell lines which have an ability to express recombinant BMAL1 or CRY proteins in fusion to fluorescent proteins which are Cerulean and Venus. Bone osteocarcoma cell line called U2OS was used. At the beginning of the study, constructions were designed and made by using recombinant DNA technology (gene cloning). After the confirmation analysis of the construction by using fluorescence microscopy, restriction analysis and westerblotting techniques, they transferred into the cell and stable cell lines were successfully generated.

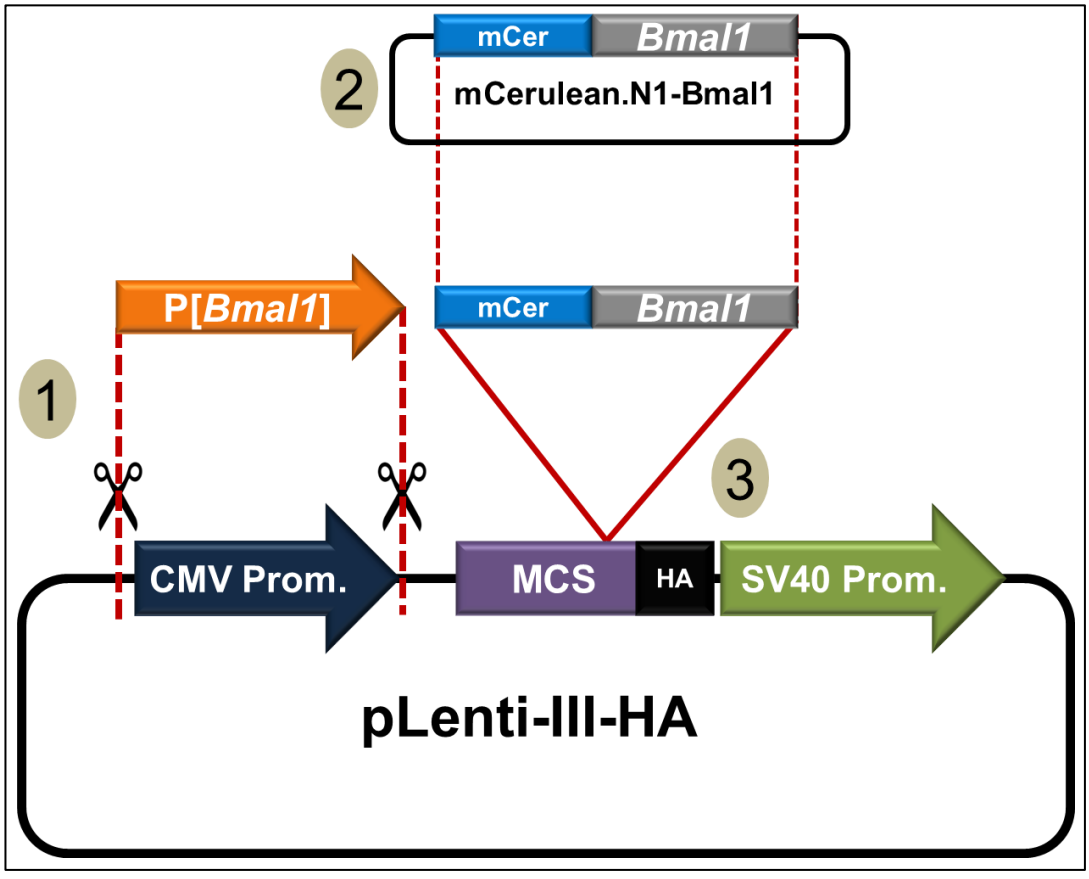

Figure 5.1: Model of Cloning Strategy of pLenti.III.P(Bmal1).mCerulean.Bmal1 plasmid.

5.1. Analysis of Constructions

5.1.1. *pLenti.III.P(Bmal1).mCerulean.Bmal1.HA*

To generate a stable cell have an ability to express Cerulean tagged Bmal1 from its natural promoter, the construct is designed as shown in figure 5.1. At the first step, (1) promoter of Bmal1 gene (P[*Bmal1*]) was replace with CMV promoter inside the pLenti-III-HA plasmid. Then, (2) mCerulean.N1-Bmal1 construct was generated by the cloning of PCR amplified Bmal1 gene into mCerulean.N1 plasmid. Finally, (3) mCerulean-Bmal1 sequence was cloned into pLenti-III.P[Bmal1]-HA plasmid.

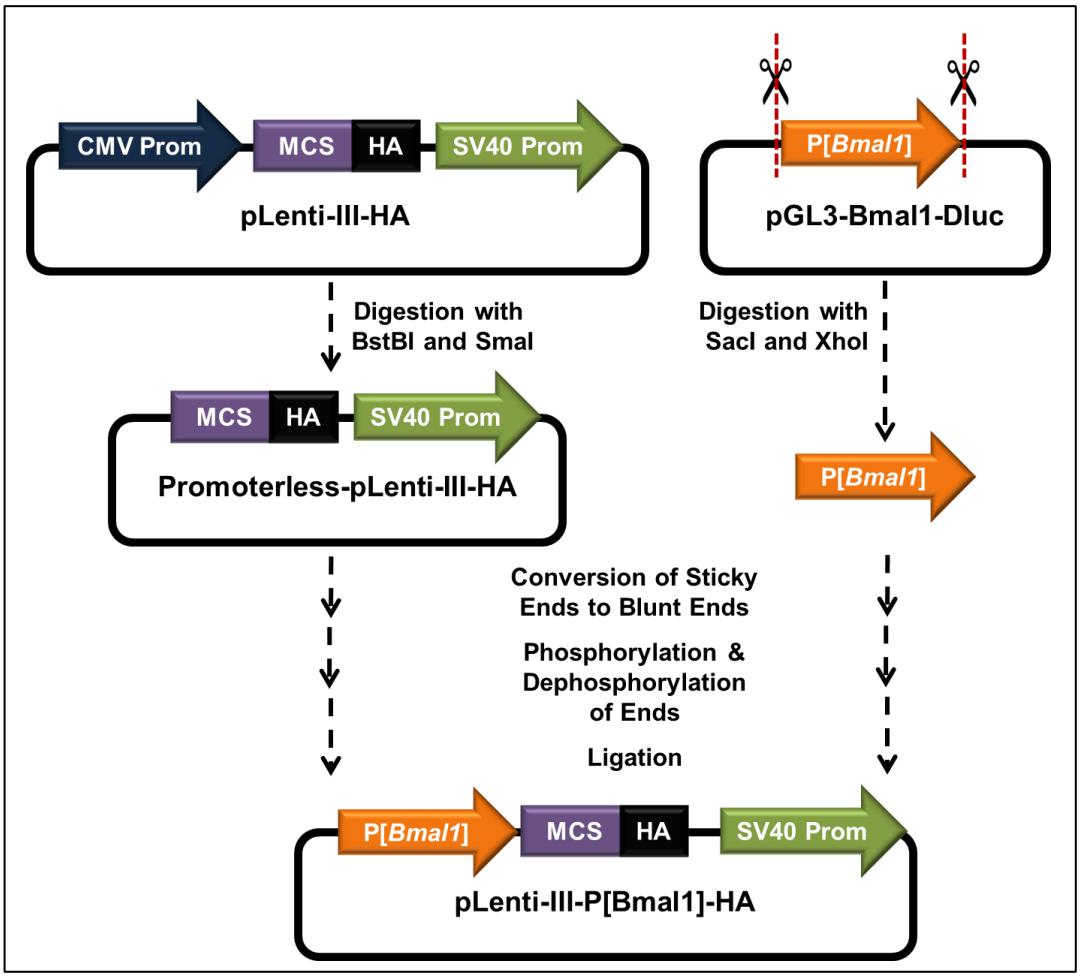

Figure 5.2: Schematic Explanation of the designing process of pLenti-III-P[Bmal1]-HA construct.

5.1.1.1. *pLenti.III.P[Bmal1].HA*

Promoter of mouse Bmal1 gene, P[Bmal1], which was cut out from the pGL3-Bmal1-Dluc plasmid was cloned inside the pLenti-III-HA plasmid in which CMV promoter of it was removed by digestion. The duration of this cloning was explained with a schematic model in figure 5.2.

