ウェブサイト公開用

（様式 10）
（海外特別研究員事業）
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海 外 特 別 研 究 員 最 終 報 告 書

独立行政法人 日本学術振興会 理事長　殿

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受 付 番 号　488
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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派遣期間を終了しましたので、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）用務地：　ハイデルベルグ　（　ドイツ連邦共和国　）

2. 研究課題名（和文）※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。
メタボロームとパスウェイ解析を応用した乳癌の脂肪組織浸潤メカニズムの解明

3. 派遣期間：平成　29 年　8 月　1 日　～　平成　31 年　7 月　31 日

4. 受入機関名及び部局名
Joint IDC-Heidelberg Translational Program Unit, Institute for Diabetes and Cancer (IDC)
Helmholtz Zentrum München, German Research Center for Environmental Health,
Professor and Director, Stephan Herzig

5. 所期の目的の遂行状況及び成果…書式任意　**書式任意（A4 判相当 3 ページ以上、英語で記入も可）**

（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）

（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。

In the past decades, a number of prognostic studies have indicated that higher body mass index or diabetic background are associated with worse outcome of disease free survival and overall survival rate among breast cancer patients across countries. However, there is no enough evidence to elucidate the mechanism of tumor formation linking diabetes, partially due to the lack of multi-disciplinary approaches bridging the two different diseases. Indeed, my previous work at Tohoku University indicating a potential regulatory protein of cancer-associated adipocytes (CAA) was limited to the tumor microenvironment in the advanced stage of breast cancer (Mayama et al, *Cancer Sci., 2018;* Sakurai *et al, Breast Can,* 2017). Thus, the purpose of this project focused on how the fat storing cells can educate adjacent epithelial cell to recruit into malignant or disease state, by evaluating models involving diabetic complications, which can potentially mediate cancer development from normal diabetes at Institute of Diabetes and Cancer (IDC). IDC consists of seven divisions of research groups covering various filed of studies involving diabetic complications and cancer, mainly focusing on biological process of metabolism using transgenic mouse models. Among IDC divisions, Joint Heidelberg-IDC translational diabetes program resides at Heidelberg University hospital, where we conduct multi-disciplinary translational diabetic research in collaboration with scientists and physicians recruited by a director of endocrinology department, prof. Peter Nawroth.

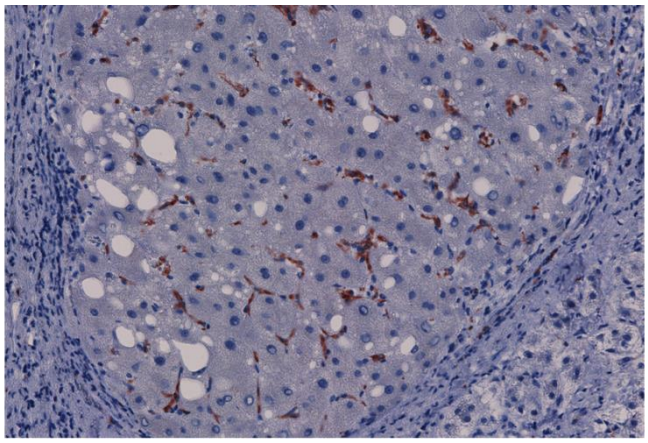


Figure 1. TSC22D4 immunostaining in non-paranchymal cells of human liver tissues (x20)

Interestingly, colleagues at IDC has reported that TSC22D4 expression was significantly elevated in cancer cachectic mice and human diabetic liver, and regulates hepatic lipid metabolism as well as triglyceride homeostasis (Jones *et al*, *EMBO Mol Med*, 2013; Ekim Ustunel *et al*, *Nat Comm.*, 2016). TSC22D4 is a transcriptional regulator known to be stimulated by TGFβ1, yet only a few studies indicated its possible roles mainly in nerve tissues. TGFβ1 is a widely known fibrosis-inducing cytokine in various organs such as liver, lung and breast by promoting collagen production. Excess fat accumulation in adipose tissues or fat-storing cells is known to cause inflammation which alternates their physiology by decreased lipid droplets and production of cytokines such as TGFβ1. TGFβ1 also plays a central role in activation of Hepatic stellate cells (HSCs), also known as fat storing cells in liver which secretes extracellular matrix components (ECM) upon its activation, comparable to formation of CAA.

