

*Full Length Research Paper*

# Key region of laminin receptor 1 for interaction with human period 1

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The 67 kDa laminin receptor 1 (Lamr1) is a novel protein that interacts with human circadian clock protein period 1 (hPer1). We confirmed the interaction between hPer1 and complete Lamr1 (295 amino acids) through yeast two-hybrid system in the present study. And we identified the interaction between hPer1 and hLamr11-190/hLamr1201-295 with yeast two-hybrid system. The results showed that hPer1 could interact with two partial Lamr1, which each contained a laminin-binding region, suggesting that both two partial sequences contained the binding region for hPer1. To define the key region of Lamr1 to interact with hPer1, pGADT7-Rec/hLamr11-190 was mutated with the palindromic sequence LMWWML, part LMW and WML, respectively. With yeast two-hybrid system, we found that hPer1 could not interact with Lamr1 mutated with LMWWML and LMW, but could interact with Lamr1 mutated with WML. It suggested that the palindromic sequence LMWWML in peptide G of Lamr1, especially LMW of it, was necessary for the interaction. Although, the palindromic sequence LMWWML is just the actual binding site for laminin. Together, these findings suggested that hPer1 might interact with Lamr1 by occupying the laminin-binding sites. It will be beneficial for studying the mechanism of hPer1 interaction with Lamr1.

**Key words:** Laminin receptor 1 (Lamr1), human circadian clock protein period 1 (hPer1), interaction, yeast two-hybrid, key region.

## INTRODUCTION

Circadian clocks are molecular time-keeping mechanisms that reside in a diverse range of cell types in a variety of organisms. The primary role of these cell-autonomous clocks is to maintain their own approximately 24 h molecular rhythms and to drive the rhythmic expression of genes involved in physiology, metabolism and behavior (Andretic and Hirsh, 2000; Dunlap, 1999). In mammals, a master circadian clock resides in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus.

The SCN is composed of multiple, single-cell circadian oscillators, which, when synchronized, lead to coordinated circadian outputs that ultimately regulate overt rhythms (Reppert, 2000). On the molecular level, the oscillator consists of an autoregulatory transcriptional-/translational-feedback loop involving a set of clock genes.

Central components of the mammalian circadian clock are the period (Per) genes which are expressed in a diurnal manner in various tissues on the RNA and protein levels (Field et al., 2000; Tei et al., 1997). Human Period 1 (hPer1), expressed widely in brain regions, non-neuronal tissues and cell lines, not only regulates the circadian oscillator but also plays some additional roles, such as drug dependence and tumor development (Andretic et al., 1999; Liu et al., 2005; Filipski et al.,

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**Abbreviations:** SCN, Suprachiasmatic nucleus; hPer1, human circadian clock protein period 1; Lamr1, laminin receptor 1.

2002). As a member of the basic helix-loop-helix (HLH)/Per/Arnt/Sim (PAS) family of proteins, hPer1 also can form multiple heterodimers and their available subunit could directly influence many multiple pathways. Therefore, according to Per1's complex functions, many people speculate that there were some other proteins that interact with Per1. Previous study finds that the 67 kDa laminin receptor 1 (Lamr1) is a novel protein that interacts with human circadian clock protein hPer1 and Lamr1 is not a direct efferent element of circadian clock (Wang et al., 2007). But how does hPer1 interact with Lamr1? We still have not known the key region of Lamr1 to interact with hPer1.

The 67 kDa Lamr1, a membrane-bound mature form of 37 kDa laminin receptor precursor (37LRP), is present on the cell surface and functions as a membrane receptor for the adhesive basement membrane protein laminin (Hundt et al., 2001; Menard et al., 1998). The 67 kDa receptor subunit and its precursor appear to play an important role in several physiologic as well as pathologic processes, including cell differentiation, growth, migration and cancer invasion (Berno et al., 2005; Faury, 1998; Orihuela et al., 2009; Qiu et al., 2008). Peptide G (20 amino acids, IPCNNKGAAHSVGLMWWMLAR), according to the amino acid residues 161-180 of Lamr1, binds specifically with high affinity to laminin (Castronovo et al., 1991; Magnifico et al., 1996; Taraboletti et al., 1993). Another binding site is at the carboxyl terminal (RDPEEIEKKEEQAAAEKAVTKKEEFQG, amino acids 205 - 229) (Gloe et al., 1999; Landowski et al., 1995). Wang et al. (2007) find that the sequence is encoded amino acids from 56 to 295 of *Homo sapiens* Lamr1 including the sequence which is shown to bind laminin. We suspected that hPer1 might interact with Lamr1 by occupying the laminin-binding sites. Because most functions of Per1 were based on protein-protein interaction, we utilized the classical techniques, yeast two-hybrid system and site-directed mutagenesis, to define the key region of Lamr1 to interact with hPer1.