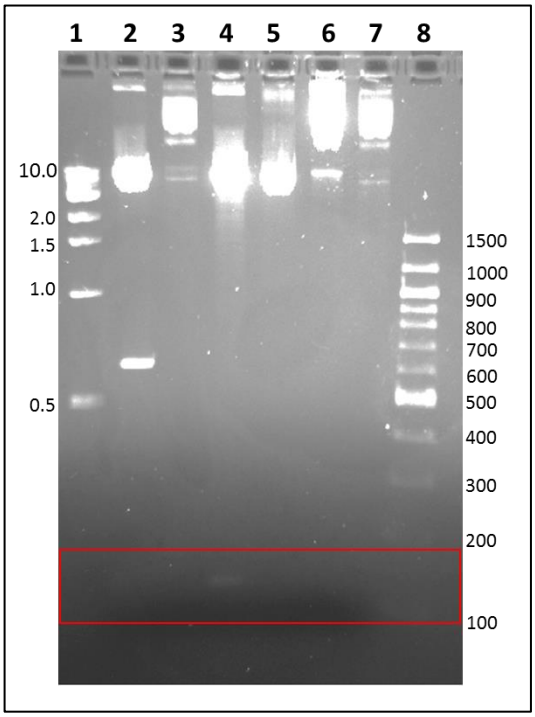

Figure 5.3: Restriction Analysis of P[*Bmal1*] inserted CMV promoterless pLenti-III-HA vector by BamHI digestion. Line 1: 1kb DNA ladder. Line 2-7: Possible pLenti-III-P[Bmal1]-HA plasmids. Line 8: 100bp DNA ladder. In the correct array, a band at 130bp (Bordered with red line) was expected as in Line 4 which is the only positive result that was used in advancing studies. Bands in line 2 was due to ligation in reverse array. The others (Line3, 5, 6 and 7) were non-ligated plasmids.

After the cloning ligation, final version of the plasmid will be used for further clonings. As shown in figure 5.3, if ligation occurs in reverse array, a band must be around 560bp but If it happens in correct array, a band must be around 130bp. Here, sample at line 2 represent reverse array pattern while sample at line 4 is in correct. Thus, further cloning experiments were continued by using sample at line 4. The end

product was designated as mCerulean-N1.Bmal1. The schematic explanation of the process is explained with visual image in figure 5.4.

5.1.1.2. *mCerulean.N1.Bmal1*

The step by step explanation of insertion of Mouse *Bmal1* gene which was obtained from MCS-BMAL1-Linker-BioID2HA plasmid by PCR amplification into mCerulean.N1 plasmid is done in figure 5.4.

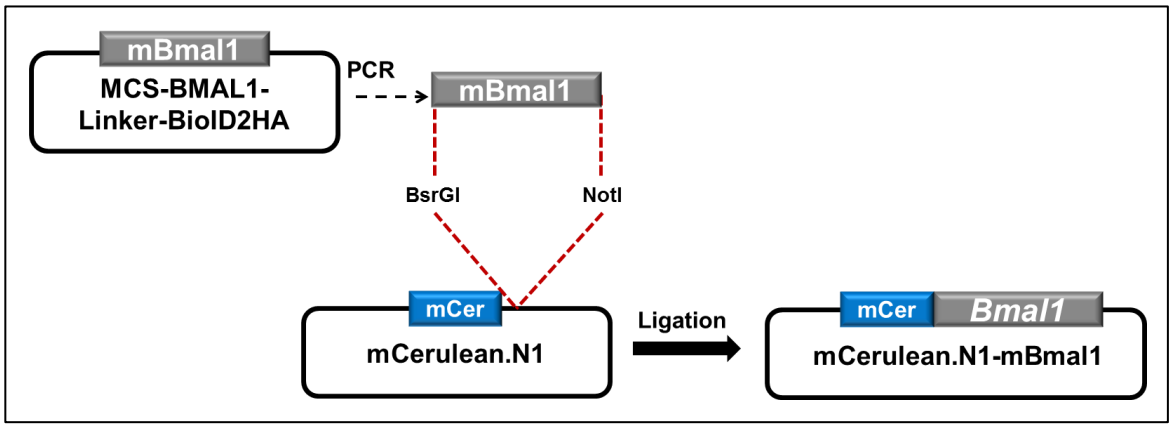

Figure 5.4: Models of the development of mCerulean.N1-mBmal1 plasmid.

PCR analysis of mouse Bmal1 gene (1780bp lenght) is shown in figure 5.5-A. A gradient PCR was done due to different Tm point of the primers. It was worked in both 57.5°C and 5 °C which are in lanes 4 and 5. Amplified mouse Bmal1 gene was then cut out from the gel and used for the next step. On the other hand, restriction digestion analysis of *Bmal1* gene inserted mCerulean.N1plasmid is found in figure 5.5-B.

Secondly, fluorescence analysis was done to confirm whether there is frameshift or not during their expression. For this purpose, constructs were transiently transfected into HEK293T cells. Then, transfected cells and a non-transfected cell (control) were imaged under fluorescence microscopy as shown in Figure 5.6. These results clearly state that C2, C3 and C4 plasmids are designated as mCerulean.N1-Bmal1.

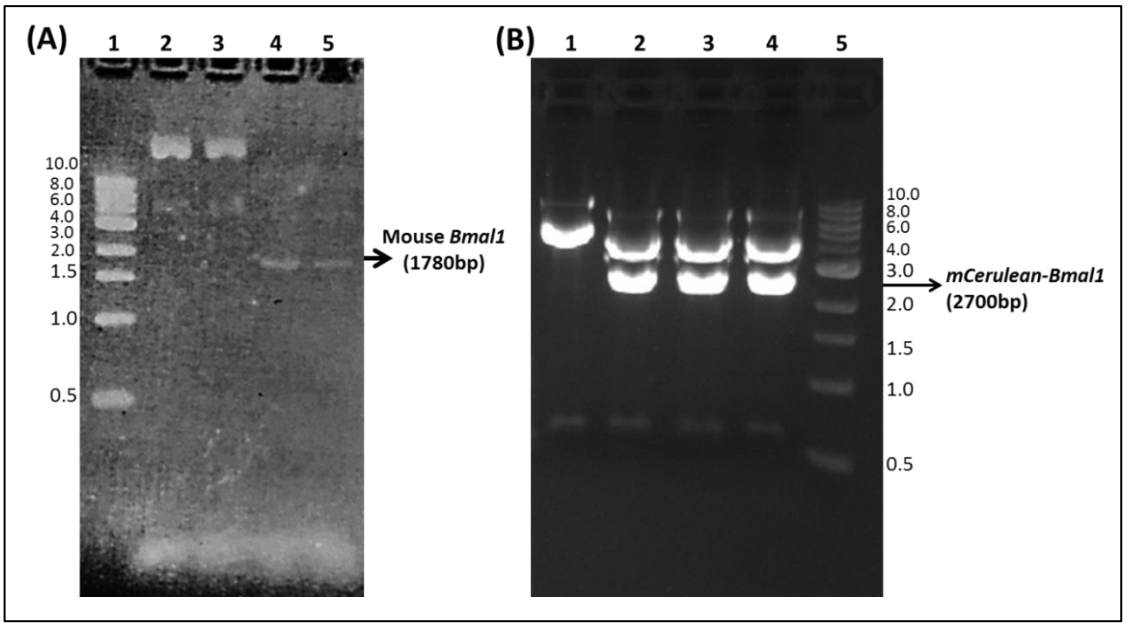

Figure 5.5: Gel Electrophoresis results that taken during the development of mCerulean.N1-Bmal1 plasmid (A) Mouse Bmal1 fragment. Lane 2-5: 62.5°C, 60 °C, 57.5 °C and 55 °C was applied in the same reaction respectively. (B) mCerulean.Bmal1 fragment. Lane 1-4:Possible mCerulean.N1-Bmal1 vectors digested with AgeI and NotI. Lane 5: 1kb DNA marker.

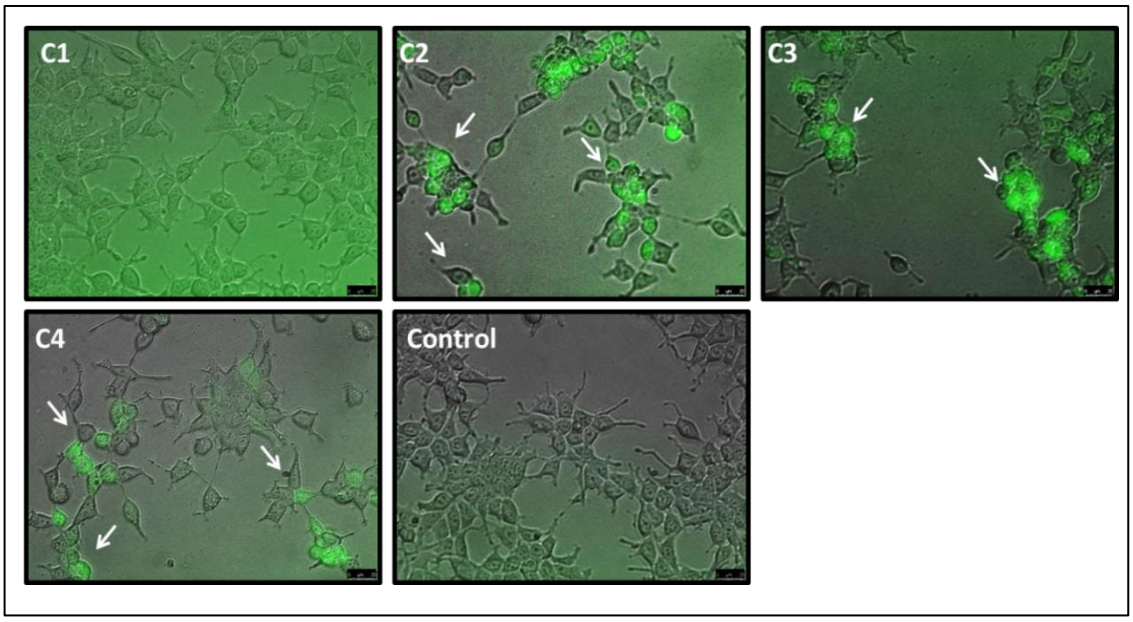

Figure 5.6: Fluoresence Imaging Analysis of Bmal1 gene inserted mCerulean.N1 vector transfected HEK293T cells. C1, C2, C3 and C4 are possible mCerulean.N1-Bmal1 clones that analysed with digestion enzymes as shown in Figure R3. Also, non-transfected HEK293T cells were used as a negative control group.