Dysregulation of lipid metabolism leading to caner formation has been extensively studied to distinguish the disease state in liver. For instance, non-alcoholic fatty liver disease (NAFLD) is widely known metabolic diseases, which are mostly caused among obese patients by accumulation of fat cells, developing insulin resistance, increasing free fatty acids and high cholesterol level. 10-25% of NAFLD patients then progress a reversible non-alcoholic steatohepatitis (NASH), induced by excessive hepatic fat accumulation causing increased oxidative stress, mitochondorial dysfunction, and inflammatory cytokine production (Bataller and Brennner, *J Clin Invest.* 2005). When scarring continues to develop, excess fibrous tissue in inflamed liver is recognized as "liver fibrosis" stage, often followed by irreversible chronic liver disease, Cirrhosis. Patients with Cirrhosis are required to transplant a new liver before facing liver failure or development of hepatocellular carcinoma. Though liver fibrosis is one of the most common diabetic complications and a key stage of the transition between fatty liver and cancer, no standard antifibrotic therapies is established due to its unknown molecular mechanisms. In order to understand the interplay of inflammatory fat to fibrosis, which then leads to cancer formation, we targeted a potential mediator of the diseases, TSC22D4 in liver fibrosis model.

In human liver tissues, TSC22D4 is primarily expressed in cytoplasm of non-parenchymal cells (Figure 1). To test the hypothesis of TSC22D4 regulation in liver fibrosis, transwell migration assay and proliferation assay was employed with TGFβ1 treatment upon TSC22D4 siRNA knockdown using immobilized human HSCs, LX2 cells (Figure 2A and 2B). Indeed, TSC22D4 knockdown inhibited TGFβ1-induced migration and proliferation of LX2. In addition, fibrosis markers, upregulation of αSMA and COL1α1 expressions upon TGFβ1

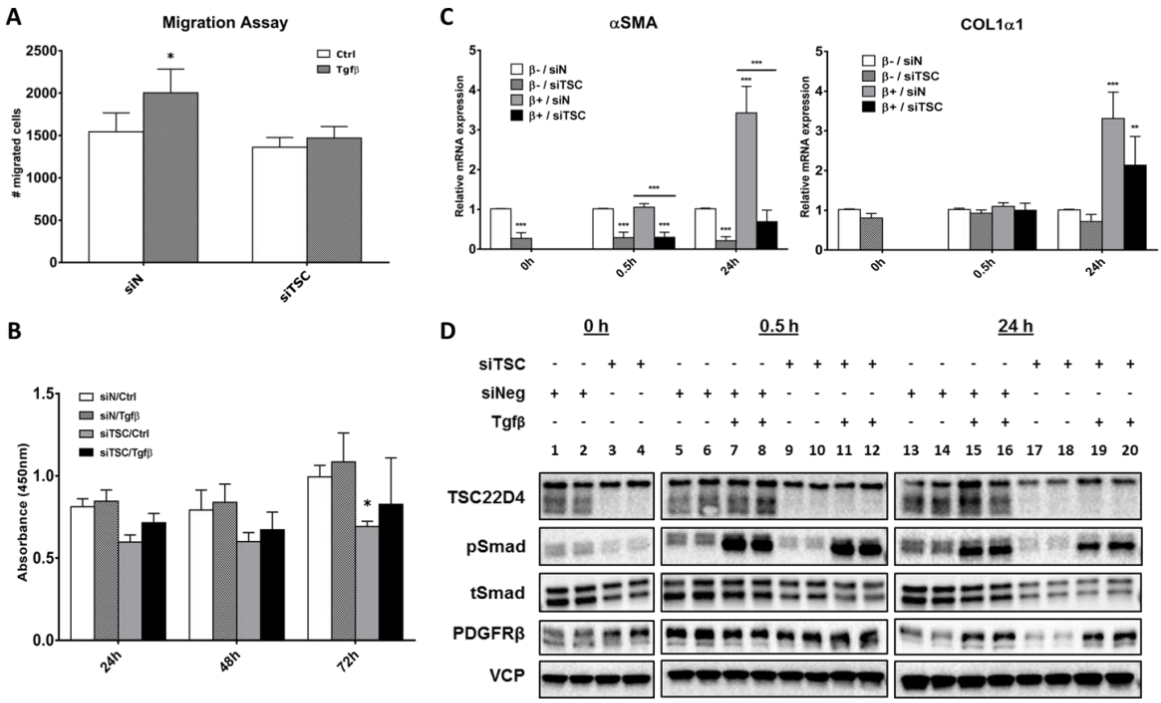
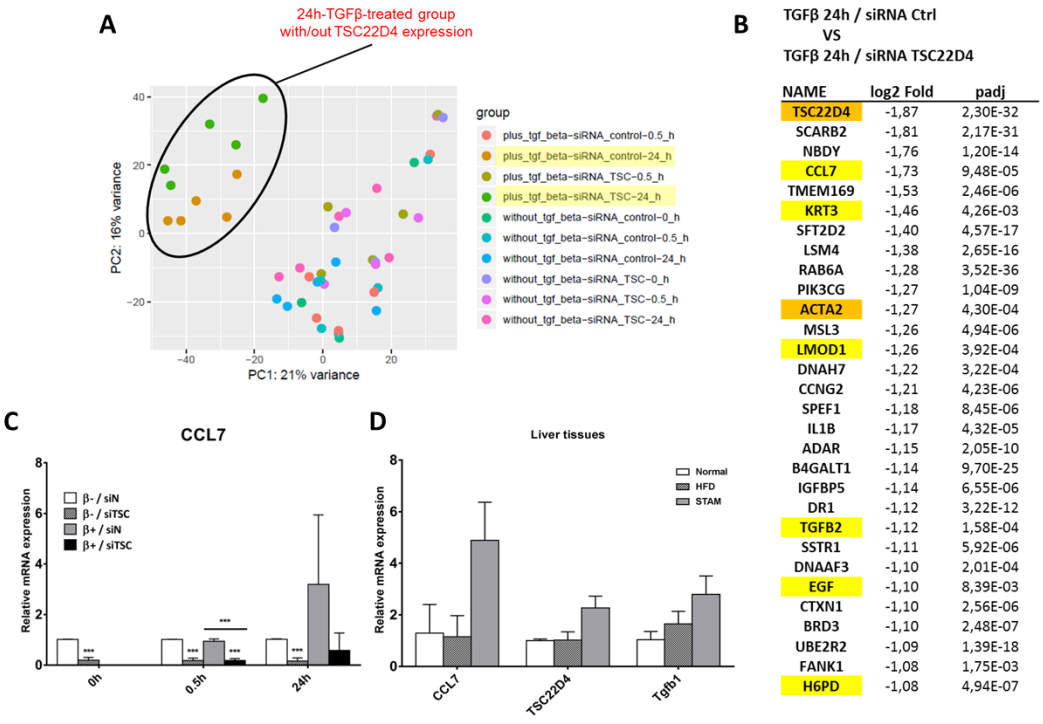