## MATERIALS AND METHODS

### Cell culture

A549 cells (Human lung adenocarcinoma epithelial cell line) were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) supplemented with antibiotics (BioWhittaker, USA) and 10% fetal calf serum (Hyclone, USA) in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C.

### Isolation of the specific DNA fragments of hLamr1

Total RNA of cells was isolated with Trisol reagent (Invitrogen, USA). The specific DNA fragments of hLamr1 were cloned from cellular total RNA by reverse transcription-polymerase chain reaction (RT-PCR) according to the RNA PCR kit manual (TaKaRa, Japan) using hLamr1 gene specific primers based on the hLamr1 cDNA sequence (gi: 59859884). The aimed sequences were encoded amino acids from 1 to 295 (Complete sequence), 1 to 190

and 201 to 295 of hLamr1, respectively. The primer sequences were 5'-GTACATATGATGTCCGGAGCCCTTGAT-3' and 5'-CGCCAGTCTAGATTAAGACCAGTCAGTG-3' for hLamr11-295; 5'-GTACATATGATGTCCGGAGCCCTTGAT-3' and 5'-GCATCTAGAGGAAATGGTGCCACGC-3' for hLamr11-190; 5'-GCGGCAATGCTGTACTTCTACAGAGAT-3' and 5'-CGCCAGTCTAGATTAAGACCAGTCAGTG-3' for hLamr1201-295. To construct the pGADT7-Rec/hLamr11-295, pGADT7-Rec/hLamr11-190 and pGADT7-Rec/hLamr1201-295 plasmids, the specific amplified DNA sequences of hLamr1 were subcloned into pGADT7-Rec prey vectors (BD Biosciences Clontech, USA) which contained the GAL4 activation domain (AD) and their sequences were analyzed, respectively.

### Screening partial Lamr1 interacting with hPer1 with yeast two-hybrid system

To initially investigate the key region of Lamr1 to interact with hPer1, we adopted yeast two-hybrid system. The different parts of hLamr1 were cloned in-frame in the yeast expression vector pGADT7-Rec, respectively. Previously the bait plasmid pGBKT7/hPer1132-497 has been successfully constructed by Wang et al. in our lab (Wang et al., 2007). The HLH-PAS domain of hPer1 cDNA was cloned in-frame in the yeast expression vector pGBKT7, which contained the GAL4 DNA-binding domain (DNA-BD). Then the constructed vector pGBKT7 was introduced into the yeast strain AH109 by lithium acetate transformation. Transformed yeasts were selected on tryptophan-deficient medium. To identify the interaction between hPER1 and LAMR1, we adopted the Matchmaker Library Construction and Screening Kits (BD Biosciences Clontech, USA).

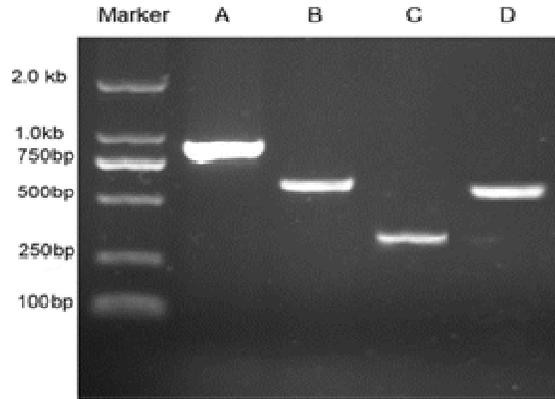
The prey plasmids pGADT7-Rec/hLamr11-295, pGADT7-Rec/hLamr11-190 and pGADT7-Rec/hLamr1201-295 were transformed into the yeast strain AH109 which contained the bait plasmid pGBKT7/hPer1132-497. Because the prey vector pGADT7-Rec carried the leucine gene, it was conferred to have the capacity to grow in leucine-deficient medium. In addition, when bait and prey fusion proteins interact in AH109 yeast reporter strain, the DNA-BD and AD are brought into proximity and activate transcription of ADE2 and HIS3 reporter genes. These two genes allowed the yeast to grow in histidine-deficient medium and adenine-deficient medium. The positive clones were selected by -Ade/-His/-Leu/-Trp dropout (DO) supplement yeast selection medium (BD Biosciences Clontech, USA).