5.1.1.3. *pLenti.III.P(Bmal1).mCerulean.Bmal1.HA*

General cloning strategy is represented in figure 5.7. In this plasmid, mCerulean.Bmal1 expression is regulated by the promoter region of mouse Bmal1 (P[Bmal1]). The confirmation of the cloning was firstly done by restriction analysis.. The figure 5.7 represents the result of it. Expected band is shown at lane 3, 5 and 7 but there is no upper band at lane 7, so colonies lane 3 and 5 were shown positive. These are entitled C3 and C5 respectively. These colonies were also checked with fluorescence imaging and westernblotting.

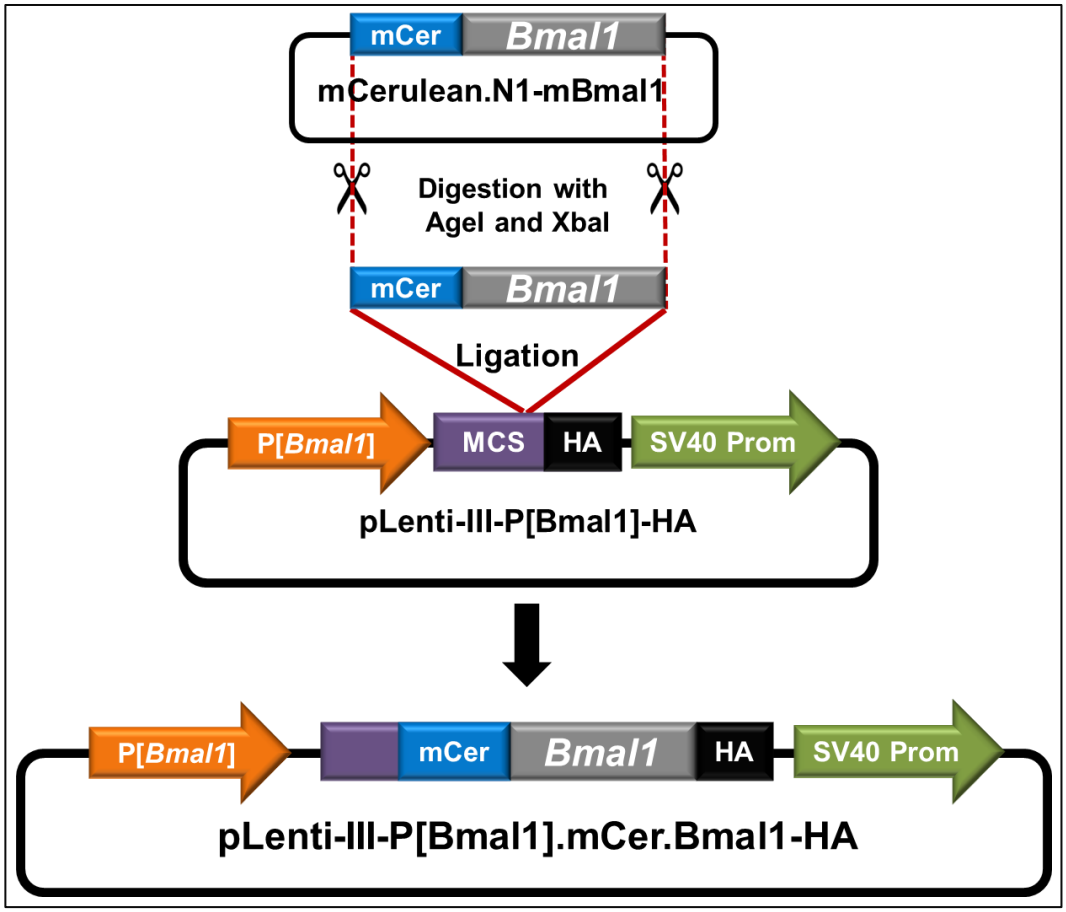

Figure 5.7: Schematic explanation of the creation of pLenti-III-P[Bmal1].mCer.Bmal1-HA construct. mCerulean.Bmal1 sequence that taken from pre-produced mCerulean.N1-mBmal1 was cloned into pre-designed pLenti-III-P[Bmal1]-HA plasmid.

Secondly, fluorescence analysis was done to confirm whether there is frameshift or not during their expression. For this purpose, constructs were transiently

transfected into HEK293T cells. Then, transfected cells and a non-transfected cell (control) was imaged under fluorescence microscopy as shown in figure 5.9-A. Finally, transiently transfected cells were harvested and analyzed with westernblotting. Bmal1 and GFP antibodies recognizes the mBmal1 and mCerulean respectively. Only positive samples, C3 and C5 were used for this analysis. As shown in figure 5.9-B, mCerulean.Bmal1 protein expresses from the cells. Both GFP and Bmal1 give signal at the same region and upper side of the endogenous BMAL1 protein. This proves that Bmal1 is successfully expressed as tagging with mCerulean protein. These results determine that C3 and C5 plasmids are designated as pLenti-III-P[Bmal1].mCer.Bmal1-HA and used for further studies.

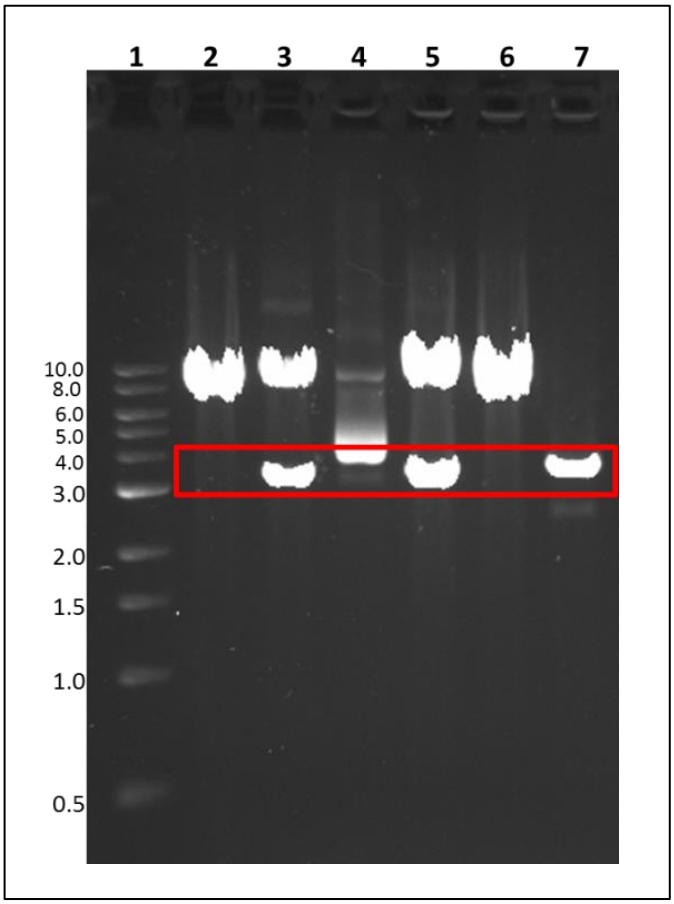

Figure 5.8: Restriction Analysis of the pLenti-III-P[Bmal1].mCer.Bmal1-HA by digesting with BsrGI enzyme. Red line indicates the region of expected band (between 3.0 and 4.0). Lane 1: 1kb DNA marker.

