論 文 の 内 容 の 要 旨
Thesis Summary

論文題目
Chemical synthesis of ubiquitin derivatives to understand the ubiquitin code and its role with nucleosome stability
（ユビキチンコードおよびヌクレオソーム安定性における役割を理解するためのユビキチン誘導体の化学合成）

氏　　名　シヴァニ　ディキシット

In this research we focused on the one of the most important epigenetic modification “ubiquitin”. Ubiquitin is a 76 amino acid long protein of 8.5kDa, which attaches to intracellular proteins through a cascade of the enzyme complex. Ubiquitination is an important recognition mark inducing protein degradation in case of polyubiquitin chain formation and different biological processes in case of mono ubiquitination and thus, affecting every pathway in the eukaryotic cell. Ubiquitin regulates critical functions such as response to inflammation, degradation of proteins via proteasome-mediated degradation or lysosome mediated degradation, DNA repair, and functioning of certain transcription factors.

Ubiquitination involves the bond formation between the C-terminus of Ubiquitin G76 and the ε-amino group of the target lysine residue. The reaction involves a series of enzymes, i.e., an activating enzyme E1 which activates the carboxyl group of ubiquitin by formation of the thiol ester for the nucleophilic attack. The E1 reactions begins with the binding of MgATP and then the ubiquitin forms the ubiquitin adenylate which serves as the intermediate donating the ubiquitin to the active site of E1. The E1 carries two forms of ubiquitin, one is thiol form and other the adenylate form. The thiol form is transferred to the E2, the conjugating enzyme that transfers the activated ubiquitin. Although the number of E1 is less than the E2, the E1 has high catalytic activity in comparison with the E2 enzyme. The E2 binds tightly to the E1-Ubiquitin complex than free ubiquitin and

free E1. E3 ligase plays an important role in transferring the activated ubiquitin from E2 to the target lysine on the substrate. The recognition of the substrate is determined by the sequence or structural motif which is known as the “ubiquitination signals” and is recognised by the E3 ligase. Thus, the E3 ligase plays an important role in guiding the ubiquitination process towards the target substrate. The E3 ligase has two main families which are known so far: Ubox/RING(Really Interesting New Gene)- and HECT(Homologous to the E6-AP Carboxyl Terminus). These families are categorised on the basis of the domain present in the protein to conduct the ligase function. Many diseases are associated with the miss regulation of these enzyme cascade. There are two mechanism for the ubiquitin assembly formation: 1. Sequential model. This is the most common and predominant method. According to this method, only one ubiquitin molecule is transferred at a time to the substrate molecule. 2. En-bloc model. According to this method a preassembled complex of ubiquitin chains on E2/E3 is transferred to the substrate. Mass spectrometry could be used to determine the ubiquitin chain assembly pathway on the target substrate. The mechanisms of ubiquitin chain assembly contribute in several factors such as determining the rate of proteasome mediated degradation. The rate of substrate undergoing proteasome mediated degradation is determined by the rate of attachment of first ubiquitin molecule to the substrate.

To study ubiquitination and its role, it is necessary to synthesis enough amount of each ubiquitin chain. Enzymatic method for production of ubiquitin chain is difficult as all the enzyme known for each ubiquitin chains are not known and it is difficult to control polyubiquitination and isolate enough amount of each chains. The NCL was introduced by Dawson and co-worker in 1994. The first chemical synthesis of ubiquitin was conducted by Briand *et al.* and Ramage *et al.* Using the chemical synthesis method ubiquitin can be conjugated with a small peptide such as Human influenza hemagglutinin (HA) or biotin and can be used for identification of new target substrate or enzymes. Many fluorogenic substrate have been conjugated to the ubiquitin using the chemical synthesis method for the identification of DUBs, understanding enzyme kinetics, identification of inhibitor molecules, synthesis of controlled ubiquitin chains, site specific incorporation of thiolysine.

To assist in the native isopeptide backbone formation some group have used glycyl auxiliary, δ-mercaptolysine analogue or γ-mercaptolysine analogue. These analogues provide advantage of chemical synthesis over biological expression as they generate the native isopeptide bond which is difficult to obtain for biologically expressed derivatives. Using δ-mercaptolysine analogue Brik group is successful in the synthesis of K48 linked tetra Ubiquitin α-Syn. Studies have been conducted to increase the length in a controlled

manner by using a less reactive alkylthioester for ligation. The major drawback of this method for synthesis of long ubiquitin is slow efficiency of the reaction.

Glycyl auxiliary having thiol group are the most often used analogue for the synthesis of ubiquitin moieties using the native chemical ligation. Using this auxiliary allows the site-specific ubiquitination with the formation of native isopeptide bond. The auxiliary deprotection can be achieved by either irradiation of light in case of photocleavable auxiliary or by treatment of acid in case of glycyl auxiliary.

For this study we used the above-mentioned methods for the chemical synthesis of different ubiquitin derivative. These derivatives mimic the different ubiquitin chains as homogenous chains and heterogenous chains. The homogenous chains have the attachment site of next chain at a particular lysine. While, the heterogenous chains have the attachment site at different lysine leading to a branched structure. In the previous method the ubiquitin chains after been isolated from cells are identified using the UbiCREST, UbFAST and other methods. However, these methods cannot differentiate between the linear or branched chains. Therefore, the main aim of this study is to develop a method which can be used for the identification of the linear and branched chain and also enable the quantification of different chains. For doing so we used the method of native chemical ligation for incorporation of the modification at the desired position. As ubiquitin chains after been isolated from cells are treated with trypsin, which cleaves the bond between Arg and Lys. After that the MS analysis is carried out. However, doing so will lead to loss of chain topology. Therefore, we aim to generate ubiquitin derivatives with the intact chain structure. The modification in this study is the signature “GG-motif” left on fragments after trypsin cleavage.

After the successful chemical synthesis of the ubiquitin chains bearing the “GG-motif” at the target positions mimicking as the ubiquitin chains with the linear and branched chains. After the successful synthesis we conducted the MS analysis which confirmed the successful synthesis of the target molecules. These molecules can be used for conducting Quantification study.

Using the above-mentioned synthesis scheme for synthesis of another derivative of ubiquitin was synthesized, the ubiquitinated histone. The target substrate for the current synthesis is H2AK119ubiquitin. H2A ubiquitin comprises approximately ~10% of the total ubiquitinated histone. This modification plays an important role in gene repression. In this study we used glycyl auxiliary to be conducted chemical synthesis of ubiquitinated histones. After the successful synthesis of ubiquitinated histone the thermal stability of nucleosome is checked.