### Colony-lift filter β-galactosidase assay

Besides ADE2 and HIS3 genes, lacZ is also a typical reporter gene in AH109 yeast strain to identify protein-protein interaction. And it could create a blue stain in the presence of 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal). X-gal must be used as the β-galactosidase substrate for the colony-lift filter β-galactosidase assay. Transformants to be assayed were lifted onto a Nitrocellulose filter (Pall Gelman, USA). Then the filter was treated with at least one freeze/thaw cycle in liquid nitrogen to lyse the yeast cell walls. Freeze-thaw cycles are a rapid and effective cell lysis method which permits accurate quantification of β-galactosidase activity. Carefully place the filter, colony side up, on the filter presoaked with Z buffer/X-gal solution (Sigma-Aldrich, USA). Incubate the filters at 30°C (or room temperature) and check periodically for the appearance of blue colonies.

### Site-directed mutations in partial Lamr1

To further investigate the key region of Lamr1 to interact with hPer1, several site-directed mutations were induced in pGADT7-



**Figure 1.** Expression of hLamr1 with RT-PCR. Complete sequence and two partial sequences targeting Homo sapiens Lamr1 were cloned from total RNA of A549 cells by RT-PCR. The electropherogram showed the expression of three kinds of Lamr1 and the housekeeping gene GAPDH. GAPDH was used as the internal control. Marker: DL2000 DNA Marker; A: Lamr11-295; B: Lamr11-190; C: Lamr11-295; D: GAPDH.

Rec/hLamr11-190. Because of too many mutated sites, we selected two suitable single restriction enzyme cutting sites *Dra* III and *Xba* I (New England Biolabs, USA), and then cut the partial sequence which contained the amino acids to be mutated from pGADT7-Rec/hLamr11-190 plasmid with the two sites. While the different DNA sequences which contained the different mutations were synthesized and ligated to the cut plasmid DNA ligation Kit Ver.2.1 (TaKaRa, Japan). The ligation products were transformed into *Escherichia coli* DH5a host cells, respectively. Three kinds of mutant plasmids were obtained: a. AD/Lamr1-LL: the mutation of LMWWML (amino acid residues 173 -178); b. AD/Lamr1-LMW: the mutation of LMW (amino acid residues 173 - 175); c. AD/Lamr1-WML: the mutation of the WML (amino acid residues of 176 - 178). The sequences of these mutant plasmids were analyzed. The mutated sites are listed below: 173Aa TTA (Leucine, L) →TTC (Phenylalanine, F); 174Aa ATG (Methionine, M)→ACG (Threonine, T); 175Aa TGG(Tryptophan, W)→TCG (Serine, S); 176Aa TGG(Tryptophan, W)→TGC (Cysteine, C); 177Aa ATG (Methionine, M) →ACG 530bp(Threonine, T); 178 CTG (Leucine, L) →CCG (Proline, P).

#### Screening key region of Lamr1 to interact with hPer1 and yeast two-hybrid system

The interaction between hPer1 and mutant Lamr1 was identified by yeast two-hybrid system. Three kinds of mutant plasmids AD/Lamr1-LL, AD/Lamr1-LMW and AD/Lamr1-WML were transformed into the yeast strain AH109 which contained the bait plasmid pGBKT7/hPer1132-497. The positive clones with reporter genes ADE2, HIS3 and lacZ were selected by -Ade/-His/-Leu/-Trp dropout (DO) supplement yeast selection medium and colony-lift filter  $\beta$ -galactosidase assay.