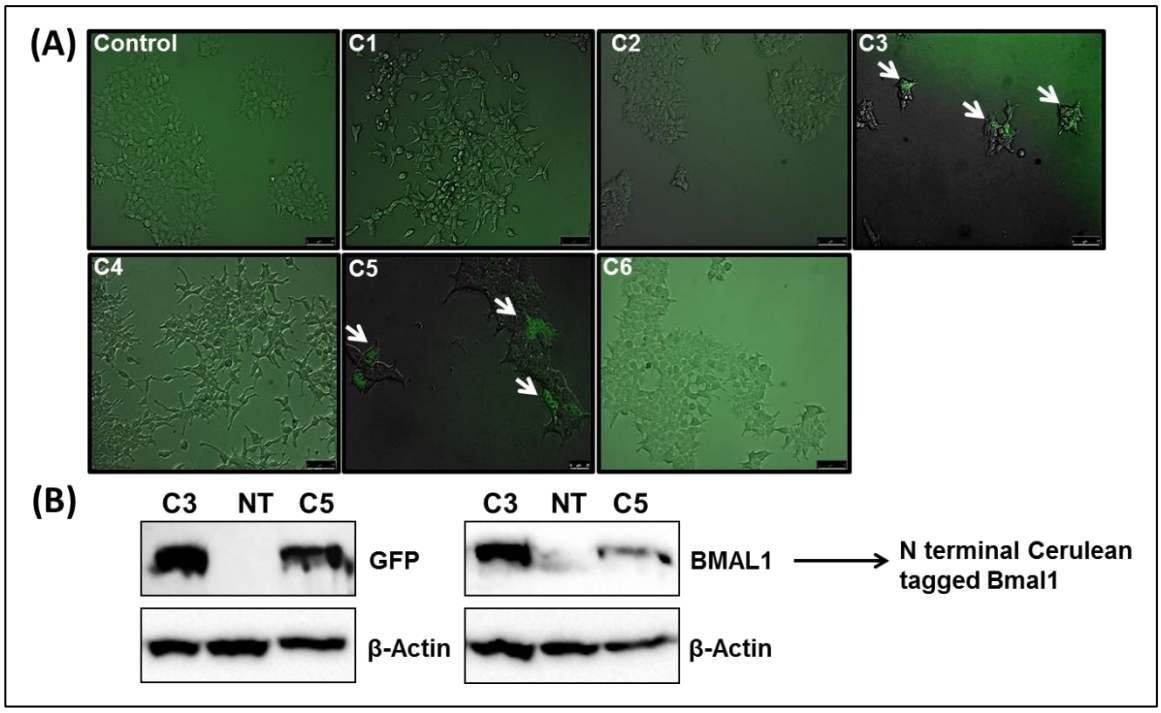

Figure 5.9: Analysis of pLenti-III-P[Bmal1].mCer.Bmal1-HA transfected HEK293T cells. Colonies in figure R8 were transiently transfected into HEK293 cells. Fluorescence imaging and westernblotting analysis of the colonies were done. C3 and C5 were selected as positive clones like in restriction analysis. Westernblotting analysis of them also confirm their positivity. B-Actin was used as a loading control.

5.1.2. pMU2-P[Cry1].)-(Cry1 intron 336).mVenus.Cry1 Plasmid

Cloning of pMU2-P[Cry1].)-(Cry1 intron 336).mVenus.Cry1 was started with PCR amplification of *mVenus* gene as shown in figure 5.10 and continue with digestion of both mVenus fragment and pMU2-P[Cry1]-Cry1 intron336)-Cry1 plasmid. After digestion, both fragments were ligated.

The full length coding sequence of mVenus (747 bp) was amplified. The result was shown in figure 5.11. Then it was ligated. There are also non-specific bands but mVenus is away from them, so it easily cut from the gel without any contamination.

The confirmation of selected colonies was done by restriction analysis, fluorescence imaging and westernblotting as represented in figure 5.12. First, clones were digested with BsrGI and SalI enzymes. BsrGI recognizes a site on mVenus gene

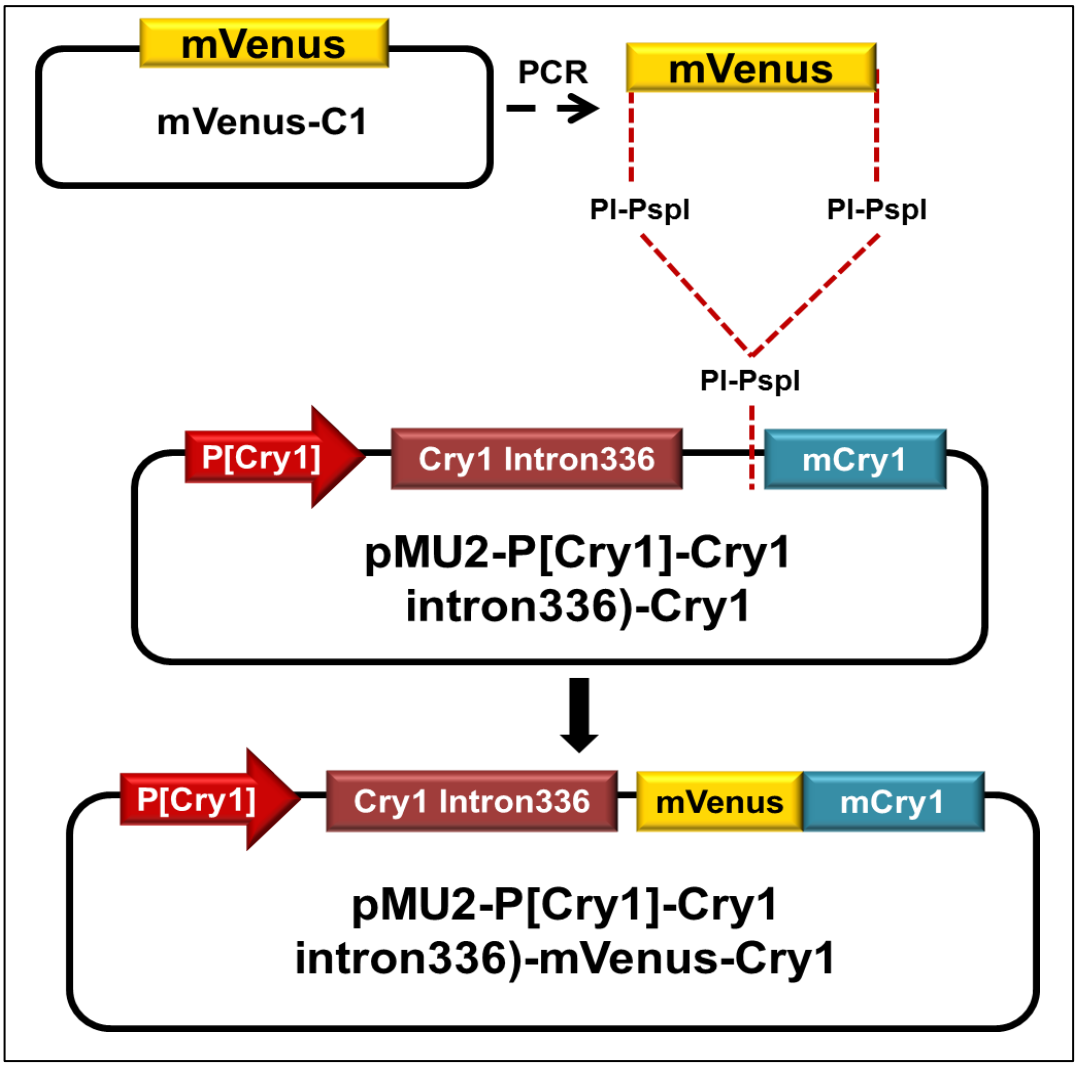

Figure 5.10: Schematic explanation of the cloning strategy of pMU2-P[Cry1].)-(Cry1 intron 336).mVenus.Cry1 Plasmid. PCR amplified mVenus gene inserted into upstream of Cry1 sequence in pMU2-P[Cry1]-Cry1 intron336)-Cry1 plasmid.

while SalI is on the plasmid. Thus, a band between 1.5bp and 2.0 as indicated in figure 5.12-A was expected from positive clones. Samples at lane 1 and 2 are shown positive. Secondly, the clones were transiently transfected into HEK293T cells. Then, fluorescence images were taken as shown in Figure 5.12-B. Sample 1 and 2 gives fluorescence signal as expected from gel electrophoresis results. Finally, these transfected cells were analyzed by westernblotting as observed in figure 5.12-C. According to whole analysis, Sample 1 and Sample 2 give positive results in all of them. Therefore, they were used in further experiments.

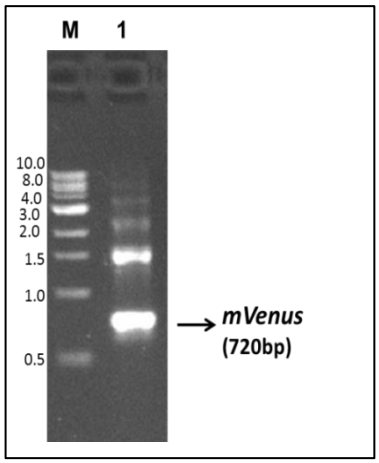

Figure 5.11. Gel Electrophoresis result of *mVenus* gene PCR product (720bp). M: 1kb DNA marker.

