

## 論文の内容の要旨

論文題目      Development of an intrabody selection platform in mammalian cells  
(哺乳動物細胞での細胞内抗体選択プラットフォームの開発)

Name: Thuy Duong Nguyen

氏    名: トウイ    ズオン    グエン

Intracellular antibody (intrabody), an antibody that can function intracellularly, is a promising tool for analyzing protein functions and therapeutic use. However, since the reducing cytoplasmic environment generally leads to low antibody stability, it has been very challenging to generate effective intrabodies and the current methods of intrabody generation have poor efficacy and remain a lot of issues. In conventional methods for intrabody selection based on antigen-binding *in vitro*, scFvs can be obtained from hybridoma monoclonal antibodies by PCR, otherwise scFvs specific for a particular protein can be selected from phage display, bacterial, yeast, and ribosome displays. However, most of the selected scFvs from these methods did not function in cytoplasm since their conformation change due to the loss of disulfide bonds. Therefore direct *in vivo* selection method of intrabodies is required. Regarding to this, there are two approaches for selecting intrabodies *in vivo*: phenotype-directed and target-directed selections. Overall, in both approaches, antibody expressing cells are selected to isolate functional antibody genes. The phenotype-directed systems in mammalian cells are useful to select functional intrabodies that target specific phenotypes of cells, but the number of cell phenotypes is limited. Additionally, their unknown target antigens require being identified using immunoprecipitation and mass spectrometry. In contrast, the target selected systems are able to select functional intrabodies to known target, but they are often not performed in mammalian cells. Therefore, it would be highly desirable to develop a direct system to select intrabodies against a known specific antigen in mammalian cells.

In this Ph.D. dissertation, I have developed a direct intrabody selection platform in the cytoplasm of mammalian cells against a given antigen. To achieve this, I investigated the following items: i) demonstrating the feasibility of the proposed intrabody selection method, ii) upgrading the response of the engineered protein, and iii) constructing an alternative suicide switch for improving quality of single-chain Fv (scFv) libraries. This Ph.D. dissertation may enable the development of intrabodies for drug discovery as well as academic research.

My idea is to engineer scFv-c-kit as a biosensor for detecting the intracellular antigen - antibody interaction. This biosensor is constructed by linking an scFv fragment and the

cytoplasmic domain of c-kit as a receptor tyrosine kinase. When scFv-c-kit binds to an oligomer antigen, it is activated and ultimately able to transduce a growth signal; therefore I term it as a growth sensor. Detection of growth signals allows selecting functional intrabodies against target antigens. Here, the interleukin-3 (IL-3)-dependent Ba/F3 cells were used as the selection platform. In the culture medium without IL-3, the cells with their own growth signals due to antigen - antibody interaction can be selected as growing cells. In library screening, firstly, the Ba/F3 cell expressing the target antigen was established. The selection flow starts from transducing scFv-c-kit library to Ba/F3 expressing the target antigen. Then, the library screening is performed in the multi-well plates. Thereafter, the growing cells were collected to analyze scFv sequences. Finally, the antigen specificity characterization was performed to determine the specific scFv against the target antigen. I exploited the proposed method to select scFv clones against a rabies virus nucleoprotein from a singly panned phage library in order to demonstrate the feasibility of the method.

After the library transduction, library screening was performed to select cells having scFv-c-kit growth signals. Because growing cells were observed, the genomic DNA was extracted from these cells to identify the scFv genes. Sequence analysis revealed that twelve clones have full-length scFv containing both heavy chain and light chain. In order to characterize these selected scFv clones, I firstly sought to investigate their antigen-dependent growth signals. To do so, the expression vectors encoding these twelve scFv-c-kit clones were introduced to Ba/F3 expressing the target antigen to obtain cells stably co-expressing the antigen and each scFv-c-kit clone. As for negative controls, these scFv-c-kit clones were also introduced to parental Ba/F3 to obtain cells stably expressing each scFv-c-kit clone only. The antigen and scFv-c-kit expression in these cells were confirmed by western blot. The result showed similar expression level of scFv-c-kit for each clone. I next used these cells to examine whether the scFv-c-kit-derived growth signals are antigen-dependent by performing the proliferation assay. The result revealed that one scFv clone (specific clone: S#1) showed antigen-dependent proliferation, and three scFv clones (constitutive clones: C#1, C#2, and C#3) showed antigen-independent proliferation. The other eight clones acted as non-functional clones which did not induce cell proliferation significantly. Furthermore, the result of immunoprecipitation assay showed that in Ba/F3 co-expressing the antigen and S#1-c-kit, the antigen was co-precipitated with scFv-c-kit, indicating that S#1 interacts with the antigen selectively. Surprisingly, in Ba/F3 co-expressing the antigen and C#1-c-kit, the interaction of C#1 and the antigen was also detected. This revealed that at least two antigen-binding specific clones among twelve screened clones were obtained. Collectively, these results demonstrated the feasibility of the proposed selection platform.

However, through the above experiments, I noticed that whereas the growth response of the S#1 growth sensor was consistent with its antigen-specific binding, that of the C#1 growth sensor was not correlated with its antigen binding. It indicated that among selected clones, there were growth sensors of constitutive clones leading to proliferation in the parental

Ba/F3 cells without the existence of the target antigen. It seems that they somehow formed oligomers and transduced growth signals. There are three possible reasons of the target antigen-independent growth signals: i) the scFv-c-kit fusion strategy, ii) intracellular scFv oligomers in the used library, and iii) binding of scFv-c-kit and endogenous oligomer proteins.