## RESULTS

### Confirmation of the interaction between hPer1 and Lamr1

To further confirm the interaction between hPer1PAS and

Lamr1, complete sequence of hLamr1 encoded by 295 amino acids was cloned from total RNA of A549 cells by RT-PCR (Figure 1A). The prey plasmid pGADT7-Rec/hLamr11-295 was constructed successfully. DNA sequence analyses showed that the DNA sequence of the insert fragment was completely homologous with that of Homo sapiens Lamr1 (gi: 59859884). The interaction between hPer1 and Lamr1 was further confirmed with yeast two-hybrid system. Clones containing bHLH-PAS domain of hPer1 and hLamr1 could be seen in SD (synthetic dropout) -Leu/-Trp and SD-Ade/-His/-Leu/-Trp selection mediums (Figure 2a). The blue stain was also obviously observed in colony-lift filter  $\beta$ -galactosidase assay (Figure 3a). These results confirmed that Lamr1 could interact with hPer1 definitely.

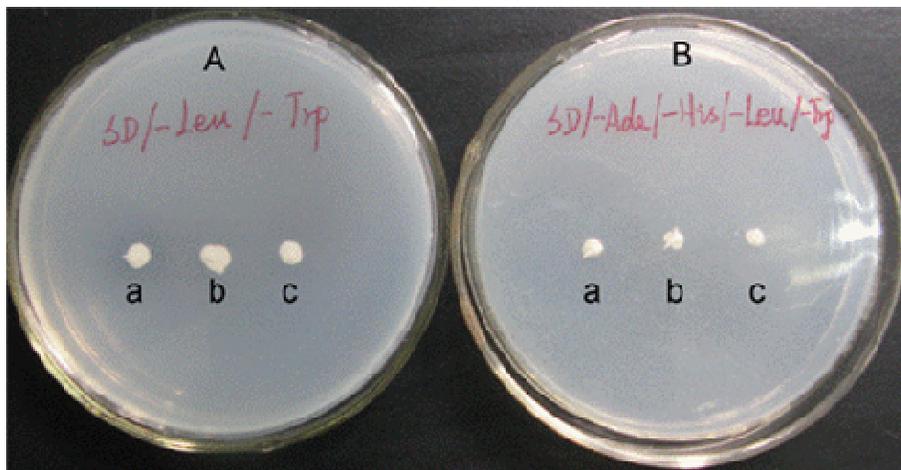
### Identification of two partial Lamr1 interacting with hPer1

To initially investigate the key region of Lamr1 to interact with hPer1, two partial DNA fragments of hLamr1, each containing one interaction region between laminin and Lamr1, were isolated from total RNA of A549 cells by RT-PCR (Figure 1). The specific DNA sequences of hLamr1 were subcloned into pGADT7-Rec prey vectors and their sequences were analyzed. The amino acid sequences of the two partial hLamr1 were identical with that of hLamr1 (amino acid residue: 1-190 and 201-295). With yeast two-hybrid system, both clones contained hPer1 PAS and hLamr11-190 and clones contained hPer1PAS and hLamr1201-295 could grow in SD/-Leu/-Trp and SD-Ade/-His/-Leu/-Trp selection mediums (Figure 2). And the colony-lift filter  $\beta$ -galactosidase assay also showed the same results. The results revealed that both two partial Lamr1 could interact with hPer1.

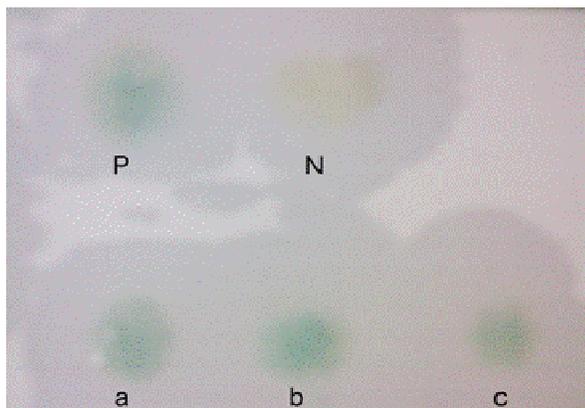
### Definition of the key region of Lamr1 to interact with hPer1