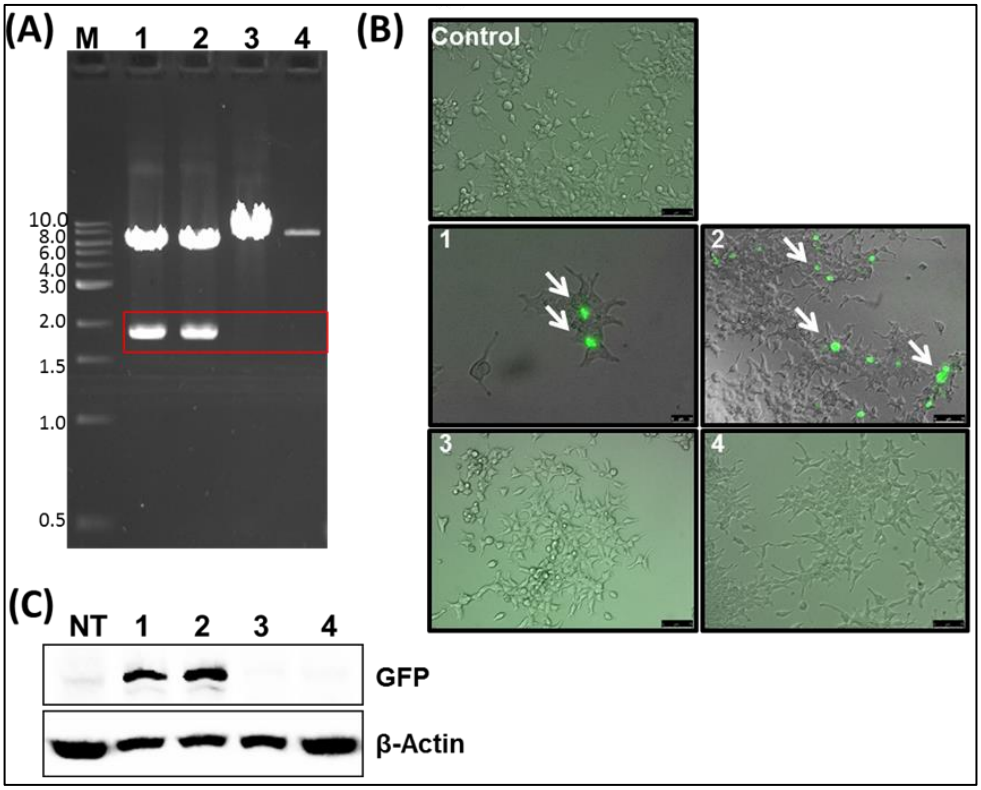

Figure 5.12: Confirmation analysis of plasmids after ligation. 4 plasmids were selected and analyzed. (A) Restriction analysis by BsrGI and SalI enzymes. Expected bands must be between 1.5 and 2.0kb (bordered with red line). M:1kb DNA marker. (B) Fluorescence imaging of transiently transfected HEK293 cells.Signals were signed with arrow. (C) HEk293cells were analyzed by westernblotting. GFP antibody was used for this assay. Only sample 1 and 2 respond to GFP antibody. B-Actin was used as loading control.

5.2. Analysis of U2OS Cell Lines Carrying the Constructs

5.2.1. *mCer/Bmal1* Cells

Lentivirus was produced from pLenti-III-P[Bmal1].mCer.Bmal1-HA plasmid that was transiently tranfected into HEK293T cells by transduction. Collected lentiviral particles were exposed to wild type U2OS cells. In this protocol, cells were grown in antibiotic-included medium, so non-infected cells were eliminated.

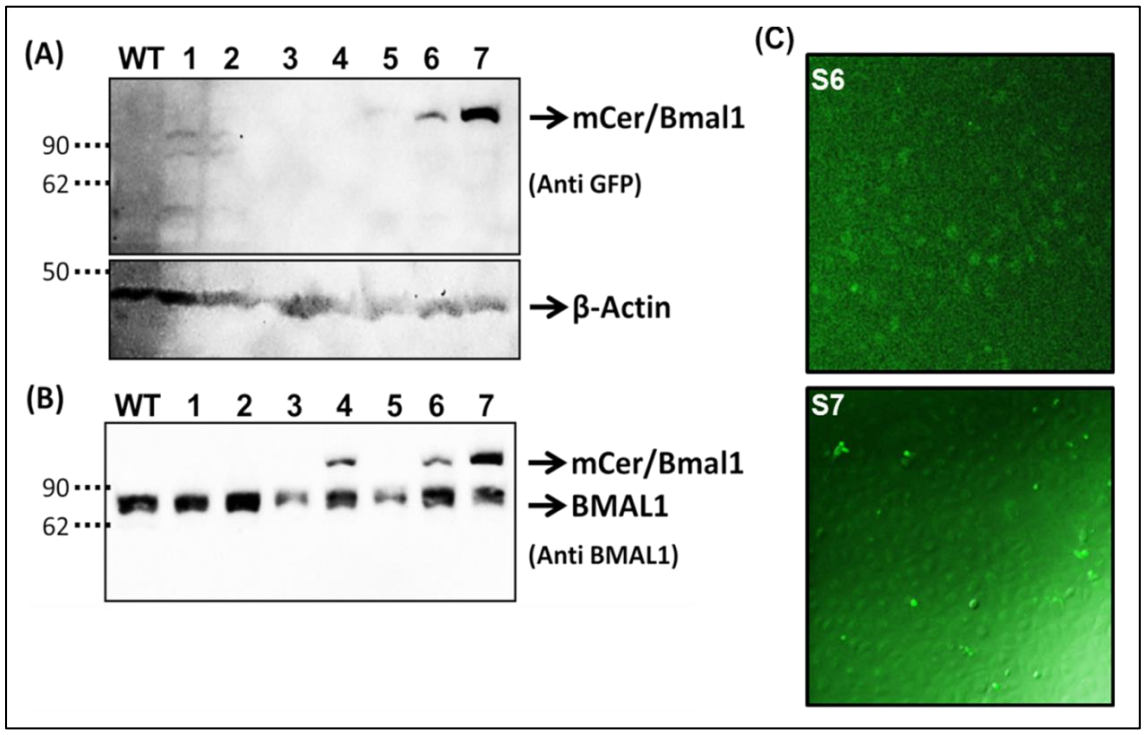

Figure 5.13: Screening of selected mCer/Bmal1 clones 1-2-3-4-5-6 by westernblotting. mCerulean fused Bmal1 protein was expected at 95 kDa. (A) U2OS clones were analyzed by anti-GFP antibody. Sample 6 and 7 respond to GFP antibody and show positive result. (B) U2OS clones were analyzed by anti-BMAL1 antibody. Sample 4, 6 and 7 display positive result. There is endogenous BMAL1 at 80kDa. Samples were compared with wild type profile and B-Actin was used as loading control in both experiments. (C) Fluorescence microscope images of sample 6 (S6) and sample 7 (S7).

Also, homogenous cell line was obtained by serial diluting methods in 96-well plate. mCerulean tagging Bmal1 protein expression was investigated in collected single colonies. mCerulean was detected with GFP antibody while Bmal1 with BMAL1.

According to westernblotting, the protein at 95kDa was examined as shown in figure 5.13-A&B. Moreover, cells were observed under fluorescence microscope to understand whether cell populations are homogenous or not. In the light of western and microscope results, sample 7 (S7) was selected and used for further studies.

5.2.2. *mVen/Cry1* Cells

Due to lack of eukaryotic selection marker on pMU2-P[Cry1].)-(Cry1 intron 336).mVenus.Cry1, it was co-tranfected with empty pLenti.III.HA plasmid to wild type U2OS cells. pLenti.III.HA has puromycin resistance gene, so non-transfected cells were eliminated by this way.

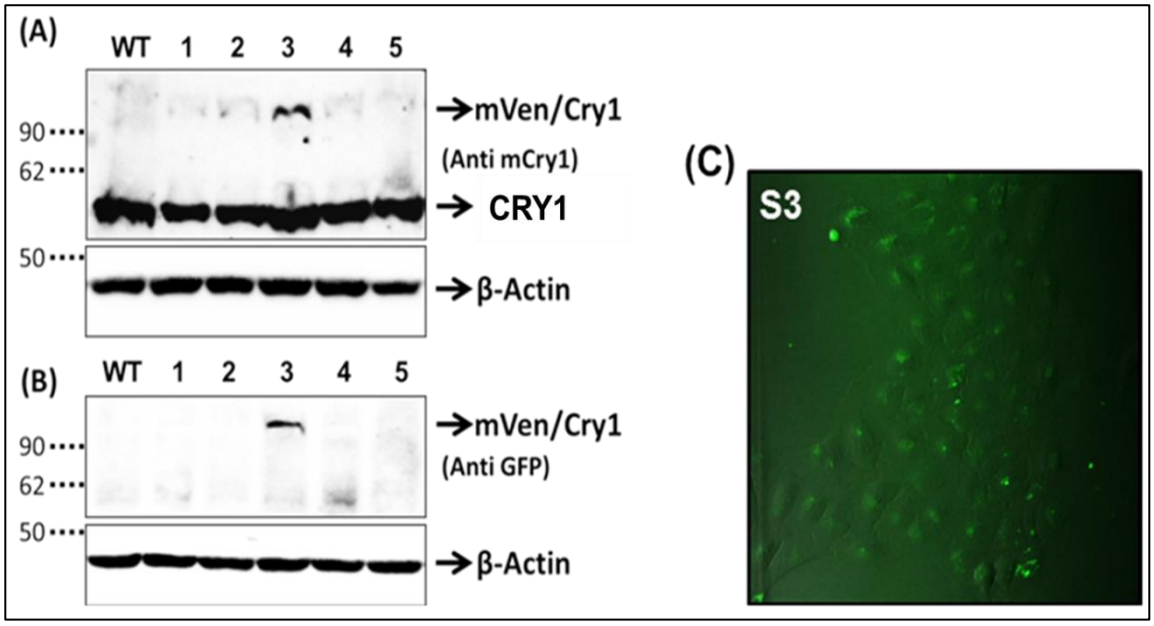

Figure 5.14: Screening of selected mVen/Cry1 clones 1-2-3-4-5 by westernblotting. mVenus fused Cry1 protein was expected at 108 kDa. (A) U2OS clones were analyzed by anti-mCry1 antibody. Sample 3 displays positive result. There is endogenous CRY1 at 55kDa. (B) U2OS clones were analyzed with anti-GFP antibody. Sample 3 responds to GFP antibody and shows positive result. Samples were compared with wild type profile and B-Actin was used as loading control in both experiments. (C) Fluorescence microscope image of sample 3 (S3).