Figure 2. TSC22D4 is involved in activation of HSCs and regulation of fibrogenesis. LX2 were treated with negative control (siN) or TSC22D4 (siTSC) siRNA 20μM and Tgfβ 2ng/ml with various time points. A) Transwell migration assay upon 24h incubation with Tgfβ. B) Proliferatin assay with indicated Tgfβ treatment time. C) mRMA expression of fibrosis markers, αSMA and COL1α1, D) Western blot analysis using indicated antibodies upon 0, 0,5, 24h Tgfβ treatment. Student's t-test. *P < 0.05; **P < 0.01; ***P < 0.001.

stimulation are also demolished with TSC22D4 inhibition, indicating that TSC22D4 plays a role in fibrogenesis (Figure 2C). TSC22D4 knockdown also reduced TGFβ1-induced pSmad3 and PDGFRβ expressions, suggesting its potential role in HSCs activation via regulating TGFβ1 signaling pathway (Figure 2D).

Since the role of TSC22D4 has not been studied in liver fibrosis model, we then conducted transcriptome analysis using LX2 cells to identify its regulated gene expressions. Principle component analysis indicated significant gene expression changes in 24h TGFβ1-treated group with/out TSC22D4 expressions (Figure 3A). Among the significantly regulated genes, several genes follow the same pattern as αSMA upon TGFβ1 stimulation and TSC22D4 knockdown as indicated in figure 3B. Notably, CCL7, a chemokine acts as a ligand of CCR1, which is known to promote hepatic fibrosis in liver injured mice model (Seki, *et al*, *JCI*, 2009). qPCR analysis has shown CCL7 is promoted by TGFβ1 but not in absence of TSC22D4, as well as significant upregulation in mouse STAM model compared to the HFD or normal diet (Figure 3C and 3D). Of note, integrated motif analysis has also shown significant changes of several transcription factors that are associated with TGFβ1 downstream pathways such as Smad, MAPK, and ARK. For instance, we detected upregulation of c-Jun/c-Fos family, which is reported to cooperate with Smad3 to promote fibrosis upon TGFβ1 signaling activation (Zhang *et al*, *Nature*, 1998). We confirmed that not only phosphor-Smad3, but also phosphor-ERK and phosphor-ARK expressions were altered upon TSC22D4 knockdown in LX2 cells, consistent with ARK interaction in hepatocytes confirmed by my colleague (data not shown). Though, we are still in process of additional experiments to clarify further molecular mechanism such as direct protein-protein interaction with TSC22D4 by proteomics, phenotype change by metabolome analysis and identify localization of its action by immunofluorescence.