To further study the key region of Lamr1 to interact with hPer1, three kinds of different site-directed mutated pGADT7-Rec/hLamr11-190 plasmids were constructed. Their sequences were analyzed. All the mutated sites were just the mutations we needed, and other sequences were identical with the origin. The palindromic sequence LMWWML (amino acid residues 173 -178) of hLamr1 was mutated to FTSCPT in AD/Lamr1-LL, LMW (amino acid residues 173 -175) were mutated to FTS in AD/Lamr1-LMW, and WML (amino acid residues 176 -178) were mutated to CTP in AD/Lamr1-WML (Figure 4).

The interaction between hPer1 and mutant Lamr11-190 were identified with yeast two-hybrid system. The results showed that the clones contained hPer1PAS and Lamr1-LL also hPer1 and Lamr1-LMW which could grow in SD/-Leu/-Trp selection mediums, but not in SD-Ade/-His/-Leu/-Trp mediums. However, the clones contained



**Figure 2.** hPer1-Lamr1 interacting detection on SD selection mediums. The interaction between hPer1 and three kinds of Lamr1 were identified with yeast two-hybrid system. Positive clones were grown in SD/-Leu/-Trp (A) and SD/-Ade/-His/-Leu/-Trp (B) selection mediums. Three kinds of clones which contained hPer1 PAS and hLamr11-295 /hLamr11-190/ hLamr1201-295 could grow both in SD/ -Leu/-Trp and SD-Ade/-His/-Leu/-Trp selection mediums. The results revealed that all complete Lamr1 and two partial Lamr1 could interact with hPer1. a: hPer1+Lamr11-295; b: hPer1+Lamr11-190; c: hPer1+Lamr11-295.



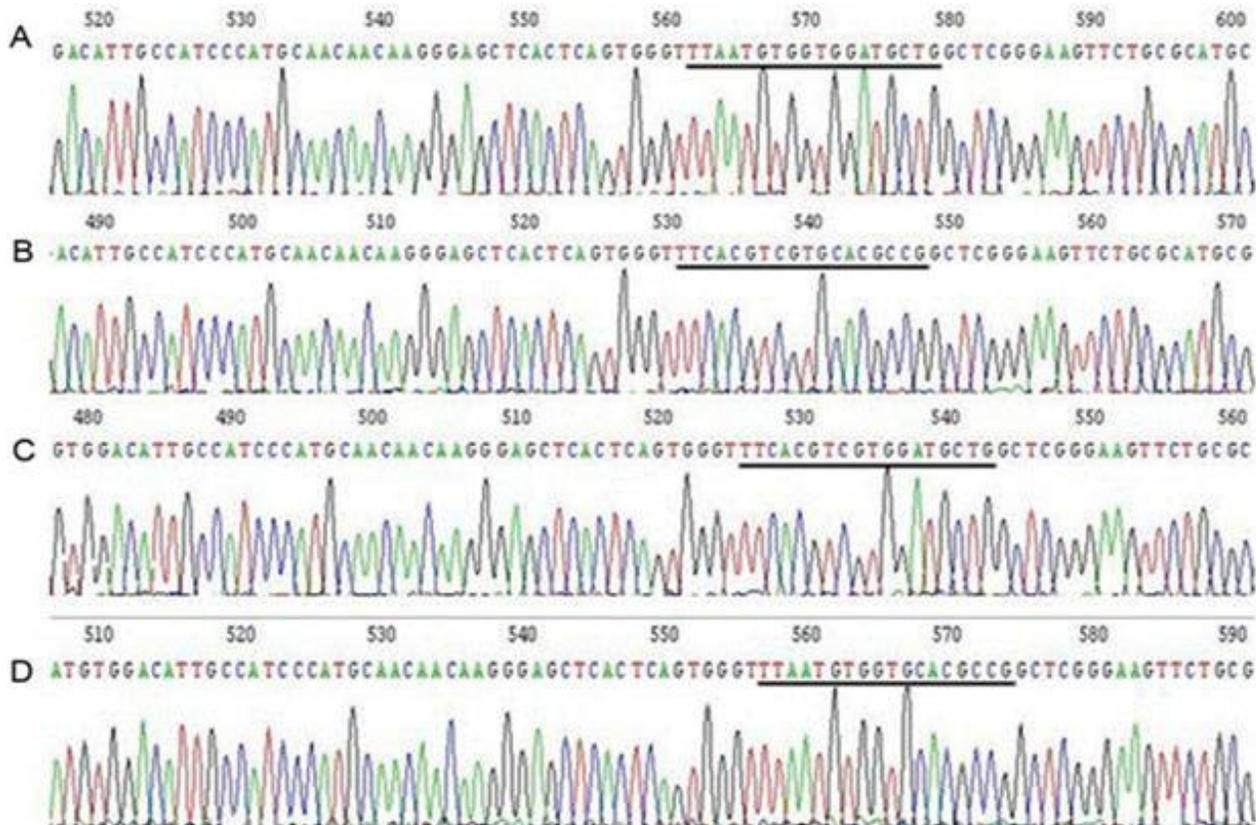
**Figure 3.** hPer1-Lamr1 interacting detection with colony-lift filter  $\beta$ -galactosidase assay. Colony-lift filter  $\beta$ -galactosidase assay was used to detect the expression of lacZ reporter gene to identify protein-protein interaction. Three kinds of clones which contained hPer1 PAS and hLamr11-295 /hLamr11-190/ hLamr1201-295 performed blue stains in the presence of X-gal, which suggested that all complete Lamr1 and two partial Lamr1 could interact with hPer1. P: Positive Control; N: Negative Control; a: hPer1+Lamr11-295; b: hPer1+Lamr11-190; c: hPer1+Lamr11-295.