Furthermore, homogenous colonies were obtained and screening as explained below. mVenus fused Cry1 protein expression was investigated by westernblotting (Figure 5.14-A&B) and imaging analysis analysis (Figure 5.14-C). Overall, S3 was used for further studies.

5.3. Temporal Behaviors of Clock Proteins

5.3.1. Oscillation Profile Analysis

Temporal changes of newly generated stable U2OS cells were monitored by westernblotting. For each cell line, samples were collected in each 6 hours in a timeline after resetting with dexamethasone. Then, they were analyzed by blotting with selected antibodies. Also, the results were quantitatively analyzed by normalizing to B-actin. mCer/Bmal1 and mVen/Cry1 proteins exhibit a circadian rhythm in protein level. Also, they display a similar expression pattern with their internal homologs. The synchronization between internal and recombinant proteins suggests that our fluorescence fusion proteins clearly mimic to their endogenous proteins. In these results, both of the recombinant protein have low level at 28H and between 46-52 H while high level at 40 H. These times are used for the investigation of protein dynamics. On the other hand, the proteins interestingly represent similar expression pattern even though they are known as antiphase.

5.3.2. Subcellular Intensity Analysis of the Stable U2OS cells.

To confirm the correct processing of our recombinant proteins, we analyzed the subcellular distribution of the proteins by using live cell imaging techniques. Fluorescent tagged proteins were followed under confocal microscope at 28 H and 40 H after dexamethasone treatment shown in Figure 5.16. Higher than 400 cells from minimum four independent experiments were counted and analyzed for each value.

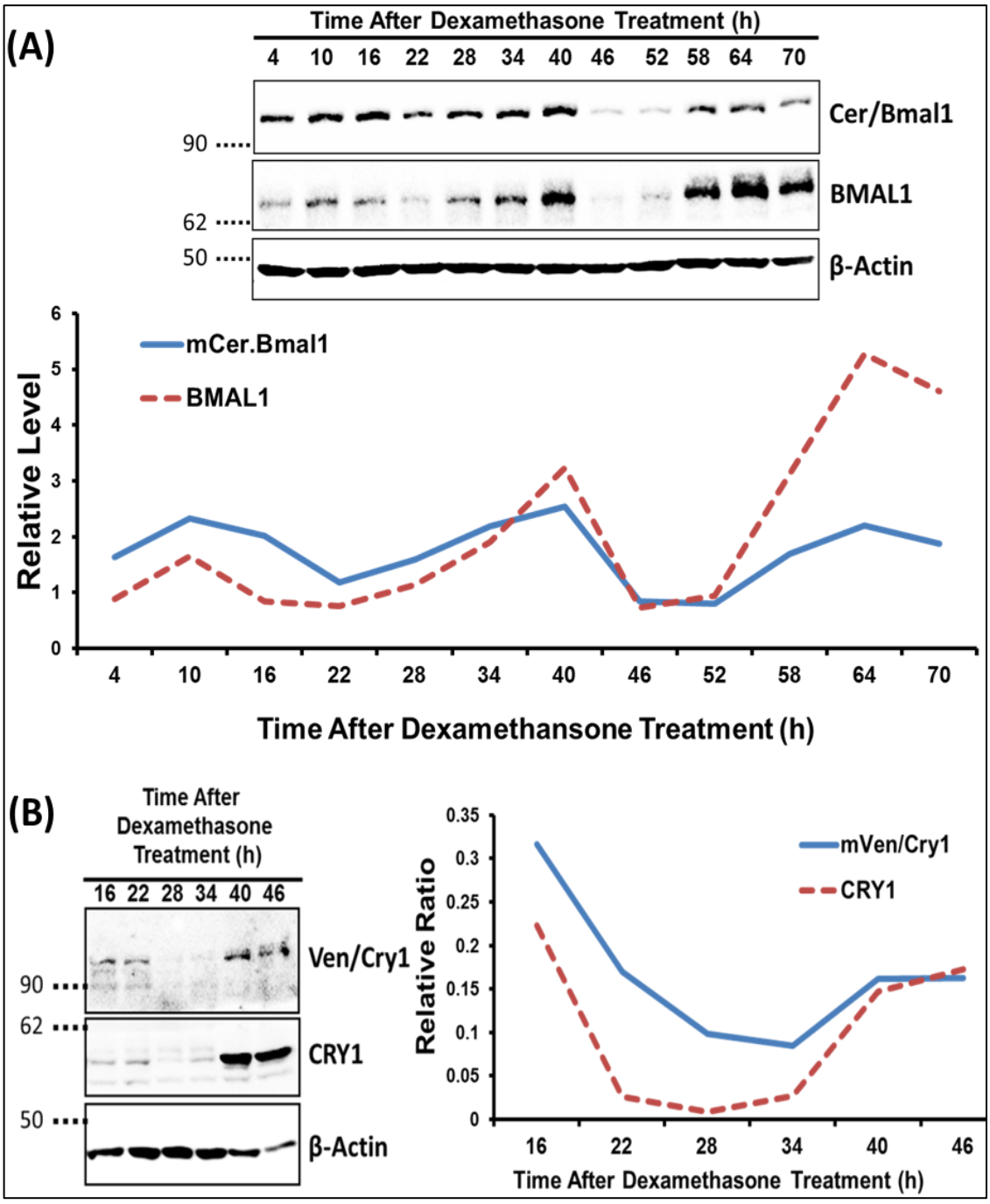

Figure 5.15: Measurement of protein levels of both recombinant and endogenous proteins in a timeline after resetting the circadian clock of cells by dexamethasone treatment. Quantitation analysis is represented with line graph.Each value was normalized to β-actin. Mean values for two independent experiments are shown. (A) Internal BMAL1 (human) and Cerulean tagged Bmal1 (mCer/Bmal1) levels were analysed at 6 hours intervals between the 4th and 70th hours. (B) mVen/Cry1 and internal CRY1 were shown. Quantitations of them are also shown in line graph.

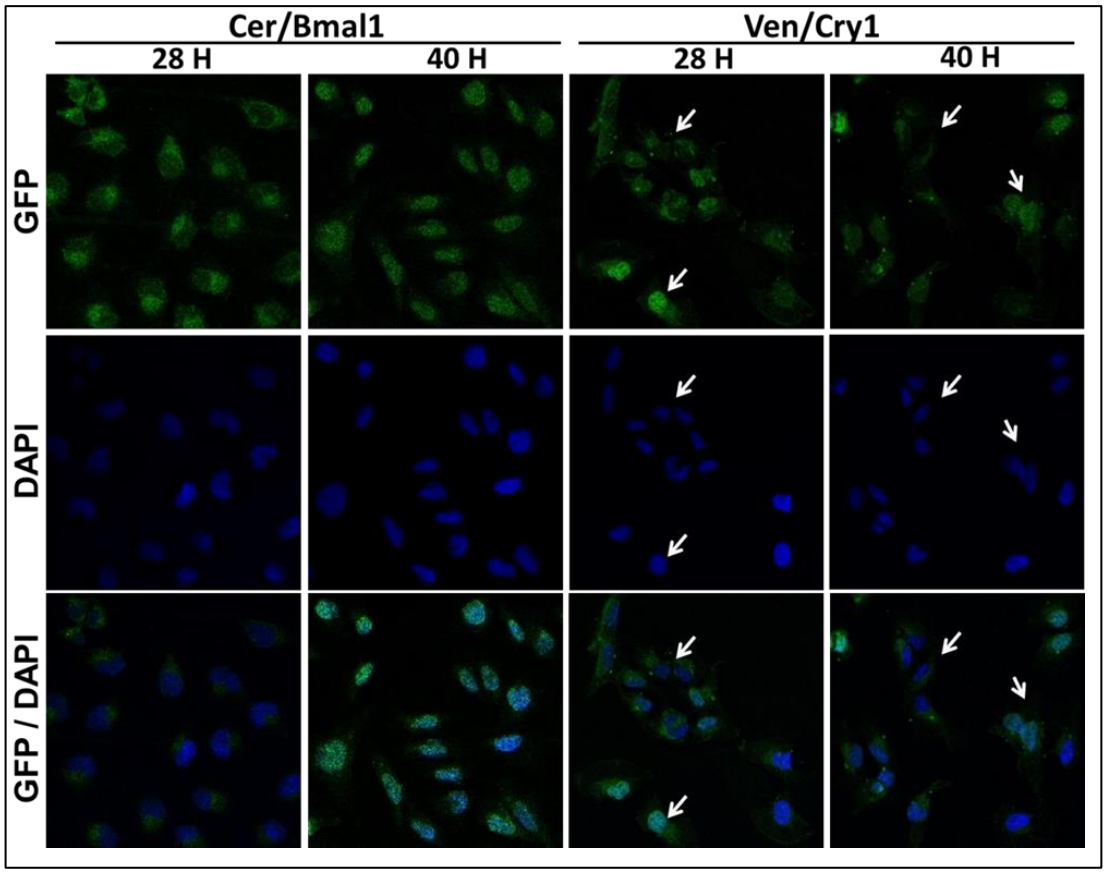

Figure 5.16: Subcellular distribution of mCer/Bmal1 and mVen/Cry1 proteins at 28 and 40 H after dexamethasone treatment (200uM Dexamethasone for 2 hours). After 28 H and 40 H, fixation was performed with 4% Paraformaldehyde. Cells were observed under confocal microscope (Zeiss LSM 880). 40X-oil objective was used. Zen 2.1 software was used for measuring the overlap coefficients. The data were obtained from 4 independent experiments and in each experiment more than 100 cells were counted according to their overlap coefficient values.

