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（氏名は必ず自署すること）

海外特別研究員としての１年間の派遣期間について下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）　バック研究所　（　米国　）

2. 研究課題名（和文）
ヒト角膜上皮幹細胞の細胞老化メカニズムの解明

（※研究課題名は申請時のものと違わないように記載すること。）

3. 派遣期間
平成 31 年 4 月 1 日 ～ 令和 3 年 9 月 30 日
（ 914 日間）

4. 受入機関名及び部局名 受入機関名:

受入機関名：Buck Institute for Research on Aging,

受入部局名：Campisi lab

5. 所期の目的の遂行状況及び成果. . . **書式任意（A4 判相当 3 ページ以上、英語で記入も可）**
【記載事項】
・ 研究・調査実施状況及びその成果の発表・関係学会への参加状況等
・ 新型コロナウイルス感染症の影響にかかる特例措置のうち、国内採用開始・採用期間延長・翌年度渡航のいずれかの適用を受けた場合は、当該措置の適用による影響等
（注）「6. 研究発表」以降については様式 10-別紙 1-4 に記入の上、併せて提出すること。

The ocular surface is the part of the visual system that is directly exposed to the environment. The ocular surface comprises the cornea, the first refractive tissue layer, and its surrounding structures, including the conjunctiva and lacrimal glands. The ocular surface evolved to protect the cornea and keep it smooth and wet, a prerequisite for proper eyesight. With age, the ocular surface becomes more vulnerable to external stimuli, as is the case for many other tissues. For example, dry eye disease (DED), which affects millions of people worldwide with varying severity, is a common aging disorder that entails instability of the tear film on the ocular surface. DED has been shown to result in the activation of mitogen-activated protein kinases (MAPKs) and increased secretion of pro-inflammatory cytokines (e.g., interleukin [IL]-1β, tumor necrosis factor [TNF]-α and IL-6), chemokines, and matrix metalloproteinases [MMPs] (e.g., MMP-3 and MMP-9). An even more severe age-related pathology is corneal opacity, with robust angiogenesis in patients with ocular cicatricial pemphigoid (OCP). OCP is an inflammatory autoimmune disorder characterized by chronic, bilateral, progressive scarring and shrinkage of the conjunctiva, accompanied by corneal opacity due to stem cell deficiency at the ocular surface. Ocular surface inflammation promotes conjunctival squamous metaplasia and inflammation-associated barrier dysfunction, leading in OCP progression, resulting in blindness. The first symptoms of OCP usually appear when patients reach their 60s, and worsens in the 70s and 80s.

It is well accepted that chronic inflammation drives many diseases, including both DED and OCP. However, most research has focused on the downstream effects of inflammation, and little has been done to determine its origin during the

development of age-related eye diseases. Cellular senescence is a cell fate in which both intrinsic and extrinsic signals can cause an irreversible cell cycle arrest, accompanied by many phenotypic changes, including an enlarged cell morphology, chromatin reorganization, altered gene expression, increased glycolytic metabolism, and increased production of reactive oxygen species. Further, senescent cells acquire a complex, often pro-inflammatory, secretory phenotype termed the senescence-associated secretory phenotype (SASP); among the molecules often secreted by senescent cells are pro-inflammatory cytokines (e.g., IL-6, IL-8 and IL-1β), chemokines (e.g., CXCL5 and CXCL10) and MMPs (e.g., MMP1, MMP3 and MMP9). These molecules are frequently associated with a variety of age-associated pathologies, including many that are characterized by chronic inflammation. Importantly, senescent cells have been shown to increase with age in many vertebrate species and in most, if not all, tissues examined. Given that DED and OCP are more prevalent in elderly people, cellular senescence is a prime candidate for being a driver of these diseases. Many molecules that are secreted by senescent cells (the SASP) can act in a paracrine manner to alter the organization and function of neighboring non-senescent cells. So far, nothing is known about the potential role of cellular senescence and the SASP at the ocular surface.

We obtained preliminary data showing that ocular surface cells are induced to undergo senescence upon treatment with a DNA damaging agent such as the chemotherapeutic drug doxorubicin (DOXO), and that senescent ocular surface cells (SOCs) secrete pro-inflammatory factors that are part of the SASP expressed by many other senescent cells. These factors include IL-6, MMP-3 and MMP-9. DOXO also caused the expected upregulation of the cell cycle inhibitors $p16^{INK4a}$ (p16) and $p21^{WAF1/Cip1}$ (p21) and down-regulation of the nuclear protein LMNB1, as we previously reported for other cell types induced to senesce.