hPer1PAS and Lamr1-WML which could grow in both kinds of selection mediums (Figure 5). And the colony-lift filter  $\beta$ -galactosidase assay also revealed that the clones contained hPer1PAS and Lamr1-WML which performed the positive results, while the other two were negative

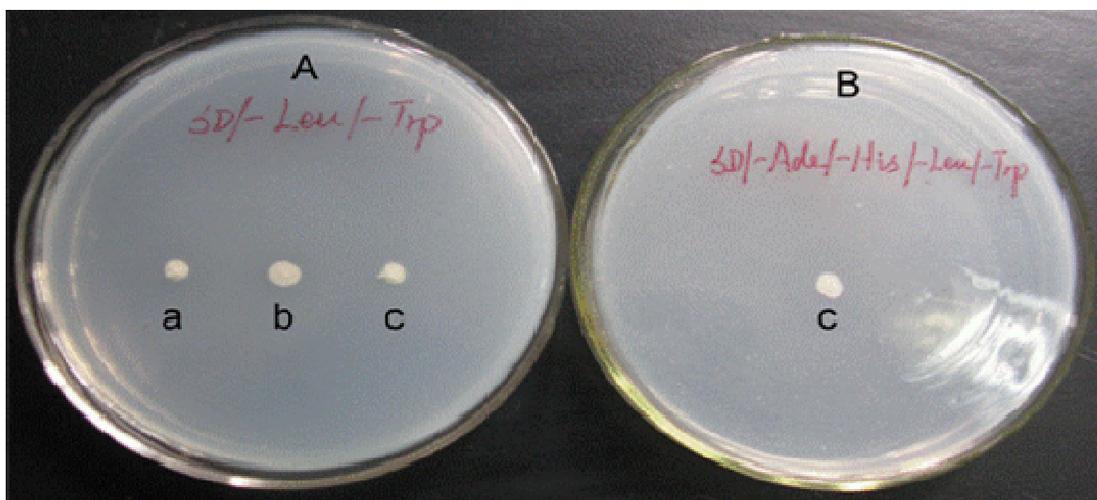
(Figure 6). The results indicated that Lamr1-WML could interact with hPer1, but Lamr1-LL and Lamr1-LMW could not. It suggested that the palindromic sequence LMWWML in peptide G of Lamr1, especially LMW of it, was necessary to the interaction between hPer1 and Lamr1.

