学位論文の要旨

The Roles of Cancer-Associated Fibroblasts in Colorectal Carcinogenesis

（大腸癌発癌における癌関連線維芽細胞の役割）

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【Introduction】

Cancer-associated fibroblasts (CAFs), key constituents of the tumour microenvironment, play a vital role in colorectal carcinogenesis. CAFs contribute to colorectal cancer (CRC) progression via secretion of growth factors, cytokines and pro-angiogenic factors as well as via remodelling of the extracellular matrix. Recent studies have revealed that CAFs are composed of functionally heterogeneous cell populations and either promote or retard tumour growth. Attempts to therapeutically target CAFs have been hampered by our rudimentary understanding of the functions and origins of these heterogeneous cells.

Key growth factors in the intestinal epithelial niche, bone morphogenetic proteins (BMPs), also play a critical role in colorectal cancer progression. However, the crucial proteins regulating stromal BMP balance and the potential application of BMP signalling to manage CRC remain largely unexplored. In this thesis, I first addressed the functional heterogeneity of CAFs involving BMP signalling.

Theoretically, CAFs could arise through at least four non-mutually exclusive mechanisms; proliferation, activation, transdifferentiation and recruitment. However, to our knowledge, no previous CAF studies have comprehensively performed lineage-tracing experiments to track the four possible CAF sources. The cellular origins of CAFs remain to be fully elucidated. Here, the second study in this thesis aimed to identify the origins and contribution of colorectal CAFs associated with poor prognosis.

【Methods】

Using human CRC RNA expression data, we identified CAF-specific factors involved in BMP signalling, then verified and characterized their expression in the CRC stroma by *in situ* hybridization. $Apc^{\Delta/\Delta}$ and $Trp53^{\Delta/\Delta}$ CRC tumoroids and $Apc^{\Delta/\Delta}$, $Trp53^{\Delta/\Delta}$ and $Smad4^{\Delta/\Delta}$ CRC tumoroids were generated using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genome engineering. Conditioned medium from colonic fibroblasts was added to the organoids to assess the BMP-mediated crosstalk between colorectal cancer cells and fibroblasts. We injected the CRC organoids into the portal vein to generate a mouse model of CRC hepatic metastasis. This mouse model was used to test approaches to modify BMP signalling and treat CRC.

To elucidate CAF origins, we used an azoxymethane (AOM)/dextran sulfate sodium (DSS) mouse model of CRC in 5 different fate-mapping mouse lines with 5-bromodeoxyuridine (BrdU) dosing. RNA-sequencing of fluorescence-activated cell sorting (FACS)-purified CRC CAFs was performed to identify a potential

therapeutic target in CAFs. To examine the prognostic significance of the novel stromal target, CRC patient RNA-sequencing data and tissue microarray were used. CRC organoids were injected into the colon of knockout mice to assess the mechanism by which the stromal gene contributes to colorectal tumorigenesis.

【Results】

Using human CRC RNA expression data, I identified Gremlin 1 (*Grem1)* and immunoglobulin superfamily containing leucine-rich repeat *(Islr)* as CAF-specific genes involved in BMP signalling. Functionally, GREM1 and ISLR acted to inhibit and promote BMP signalling, respectively. *GREM1* and *ISLR* marked $ACTA2^{high}$ and $ACTA2^{low}$ colorectal CAFs, respectively. *Grem1* and *Islr* expression were differentially regulated by transforming growth factor-ß (TGF-ß) and the forkhead box L1 (FOXL1) transcription factor, providing an underlying mechanism to explain fibroblast biological dichotomy. In CRC patients, high *GREM1* and *ISLR* expression were associated with poor and favourable survival, respectively. Treatment with a GREM1-neutralizing antibody reduced CRC tumoroid growth and promoted $Lgr5^{+}$ intestinal stem cell differentiation through restored BMP signalling. Similarly, conditioned medium from *Islr*-overexpressing fibroblasts increased BMP signalling, attenuated CRC tumoroid growth and facilitated $Lgr5^{+}$ stem cell differentiation. Finally, adeno-associated virus 8 (AAV8)-mediated delivery of *Islr* to hepatocytes increased BMP signalling and improved mouse survival in our preclinical model of hepatic metastasis.

Next, I examined the origins and contributions of colorectal CAFs. Using five different fate-mapping models with BrdU dosing, this study revealed that half of $ACTA2^{+}$ CAFs emerge through proliferation in the AOM/DSS mouse model of CRC. No $ACTA2^{+}$ CAFs were derived from *Krt19*-lineage epithelial cells or bone marrow-derived cells, indicating no involvement of epithelial-mesenchymal transition and bone marrow recruitment to the tumour in this model. Intestinal pericryptal Leptin receptor $(Lepr)^{+}$ cells were the major origins of the proliferating CAFs. These *Lepr*-lineage CAFs, in turn, express melanoma cell adhesion molecule (MCAM), a CRC stroma-specific marker we identified using RNA-sequencing. High MCAM expression was inversely associated with patient survival in human CRC. In mice, stromal *Mcam* knockout attenuated orthotopically injected colorectal tumoroid growth and improved mouse survival through the altered immune microenvironment.

【Conclusion】

Stromal BMP signalling, inhibited by GREM1 and promoted by ISLR, is

biologically relevant in CRC growth, spread, and survival (Figure 1). This can be therapeutically targeted by novel AAV-directed gene delivery to the liver. By targeting the upstream determinants of mesenchymal expression, such as TGFß and FOXL1, or by targeting the downstream drivers of BMP signalling, such as GREM1 and ISLR, one may identify novel approaches to treat CRC.

Lepr-lineage intestinal stromal cells, resident at the pericryptal base in the normal colon, proliferate in colorectal carcinogenesis to generate $MCAM^+$ CAFs. We also demonstrate that MCAM is an important factor in sculpting the detrimental immune microenvironment responsible for driving colorectal carcinogenesis and the associated poor patient outcome. Preventing the expansion/differentiation of *LEPR*-lineage CAFs or inhibiting MCAM activity could be effective therapeutic approaches for CRC.

These data indicate that targeting these CAF subpopulations could be novel potential therapeutic strategies to inhibit CRC progression.