NAME	log2 Fold	padj
TSC22D4	-1,87	2,30E-32
SCARB2	-1,81	2,17E-31
NBDY	-1,76	1,20E-14
CCL7	-1,73	9,48E-05
TMEM169	-1,53	2,46E-06
KRT3	-1,46	4,26E-03
SFT2D2	-1,40	4,57E-17
LSM4	-1,38	2,65E-16
RAB6A	-1,28	3,52E-36
PIK3CG	-1,27	1,04E-09
ACTA2	-1,27	4,30E-04
MSL3	-1,26	4,94E-06
LMOD1	-1,26	3,92E-04
DNAH7	-1,22	3,22E-04
CCNG2	-1,21	4,23E-06
SPEF1	-1,18	8,45E-06
IL1B	-1,17	4,32E-05
ADAR	-1,15	2,05E-10
B4GALT1	-1,14	9,70E-25
IGFBP5	-1,14	6,55E-06
DR1	-1,12	3,22E-12
TGFB2	-1,12	1,58E-04
SSTR1	-1,11	5,92E-06
DNAAF3	-1,10	2,01E-04
EGF	-1,10	8,39E-03
CTXN1	-1,10	2,56E-06
BRD3	-1,10	2,48E-07
UBE2R2	-1,09	1,39E-18
FANK1	-1,08	1,75E-03
H6PD	-1,08	4,94E-07

Figure 3. Profiling TSC22D4 regulated genes by transcriptome analysis A) Principle component analysis of mRNA samples B) Significantly downregulated genes upon 24h Tgfβ treatment with/out TSC22D4 expression. C)RT-qPCR analysis of CCL7 in LX2 and D) in mouse liver tissues in comparison to TSC22D4 and Tgfβ1.

Importantly, we are also establishing a new transgenic mouse model specifically targeting HSCs for Tsc22d4 knockdown. It is challenging to control the disease state to induce liver steatosis without liver injury and cancer, though we have managed to monitor type-II diabetic phenotype by methionine choline-deficient (MCD), Streptozotocin plus high fat (STAM), or fructose, palmitate, cholesterol and trans-fat (FPC) diet to examine non-alcohol steatohepatitis. Due to the complexity of HSCs-targeted LratCre transgenic mouse generation, our first experimental model is still ongoing until the next year. In the meantime, another cohort of Tsc22d4 knockout (KO) mice targeting hepatocytes (AlbCre) has shown changes in triglyceride level, suggesting a possibility of Tsc22d4 in regulation of lipogenesis. Overall, understanding the lipid metabolism together with its associated signaling molecules seems to be the key to elucidate the fat-induced tumor formation. As lipid metabolism of CAAs especially in the breast tissues are not well understood, it is important to explore the key regulator to link diabetes and cancer such as TSC22D4.

A crosstalk of diabetes and cancer has caught increased attentions over a decade, and several projects at IDC focus on the topic with various targeted organs such breast and prostate cancer, as well as adipocytes and liver in diabetes. Interestingly, several genes causing hepatic dysregulation belong to the same family of cancer-associated adipocyte induced genes such as Lipocalin and S100 family. For instance, S100A4 was associated with TGFβ1 induced Smads activation in endometrial cancer (Xie *et al, Lab. Invest.* 2009), while my colleagues at Tohoku University and I have shown that S100A7 expression in breast cancer cells are significantly involved with cancer-associated adipocytes (CAA) regulation in tumor microenvironment, as well as its positive correlation in the late stage breast cancer (Mayama et al, *Cancer Sci., 2018;* Sakurai *et al, Breast Can,* 2017). In liver tissues, S100A6 is suggested as a novel therapeutic target as it enhances HSC activation in CCl4 induced hepatic fibrosis (Xia *et al, Euro J Pharm.,* 2018). Once a target molecule is extensively studied in a basic model such as transgenic mouse liver, the idea can be applied to different organs such as breast, as long as both are confirmed to have the same mediator, such as TGFβ1. Hence, conducting an interdisciplinary study across diabetes and cancer could potentially provide a key finding applicable to systemic homeostasis across diseases.