## DISCUSSION

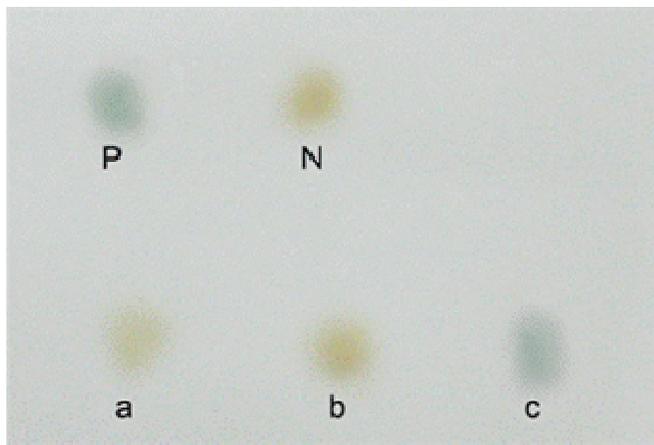
Circadian rhythms are the overt consequences of biological clocks – endogenous timers acting within cells. At the molecular level, circadian clocks are constructed from ‘clock genes’, some of which encode proteins able to feedback and inhibit their own transcription (Dunlap, 1999). The mammalian clock system is hierarchical, with a master clock located within the neurons of the supra-chiasmatic nucleus (SCN) in the hypothalamus. The SCN receives signals from the environment and provides the principal timing cues for synchronizing the daily oscillations in peripheral tissues (Cermakian and Sassone-Corsi, 2000; Panda et al., 2002). The components of the mammalian circadian oscillator involve a complex transcriptional feedback circuit of three period genes (per1, per2 and per3), two cryptochrome genes (cry1 and cry2), a clock gene (clk) and the gene encoding brain-muscle Arnt-like protein 1 (bmal1) (Cermakian and Sassone-Corsi, 2000; Allada et al., 2001; Young and Kay, 2001). As a mammalian ortholog of the drosophila period gene, human period genes (hPer1, hPer2 and hPer3) are considered to be an important component in the mechanism of circadian rhythm (Hida et al., 2000; Shearman et



**Figure 4.** Sequence analysis of mutant plasmids. The DNA sequences of three kinds of mutant plasmids: AD/Lamr1-LL, AD/Lamr1-LMW and AD/Lamr1-WML were analyzed. Sequencing of pGADT7-Rec/hLamr11-190 was performed as control (A). With the mutation, the palindromic sequence LMWWML of hLamr1 was observed as FTSCPT in AD/Lamr1-LL (B), LMW as FTS in AD/Lamr1-LMW (C), and WML as CTP in AD/Lamr1-WML (D). The bars showed the aimed sequences. All the mutate sites were just the mutation we needed, and other sequences were identical with the origin.



**Figure 5.** hPer1-mutant Lamr1 interacting detection on SD selection mediums. The interaction between hPer1 and three kinds of mutant Lamr1 were identified with yeast two-hybrid system. Positive clones were grown in SD/-Leu/-Trp (A) and SD/-Ade/-His/-Leu/-Trp (B) selection mediums. The clones that contained hPer1PAS and Lamr1-LL (a) and the clones that contained hPer1 and Lamr1-LMW (b) could grow in SD/-Leu/-Trp selection mediums, but not in SD/-Ade/-His/-Leu/-Trp mediums. However, the clones containing hPer1PAS and Lamr1-WML (c) could grow in both two kinds of selection mediums. The results indicated that Lamr1-WML could interact with hPer1, but Lamr1-LL and Lamr1-LMW could not. a: hPer1+ Lamr1-LL; b: hPer1+ Lamr1-LMW; c: hPer1+ Lamr1-WML.



**Figure 6.** hPer1-mutant Lamr1 interacting detection with colony-lift filter  $\beta$ -galactosidase assay. The clones that contained hPer1PAS and Lamr1-WML (c) performed blue stains in the presence of X-gal, whereas the clones that contained hPer1PAS and Lamr1-LL (a) and the clones containing hPer1 and Lamr1-LMW (b) did not. It revealed that Lamr1-WML could interact with hPer1, but Lamr1-LL and Lamr1-LMW could not. P: Positive Control; N: Negative Control; a: hPer1+ Lamr1-LL; b: hPer1+ Lamr1-LMW; c: hPer1+ Lamr1-WML.

al., 1997). Besides circadian function, Per1 seems to involve many other functions, such as drug dependence and tumor development (Andreic et al., 1999; Liu et al., 2005; Filipski et al., 2002). Because of its complex functions, Per1 may interact with more other proteins to involve intricate functions.