Bmal1 localizes in nucleus at 40 H or cytoplasm at 28 H. On the other hand, Cry1 localization has distributed profile. Its localization is not certain in different time points.

Quantitation analysis in figure 5.17 shows that mBmal1 specifically localize in different region of the cell. In addition, its nuclear import and export display a 24h-oscillation pattern. This might be dependent on its activity or protein level. Furthermore, cytoplasmic level of mCry1 does not change while its nuclear intensity has circadian profile. Its nuclear intensity changes in different time points but cytoplasmic level is stable.

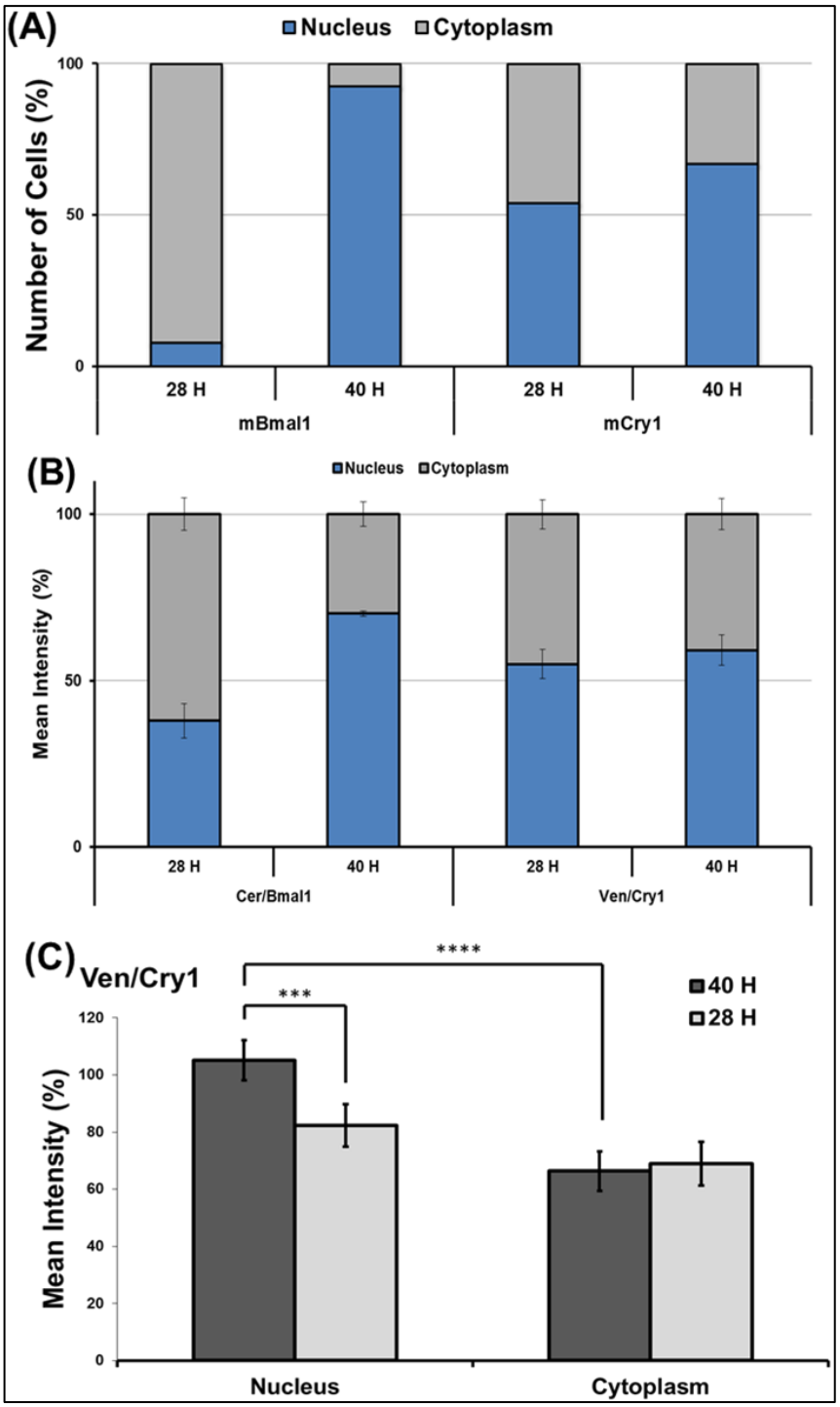

Figure 5.17: Quantitation analysis of nucleocytoplasmic distribution of Ven/Cry1 protein at 28H and 40H. (A) Cells were counted according to their overlap coefficient values. 80% < were determined as nuclear localization. (B) Nuclear and cytoplasmic fluorescence intensities of cells. % Values were determined in each time point. (C) Relative fluorescence intensities of Ven/Cry1 protein in different localization of the cell in different time points. ***P < 0.001, ****P < 0.0001

6. DISCUSSION

At the end of the twentieth century, molecular biologist completed genome sequence analysis of many organisms and they started to investigate functions of proteins that expressed from these discovered sequences. Dynamics of a protein including its subcellular localization, interactions, bindings, roads inside the cell and mobility represent tremendous information from the beginning of its expression to the degradation. All of these can be well-studied by using advances of fluorescence proteins. In this study, we labelled the selected clock proteins with fluorescence proteins in live cells to follow their behavior like in vivo. In this purpose, we generate two cell lines have an ability to express fluorescent protein tagged BMAL1 and CRY1 proteins from their native promoter regions. By this way, we provide an studying tool for the investigation of BMAL1 and CRY1 dynamics in further studies both my own and other laboratories.

Circadian clock is one of the crucial regulators of many physiological and chemical events in biological systems. It directly or indirectly associated with many molecular pathways. Its disruption by any reason such as jet lag, old age or shift work are strongly related with many physical and mental problems such as sleep disorders, diabetes, heart disease, depression and cancer. For example, Dr. Korkmaz et al. reported that lack of BMAL1 increases the apoptotic response of breast cancer cells in recent study while Dr. Ozturk suggested that Cryptochrome-deficiency enhances the life span of *p53*-mutant mice. That's why it is important to understand the molecular mechanism of circadian clock and its components. On the other hand, a clear role of these processes from macro to micro-world is still elusive even though many studies have pursued on functions of biological rhythms. For example, there are still gaps in how photic information taken from the retina synchronizes the neuronal cellular clocks and initiates the internal day or the kinetics of the oscillation processes. Although the importance of nuclear import and export signals of clock proteins well established with

recent studies, little is known about their dynamics and effect of their dynamics on circadian oscillation.

In this study, we specifically focused on BMAL1 and CRY1 proteins because their cancer-related studies had been reported with recent studies from our laboratory and we aim to reveal their kinetics and interactions in future studies. Thus, we aimed to generate two biosensor cells to follow the behavior of clock proteins like *in vivo*. These cells have ability to express fluorescence tagged BMAL1 and CRY1 proteins from their own native promoter region. We tried to improve these cells as close as *in vivo* by considering recent studies. For this purpose, two different plasmids had been designed and generated. One of them consist of mouse Bmal1 promoter, mCerulean fluorescent protein sequence and mouse Bmal1 gene sequence while the other has mouse Cryptochrome-1 promoter, intron 336 site, Venus fluorescent protein sequence and mouse Cryptochrome-1 gene sequence respectively. Previous studies demonstrates by in vivo studies that the concentration of BMAL1 protein into cell is low while relatively comparing with CLOCK protein (Choogon Lee et al., 2001). This means that concentration of BMAL1 is a rate limiting effect for the formation of complex and functional activation. Therefore, we improved our BMAL1 and CRY1 stable cells as processing their expression from their native promoter not overexpression vector. In this way, we prevent high abundance of BMAL1 or CRY1 protein into cells which may cause any abnormal results about their dynamics. On the other hand, N-terminal and PAS domain of BMAL1 protein have nuclear localization signal (NLS) which allows its localization into nucleus and nuclear export signals (NES) which blocks its accumulation in nucleus regions respectively. Thus, full-length expression of BMAL1 is also other important point that has to be careful during studying its dynamics (Ilmin Kwon et al., 2006). In another point, Cryptochromes, mCry1 and mCry2 has a repressor role in circadian mechanism. After the translation, they translocate into nucleus to repress their transcriptionally activator CLOCK/BMAL1 complex by binding them. Besides its many regulatory roles, its C-terminal tails are crucial for its nuclear localization. In this process, there is a NLS area between the 274 and 278 amino acid residues of

mammalian CRY1 protein and absence of the region decrease the nuclear accumulation of the protein (Ineˆs Chaves et al., 2006). Thus, the full-length expression of Cry1 protein is also important for its nuclear localization, so fully expression of it tagging with mVenus is provided by the construct.