I first tried to figure out the problem of scFv-c-kit fusion protein. When scFv-c-kit is expressed from a recombinant synthetic library, a part of scFv-c-kit fusion proteins could be prone to aggregation. Moreover, fusing the scFv fragment to the c-kit cytoplasmic domain could also induce conformation changes of the c-kit domain, and result in constitutive activation of c-kit which is also observed in certain c-kit-related cancers. These possibilities could lead to proliferation without the interaction of scFv and the antigen, resulting in a low signal-to-noise ratio. To overcome this problem, it is necessary to optimize the construction of the scFv-c-kit growth sensor to increase its signal-to-noise ratio.

Here, I attempted to re-engineer the construction of the scFv-c-kit growth sensor by tuning its conformation. One of three flexible linkers ( $G_4S$ ,  $(G_4S)_2$ , and  $(G_4S)_3$ ) was inserted between scFv and c-kit. In this experiment, a rabies virus nucleoprotein was used as the model antigen, and scFv S#1 and C#1 were used as its specific binding clones. The purpose was to determine a linker which can make the growth sensors of both S#1 and C#1 transduce antigen-dependent growth signals. The proliferation assay result for clone S#1 showed that the  $G_4S$  linker maintained the signal-to-noise ratio of the antigen-induced proliferation, while the  $(G_4S)_2$  and  $(G_4S)_3$  linkers deteriorated performance of the growth sensor. Also for clone C#1, inserting the  $G_4S$  linker diminished the noise of the C#1 growth sensor without the antigen and maintained the intensity of the growth signal when binding to the antigen, resulting in its remarkable improvement of the antigen-dependent response. Meanwhile, the  $(G_4S)_2$  and  $(G_4S)_3$  were unable to refine performance of the C#1-c-kit growth sensor significantly. Consequently, I succeeded in refining the performance of the growth sensors by insertion of  $G_4S$  linker.

In the final section, I sought to investigate the problem of scFv libraries which may contain intracellular scFv oligomers and scFvs which could bind to endogenous oligomer proteins. I pointed out a suitable library for the proposed intrabody selection platform. In order to generate the suitable library, I developed an alternative suicide switch to eliminate the scFvs which hinder the selection process. I demonstrated successfully the function of the proposed suicide switch to directly eliminate intracellular scFv oligomers in mammalian cells. Taken together, the efforts for re-engineering a growth sensor and scFv libraries may enable a highly efficient selection platform for intrabodies in the future.

The described intrabody selection platform holds distinctive advantages and novelties over conventional methods. i) The method allows direct selection of soluble intrabodies intracellularly. This platform in the cytoplasm of mammalian cells may be more accurate at mimicking the native state of antigens and antibodies because of the high potential of correct

protein folding and post-translational modifications, which overcomes the problems of selection methods in bacteria and the nucleus of yeast. ii) There is no need to fuse an antigen with a functional protein. This allows a novel selection platform for functional intrabodies against a target antigen with a native structure. iii) The screening procedures are readily performed by removing IL-3 and then seeding cells into multi-well plates. It is easy to isolate potential antigen-specific clones by collecting the growing cells after library screening without labor-intensive panning or sorting.

In this work, an scFv Tomlinson I library as a model was used to isolate intrabodies which bind to RV-N. It is quite surprising that using the growth sensor, two intrabodies against the target antigen were able to be selected, because Tomlinson I library was not originally generated for the purpose of intrabody selection. Even with one round of selection flow, I succeeded in isolating specific scFvs from a library. This might be reasoned that the growth sensor is composed of c-kit, an upstream signaling protein, which is able to amplify the signal due to the signaling cascades. Consequently, the scFv-c-kit growth sensor induces a strong growth signal when turning from the non-activated state to the activated state. This feature greatly contributes to library selection, in which the signals of antigen-binding scFvs should be significantly higher than the signals of non-binding scFvs so that potential antigen-binding scFvs are easily isolated even within one round of selection. If it is unable to isolate specific scFvs with sufficient affinities after one round selection, affinity maturation could be performed to obtain scFvs with higher affinities.

In this dissertation, I also developed a quick assay based on the target antigen-c-kit fusion protein for investigating whether the target antigen forms a homo-oligomer directly in the used Ba/F3 cells, which is one of the requirements for applying to the proposed intrabody selection platform. If the antigen is not expressed in an oligomeric format intracellularly, it can be engineered to form a conditional dimer by fusing the antigen with a dimer-regulated FKBP domain or with a dimer-forming peptide such as leucine zippers. Since this antigen compatible assay is performed in living cells, it can provide *in vivo* evidence of oligomerization, which might be more straightforward for the platform than the evidence only provided from crystal structures. Regardless of the availability of crystal structure information, this *in vivo* assay can facilitate the screening of target proteins for the platform.

There are a lot of disease-related proteins can be targeted by this platform. Pathogens such as viral proteins can be applied to select a specific intrabody which may inhibit the virus replication in host cells as a treatment of infectious diseases. Finding an intrabody which can block that constitutive state of signaling proteins due to the incorrect dimerization in cancers at the protein level allows other functions of the target protein continue to work, leading to a promising treatment with very low side effect. The efficient platform of intrabody generation together with the development of the delivery of intrabodies into the target cells may become a break-through of disease treatment in the near future.