博士論文(要約)

Application of ES cell-derived gastric organoids for the functional analysis of the *Helicobacter pylori* CagA oncoprotein

(ES 細胞由来胃オルガノイドを用いたピロリ菌がんタンパク質 CagA の機能解析)

橋　香奈

博士論文の要約

論文題目 Application of ES cell-derived gastric organoids for the functional analysis of the *Helicobacter pylori* CagA oncoprotein

（ES 細胞由来胃オルガノイドを用いたピロリ菌がんタンパク質 CagA の機能解析）

氏名　橋　香奈

Helicobacter pylori strains producing a bacterial oncoprotein CagA (CagA-positive *H. pylori*) is critically involved in the development of gastric cancer. Delivery of the *H. pylori* CagA protein in host gastric epithelial cells has been thought to play a crucial role in the neoplastic transformation of gastric epithelial cells, as delivered CagA promiscuously interacts with multiple host proteins and thereby exerts pro-oncogenic actions. Upon delivery into gastric epithelial cells, CagA induces deregulated cell proliferation and enhanced cell motility by binding to and thereby deregulating SHP2 (also known as Ptpn11) tyrosine phosphatase, aberrant activation of which has been shown to promote oncogenesis. CagA also binds to PAR1b/MARK2, a member of PAR1/MARK family of serine/threonine kinases, which mediate development and maintenance of cell polarity. The kinase activity of PAR1b is inhibited upon CagA binding, thereby causing disruption of epithelial cell polarity. Since transgenic expression of CagA in mice develops gastrointestinal neoplasias as well as hematologic malignancies, CagA is recognized as the first bacterial oncoprotein to be identified. To elucidate the mechanisms underlying pathogenic/oncogenic CagA actions towards gastric epithelial cells, numerous *in vitro* and *in vivo* studies have extensively been conducted. However, observations using *in vitro* study models have not always been applicable to *in vivo* infection studies using rodent that do not consistently reconstitute gastric lesions observed in humans. Given this, I sought to establish an organoid model in 3D cultures from mouse ES (mES) cells, which could develop stomach organoid tissue, and thereby investigate the oncogenic action of CagA by infecting CagA-positive *H. pylori*.

Initially, I developed the method to generate mES cell-derived organoids in 26

days, which followed the process of embryonic stomach development. The mES cells differentiated into definitive endoderm through the formation of embryonic body, which differentiated to posterior foregut. Posterior foregut was then developed into organoids in 3D matrigel culture with EGF-based medium. Hematoxylin-Eosin (HE) staining as well as immunostaining showed that the organoids comprised three distinct components; epithelial cell monolayer, mesenchymal cells, and muscularis. Epithelial cells produced gastric mucin, mucin5Ac and mucin 6. Microarray analysis of mRNA expression also indicated that the obtained organoids acquired properties of gastric tissue. The results of the study indicated that the organoids generated in this system were stomach-like organoids.

To apply the resulting organoids for *in vitro* infection with the gastric pathogen *H. pylori,* either CagA-positive or CagA-negative *H. pylori* strain was microinjected inside the organoids as the epithelial monolayer is formed as an inner face. As a result, I consistently observed massive destruction of the epithelial monolayer in organoids upon injection with CagA-positive, but not CagA-negative, *H. pylori* into the organoids. Immunostaining analysis revealed that baso-lateral membrane staining of E-cadherin was markedly diminished along polarized epithelial monolayer in CagA-positive *H. pylori* infected organoids. Such abnormality in membrane staining of E-cadherin was never observed when CagA-negative *H. pylori* was injected or mock infection was performed. Microarray analysis was then conducted to investigate whether the delivered CagA was capable of altering mRNA expression in the stomach-like organoid cells. Results of the experiment indicated that several genes were specifically up-regulated upon CagA-positive *H. pylori* infection when compared with CagA-negative *H. pylori* infection. Those genes included epithelial cell adhesion molecule (EPCAM) and matrix metallopeptidase 3 (MMP3), up-regulation of which has been reported in gastric cancer as well as breast cancer and lung cancer. Additionally, 5 of known c-Myc target genes, E2F2, Lztfl1, Eif2s1, Kpnb1 and Qrsf1, were also up-regulated, which may indicate that CagA gives rise to c-Myc activation. These results further suggested that stomach-like organoids could provide a unique experimental system in spatiotemporally investigating the effects of delivered *H. pylori* CagA in gastric epithelial cells.