The ocular surface evolved to protect the cornea and keep it smooth and wet for optimal eyesight. The ocular surface barrier must also maintain transparency. To measure the barrier function of ocular surface cells, we used a volt–ohm meter (EVOM; World Precision Instruments) as we previously reported, and determined whether SOCs could modify the ocular surface barrier. We compared the barrier level of non-senescent corneal epithelial cells with that of SOCs (SOCs and non-senescent cells seeded at a ratio of 3 to 1). With SOCs, the barrier function declined by 20 to 30% compared to control conditions. To begin to decipher the mechanisms behind this disruption, we collected the SASP-containing supernatant of SOCs, and added it to non-senescent corneal epithelial cells. The barrier function again declined, suggesting that some SASP factors secreted by SOCs are responsible for the disruption.

We also examined whether ABT263, a senolytic drug that selectively kills some senescent cells, restores the barrier function upon elimination of senescent cells. We found that the barrier function gradually recovered after 3 days of administration of ABT263, and, after 5 days, the function returned to normal levels. Immunostaining of the treated cell showed re-expression of ZO-1, a tight-junction protein. These data suggest that eliminating SOCs and reduced secretion of SASP factors can improve the barrier function.

We next focused on cell non-autonomous effects of senescent cells, i.e., the potential interaction between these cells and immune cells in changes in the ocular surface microenvironment. Our preliminary results show an increase in infiltrating macrophages at the ocular surface of old mice. Levels of Cd68, a macrophage marker, increased in the conjunctiva and cornea of old (24 months of age) compared to young (4 months of age) mice. We recently obtained preliminary data showing that SOCs not only produce proinflammatory cytokines and MMPs, but also chemokines that can attract immune cells. The expression level of CXCL10, which is involved in

the chemoattraction of monocytes, macrophages, T cells, and NK cells, were significantly elevated in SOCs from the cornea and conjunctiva, along with increased expression levels of these markers in the cornea and conjunctiva of old mice. This suggests that senescent cells could be involved in the infiltration of immune cells, leading to modifications of the ocular surface microenvironment.

Since aging is a significant risk factor for developing many eye diseases, we attempted to obtain preliminary data comparing young (4 months of age) and old (24 months of age) mice. These preliminary data show that more punctate epithelial damage is found on the cornea of old mice compared to young mice. Moreover, in old mice, a significant decrease in goblet cell density in the conjunctiva was observed. Like humans, in some cases, corneal opacity was observed in very old mice (30 months of age). We also examined tear fluid for MMP9, one of the SASP factors expressed by both human and mouse SOCs, and compared protein levels in young and old mice. MMP9 was significantly elevated in tear fluid from old mice. In addition, p16 mRNA levels showed an age-related increase in the cornea, conjunctiva and lacrimal gland, suggesting that senescent cells at the ocular surface contribute to the development of age-related pathologies.

We used our p16-3MR transgenic mice, which allows us to visualize and isolate senescent cells that express the cell cycle inhibitor and tumor suppressor $p16^{INK4a}$. Importantly, it contains a Herpes simplex virus thymidine kinase (HSV-TK), which enables the elimination of p16-expressing senescent cells by the otherwise benign drug ganciclovir (GCV). We induced dry eye by LGE in p16-3MR mice. In young mice, LGE decreased tear secretion and induced punctate corneal damage. However, the eyes of old mice, which express high p16 levels, LGE caused corneal opacity with severe angiogenesis, accompanied by lower tear secretion, which resembles manifestations of OCP. These results suggest that senescent cells can

modify the ocular surface microenvironment, and trigger severe ocular surface phenotypes such as OCP.

We asked whether SOCs that secrete SASP factors drive these ocular phenotypes. GCV enables the killing of p16-expressing senescent cells in p16-3MR transgenic mice. We administered topical GCV to LGE-treated eyes for 2 cycles of 5 days each. After GCV treatment, angiogenesis declined as did corneal epithelial hyperproliferation; PBS treatment did not trigger the same effects, but hyperproliferation of the epithelium still occurred. The SASP factors MMP9 and VEGF, as well as the macrophage marker CD68, increased in LGE-treated eyes, then declined upon GCV treatment. Interestingly, LGE in young mice did not drive macrophage infiltration, suggesting that senescent cells could be responsible for the phenotypic differences observed in young versus old mice after LGE.

Overall, we found that our results broaden our understanding of how SOCs change the ocular surface microenvironment, leading to the pathology of diseases such as DED and OCP.

In the beginning of the pandemic due to COVID-19, the Buck Institute decided to reduce the capacity of working inside the building. Since March 17, 2020, the stay-at-home order has been implemented in the entire San Francisco Bay Area, where the institute is located. All cell culture and mouse experiments were suspended. During this time, I could not do any experiments, but only did administrative work such as writing papers and research grant applications. The Buck institute resumed its activities as of June 2nd. However, the level of research activities had to be limited to 50%, and time shifts among the lab members had to be arranged to adjust the laboratory hours. I resumed breeding mice in July, and then began conducting mouse experiments in October, 2020.