Previous study by Wang et al. finds that hPer1PAS can interact with Lamr1 through yeast two-hybrid system and co-immunoprecipitation analysis, and Lamr1 transcription cannot be influenced by hPer1 (Wang et al., 2007). Lamr1 is a nonintegrin cell surface receptor that mediates high-affinity interactions between cells and laminin. It is found to be widely expressed in most brain, spinal cord neurons, lung, heart, liver, skeletal muscles, thymus, spleen, kidney, intestine and aorta, and to be particularly abundant on the cancer cell surface (Asano et al., 2004; Sobel, 1993). The 67 kDa receptor subunit and its precursor 37LRP appear to play an important role in several physiologic as well as pathologic processes, including cell differentiation, growth, migration and cancer invasion (Faury, 1998; Berno et al., 2005; Qiu et al., 2008; Orihuela et al., 2009). Previous studies show that peptide G (amino acid residues 161-180 of Lamr1) had a high affinity for laminin and Lamr1 binds to laminin, at least in part, via an amino acid sequence contained within that peptide (Castronovo et al., 1991; Magnifico et al., 1996; Tarabozetti et al., 1993). And the evolutionary analysis of the sequence identified as the laminin-binding site in the human protein suggests that the acquisition of the laminin-binding capability is linked to the palindromic sequence LMWWML (Ardini et al., 1998). Another binding site is at the carboxyl terminal (amino acids 205–229),

which binds to the peptide YIGSR on  $\beta$ 1 chain of laminin (Gloe et al., 1999; Landowski et al., 1995). Yeast two-hybrid system is a typical method to identify novel protein-protein interactions, confirm suspected interactions, and define interacting domains *in vivo*. The interactive sequence of Lamr1 to hPer1 is initially identified with yeast two-hybrid system by Wang et al. (2007). They find that the sequence was encoded amino acids from 56 to 295 of Homo sapiens Lamr1 including the sequence which was shown to bind laminin. In the present study, we confirmed the interaction between hPer1 and complete Lamr1 through yeast two-hybrid system. And they found that hPer1 could interact with two partial Lamr1, in which one contained peptide G and the other contained another laminin-binding region. It demonstrated that both two partial sequences contained the interaction region of Lamr1 with hPer1. However, the actual binding sites are not clearly identified.

To further define the key region of Lamr1 to interact with hPer1, we investigated the interaction between hPer1 and Lamr11 -190 mutated with the palindromic sequence LMWWML. The results revealed that hPer1 could not interact with partial Lamr1 mutated with LMWWML, which suggests that the palindromic sequence LMWWML was the binding region of Lamr1 to interact with hPer1. Then we found that hPer1 also could not interact with Lamr1 mutated with partial sequence LMW, but could interact with Lamr1 mutated with WML, suggesting that the amino acid sequence LMW of Lamr1 was necessary to the interaction. While, the palindromic sequence LMWWML is just the actual binding site for laminin in peptide G of Lamr1. Together, these findings suggested that hPer1 might interact with Lamr1 by occupying the laminin-binding sites. It will be beneficial for studying the mechanism of hPer1 interaction with Lamr1. However, we only proved one binding domain; more regions need to be proved to confirm the conclusion.

With yeast two-hybrid system and site-directed mutagenesis, we found that the palindromic sequence LMWWML of Lamr1, especially LMW of it, was necessary to the interaction of Lamr1 and hPer1. The finding suggested that hPer1 might interact with Lamr1 by occupying the laminin-binding sites. It will provide a new way to study the complex function of hPer1 and Lamr1. For example, the present study may offer a new theory for tumor development and therapy. But more evidence need to be provided in further studies. And though Lamr1 transcription could not be influenced by hPer1, whether hPer1 could affect the function of Lamr1, or whether Lamr1 could influence hPer1 transcription or its function. These problems need to be solved in future research.

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