In the second part of the study, we generated two different stable cell lines by lentiviral transfection of newly improved plasmids. U2OS, human bone osteocarcoma cell line was used for this purpose. This cell line has an ability to oscillate for 4-5 days after resetting and it is also commonly used for live cell imaging due to their large size. Moreover, their high transfection efficiency makes the experiment easier. Both construct were transfected into different cells. Thus, two cell lines with one mCer.Bmal1 and the other mVen.Cry1 expressed were obtained at the end. Limiting dilution method was used as explained at section 4.2.3.3, so homogenous cell populations were developed. According to results, recombinant proteins were observed at the expected size (kDa) and also, cell were shown homogenous. In these stable cells, both recombinant and endogenous homolog proteins are expressed together.

At the last part, some kinds of experiments were performed to understand whether our recombinant can mimic their internal homologs or not. First, protein levels of both recombinant and internal proteins were investigated in a timeline after resetting the circadian clock of the cells by dexamethasone treatment. By this experiment, it was revealed that both of these proteins represent the same oscillation profile. Their lowest protein level was observed at 28th hour and the highest at 40th hour. They has about 24-hour periodicity. Furthermore, interestingly, we obtained that BMAL1 and CRY1 display similar pattern while they are known to be in antiphase in theory. Although the results seem doubtful, this is not the only study that was exhibited the BMAL1 and CRY1 at the same phase. Many studies represent that there is a slight delay between the abundance of BMAL1 and CRY1, so they are not found at antiphase like BMAL1 and PER1 relationship. Ines Chaves suggested that presence of BMAL1 is important for nuclear localization of CRY1. Secondly, nucleocytoplasmic

distributions of clock proteins were observed at determined timepoints (28h & 40h) by live cell imaging with confocal microscopy. Also, quantitation of subcellular localization and mean intensity were analyzed. As a result, Bmal1 predominantly localize in nucleus at 40^{th} hour or cytoplasm at 28^{th} hour. It is well known that BMAL1 localization is strongly related with its post-translational modifications. For example, hyperphosphorylated BMAL1 localize at nucleus, not in cytoplasm. However, Cry1 localization does not depend on these timepoints. In previous studies, Cry1 was commonly observed at both nucleus and cytoplasm even though there are many studies suggested predominantly localization of mCry1 in nucleus at specific time points. For example, Choogon Lee et al claimed that mCry1 and mCry2 are expressed at high level in cytoplasm and generally exist in there, so their regulation occurs in nuclear import level. When compare the cytoplasmic and nuclear intensities of mCry1 in different time points, it is conclude that cytoplasmic mCry1 remains constant while nuclear accumulation differs according to its protein level. However, investigation of nuclear and cytoplasmic protein levels of both of these proteins in a timeline will give a clear result about their localization.

Although subcellular localization of CRY1 and BMAL1 and their movement regulation by interactions and posttranslational modification were all studied in previously studies, the common problem is the usage of cells that transiently express GFP tagged clock proteins or overexpression of the proteins. However, these systems does not display the really truth feature of clock proteins whereas localization of a protein depends on many different parameters. . The core loop of circadian clock is completely dependent on correct expression pattern of CRY1. The oscillation pattern might be disrupted under the overexpression or lack of it. On the other hand, CRY1 has a delayed gene expression pattern while comparing with others (Ueda et al., 2005). The importance and requirement of the delaying motif for the maintenance of circadian clock oscillation was well-explained by the Ueda's group (Ueda et al, 2011). Thus, the vector including Cry1 intron 336 on the downstream of mouse promoter sequence was used in this study. By this way, expression pattern of our mVenus-tagged

Cry1 protein can be delayed in stable U2OS cells. Here, we successfully generate two cell lines that express recombinant clock proteins which are BMAL1 and CRY1 from their native promoter sequence. Thus, it is expected that these proteins behave as close as to endogenous clock proteins. As a result of confirmation experiments, it is claimed that our recombinant proteins clearly mimic their internal homologs like *in vivo*. They will be used as a screening tool for interactions of or to understand the effects of small chemicals or candidate drugs or stress conditions on BMAL1 and CRY1 proteins. Therefore, in the future studies will be performed by our or other research groups, dynamics of the clock proteins can be clearly enlighten by these new tools.

In the follow-up experiments, internal homologs of our recombinant proteins can be knockout because mouse sequences found into our recombinant proteins. Also, by using these cells, photobleaching experiments like FRAP, FLIP can be performed to understand localization rates, mobilities or stabilities of clock proteins. Furthermore, responses of BMAL1 and CRY1 in different time points on different stress conditions such as treatment with DNA-break agents. Additionally, interactions of the proteins in different time points and under different conditions can be observed by immunoprecipitation studies. Fluorescence proteins tagged with clock proteins can be used for this purpose. Moreover, the interaction of mammalian clock proteins for the first time in different timeline with microscopy can be done with generation of double stable cells which have an ability to express both of our recombinant proteins. Because fluorescence proteins which are mCerulean and mVenus are available for FRET experiments. In conclusion, cells produced by this study provide large opportunities in future experiment for high throughput purposes and investigation of dynamics and interactions of clock proteins.

REFERENCES

[1] Sancar A., (2004) "Regulation of the Mammalian Circadian Clock by Cryptochrome," The Journal of Biological Chemistry, 279, (13), 34079–34082.

[2] Reppert S. M., Weaver D. R., (2002) "Coordination of circadian timing in mammals," NATURE, 418, (29), 935-941.

[3] Partch C. L., Green C. B., Takahashi J.S., (2014) "Molecular Architecture of the Mammalian Circadian Clock," Trends Cell Biol, 24, (2), 90-99.

[4] Ueda H., (2007) "Systems biology of mammalian circadian clocks.," Cold Spring Harb Symp Quant Bio, 72, (2), 365-380.

[5] Partch C., (2006) " Posttranslational regulation of the mammalian circadian clock by cryptochrome and protein phosphatase 5," Proc Natl Acad Sci USA, 103, (9), 10467–10472.

[6] Brown S. A., Elzbieta K., Dallmann R., (2012) "(Re)inventing the Circadian Feedback Loop," Cell Press, 22, (13), 477-487.

[7] Storch K. F., (2002) "Extensive and divergent circadian gene expression in liver and heart," Nature, 417, (6884), 78-83

[8] Saurabh S., Paolo S. C., (2009) "Metabolism and cancer:the circadian clock connection," Nature Reviews, 9, (12), 886-896.

[9] Toh KL., (2001) "An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome," Science , 291, (5506), 1040–1043.

[10] Bechtel W., (2015)"Circadian rhythms and mood disorders: are the phenomena and mechanisms causally related," Front. Psychiatry , 6,(118), 419-429 .

[11] Till R., Martha M. (2016) "The Circadian Clock and Human Health," Cell, 26, (10), R432 –R443.

[12] Levi F. E., (2007) " Implications of circadian clocks for the rhythmic delivery of cancer therapeutics.," Adv. Drug Deliv. Rev., 59,(10), 1015-1035.

[13] Kobayashi W. J., (2002) "Circadian chemotherapy for gynecological and genitourinary cancers," Chronobiol International, 19, (1), 237–251.

[14] Schernhammer ES., Laden F., (2001) "'Rotating night shifts and risk of breast cancer in women participatig in the nurses' health study'," Journal of the National Cancer Institute, 93, (20), 1563-8

[15] Ozturk N., (2009) "Loss of cryptochrome reduces cancer risk in p53 mutant mice," PNAS, 106, (8), 2841-6

[16] Song S.H., Ozturk N., (2007) "Formation and function of flavin anion radical in cryptochrome 1 blue-light photoreceptor of monarch butterfly.," J Biol Chem., 282, (24), 176-184

[17] Chacinska, (2009) "Importing mitochondrial proteins: machineries and mechanisms," Cell, 138, (4), 628-644.

[18] Hung M.C., (2011) "Protein localization in disease and therapy," Journal of Cell Science, vol. 124, (20), 3381–3392.

[19] Gingras A.C., (2007) "Analysis of protein complexes using mass spectrometry," Nat. Rev. Mol. Cell Biol., 8(8):645-54

[20] Morin X., Daneman R., (2001) "A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in Drosophila," PNAS, 98(26)-15050-15055

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