The C-terminal region of CagA possesses the Glu-Pro-Ile-Tyr-Ala (EPIYA) repeat region that contains variable numbers of the EPIYA tyrosine-phosphorylation motifs and the 16-amino-acid CagA multimerization (CM) sequences. *H. pylori* is

known to be quasi-species highly diverged in their genomes among distinct *H. pylori* strains. Especially, CagA is extremely polymorphic at the C-terminal EPIYA repeat region. Hence, structural analysis of the CagA EPIYA region is important in evaluating the magnitude of their binding to SHP2 and PAR1b, which may predict the degree of pathogenic CagA action. Based on the EPIYA region polymorphism, *H. pylori* CagA is geographically sub-classified in several groups such as; East Asian type, Western type, Africa type and Amerindian type, on the basis of their structural diversity at the EPIYA region. Among these, pathophysiological activities of Western and East Asian CagA have been extensively investigated, whereas little is known for Amerindian and African CagA species.

The strain v225d is the *H. pylori* strain isolated from a gastritis patient of Amerindians in Venezuelan Piaroa region where have been separated from civilization for more than 15,000 years. The v225d-derived CagA (v225d CagA) is classified as Amerindian type based on its unique structural variation in the EPIYA region of CagA. The most distinguishable feature of v225d CagA is the disruption of the CM sequence by insertion of EPIYA segment. To examine the strength of pathogenicity associated with the *H. pylori* v225d strain, biological activity of v225d CagA was investigated by concentrating on its functions mediated through the EPIYA repeat region. Consequently, v225d CagA was found to retain the ability to bind SHP2 with EPIYA tyrosine phosphorylation, but almost completely failed to interact with PAR1b.

Although human appears to be the only natural infection hosts of *H. pylori*, the bacterial pathogen can infect several animals other than human. For instance, *H. pylori* has been detected from non-human primates, house cats or pet dogs, most probably infected from human carriers. *Rhesus macaque* has been used as an animal model of *H. pylori* infection, such as cohort study, or finding methods for *H. pylori* detection. Even CagA-positive *H. pylori* strains were detected from *R. macaque*, it has not known if those *cagA* genes and their CagA products are functional and retain pathogenic actions. Accordingly, in this study, I fully sequenced the *cagA* genes of *H. pylori* isolated from the stomachs of *R. macaques* that had independently been housed in Primate Research Institute, Kyoto University. Phylogenic analysis showed that *R. macaque* derived CagA proteins (RM CagA proteins) were genetically distant from any of those found in *H. pylori* strains previously isolated from humans. Furthermore, its EPIYA repeat region had EPIYA segments that were substantially diverged from those

currently reported *H. pylori* strains isolated from humans. To investigate its pathophysiological action, an RM CagA expression vector was constructed and transfected into AGS cells. Co-immunoprecipitation experiment showed that RM CagA was capable of binding with SHP2 via the functional EPIYA motif but failed to interact with PAR1b via the CM sequence.

Finally, I wished to utilize the gastric-like organoids in studying the pathophysiological actions of *H. pylori* carrying unique CagA variants such as v225d CagA and RM CagA. To this end, The *H. pylori* v225d strain and an *H. pylori* strain isolated from *R. macaque* (named as TH2099 strain) were microinjected to the lumen of generated organoids. H-E staining experiment revealed that, in contrast to the case of the NCTC11637 strain (a standard western type CagA-positive *H. pylori* strain), that these newly isolated *H. pylori* strains, both of which cannot bind PAR1b, failed to disrupt epithelial monolayers in the stomach-like organoids. Likewise, immunostaining showed that infection with the v225d or TH2099 strain did not induce E-cadherin mislocalization. Taken together, these observations indicated that *H. pylori* strains carrying biologically attenuated forms of CagA, such as failure of PAR1b binding, may have less pro-pathogenic impact on gastric tissue.

In conclusion, this study successfully generated gastric-like organoids from mES cells, which should provide a unique experimental opportunity to spatiotemporally reconstitute *H. pylori* CagA-gastric epithelial cell interaction that underlies the development of mucosal lesions leading to gastric cancer.